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IDENTIFICATION OF AUTOANTIGENS IN MULTIPLE SCLEROSIS

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IDENTIFICATION OF AUTOANTIGENS IN MULTIPLE SCLEROSIS

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To my girls

Molly, Asta & Lovis

And now after some thinking
I'd say I'd rather be
a functioning cog in some great machinery
serving something beyond me

Fleet Foxes

POPULAR SCIENCE SUMMARY OF THE THESIS

The human immune system is an incredibly complex and finely tuned machine with many different players. Each performs its part in the body's defense against threats from the outside world, like bacteria or viruses, and from inside the body itself, like cancer. While staying ever vigilant, it can be too easy on the trigger and mistake the "self" for an intruder. Such mistakes are the basis for autoimmunity, where the immune system's weapons are turned upon the otherwise healthy body. This results in diseases like diabetes type I, rheumatoid arthritis, and multiple sclerosis (MS), which are, as a rule, chronic and incurable. In MS, the immune system targets structures in the central nervous system (CNS), i.e., the brain and spinal cord. This leads to inflammation and loss of myelin, the isolating sheaths wrapping around the wires of the CNS, the neuronal axons, which impairs the electrical nerve signals. This leads to neurological symptoms like loss of vision, sensory deficits, or motor impairment.

In the case of multiple sclerosis, evidence points towards the adaptive part of the immune system being the main culprit. The adaptive immune system is the specialized part responsible for adapting to and learning from different infections and remembering them over time, i.e., immunity. In particular, MS autoimmunity is driven by T helper cells, the highly specialized intelligence officers of the immune system army responsible for recognizing threats and directing other immune cells toward them. Each cell has one specific target, i.e., antigen, which it can recognize, and in MS, they have mistaken self-proteins as targets. Such self-targets are called autoantigens. Autoantigens can help explain why people get MS, be used for new diagnostic tools, and perhaps most importantly, be a target for new treatments. However, precisely which autoantigens are targeted in MS is not yet known. If the autoantigens are known, a strategy of re-educating the immune system, teaching it to not perceive the autoantigens as targets, to treating autoimmunity could be possible. It has shown great promise in mouse models and is already used for allergies. In MS, it has not yet shown any significant efficacy, most likely because there are still large gaps in the known autoantigen repertoire.

The aim of this thesis was, therefore, to identify previously unknown autoantigens in MS. In **Paper I**, we tried to solve a common problem when conducting these types of studies: the fact that the autoantigen targeting T cells are very rare and often obscured by the general noise of experimental assays. We developed a method where we bind the autoantigen of interest to tiny magnetic beads, filling two functions. It allows for removing contaminants, which would otherwise increase the noise of follow-up experiments, and their size triggers immune cells to target them. We then used model bacterial and viral antigens to show that we could get strong responses with low noise. We used this new method in **Paper II** to investigate an earlier described but still controversial autoantigen in MS, myelin oligodendrocyte glycoprotein (MOG). We found that half of the persons

with MS tested had proinflammatory T helper cells, which targeted MOG, cementing MOG as an autoantigen in MS. The study also worked as a proof of concept that the method was sensitive enough to detect rare autoreactive cells.

In **Paper III**, we went broader and examined a library of 63 proteins normally present in the brain and, as such, potential targets of the autoimmune attack. In this study, we found four previously unknown autoantigens which elicited inflammatory responses in persons with MS. By examining the response to several different autoantigens, both already known and the four new ones; we could see that each person with MS displayed an essentially unique pattern of autoreactivity demonstrating the underlying heterogeneity of MS. Testing autoreactivity broadly could also potentially be used to aid in diagnosis. Further, in a mouse model, we could see that T cells specific for these autoantigens invaded the CNS. Lastly, in **Paper IV**, we revisited a contentious autoantigen in MS, alpha-crystallin B, and examined antibody and T-cell responses using our methodology. Here, we could confirm it as a target associated with MS, and we could see that it was a result of misdirected Epstein-Barr virus immunity via molecular mimicry, providing a mechanistic explanation as to why Epstein-Barr virus infection increases the risk of MS.

This work presents a method for examining antigen-specific autoreactivity, confirming old and presenting four new autoantigens in MS that can be used as targets for novel diagnostic and therapeutic strategies.

ABSTRACT

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system in which cells from primarily the adaptive immune system infiltrate the brain and spinal cord, leading to inflammation and demyelination. Debuting primarily between 20-40 years of age and with a prevalence in Sweden of ~0.2%, it is one of the leading causes of disability in working-age adults. While the cause is still unknown, the risk of developing MS is influenced by an interplay of both genetic and environmental risk factors. Genetic and immunological data point towards $CD4^+$ T cells being a primary driver of the disease. While some de facto targets, i.e., autoantigens, have been identified, the known autoantigen repertoire still contains considerable gaps. This remains a critical problem for developing autoantigen-targeted diagnostic tools and autoantigen-specific treatment strategies. This thesis aimed to identify novel autoantigens in MS.

Paper I addressed a common problem when studying autoantigen-specific T-cell responses: autoreactive T cells are rare, and antigens can be either weak stimulators or contain contaminants that are challenging to remove, resulting in high assay noise and low sensitivity. By covalently coupling recombinant protein antigens to 1 μm paramagnetic polystyrene beads, we show that contaminants can be removed while the ability to stimulate T-cell responses remains. This resulted in a sensitive assay with high signal-to-noise ratios, with a threshold for detection at 1 in 18 000 cells.

In **Paper II**, we used this novel method to examine T cell responses to myelin oligodendrocyte glycoprotein (MOG), an autoantigen for which previous results have conflicted. By examining peripheral blood mononuclear cells (PBMCs) from a cohort of persons with MS (pwMS) and matched healthy controls (HC), MOG-specific $CD4^+$ T cells were detected in approximately half of all pwMS. Additionally, MOG-epitopes were presented by monocytes and restricted to HLA-DR. Lastly, using three different antibody-assays, we could not detect any significant portion of MOG-specific autoantibodies despite the presence of MOG-specific T cells.

Paper III addressed the main aim of this thesis, i.e., identifying novel autoantigens. This study combined the antigen-bead method with the Human Protein Atlas recombinant protein epitope signature tag library to screen for T-cell reactivity against a panel of 63 central nervous system-expressed proteins. In a smaller screening cohort, there were increased proinflammatory responses against four novel autoantigens targets: fatty acid binding protein 7 (FABP7), prokineticin-2 (PROK2), reticulon-3 (RTN3), and synaptosome associated protein 91 (SNAP91), as well as the previously described autoantigen MOG in pwMS. The screening results were validated using full-length versions of the targets in two larger cohorts, including pharmacologically untreated pwMS. The autoreactive profiles of individuals were heterogenous, but a panel of several autoantigens could distinguish between MS and non-MS with high accuracy. Immunophenotyping revealed MS-

specific autoreactive cells to be mainly HLA-DR-restricted $CD4^+$ T cells and responded with interferon-gamma and granulocyte-macrophage colony-stimulating factor production upon stimulation. The presence of autoantibodies was examined in a large cohort of patients and controls. Still, it was not increased in MS. Immunization of mice with the novel autoantigens induced T cell responses, leading to CNS-leukocyte migration and crossing of the blood-brain barrier, demonstrating encephalitogenic potential.

Paper IV explored a possible immunological link between Epstein-Barr virus infection and MS. We examined serological responses to alpha-crystallin B (CRYAB) and Epstein-Barr virus nuclear antigen 1 (EBNA1) in a cohort of 713 pwMS and 722 HC. Anti-CRYAB-antibodies were associated with MS with an odds ratio (OR) of 2.0, which had a synergistic effect with high EBNA1 responses (OR of 9.0). By depleting plasma of anti-EBNA1 antibodies, CRYAB responses were similarly removed, demonstrating cross-reactivity between the two antigens due to an amino acid sequence homology (RRPFF, CRYAB aa11-15 and EBNA1 aa402-406 respectively). In a mouse model, EBNA1-primed T cells were also CRYAB-reactive, and EBNA1 and CRYAB-responsive T cells were highly correlated and increased in natalizumab-treated pwMS, pointing towards a similar cross-reactivity in the T-cell compartment as well.

In conclusion, this thesis presents methods for sensitively assessing autoreactive T-cell responses, reexamining and confirming MOG and CRYAB as targets. It considerably expands the knowledge regarding the targets of the autoimmune attack in MS by adding four novel autoantigens to the known repertoire. Further, it demonstrates an underlying heterogeneity of the immunological landscape of MS and provides a mechanistic link between Epstein-Barr virus and MS. It demonstrates a first step in the development of autoantigen-specific methods for diagnostics and introduces novel targets for potentially effective antigen-specific immunotherapy.

LIST OF SCIENTIFIC PAPERS

I. Bronge M, Kaiser A, Carvalho-Queiroz C, Nilsson OB, Ruhrmann S, Holmgren E, Olsson T, Gafvelin G, Grönlund H.

Sensitive detection of antigen-specific T-cells using bead-bound antigen for in vitro re-stimulation. *MethodsX.* 2019 Jul 8;6:1635-1641. Doi: 10.1016/j.mex.2019.07.004.

II. Bronge M, Ruhrmann S, Carvalho-Queiroz C, Nilsson OB, Kaiser A, Holmgren E, Macrini C, Winklmeier S, Meinl E, Brundin L, Khademi M, Olsson T, Gafvelin G, Grönlund H.

Myelin oligodendrocyte glycoprotein revisited-sensitive detection of MOG-specific T-cells in multiple sclerosis. *J Autoimmun.* 2019 Aug;102:38-49. Doi: 10.1016/j.jaut.2019.04.013.

III. Bronge M, Asplund Högelin K, Thomas OG, Ruhrmann S, Carvalho-Queiroz C, Nilsson OB, Kaiser A, Zeitelhofer M, Holmgren E, Linnerbauer M, Adzemovic MZ, Hellström C, Jelcic C, Liu H, Nilsson P, Hillert J, Brundin L, Fink K, Kockum I, Tengvall I, Martin R, Tegel H, Gräslund T, Al Nimer F, Guerreiro-Cacais AO, Khademi M, Gafvelin G, Olsson T, Grönlund H.

Identification of four novel T cell autoantigens and personal autoreactive profiles in multiple sclerosis. *Sci Adv.* 2022 Apr 29;8(17). Doi: 10.1126/sciadv.abn1823.

IV. Thomas OG*, Bronge M*, Tengvall K, Akpinar B, Nilsson OB, Holmgren E, Hessa T, Gafvelin G, Khademi M, Alfredsson L, Martin R Guerreiro-Cacais AO, Grönlund H, Olsson T*, Kockum I*

Cross-reactive EBNA1 immunity targets alpha-crystallin B and is associated with multiple sclerosis. *Submitted manuscript.*

* Shared first and last authorship

SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Gerhards R, Pfeffer LK, Lorenz J, Starost L, Nowack L, Thaler FS, Schlüter M, Rübsamen H, Macrini C, Winklmeier S, Mader S, Bronge M, Grönlund H, Feederle R, Hsia HE, Lichtenthaler SF, Merl-Pham J, Hauck SM, Kuhlmann T, Bauer IJ, Beltran E, Gerdes LA, Mezydlo A, Bar-Or A, Banwell B, Khademi M, Olsson T, Hohlfeld R, Lassmann H, Kümpfel T, Kawakami N, Meinl E.

   Oligodendrocyte myelin glycoprotein as a novel target for pathogenic autoimmunity in the CNS. *Acta Neuropathol Commun.* 2020 Nov 30;8(1):207. Doi: 10.1186/s40478-020-01086-2.

II. Bronge M, Fink K, Ruhrmann S, Iacobaeus E, Nilsson OB, Kaiser A, Khademi M, Gafvelin G, Olsson T, Grönlund H.

   Proinflammatory autoreactive hypocretin-specific T cells are common in both narcolepsy and non-narcolepsy. *Submitted Manuscript*.

III. Lutterotti A, Docampo MJ, Ludersdorfer T, Hohmann M, Hayward-Koennecke, Bronge M, von Niederhäusern V, Cruciani C, Thomas OG, Sellés-Moreno C, Stenger R, Mueller T, Blumer C, Hilty M, Foege M, Guffanti F, Weichselbaumer V, Blanc A, Jelcic I, Kayser M, Hüllner M, Winkelhofer S, Treyer V, Goede J, Behe M, Broggini M, Scanziani E, Olsson T, Grönlund H, Sospedra M, Martin R.

   Establish tolerance in multiple sclerosis with myelin peptide-coupled red blood cells – the ETIMS^{red} trial. *Manuscript*

CONTENTS

LIST OF ABBREVIATIONS

ADEM	Acute disseminated encephalomyelitis
ANO2	Anoctamin-2
APC	Antigen-presenting cell
BBB	Blood-brain barrier
CD	Cluster of differentiation
CIS	Clinically isolated syndrome
CMV	Cytomegalovirus
CNS	Central nervous system
CRYAB	Alpha-Crystallin B
CSF	Cerebrospinal fluid
DC	Dendritic cell
DMT	Disease-modifying treatment
EAE	Experimental autoimmune encephalomyelitis
EBNA1	Esptein-Barr virus nuclear antigen 1
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
FABP7	Fatty acid binding protein 7
HC	Healthy control
HSCT	Hematopoietic stem cell transplantation
HLA	Human leukocyte antigen
HPA	Human Protein Atlas
IL	Interleukin
IFN	Interferon
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MOGAD	Myelin oligodendrocyte glycoprotein antibody associated disease

MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MS-Nat	Natalizumab treated persons with MS
MS-Un	Persons with MS without ongoing disease modifying treatment
NMOSD	Neuromyelitis optica spectrum disorders
OCBs	Oligoclonal bands
OND	Other neurological disease controls
OR	Odds ratio
PBMC	Peripheral blood mononuclear cells
PLP	Proteolipid protein
PPMS	Primary progressive multiple sclerosis
PROK2	Prokineticin-2
RTN3	Reticulon-3
RRMS	Relapsing-remitting multiple sclerosis
SNAP91	Synaptosome associated protein 91
SPMS	Secondary progressive multiple sclerosis
TCR	T-cell receptor
$T_H1/17/22$	T helper cell 1/17/22
T_{reg}	T-regulatory cell

1 INTRODUCTION

Science has allowed humans to explore the universe beyond our planet's borders and look inwards, unraveling the complicated intricacies of our bodies in health and disease. However, the expansion of our knowledge is not always fast. While the first descriptions of the pathological characteristics of the typical multiple sclerosis (MS) lesions date back as early as the 1830s, published in works by the pathologists Carswell [1] and Cruveilhier [2], it took more than 30 years for someone to identify it as a distinct disease with its own clinical features. In a series of lectures [3] in 1868, the prominent French neurologist Jean Martin Charcot described a novel neurological disease with pathologically characteristic lesions in the central nervous system (CNS). He drew the association to the decades-earlier findings of Carswell and Cruveilhier, calling it "*sclérose en plaques*" from which the English name "multiple sclerosis" is derived.

Over 150 years later, our understanding of the disease has naturally reached much further due to a combination of technological advances and countless hours of clinical, epidemiological, and laboratory research performed by the dedicated researchers of the last century. We can now diagnose MS with better accuracy, there are several high-efficacy treatments available, we know of many environmental and genetic risk and protective factors, and we understand that the adaptive immune system plays a crucial part in causing and propagating the lesions first described almost 200 years ago. While the gaps in our knowledge continue to shrink daily, several vital questions still need to be answered. There is yet no curative treatment. MS continues to instill significant morbidity in those it affects. We do not fully understand why some people get MS and the reasons for the different paths the disease takes in individual patients. We need to understand the molecular structures in the CNS that the aberrant immune cells target.

This thesis attempts to address that last question by developing and utilizing new tools to identify the target, i.e., autoantigen, of the primary immune cell culprit: T cells. Although the first evidence of T cells targeting myelin-proteins hails back to the 1980s, only a few additions have conclusively been made since, and the known autoantigen repertoire remains full of cumbersome gaps. In the last 20 years, several studies have demonstrated the potential of antigen-specific immunotherapy in mouse models of MS, showing that it is possible to ameliorate or even cure autoimmune disease when the autoantigens are known. These therapies have increased the importance of finding the autoantigens in MS and demonstrated that we still need the complete picture, as the numerous trials during the past 20 years targeting the so far known autoantigens have shown no to little efficacy.

2 LITERATURE REVIEW

2.1 MULTIPLE SCLEROSIS

MS is a complex chronic immune-mediated disease of the CNS with an unclear cause. It is considered an autoimmune inflammatory disease in which cells from the adaptive immune system cross the blood-brain barrier (BBB) and cause localized CNS inflammation, demyelination, and in the end, axonal damage [4]. Commonly debuting at 20-40 years of age and with a worldwide prevalence of around 2.8 million [5], it is the leading cause of non-traumatic neurological disability of young people in the developed parts of the world and responsible for substantial societal cost and morbidity [6,7]. Despite advancements in treatment options during the last decade, no curative treatment exists, and current strategies focus on slowing disease progression and alleviating symptoms [8].

Localized areas of inflammation, i.e., lesions, characterize MS and while preferentially present in periventricular, juxtacortical, and infratentorial brain regions and the spinal cord, they can occur essentially anywhere in the CNS. As such, MS presents with a wide variety of symptoms depending on where the lesions are located, including but not limited to optic neuritis, sensory symptoms, limb weakness, imbalance, incontinence, cerebellar ataxia, and often less anatomically specific symptoms such as fatigue and cognitive decline [9]. Early on, symptoms usually come in relapses, distinct episodes of transient neurological worsening with partial or even complete recovery in between. However, as the disease continues, recovery gradually becomes deficient, and an accumulation of neurological disability follows [8].

2.1.1 Clinical Course

Multiple sclerosis is a heterogeneous condition with varying degrees of disease activity and progression (Figure 1). Since 1996, MS has been divided into four distinct clinical disease patterns: 1) The archetypical relapsing-remitting (RRMS) type, defined by clear relapses with partial to complete recovery in between. 2) Primary-progressive (PPMS), which presents with a continuous worsening of symptoms from disease onset with a lack of relapses. 3) Secondary-progressive (SPMS), which is when a previous RRMS transitions to a progressive disease course. 4) Progressive-relapsing (PRMS), which presents with progression from onset combined with relapses [10]. This classical division based on clinical phenotypes has since been updated to consider the underlying disease mechanisms and better access to imaging [11]. The main distinction between relapsing and progressive disease is still being made, but with additional modifiers of activity (new clinical relapses or new contrast-enhancing lesions on MRI) and progression. RRMS, PPMS, and SPMS are still used (active or inactive and with progression or not), while PRMS, following the 2017 criteria, is defined as PPMS with disease activity. An addition of clinically isolated syndrome (CIS) has also

been made, where a patient has experienced one typical MS-like attack, but the repeating pattern of RRMS has not yet emerged. Most patients with CIS will eventually develop MS. Interestingly, the underlying mechanism of MS likely begins years before the first typical symptoms occur, leading to a long prodromal phase [12,13].

Most patients (85-90%) debut with an RRMS pattern. While a progressive disease at onset is substantially rarer than the relapsing form, most RRMS patients will eventually convert to SPMS, with a mean time of 10.7 years from disease onset [14]. However, to what extent the recent advances in treatment will affect this conversion is still not fully known. While there has been a substantial improvement in treatment in the past decades, leading to a considerable decrease in mortality, persons with MS are still at risk compared to the general population and suffer a 6–12-year decrease in life expectancy [15,16].

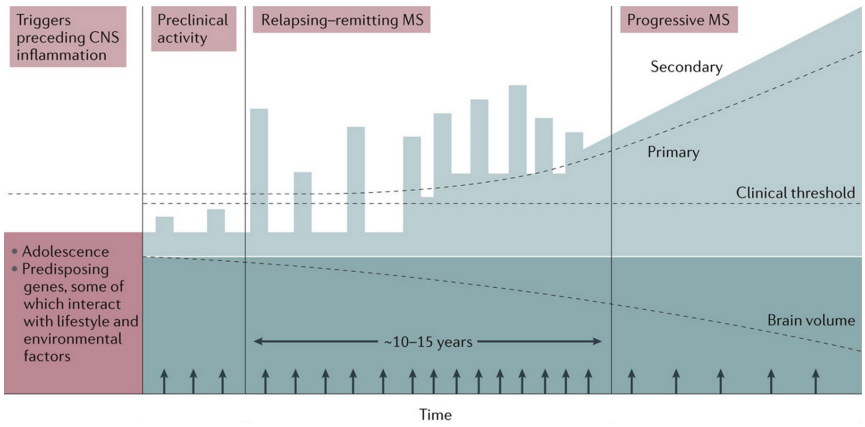

Figure 1. Schematic overview of the MS disease course. Reprinted by permission from Nature Reviews Neurology, Olsson T, et. al., © 2016.

2.1.2 Diagnosis

Diagnosing MS remains challenging, and misdiagnosis has been estimated to be as high as 10%, sometimes with detrimental consequences as inappropriate disease-modifying treatments are used [17]. The reason is partly due to the difficulty to differentiate MS from other diseases with demyelinating properties, such as neuromyelitis optica spectrum disorders (NMOSD) or acute disseminated encephalomyelitis (ADEM), and neuroinflammatory diseases like neurosarcoidosis or CNS vasculitis [9].

The diagnostic criteria for MS were revised and updated in 2017 [18]. As a core principle, accurate diagnosis relies on demonstrating the dissemination of CNS lesions in space and time, meaning that objective evidence of lesions appearing in distinct anatomical locations within the CNS and new lesions appearing over time should be demonstrated (Table 1). Essentially, two clinical attacks with different symptoms, reflecting lesions at two or more locations, is enough for a diagnosis of MS. However, the disease is rarely clear-cut, and in cases where clinical data cannot confidently assess the number of lesions or if it is the first symptomatic neurological attack, magnetic resonance imaging (MRI) can be used to demonstrate visible lesions at different sites or lesions of different

ages. In atypical cases where diagnostic criteria are not reached with the combination of clinical and MRI evaluation, the presence of oligoclonal bands (OCBs) in the cerebrospinal fluid (CSF) can be used as a substitution for the dissemination of time requirement [18]. While a robust biomarker for MS, OCBs are unfortunately unspecific and can be seen in other neuroinflammatory diseases [19]. In PPMS, these criteria are not as applicable, as distinct relapses often are absent. Instead, one year of disability progression in combination with either MS-typical MRI findings or CSF-OCBs are used as criteria [18].

Table 1. 2017 McDonald criteria for diagnosis of multiple sclerosis

Number of clinical attacks	Number of lesions with objective clinical evidence	Additional data needed for diagnosis*
≥2	≥2	None
≥2	1 (As well as clear-cut historical evidence of a lesion in a distinct anatomical location)	None
≥2	≥1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI.
1	≥2	Dissemination in time is demonstrated by an additional clinical attack or MRI or demonstration of CSF-oligoclonal bands.
1	1	Dissemination in space demonstrated by an additional clinical attack implicating a different CNS site or by MRI. AND Dissemination in time is demonstrated by an additional clinical attack or MRI or demonstration of CSF-oligoclonal bands.

*Brain MRI is recommended for all patients with suspected multiple sclerosis. Spinal MRI and CSF examination should be considered in patients with insufficient or atypical clinical and MRI evidence. Adapted from Thompson AJ, et. al., Lancet Neurology, 2018. [18]

2.1.3 Treatment

There are yet no curative treatments for MS. Rather, current treatment strategies focus on reducing the rate and severity of relapses and the following accumulation of disability. Several approved disease-modifying treatments (DMTs) exist, such as interferon beta, glatiramer acetate, monoclonal antibodies like natalizumab, alemtuzumab, daclizumab, B-cell depleting antibodies like rituximab and ocrelizumab, and oral agents like fingolimod and dimethyl fumarate [8]. While the modes of action differ between the substances, they share the common goal of immunosuppression or modulation and come with the risk of severe side-effects [20]. Evidence points to an increased risk of serious infections [21] and cancer [22] in patients treated with the more effective drugs. Additionally problematic is that most of these substances are only effective in treating relapsing disease, with little to no effect on the progressive forms. Ocrelizumab has shown some potential benefit in progressive MS, but while it is now approved as treatment with favorable safety data over time [23], the magnitude of the long-term benefit remains uncertain [24].

A completely different but promising approach is autologous hematopoietic stem cell transplantation (HSCT). Small-scale studies have shown an effect surpassing that of many traditional pharmacological treatments, with complete suppression of disease activity for up to 5 years in 70-80% of patients [25]. As the treatment has become increasingly safe in recent years, it could be a viable option for selected patient populations. Mesenchymal stem cells have also been a tempting alternative, with hopes of lowering disease activity, aiding in remyelination, and possibly reversing already accumulated damage. However, recent trials report conflicting results [26,27], highlighting the need for more research before this kind of treatment can be implemented in clinical praxis.

Another possible treatment is antigen-specific immunotherapy [28], which has shown promise in animal models. This strategy will be further discussed in subsequent sections of this review.

2.1.4 Epidemiology and etiology

While being the most common chronic inflammatory disease of the CNS, the prevalence of MS varies significantly across the globe, following a pattern of increased prevalence further away from the equator with <10 per 100 000 in Southeast Asia and South America to 50-300 per 100 000 in Western Europe [5,29]. However, the correlation between latitude and incidence is not as straightforward as for prevalence, indicating that the difference could be partly explained by socioeconomic differences leading to better healthcare and longer survival times [6]. While MS is more common in females, as is the case for many autoimmune diseases, the female-to-male ratio has increased further in the past decades, reaching above 3:1 in many countries [6]. The reason behind this trend is however not evident as of yet.

2.1.4.1 Environmental and genetic risk factors

As with most multifactorial immune-mediated diseases, the cause of MS is not fully understood. Still, genetic risk variants and environmental risk factors have been identified, and a majority of MS-risk can be explained by these identified risk factors [30,31]. Among environmental risk factors are smoking, low vitamin D levels (which in turn could be partially responsible for the latitude-prevalence correlation), adolescent obesity, night work, Epstein-Barr virus (EBV) infection (discussed further in the subsequent section), use of organic solvents and a history of concussion [32]. Conversely, alcohol, coffee, and oral tobacco (Swedish snuff) are associated with a lower risk [30,33].

Studies of monozygotic twins have shown a concordance of MS of 20-35%, meaning that while important, genetic factors fail to explain the majority of MS-cases [34]. Nonetheless, the genetic contribution to MS susceptibility is undeniable [35]. Like in many autoimmune diseases, the strongest genetic association to MS lies in the human leukocyte antigen (HLA) region (the human version of the major histocompatibility complex (MHC)) on the short arm of chromosome 6 [35]. This

association was established in the 1970s [36], but due to the strong linkage disequilibrium within the HLA-region, the exact haplotypes that carried the risk-increase would take decades and great advancements in genotyping methodology to unravel [35]. It is now well established that HLA-DRB1*15:01 confers the single highest independent risk of any genetic factor, with an odds ratio (OR) of 3.1, followed by DRB1*13:03 and DRB1*03:01 with ORs of 2.4 and 1.26 respectively. HLA-A*02:01 is the strongest protective gene with an OR of 0.73 [37].

Extensive international efforts to map the MS genetics have identified over 100 different genetic risk variants, with modest risk increases with individual ORs of 1.1-1.3 [38]. Just like the HLA-association, the vast majority of these minor susceptibility genes code for proteins involved in various immunological pathways like cytokines (interleukin (IL)-2RA, IL-7R, IL-22RA2), costimulatory signals (CD37, CD40, CD80) and signal transduction (STAT3, TYK2), altogether pointing towards the immune system being the main culprit in MS-pathology [37].

There is also a substantial interaction between genetic and environmental factors [30]. For example, the presence of HLA-DRB1*15:01 and the absence of HLA-A*02 results in an OR of MS of ~5. In combination with smoking, the risk increases substantially to an OR of ~15 [39], suggesting an interactive effect. Similar genetic-lifestyle interactions have also been noted for obesity [40].

In common for all known risk factors is that all have a plausible pathway involving the adaptive immune system, even if not as clear-cut for the environmental as for the genetic factors. For example, the protective effect of oral tobacco can be explained by the alpha-7 nicotinic acetylcholine receptor on immune cells, by which nicotine act as an immunosuppressant [41,42]. Conversely, smoking and organic solvents lead to airway inflammation, which could activate autoimmune T cells [30]. Indeed, a connection between CNS autoimmunity and the pulmonary microbiome has been made, hinting toward possible mechanistic explanations for this link [43].

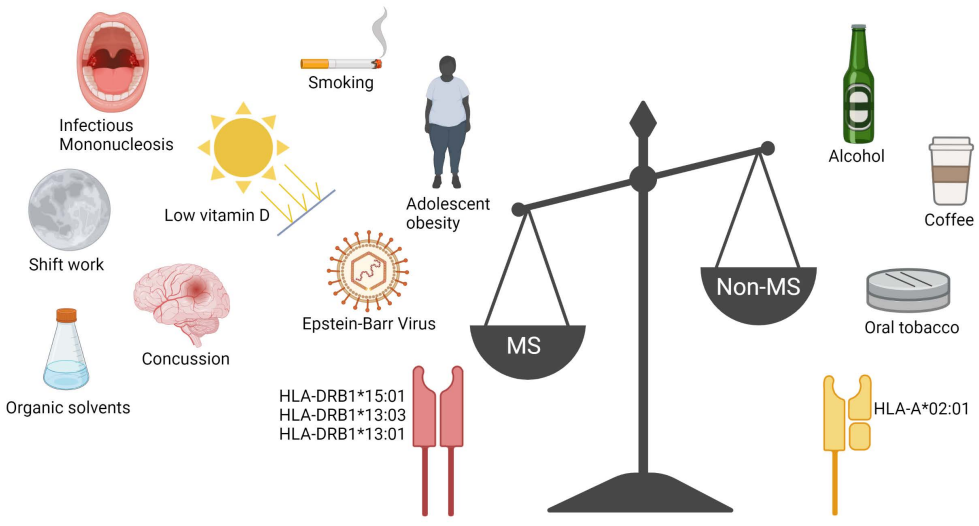

Figure 2. Risk and protective factors of multiple sclerosis. Risk factors are on the left side of the scales, protective factors are on the right. Based on Olsson T, et. al., Nat Rev Neurol. (2017) [30] and Montgomery S, et. al., Ann Neurol (2017) [32]. Created with Biorender.com.

2.1.4.2 Epstein–Barr virus and MS

Epstein-Barr virus is a double-stranded DNA virus belonging to the human herpesvirus family and is an almost ubiquitous human pathogen. While the primary infection is often very mild or even asymptomatic, the most recognized clinical manifestation is acute mononucleosis, especially if the primary infection occurs during adolescence. By adult age, more than 90% of individuals have been infected, with only a portion having a history of mononucleosis [44]. EBV primarily infects B cells, and after the acute phase, the so-called lytic phase where viral replication occurs, has been cleared, the infection enters a latency phase. Here, it resides without viral replication in B cells for life with some bouts of re-activation, and with a changed expression of the virus genome, mainly producing latency-proteins like EBV-nuclear antigen 1 (EBNA1) [45]. Besides mononucleosis, EBV has been linked to several forms of malignancies, such as Hodgkin-, Burkitt-, and Diffuse large B-cell lymphoma [46], as well as autoimmune diseases [47].

EBV infection has profound effects on the immune system, primarily in the infected B-cell compartment, which persists throughout life. It acts by deregulating host immune responses to allow for chronic latent infection. This is done by inducing regulatory responses through transcriptional modification of cytokines and receptors and a more direct effect by having viral proteins which mimics anti-inflammatory IL-10 [48]. Additionally, it induces proliferation and survival of infected B cells, a phenomenon that can be used *in vitro* to immortalize B-cell lines [49].

An epidemiological link between EBV infection and MS has been known for a long time [50,51], with a recent large epidemiological study cementing this link and indicating that EBV infection might even be a prerequisite for developing MS [52]. Interestingly, there are both environmental, age- and genetic interactions, where infectious mononucleosis in adolescence and HLA-DRB1*15:01 further increase the EBV-associated risk of MS [31,51,53] and smoking, low sun exposure, and obesity all synergize with EBV infection [54-56]. While this link is not entirely understood, it suggests that immune responses against EBV are somehow at play. As discussed previously, the synergistic risk-factors all influence the immune system. Indeed, it has been shown that persons with MS have dysregulated EBV responses [57,58]. One compelling explanation is molecular mimicry, a mechanism where EBV-targeted antigen-specific immune responses could mistakenly lead to an immune attack against CNS-expressed proteins due to similarities in amino acid (aa) sequences [59]. This phenomenon in the context of EBV will be further discussed in subsequent sections.

2.2 MS IMMUNOLOGY

MS lesions, i.e., areas of inflammation and demyelination, are present in the grey and white matter of persons with MS, with no evident attack of structures outside of the CNS. These lesions are characterized by the infiltration across the BBB of monocytes, T- and B-cells, and dendritic cells (DCs), and activation of resident macrophages, i.e., microglia. This subsequently leads to a loss of

oligodendrocytes and their product, myelin [8,60] (Figure 3). After such an attack, whether ameliorated by itself or by pharmacological intervention, some re-myelination usually occurs, but as the disease progresses lasting axonal damage develops.

Exactly how the initial activation of autoreactive cells occurs is not known. One hypothesis is that the activation occurs in the periphery, where CNS-autoreactive T cells are triggered in other organs by an immune reaction against non-CNS antigens via mechanisms like molecular mimicry or bystander activation [4]. After clonal expansion in lymph nodes, a few of these cells then crosses the BBB, encounter their antigen presented by resident antigen-presenting cells (APCs), activate, and release their inflammatory mediators leading to a disruption of the BBB, recruitment of more lymphocytes and monocytes. As more cells arrive, inflammation increases, leading to tissue damage and oligodendrocyte death, increased phagocytic activity, and the formation of a lesion. Another hypothesis is that a primary initiating event like spontaneous oligodendrocyte death or a viral infection occurs in the CNS, activating resident microglia. Autoantigens then drain to cervical lymph nodes, leading to a secondary adaptive immune response of autoantigen-specific cells that migrate to the CNS and drive additional inflammation [4].

As the disease continues, especially in the progressive stages, T and B cells exhibit more diffuse infiltration patterns, and resident phagocytes show chronic activation. Pathologically, the CNS undergoes generalized atrophy of grey and white matter with neurodegeneration rather than inflammation as the key feature [61]. Exactly how or why this change occurs is unknown, but the degeneration is believed to be a self-sustaining chronic process resulting from chronic inflammation leading to neuro-axonal and mitochondrial injury, and subsequent metabolic stress of neurons [61]. A supporting observation is that the many different pharmacological treatments targeting the immune system lose effectiveness as the disease enters the progressive phase. However, CNS-resident cells have been shown to produce neurotoxic substances that promote neuronal injury, indicating that there might be a switch from the adaptive to the innate immune system as the primary mediators of the progressive disease [62].

While phagocytes are essential for myelin damage, there is much evidence for the pivotal role the adaptive immune system plays in multiple sclerosis lesions apart from the abundant infiltration, especially at the early stages of the disease. The strong association between MS and HLA and other adaptive-immunity-related loci, the efficacy of pharmacological treatments targeting T and B cells in various ways, as well as the vast amount of research done on experimental autoimmune encephalomyelitis (EAE) in rodents and marmosets are factors that all point towards the adaptive immune system being essential in driving MS-like neuroinflammation [4,37,63]. While this review will primarily focus on the cells of the adaptive immune system and their target autoantigens, the role of the innate arm of the immune system, while less well defined, has gained more interest in recent

years and seem to be important both for initiating and maintaining adaptive immune responses, disease progression, and recovery after relapses [62,64,65].

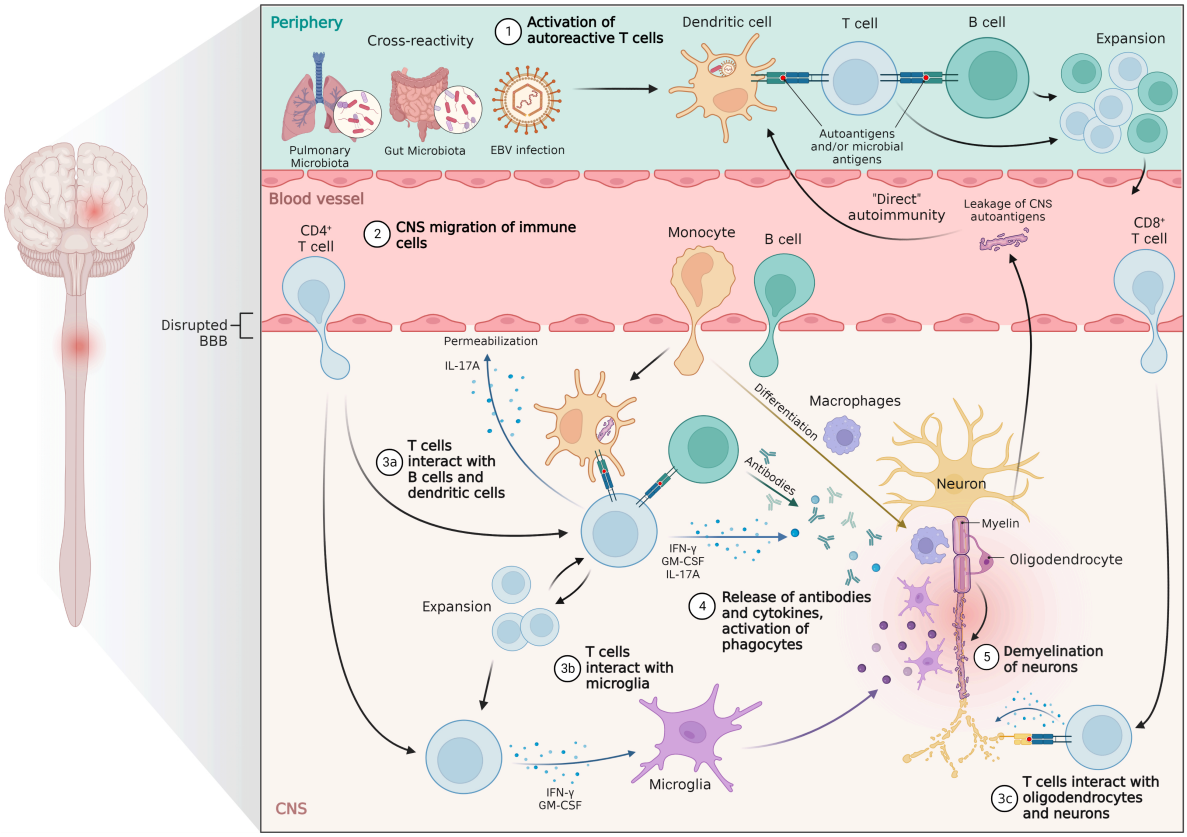

Figure 3. Overview of MS immunology. Simplified schematic overview of the immunopathology of MS. EBV: Epstein-Barr Virus. CNS: Central nervous system. BBB: Blood-brain barrier. Created with Biorender.com.

2.2.1 The innate immune system

The innate immune system is tasked with fast action against dangers by several pathways like complement factors, antimicrobial peptides, and phagocytes. Phagocytes are cells that fight infections by phagocytosis and intracellular degradation of pathogens and perform tissue homeostasis by clearing dead cell debris. Unlike the adaptive immune system, these cells are more readily available and do not exhibit memory capabilities. Instead, they utilize the expression of broad pathogen-associated molecular pattern (PAMP) receptors, capable of recognizing common dangerous molecules like bacterial lipopolysaccharides, flagellin, or double-stranded RNA.

Some phagocytes act more like a bridge between innate and adaptive immunity, such as monocytes, macrophages, and dendritic cells, defined as professional APCs. After phagocytosis, they process engulfed proteins and present the resulting antigen-epitopes to T cells via the MHC class II molecule. While MHC class II is primarily associated with APCs, some cross-presentation can also occur via MHC class I molecules [66], although MHC class I is expressed on all nucleated cells.

Monocytes typically reside in the blood, surveilling the body for signs of inflammation or infection, and are characterized by their expression of CD14. After migrating to tissue, they usually

differentiate into macrophages or dendritic cells. Macrophages are the more phagocytosis-focused, tissue-residing cell with high antimicrobial and proinflammatory capabilities. While macrophages are capable APCs, dendritic cells focus more on this part of the innate-adaptive bridge. After engulfing antigens, they migrate to lymph nodes, where they help develop and recruit T cells.

2.2.1.1 Innate immune cells in MS

Mononuclear phagocytes are the dominant immune cells in multiple sclerosis lesions [60]. Their role in inflammation is twofold. One, by causing direct tissue and myelin damage via oxidative stress and secretion of proinflammatory cytokines and chemokines [4]. Secondly, by their ability to recruit and drive the autoreactive adaptive immune responses, as T-cell responses are hinged on antigen presentation by APCs. However, while essential for causing tissue damage, they also play a regulatory function and are necessary for tissue repair after CNS-injury [65].

Microglia are the CNS-resident and exclusive macrophages tasked with immune homeostasis and surveillance and have been implicated as important cells in MS inflammation by large genetic studies [64]. However, despite their expression of HLA class II and theoretical antigen-presenting role, their APC function is dispensable for disease induction, at least in EAE, [67]. Rather than innate microglia, dendritic cells seem important in initiating disease by licensing autoreactive encephalitogenic T cells [68]. Instead, microglia influence inflammation and, via interactions with infiltrating peripheral immune cells, switch to an activated and tissue-damaging role [69,70].

2.2.2 The adaptive immune system

The adaptive immune system comprises highly specialized cells, each specific to a particular pathogenic structure, i.e., antigen. Additionally, the adaptive immune system has a memory function, in which the defense gets quicker and more effective after repeated exposures to the same pathogen. This memory contrasts the innate immune system, which recognizes general pathogen-like structures with high speed but at the cost of a less specific and effective response. There are two broad classes of immune cells under the adaptive umbrella, B cells and T cells. T cells are further divided into subsets, the main ones being T helper and cytotoxic lymphocytes.

The primary role of T helper lymphocytes, also called $CD4^+$ T cells, is to recognize foreign pathogens. They do this via the T-cell receptor (TCR), which recognizes specific peptides presented by MHC class II molecules (HLA-DR, -DP, –DQ, -DM, and -DO in humans) of professional APCs. This contrasts with T cytotoxic ($CD8^+$) cells which, with their TCR, monitor all nucleated cells for their protein expression and recognize abnormal peptides, e.g., of viral or neoplastic origin, presented by MHC class I molecules (HLA-A, -B, and -C in humans). Apart from the different MHC-restriction, they also differ in functionality. $CD4^+$ T cells use cytokines and chemokines to recruit and orchestrate other immune cells in order to mount a defense, while $CD8^+$ T cells use

cytotoxic substances to kill aberrant cells directly (although the canonical helper versus cytotoxic distinction is not as strict as previously believed [71]). B cells with their B-cell receptor and soluble version of it, antibodies, directly recognize antigens, do not require MHC presentation, and are not limited to peptide-antigens. The antibodies then directly facilitate the immune response by inactivating the pathogen, facilitating phagocytosis, or activating complement factors.

The TCR is made up of an α- and β-chain and gains its diversity by somatic recombination of its building gene fragments (V, D, and J-fragments), which amounts to ~$6x10^6$ possible combinations. The possible addition or removal of nucleotides at the V-J (α) or V-D-J (β) junctions results in ~10^{15} different possible TCRs [72,73]. While slightly different in structure, similar mechanisms give rise to the variety of BCR and antibody specificity, resulting in ~10^{11} possible variants. This diversity does not necessitate exclusive specificity, and TCRs can have broad specificity and recognize several different antigens with varying affinity [73]. This promiscuous recognition enables cross-reactivity, which is one of the possible explanations for how autoimmunity arises. Briefly, an immune response against a pathogen can give rise to T cells targeting self-proteins due to structure similarity, i.e., molecular mimicry [73,74]. In addition to the primary TCR recognition of antigens, numerous co-stimulatory molecules affect the downstream intracellular signaling and influence the fate of the antigen-recognizing T cell [75]. In short, T cells require further signals via surface receptors for full T-cell activation in addition to the TCR signaling, a classic example being via their CD28 receptor. Similarly, co-inhibitory signals, e.g., CTLA-4 or PD1, or anti-inflammatory cytokines, can downregulate the response, despite the antigen recognition.

2.2.2.1 CD4⁺ T cells in MS

The role of $CD4^+$ T cells as major players in MS pathogenesis is supported by extensive evidence, although indirect in humans [69]. The strong connection between MS and genes coding for MHC class II points towards antigen presentation to autoreactive $CD4^+$ T cells lying in the center of the disease pathogenesis [38]. Data from EAE and similar models in primates provide a more direct link between $CD4^+$ T cells and neuroinflammation. Here it has been demonstrated that transfusion of myelin-reactive $CD4^+$ T cells into immunologically naïve animals results in MS-like CNS inflammation, meaning they are themselves sufficient for inducing demyelinating neuroinflammatory disease [76]. Myelin reactive $CD4^+$ T-cell clones can be isolated and expanded from persons with MS [4,63,74]. $CD4^+$ T cells migrate to the CNS and are found in active inflammatory lesions. Inhibiting this migration, as with natalizumab treatment, is very effective in ameliorating disease. Similar apparent pathological properties have not been reported for other cell types. In MS, the prevailing theory states that autoreactive $CD4^+$ T cells somehow escape tolerance and home to their target autoantigen, located in the CNS. Upon encountering their antigen, they get activated, release proinflammatory cytokine and chemokines, and start an inflammatory cascade, leading to the activation of phagocytes and subsequent myelin and axonal damage [4,69,74].

$CD4^+$ T cells are further divided based on distinct cytokine profiles and functions, with the most prominent subclasses being T-helper type 1 (T_H1), T_H2, T_H17, and T-regulatory cells (T_{reg}). Classically, T_H1 cells were believed to be the most critical subpopulation in MS. T_H1 cells, and their signature expressed cytokine interferon-gamma (IFN-γ), are involved in the defense against intracellular pathogens via promoting the cytotoxic activities of other cells, mainly macrophages, regulating the expression of MHC molecules and driving further T_H1 differentiation of naïve $CD4^+$ T cells [77]. T_H17 cells, on the other hand, are more involved in the defense of extracellular pathogens and act mainly via the proinflammatory cytokine IL-17. IL-17 recruits neutrophils and monocytes, and upregulates other cytokines, chemokines, and metalloproteases. IL-22, previously ascribed to T_H17 cells, promotes the integrity and repair of epithelial barriers [78]. However, in the last decade, IL-22 has been primarily associated with its own T-cell subclass, T_H22 cells.

In multiple sclerosis, the role of $CD4^+$ T cells lies mainly with maintaining inflammation and activating CNS-resident immune cells [69,79,80]. T_H1 cells and IFN-γ are the most studied in MS, as T_H1 cells have been implicated in both MS and EAE and are potent drivers in inflammation. In particular, a signature T_H1 population characterized by expression of GM-CSF, IFN-γ, and CXCR4 has been identified in MS [81]. However, other studies point towards T_H17 cells being equally or possibly more important in MS pathogenesis, especially those which express more than one cytokine [82-84]. This seems to lie in the T_H17 cells' ability to weaken BBB integrity, enabling other inflammatory cells to infiltrate the CNS lesions [83-85]. Similarly, IL-22 and T_H22 cells have been associated with inflammatory and autoimmune diseases, including MS [86-88]. However, the role of IL-22 is not entirely understood, as conflicting evidence shows that increased levels of IL-22 binding protein, an antagonist, are possibly pathogenic in MS due to a lesser level of IL-22-mediated inhibition of IFN-γ expression [89,90].

2.2.2.2 $CD8^+$ T cells in MS

While $CD4^+$ T cells are essential in MS pathogenesis, $CD8^+$ T cells are the most abundant lymphocyte subset in MS lesions, and studies have found that the infiltrating cells are clonally expanded, suggesting a local antigen-driven expansion and an active role of $CD8^+$ T cells in MS [91]. Similarly, the fact that HLA-A*02 is protective in MS indicates that they play a part [38]. Support for this notion has been found in EAE, where APCs can activate myelin-reactive $CD8^+$ T cells via cross-presentation of phagocytosed antigens on MHC class I [66]. Astrocyte-derived antigens can activate memory-like $CD8^+$ T cells, triggering a relapse-remitting type disease [92]. Evidence from prodromal MS also shows that $CD8^+$, rather than $CD4^+$, T cells are the first to expand locally in the MS brain. CNS-infiltrating $CD8^+$ T cells can also, besides their canonical cytotoxic effector molecules like granzyme B and perforin, produce the proinflammatory MS-associated cytokines IFN-γ and IL-17 [71,93]. Depletion of $CD8^+$ T cells via HSCT results in efficient disease elimination [61,94]. Still, compared to $CD4^+$ T cells, while several candidates have been proposed, even less is known about their

antigenic targets as human data is lacking [91]. One reason for this has been the difficulty in establishing antigen-specific $CD8^+$ T-cell clones, a strategy used for much of the research surrounding $CD4^+$ T cells [91]. To complicate matters further, $CD8^+$ T cells have also been implicated as CNS-protective by expanding in response to the induction of pathogenic myelin-reactive $CD4^+$ T cells and acting as regulatory cells that limit the proinflammatory responses in CNS inflammation [95,96]. In summary, the role of $CD8^+$ T cells in MS remains elusive and is likely more multifaceted than initially thought, with both pathological and protective functions played out by different populations.

2.2.2.3 B cells in MS

The interest in B cells originally came from the observation that OCBs were present in the CSF in up to 95% of persons with MS [97]. The exact role of these B cells in MS has been difficult to decipher, as it proved difficult to find distinct autoantigen targets of the intrathecal antibodies. In similar autoimmune diseases, like NMO, autoantibodies targeting CNS-autoantigens are closely related to the disease pathogenesis, but such a clear link has been missing in MS [91,98,99]. The complex relationship between autoantibodies and MS, and autoimmune disease in general, have been challenging to unravel because autoantibodies are not necessarily pathogenic, as a repertoire of autoantibodies can be readily found in otherwise healthy individuals [100]. Still, the presence of OCBs remains an important biomarker for MS diagnosis.

Lately, the interest in B cells has risen again, owing a major part to the discovered effectiveness of B-cell depletion therapies and the identification of antibody-targeted autoantigens. Findings regarding autoantibodies targeting one classical myelin autoantigen, myelin oligodendrocyte glycoprotein (MOG), associate them with related neuroinflammatory diseases rather than MS. However, Anoctamin-2 (ANO2) and the inwardly rectifying potassium channel KIR4.1 have been identified as potential antibody-targeted autoantigens [59,101]. However, follow-up studies have failed to confirm the results regarding the latter, leaving KIR4.1 autoreactivity controversial [102-104]. The encephalitogenic potential of such CNS-targeting autoantibodies is well studied in EAE and, while not enough to cause disease on their own, have been shown to accelerate the disease course and demyelination if administered in conjunction with myelin-antigen immunization [91,105]. However, whether such autoantibodies are pathogenic in MS is still unknown, and their presence may constitute associated epiphenomena or markers for autoreactive T-cell responses rather than a critical immunological process.

In recent years the hypothesis that B cells act as antigen-presenting and regulatory cells and facilitate the pathological $CD4^+$ T-cell response rather than influence the disease via pathological humoral responses has gained traction [91,106-108]. In EAE, this APC-role of the B cells has been demonstrated by transgenic mice lacking the MHC class II on B cells being protected from disease after

immunization with MOG, despite functional antibody production [109]. In MS, in support of this notion, it has been observed that while B-cell depletion therapies reduce the number of intrathecal B cells, clinical improvement happens regardless of changes in intrathecal antibody levels [110,111]. B cells have also been shown to drive the proliferation of CNS-infiltrating $CD4^+$ T cells, further supporting this hypothesis [106]. The therapeutic effect of B-cell depletion therapies might not be entirely due to the depletion of B cells. Recent findings demonstrate that a subpopulation of myelin-specific $CD20^+$ T cells is also reduced following treatment [112]. In conclusion, while B cells play an important role in MS, the relative importance of antibody production versus T-cell interaction remains contested.

2.2.2.4 Tolerance and autoimmunity

The enormous underlying variation in possible TCR sequences and specificities is crucial for the ability of T cells to defend against all possible pathogens in our environment. However, this inherently introduces a problem. A large portion of the theoretically possible TCRs will not be able to interact appropriately with MHC-antigen complexes, and some might automatically target self-proteins. To mitigate these problems, T cells undergo maturation in the thymus in two critical steps. First, T cells that can distinguish self-MHC are positively selected to ensure functionality. Secondly, T cells undergo a negative selection, where T cells that respond to MHC-self antigen complexes are forced to undergo apoptosis to eliminate potential autoreactive cells, a process also referred to as central tolerance [113].

This central tolerance mechanism is not perfect, and the escape of autoreactive cells is a common occurrence, evidenced by the frequent identification of autoreactive T cells and autoantibodies in the healthy immune repertoire [74,100]. A secondary system of peripheral tolerance is also in place, mainly driven by T_{reg} cells. T cells with high TCR affinity for autoantigens are depleted in the negative thymic selection. In contrast, some cells with a medium-to-high affinity differentiate into anti-inflammatory T_{reg} cells, characterized by expression of the transcriptional factor FOXP3 and cytokine IL-10 [114,115]. These cells circulate the body and suppress inflammation, a function which is essential for maintaining tolerance, evidenced by the severe multi-organ autoimmunity in persons with dysfunctional T_{reg} cells [116]. APCs, while vital for inducing and maintaining adaptive immunity, can also act in a tolerogenic fashion. Tolerogenic dendritic cells present self-antigens to T cells without co-stimulatory factors necessary for activation. This direct and isolated TCR stimulation induces anergy in autoreactive T cells [116].

Exactly why T cells break tolerance in MS is still not known. The widely used EAE model requires exogenous priming of cells and is, as such, a poor model for studying the underlying cause and early immunological events of MS. Molecular mimicry is one explanation, where a pathogen leads to priming of proinflammatory T cells targeting an extrinsic antigen but then cross-reacts to a similar

self-antigen and overwhelm the regulatory barriers. Indeed, several such cross-reactivities have been implicated in MS [59,117-119] and in other autoimmune neurological diseases as well [120]. The most likely culprit for this link in MS is EBV, which some argue is a prerequisite for MS development. However, as almost all adults are infected, and only a fraction develop MS, other mechanisms must also be at play. A second possible explanation is bystander activation, where an event such as trauma [32] induces a proinflammatory milieu in the presence of thymus-escaped autoreactive T cells, possibly overwhelming the tolerogenic mechanisms. The link between MS and various infections during an "autoimmune susceptible" age [121] and the presence of pulmonary and gut microbiota-neuroinflammation axes makes a case for bystander activation playing a part in the development of the aberrant immune response [43,122]. As MS is a heterogeneous disease in its clinical presentation, underlying genetic associations, and immunological landscape, no single ubiquitous trigger for breaking tolerance likely exists.

2.2.3 Autoantigens in MS

As the adaptive immune system is believed to be the driver of MS, the question of what the autoantigens are, i.e., the targets of the immune attack, has been a central research point in the field of MS immunology. It is pivotal in understanding pathogenesis, as knowledge of autoantigens could solve unanswered questions as to why MS arises, as well as from a diagnostic and therapeutic standpoint. More precisely, antigen-specific antibodies or T cells could be used both as disease biomarkers and treatment targets [123]. Many aim towards antigen-specific immunotherapies, which only target the pathological autoreactive T cells, as the next step in MS treatment. However, knowledge of the autoantigen repertoire is vital for effective antigen-specific treatment. As the inflammation in MS is strictly limited to the CNS, the targeted autoantigens likely consist of CNS-expressed proteins. Several autoantigens, mostly myelin-, astrocyte- or neuronal-derived proteins, have been proposed and studied in MS (Table 2) [124]. Among these are Myelin Basic Protein (MBP) [125], Proteolipid Protein (PLP) [126], Myelin Oligodendrocyte Glycoprotein (MOG) [127], Myelin Associated Glycoprotein (MAG) [128], and Transaldolase [129,130].

Many of these candidates come from the EAE model, initially induced by active immunization with CNS tissue homogenates and later shown to be inducible by immunization with specific myelin proteins, especially MBP, PLP, and MOG. The essential proof of their relevance, at least in EAE, came when it was shown that the adoptive transfer of purified myelin-specific $CD4^+$ T cells was sufficient for EAE induction [74]. A strong case for the relevance of MBP in MS was also made when a humanized transgenic mouse model expressing an MBP-specific human TCR spontaneously developed EAE [131]. Similar observations have since been found for PLP and MOG as well. It has, unfortunately, been challenging to make a definitive case for these myelin-derived autoantigens in MS. Even though early on, it proved possible to isolate and expand MBP-specific T cells from persons with MS, it was equally possible to derive these clones from healthy controls, meaning that

the mere presence of MBP-specific T cells does not cause disease [132]. Instead, some other property must be essential, whether being a distinct phenotype, migratory potential, or sheer frequency of the autoreactive cells in circulation. So far, the strongest case has (accidentally) been made for MBP, where a failed intervention study led to MBP-immunity-induced demyelination [133].

Table 2. Studied autoantigens and key papers

Autoantigen candidate	Key Studies	Study conclusion of T-cell response	Overall conclusion
Myelin basic protein	Olsson et al. [125] Bielekova et al. [133] Pette et al. [132]	Increased in MS Encephalitogenic in human In both MS and controls	- T-cell reactivity increased in MS. - Encephalitogenic in humans. - Encephalitogenic in EAE [134]
Proteolipid protein	Greer et al. [135] Pender et al. [136] Trotter et al. [126]	Increased in MS Increased in MS Increased in MS	- T-cell reactivity increased in MS. - Encephalitogenic in EAE [137]
Myelin oligodendrocyte glycoprotein	Sun et al. [138] Wallström et al. [127] Varrin-Doyer et al. [139] de Rosbo et al. [140] Hellings et al. [141] Van der Aa et al. [142] Johnson et al. [143] Lindert et al. [144]	Increased in MS Increased in MS Increased in MS Increased in MS Not increased in MS Not increased in MS Increased in subgroup MS In both MS and controls	- T-cell reactivity increased in MS but conflicting results. - Encephalitogenic in EAE. [145] - Autoantibodies in MOGAD diseases.[146]
GDP-l-fucose synthase	Planas et al. [147] Cruciani et al. [148]	Increased in MS Increased in MS	- T-cell reactivity increased in DRB3*02:02/03:01^{+} MS
RAS guanyl-releasing protein 2	Wang et al. [119] Jelcic et al. [106]	Increased in MS Increased in MS	- T-cell reactivity increased in MS
β-synuclein	Lodygin et al. [149]	Increased in MS	- Autoreactivity-induced grey matter degeneration in mice.
CNPase	Muraro et al. [150]	Reactive clones in some MS	- T-cell reactivity might be increased in MS
Myelin associated glycoprotein	Andersson et al. [128]	Increased in MS and OND	- Possible neuroinflammatory but not MS-exclusive autoantigen
Transaldolase	Banki et al. [130] Niland et al. [129]	Increased in MS CD8^{+} target in MS	- Possible CD8^{+} T-cell target in MS
Anoctamin 2	Ayoglu et al. [151] Tengvall et al. [59]	T cells not studied	- AutoAbs in MS, which cross-react to EBNA1 epitope.
KIR 4.1	Srivistava et al. [101] Brickshawana et al. [102]	T cells not studied T cells not studied	- Conflicting autoantibody data.
Oligodendrocyte myelin glycoprotein	Gerhards et al. [152]	Possibly increased in MS	- AutoAbs in a subgroup of MS.
GlialCAM	Lanz et al. [117]	Possibly increased in MS	- AutoAbs in MS, which cross-react to EBNA1 epitope.
Alpha-crystallin B	Van Noort et al. [153,154] Rothbart et al. [155]	Increased in MS Not an antigen	- AutoAbs and T-cell responses. - Contentious results.

EAE: Experimental autoimmune encephalomyelitis. AutoAbs: Autoantibodies.

Despite a vast amount of research in the past decades, data regarding the difference in frequency and phenotype of autoreactive T cells in persons with MS and healthy controls have generated inconclusive and often contradicting data [124]. Still, MBP, PLP, and MOG remain the most suspected autoantigens in MS due to their clear encephalitogenic potential in EAE.

2.2.3.1 Myelin Oligodendrocyte Glycoprotein

MOG, which is a member of the immunoglobulin superfamily, is selectively expressed on the surface of CNS-myelin and thus a prominent potential immune target in MS. It was identified as a target in EAE and has since been well established as MOG-specific T cells and MOG-autoantibodies have both been shown to be pathogenic, with capabilities of inducing MS-like neuroinflammation [145,156,157]. It was long thought to be an autoantigen in MS as well and is now one of the most studied candidates. Initially, a few studies managed to identify increased frequencies of MOG-specific T cells in pwMS [138,140], but subsequent studies aiming to replicate these findings were unsuccessful. Instead of a difference, MOG-reactivity was found in both patients and controls or in neither [141,142,144]. Additionally, it proved difficult to find MOG-autoantibodies due to the limited available methodology, as biologically relevant MOG-antibodies are conformationally dependent [105,146]. As better assays to detect anti-MOG-antibodies were developed, it was discovered that while seemingly CNS-pathogenic [105], they were only present in approximately 5% of persons with MS. Instead, they were more associated with other CNS-inflammatory diseases such as acute disseminated encephalomyelitis and aquaporin-4 seronegative neuromyelitis optica, also called MOG-antibody-associated disease (MOGAD) [158,159]. As such, the role of MOG as an autoantigen in MS remains controversial.

2.2.3.2 Non-myelin autoantigens

Several new autoantigen candidates have been described in the last couple of years. For example, ANO2 has been identified as an autoantibody target in ~15% of MS patients [151]. Interestingly, this immune response interacted with other risk factors. Anti-EBV- and ANO2-antibodies in combination with HLA-DRB1*15:01 and the absence of HLA-A*02 resulted in an OR for MS of ~26, compared to those without any of these risk factors [59]. Additional evidence suggests molecular mimicry between EBV epitopes and ANO2 as antibodies cross-react between these antigens, which provides a compelling explanation of a potential biological pathway and could explain (at least a part of) the connection between EBV and MS-risk [59].

A recent study similarly found cross-reactivity on the antibody level of another EBV-epitope and the CNS-expressed protein glialCAM, further giving weight to the molecular mimicry hypothesis [117]. Moreover, more ubiquitous, non-CNS-specific autoantigens have also been identified as targets. RASPGR2, presented via B cells to T cells, has been shown to induce proliferation of CNS-infiltrating T cells and thus also explain the B-T-cell interaction in MS [106]. GDP-L-fucose-synthase

has similarly been shown to be an autoantigen, restricted not to the central HLA-DRB1*15:01 haplotype but rather present in DRB3*02:02 positive pwMS [147,148]. Moreover, β-synuclein has been shown to induce grey-matter degeneration in mice, mimicking the degenerative and progressive phases of MS more accurately than classic, myelin-antigen-induced EAE [149]. While the human data regarding β-synuclein remains limited, it is an attractive novel candidate that could explain more of the progressive MS phases, which classical mouse models of myelin autoreactivity have failed to do.

Alpha crystallin B-chain (CRYAB) is a heat-shock protein expressed in oligodendrocytes in MS-lesions and is another contentious autoantigen that was first reported as a T-cell autoantigen 25 years ago [153]. While initially a promising target, subsequent studies failed to confirm it as an encephalitogenic target in mouse models [160,161]. Further, it was later shown to have a therapeutic effect in neuroinflammation [162-164]. One explanation for the conflicting results is that the chaperone-properties influenced assays, leading to incorrect conclusions regarding CRYAB being a target [155]. However, other studies have demonstrated that it is both a T cell and antibody target, and autoimmunity alters the reported protective effect [154,165].

2.3 ANTIGEN-SPECIFIC IMMUNOTHERAPY

Antigen-specific immunotherapy aims to directly inhibit autoreactive T cells by inducing peripheral immune tolerance to the targeted autoantigens in the particular autoimmune disease. Unlike broader immunotherapy, in theory, only pathogenic disease-driving T-cell responses should be affected without influencing the healthy physiological immune responses [166]. To date, several different methods to induce tolerance have been tested [167,168]. The first family of strategies encompasses direct administration of myelin peptides, either by oral route [169,170], intravenous administration [171], or subcutaneous/intradermal administration [172] to induce clonal anergy of autoreactive T cells and suppression via induced T_{regs}. A variation of this strategy is altered peptide ligands, modified versions of the immunogenic epitopes acting as partial agonists to the T-cell receptor [173]. However, in one study evaluating this approach, the treatment induced severe disease activity, and the trial was halted [133]. Other tested strategies include DNA-vaccination, with the idea of inducing a low-level expression of the target autoantigen in otherwise healthy tissues, inducing regulatory responses [174,175] and forms of autologous irradiated myelin-specific T-cell vaccinations [176,177]. Lastly, peptide-coupled blood cells have been tried, with the idea of inducing tolerance by direct TCR stimulation by presenting the autoantigen on chemically fixed APCs without co-stimulation and by processing apoptotic cells containing autoantigens, leading to presentation of autoantigens in a tolerogenic milieu [178].

These approaches have been very promising in initial animal models and pre-clinical studies [179-183]. However, despite successful animal models dating back 30 years, the follow-up clinical trials in MS

have shown little efficacy, if mostly favorable regarding tolerance and side effects (with notable exceptions [133]). The prevailing hypothesis for the disappointing results lies in the autoantigens which have been targeted. Animal models are induced by active immunization with a limited number of antigens, most commonly one, which can then be targeted for treatment. In MS, the actual autoantigen repertoire is most likely much broader. Conversely, trials have used primarily one myelin antigen, i.e., MBP or MOG, while the full or at least a larger proportion of the underlying pathogenic responses must be targeted for the treatment to show efficacy [74,167]. Herein lies the crux of the problem; the autoantigen repertoire in MS is full of cumbersome gaps.

2.4 METHODS FOR IDENTIFYING ANTIGEN-SPECIFIC T CELLS

While research on creating encephalitogenic antigen-specific T-cell clones has provided important insights into possible pathological mechanisms, these approaches have proved somewhat inefficient in deciding whether an autoantigen is genuinely relevant since T-cell clones specific for autoantigens have also been derived from healthy controls. Indeed, the more important questions might be the frequencies and functional properties of the autoreactive T cells or the overall autoreactive repertoire. These questions have been difficult to answer because the cells of interest are rare, and the commonly available methods are not sensitive enough and suffer from unspecific noise. There are essentially two methodological strategies: functional assays of T cells after antigenic stimulation or direct analysis of the TCR-specificities or HLA-bound peptides [184].

2.4.1 Functional T-cell assays

In functional assays, T cells are analyzed based on effector molecules like cytokine production or expression of activation markers or cellular function such as proliferation after stimulation with antigens [184]. Many different methods are available for measuring these antigen-induced T-cell responses (Figure 4). Proliferative responses can be analyzed with radiolabeled nucleoside (e.g., thymidine or bromodeoxyuridine) incorporation, diluting cell staining dyes (e.g., carboxyfluorescein succinimidyl ester (CFSE)) or expression of proliferative markers like Ki67 [185]. These methods focus on detecting cell divisions or surrogates thereof. Incorporation relies on the duplication of DNA during cellular division, as the nucleoside analog present during cell culture is incorporated in newly formed DNA and can later be detected, correlating with the proliferative response.

Conversely, diluting dyes like CFSE relies on staining cells before culture, and with each cell division, the stain is diluted, effectively reduced by half. The dilution of the signal can be analyzed using flow cytometry. An advantage of diluting dyes over thymidine incorporation is the ability to analyze the phenotype of the dividing cells in more detail by simultaneously staining the cells for other relevant markers like surface receptors or intracellular cytokines. Similarly, the expression of activation markers can be utilized by staining and analysis using flow cytometry.

Another strategy is focusing on the functional response rather than cell division. Cytokine production can, in addition to flow cytometry, be analyzed by cytokine secretion assays like ELISA or ELISpot/FluoroSpot [186]. The main advantage of analyzing functional cell responses over proliferation assays is that more information regarding the phenotype and function of the responding cells is obtained. ELISpot/FluoroSpot is generally considered the most sensitive functional assay, as it can detect cytokine production in single cells, while flow-cytometry-based assays or ELISA need more robust responses to separate positive events from assay noise [187,188].

On the other hand, flow cytometry-based assays are not as limited regarding readout and can give a much deeper analysis of the response characteristics and cell phenotype. For example, identifying responding cells by strategies like CFSE dilution, expression of activation markers like Ki67, CD69, or intracellular cytokine staining allows for simultaneous detection of $CD4^+$/$CD8^+$, memory markers like CCR7 and CD45RA, and chemokine receptors like CXCR4. The response can be investigated in much more detail, and subtler differences in responding cells can be detected. However, such detailed phenotyping requires a massive cell material when analyzing the scarce autoreactive T-cell population. It is generally more suited for comparisons at a more general level, like T cells in different types of lesions [189], or before and after initiation of a therapy [190]. Another strategy to overcome the scarcity of autoreactive T cells is to culture and expand the autoreactive T-cell population *in vitro* before the analysis. However, culture-induced changes in T-cell phenotypes make this kind of data less translatable to the true *in vivo* situation.

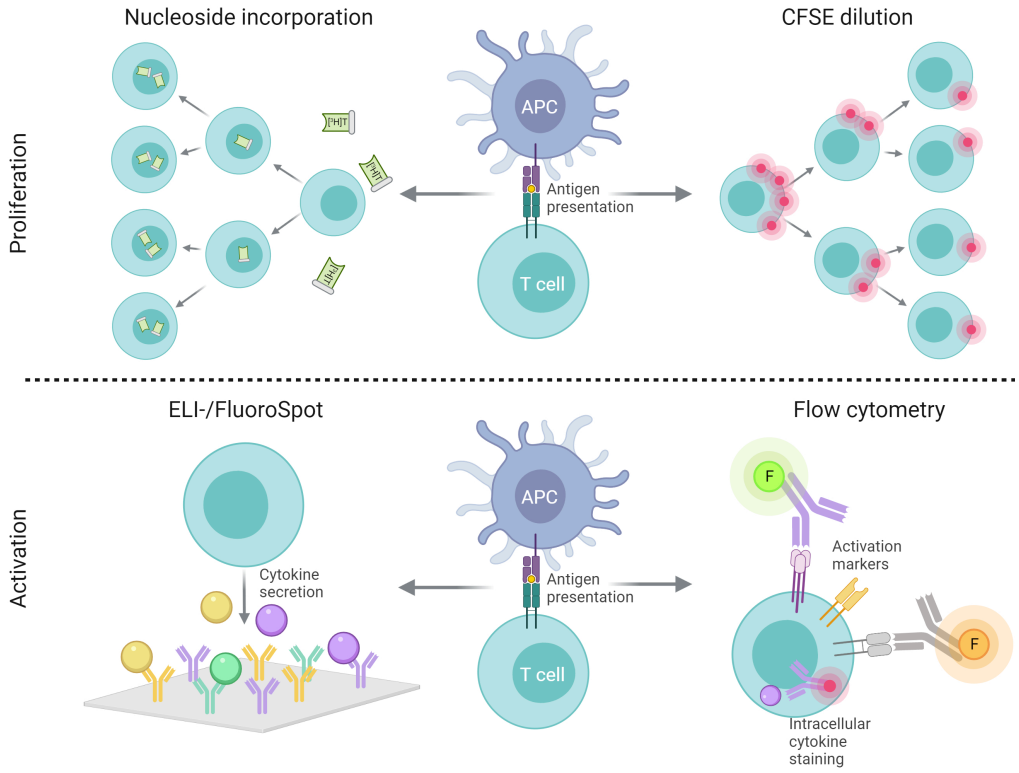

Figure 4. Principles of different functional T-cell assays. Methods targeting cell proliferation (Thymidine incorporation, CFSE dilution), cytokine production (ELI-/FluoroSpot) or changes in expression of certain proteins (Flow cytometry). APC: Antigen presenting cell. [^{3}H]T: Tritiated thymidine. F: Fluorophore. Created with Biorender.com.

For all these strategies, problems arise due to the need for antigen stimulation, as it can be difficult to obtain antigens pure enough to avoid non-antigen-specific stimulation. Combined with the low frequency of the autoantigen-specific cells of interest, this can result in low signal-to-noise ratios. This problem is less pronounced when studying the relatively abundant autoreactive cells in EAE models or pathogen-specific T cells. A possible approach to eliminate this problem is stimulation with synthetic peptides instead of recombinant proteins. This solution, however, comes with the drawbacks of a lower possible number of simultaneously tested antigens and decreased sensitivity due to factors such as non-optimal peptide designs and lack of co-stimulation [191-193].

2.4.2 Direct TCR-specificity analysis

Another approach is to analyze the antigen specificity more directly. One commonly used powerful tool is the direct staining of TCRs by MHC-oligomers, multimerized MHC molecules loaded with antigenic peptides, and follow-up flow cytometry-analysis [194]. This strategy can also facilitate deeper phenotyping of the antigen-specific T cells and help with isolation by flow-based or magnetic bead separation techniques [184]. This method has also been further developed to simultaneously allow for the analysis of larger peptide arrays to increase throughput [195]. Despite these advances, the work required to produce peptide-MHC-oligomers and the limit to the number of samples tested generates the same problem as using synthetic peptides for stimulation in that it is not feasible when screening for a large number of possible epitopes. Additionally, the existence of MHC-peptide binding TCRs does not necessarily mean that the T cell would respond to the antigen *in vivo*. In general, TCR-binding is promiscuous and could in an experimental setting bind to specific peptides without leading to an activation signal in the cells [191,192,196]. Lastly, a particular synthetically or library generated peptide present in the oligomers might not be presented *in vivo* at all and could ultimately be irrelevant despite TCR-recognition.

A powerful tool in recent years is single-cell genomic sequencing, focusing on specific TCR sequences. Using this method, one can identify clonally expanded T cells on a genetic level, as the somatic recombination of the underlying gene fragments informs TCR-specificity. This method can compare TCR sequences of brain infiltrating T cells and those that expand in response to antigen stimulation [106].

2.5 CONCLUSIONS

While there are still gaps in the knowledge regarding the exact role of the different immune-cell subsets in MS pathogenesis, $CD4^+$ T cells are believed to be the primary driver of MS pathology and constitute an ideal target for therapy. The autoantigen repertoire of these cells, which must be known to design and create efficient antigen-specific immunotherapies, remains severely incomplete. Despite the first evidence of myelin-reactive T cells hailing back to the 1980s, only a few conclusive additions have been made since. One of the reasons why is the difficulty of detecting

rare autoantigen-specific T cells and the limits in available methodology. In the last 20 years, several studies have demonstrated the potential of antigen-specific immunotherapy in mouse models of MS, showing that it is possible to ameliorate autoimmune diseases where the autoantigens are known. This has increased the interest in finding the autoantigens in MS and demonstrated that we do not yet have a complete picture, as the numerous trials targeting the so far known autoantigens during the past 20 years have shown no to little efficacy.

3 RESEARCH AIMS

The overall purpose of this thesis was to identify previously unknown T-cell autoantigens in MS and evaluate them as possible markers for diagnosis.

Specifically, the aims were:

Paper I Develop and evaluate a novel method of T-cell stimulation that can detect antigen-specific cells with high sensitivity.

Paper II Determine if there is an increase in MOG-reactive T cells in persons with MS and evaluate the functionality of the method in detecting autoreactive T cells.

Paper III Identify previously unknown T-cell autoantigens in MS by extensively screening T-cell autoreactivity to CNS-expressed proteins. Sub-aim to characterize the autoreactive response, evaluate autoreactivity as a possible diagnostic marker and explore the encephalitogenic potential of autoreactive T cells.

Paper IV Evaluate serological and T-cell responses to CRYAB and examine possible cross-reactivity with EBNA1.

4 MATERIALS AND METHODS

This thesis section will focus on the methodology used in the included papers. Rather than repeating specific technical aspects and protocols of used assays, which can be found in the material and methods section of the respective paper, this section will focus on the function and rationale behind the main methods used. Table 3 lists the specific methods and in which paper they were utilized, where the protocols can also be found.

Table 3. Methods used and the corresponding paper.

Method type	*Method name*	*Paper utilization*
Clinical data	Electronic health records	II–IV
	Multiple Sclerosis registry	II–IV
In vitro techniques	Peripheral blood mononuclear cell isolation	I–IV
	Cryopreservation of cells	I–IV
	Cell depletions	II
	Recombinant protein expression	I–IV
	Antigen bead coupling	I–IV
	Endotoxin removal	I–IV
	Coupling quality control	I–IV
	Limulus amebocyte lysate assay	I–III
	Cell culture	I–IV
Immune analyses	FluoroSpot	I–IV
	Flow cytometry	II–IV
	HLA-blocking	II–III
	ELISA	II, III
	Cell-based antibody assay	II
	Suspension bead-array	III–IV
Animal Model	Autoantigen immunization	III–IV
	EAE-assessment	III
	Recall stimulations	III–IV
	Immunohistochemistry	III
Software	Graphpad Prism	I–IV
	FlowJo	I–IV
	AID EliSpot Reader	I–III
	Mabtech SpotReader	III–IV
Statistical analyses	(In) dependent (non) parametric comparisons	I–IV
	Correlations	I–IV
	Contingency analyses	III–IV
	Analysis of variance tests	III–IV
	False Discovery Rate compensations	III
	Receiver Operating Characteristics	II, III

4.1 T-CELL DETECTION

For the detection of T cells in **Papers I-IV**, we opted to use FluoroSpot as the primary assay in this thesis. This method detects cytokine-secreting cells at the single-cell level, as explained in section 2.4. Briefly, cells are cultured in the presence of antigens in a well with a membrane coated with cytokine-specific antibodies in the bottom. As a cell is secreting a cytokine, it is immediately captured by the membrane-bound antibodies in the proximity. After the culture, secondary antibodies for that same cytokine conjugated with a probe (either a fluorescent compound or enzyme) are added, resulting in a "spot" on the membrane where the cytokine-secreting cell was located. These spots can then be detected and counted using dedicated spot-readers and semi-automated softwares. ELISpot uses an enzyme-substrate system that visually creates a colored spot, while FluoroSpot uses fluorescently labaled antibodies, which allows for several cytokine-antibody-fluorescence systems to be used in parallel for multiplex analyses [186,197].

FluoroSpot has several advantages. First, it is extraordinarily sensitive due to its single-cell detection. Theoretically, the sensitivity is as high as 1 in 500 000 cells, although in practice, unspecific background noise lowers this number considerably. Its sensitivity and 96-well format allow for parallel analyses using different stimuli in a high-throughput fashion without using too much of the limited cell source, which is essential for screening purposes. It gives a semi-high-dimension functional readout by analyzing a few different cytokines and can focus on proinflammatory responses, while, for example, thymidine incorporation fails to answer if proliferating cells are pro- or anti-inflammatory. Additionally, it works well with peripheral blood mononuclear cells (PBMC), which contain both APCs and T cells, and can be readily isolated from venous blood samples.

For these studies, we opted to use an IFN-γ/IL-17A/IL-22 assay. The main reason for looking primarily at these cytokines is that they are proinflammatory cytokines that have all been implicated in MS pathogenesis, particularly IFN-γ and IL-17 [82-84,86,87]. A second reason is that they are all mainly expressed in T cells, which makes them compatible with the FluoroSpot assay [78]. Another cytokine of interest in MS, GM-CSF [81], is more ubiquitously expressed in cells of the innate immune system, which makes detection of GM-CSF T cells difficult in mixed cell population cultures. GM-CSF and IL-10 FluoroSpots were evaluated in method development but were ultimately not usable in this setting.

Flow cytometry theoretically also detects cells at the single-cell level and can give the same functional answers as FluoroSpot with intracellular cytokine staining. However, the background is generally higher, and in practice, it does not provide the same level of sensitivity as ELI-/FluoroSpot [188,198]. This is especially true when looking for low-level responses, which we hypothesized autoreactive responses to be, making FluoroSpot superior as the primary method of this thesis [187].

Flow cytometry provides more information and a higher dimensional analysis of the cells of interest. For this purpose, in **Papers III** and **IV**, it was used as a complimentary analysis to phenotype the autoreactive cells further. We used flow cytometry in the mouse model recall experiments in **Papers III** and **IV**, where antigen-specific responses were expected to be stronger than in spontaneous autoimmunity.

However, there are limitations to FluoroSpot as well. It is an indirect detection method that detects cytokines, not the cells themselves. If analyzing cytokines with broader expression patterns, there is no guarantee that spots represent T cells. This problem is inherent in the assay, as both T cells and antigen-presenting cells are needed in the culture. As such, one is limited to cytokines with selective T-cell expression. Also, it is not possible to perform deeper phenotyping of the cells.

The FluoroSpot-assay demonstrated high repeatability, with a correlation r-value of 0.89 between two assays performed seven months apart (Figure 5). FluoroSpot and flow cytometry were also well correlated, strengthening the findings of each and demonstrating that the cytokine responses seen in FluoroSpot reflected activated T cells.

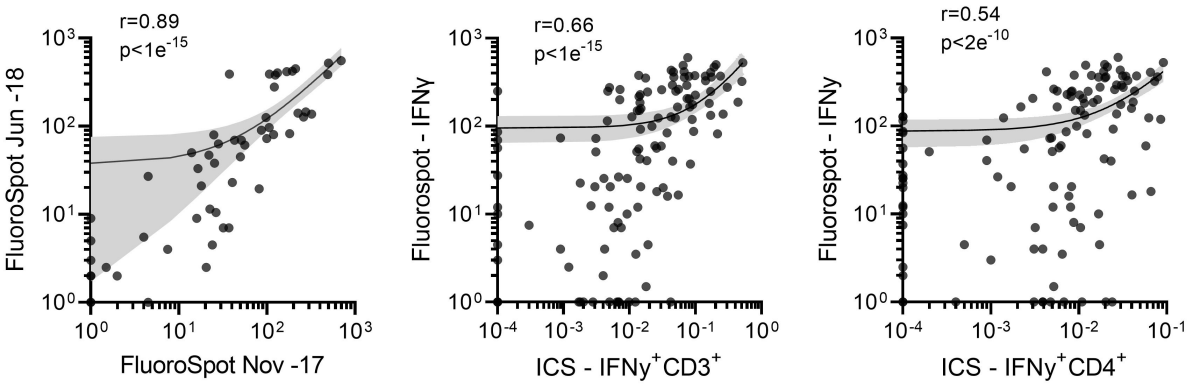

Figure 5. Intra- and inter-method correlation. The left-hand plot shows the correlation of IFN-γ antigen responses (56 responses, 8 different antigen-beads in 7 donors) in two FluoroSpot assays ran 7 months apart. The middle and right-hand plots show correlations between IFN-γ FluoroSpot and IFN-γ intracellular cytokine staining (ICS) and flow cytometry analysis of $CD3^+$ (middle) and $CD4^+$ (right) cells. Spearman r and p-values are written. Line and red area denote linear regression curve and 95% CI.

4.1.1 Solving limitations

Two methods were utilized to solve the problem of not knowing if the detected cytokine was secreted by T cells. In **Paper II**, specific cell populations were depleted via mixing PBMCs with magnetic beads linked with antibodies specific for surface markers, e.g., CD4 for $CD4^+$ T cells, which allows magnetic depletion of that particular cell type before the FluoroSpot assay. In **Papers III** and **IV,** we used flow cytometry with intracellular cytokine staining to validate that the responses seen in the FluoroSpot came from T cells and to phenotype the activated T cells further.

Similarly, in **Papers III** and **IV,** we used flow cytometry to investigate GM-CSF-producing T cells, which was not possible in the FluoroSpot due to high background production in monocytes, which can easily be gated away in flow cytometry.

The mixed population of cells required for the FluoroSpot, especially the necessary presence of innate immune cells (monocytes), introduces the problem of antigen nonspecific activation. For this, we carefully considered the antigenic material and introduced stringent washes of our antigen to remove contaminants like lipopolysaccharide (LPS).

4.2 ANTIGENS

The source and processing of antigens used for T-cell and antibody detection assays were carefully considered in this thesis and are the subject of **Paper I** and part of **Paper II**. As such, some details and method evaluations will be discussed under the results section rather than the material and methods section.

Four different kinds of antigens were used: synthetic peptides (**Paper IV**), protein epitope signature tags (PrESTs) [199] (**Papers III** and **IV**), full-length proteins (**Papers II-IV**), and designed artificial proteins (**Papers I-IV**).

4.2.1 Peptides

Synthetic peptides are short oligomers, commonly 15-25aa long, selected to represent specific areas of interest or the entire length of proteins, usually overlapping. In **Paper IV**, overlapping 15-mere synthetic peptides with only one aa-step in between were used to high-resolution map the antibody epitope of the N-terminal end of CRYAB. The advantages of peptides are that they are pure and (often) easily synthesized and can be used to map epitopes in detail. However, in antibody studies, they only represent linear epitopes and will not bind conformationally dependent antibodies. In T-cell studies, they depend on direct binding to surface-expressed MHC molecules. As such, problems like stability, solubility, multimerization, co-stimulation, competition with already bound peptides, and the astronomical number of possible peptides one can derive from just one protein severely limit the sensitivity of this strategy. The peptides used in this study were purchased from a commercial vendor.

4.2.2 Full-length proteins

Full-length proteins represent the whole natively expressed protein and can be either purified from tissue samples or produced in *E. coli* or mammalian cell lines via the transformation of recombinant DNA. In this thesis, recombinant full-length proteins produced in *E. coli* were used for most T-cell studies in **Papers II-IV**. The advantage of using full-length proteins in T-cell studies is that all possible epitopes are represented, increasing the assays' sensitivity. Further, it must go through intracellular processing and degradation in APCs before being presented via an MHC molecule, mimicking the *in vivo* pathway and resulting in more biologically relevant epitope presentation. The main disadvantages are purity, as *E. coli* production contaminates the protein with bacteria-derived molecules like lipopolysaccharide (LPS), potent stimulators of innate immune cells [200], and

production. Sufficient protein expression and solubility can be problematic, especially for large and transmembrane proteins. Another problem is that *E.coli*-expressed proteins do not have mammalian post-translational modifications like glycosylation, which could potentially be important for epitope recognition. In antibody studies, due to the tertiary structure, linear epitopes can be structurally inaccessible by antibodies.

As sensitivity was essential to detect rare autoreactive T cells, full-length proteins were chosen as the primary tool in T-cell assays. They were designed and produced in-house. Briefly, DNA covering the designed protein is amplified and cloned into a recombinant plasmid, which is transformed into *E. coli*. A plasmid was constructed for this project containing a histidine repeat tag for purification and an albumin binding domain for quality control and expression purposes [201]. Additionally, the plasmids contain an antibiotic resistance and expression induction gene to ensure that follow-up culture conditions select for successfully transformed *E. coli* and high protein expression.

4.2.3 Protein epitope signature tags

PrESTs are a hybrid between peptides and full-length proteins. Here, recombinant protein subfragments are designed to cover shorter parts of proteins, typically between 50-100 aa, based on the lowest sequence similarity to other human proteins and for predicted efficient production in *E. coli* [199]. The advantages and disadvantages are similar to full-length proteins. However, as payoff for ease of production, PrESTs confer lower sensitivity, as disease-relevant epitopes are not necessarily represented in unique parts of proteins.

PrESTs were used for the screening panel in **Paper III** and antibody detection in **Paper III** and **IV** and was provided by the KTH royal institute of technology and their Human Protein Atlas (HPA) project [202]. In this project, PrESTs covering the whole human proteome were produced to obtain antibodies for detailed protein expression analyses, i.e., creating a map of the human proteome. A vast library of PrESTs was available, paired with detailed expression data, which allowed us to select PrESTs representing proteins with selective CNS expression.

4.3 ANTIBODY DETECTION

For this thesis, three different methods for antibody detection were utilized: Enzyme-linked immunosorbent assay (ELISA) [203], suspension bead array [100], and cell-based antibody detection [159] (Figure 6).

4.3.1 ELISA

ELISA [203] is a method where proteins of interest are adsorbed to a microwell's bottom surface. Serum or plasma samples are then incubated in the wells where antibodies bind specifically to the

adsorbed protein. Afterward, enzyme-conjugated secondary antibodies are added, which can be detected by adding the enzyme's substrate and quantifying the reaction. It is generally an easy and inexpensive method; however, it does not allow for multiplexing, which limits the number of targets that can be investigated. ELISA was used in **Paper II** to detect MOG-antibodies and in **Paper III** to validate the results of the suspension bead-array.

4.3.2 Suspension bead-array

Suspension bead-array [100] is a step up from ELISA regarding multiplexing and throughput but is essentially based on the same underlying principles. Here, targets of interest are linked to microbeads containing unique fluorescent signatures, which can later be mixed for multiplexing. Antibody-containing samples, i.e., plasma, are incubated with the beads before fluorescently labeled secondary antibodies are added. By analyzing the beads using flow cytometry, it is possible to simultaneously evaluate how much antibodies have bound to the bead while analyzing the beads' unique fluorescent signature and de-convolute the bead mix computationally. As such, it is possible to investigate as many different targets in one test as there are bead signatures, which is well over 100 in commercially available kits. This method was primarily chosen for **Papers III** and **IV**, which aimed to analyze many different specificities in large cohorts.

4.3.3 Cell-based antibody detection

Lastly, a cell-based assay was used in **Paper II** for investigating MOG-reactivity [159,204]. As relevant anti-MOG antibodies depend on natively folded MOG [146], assays must account for this, which

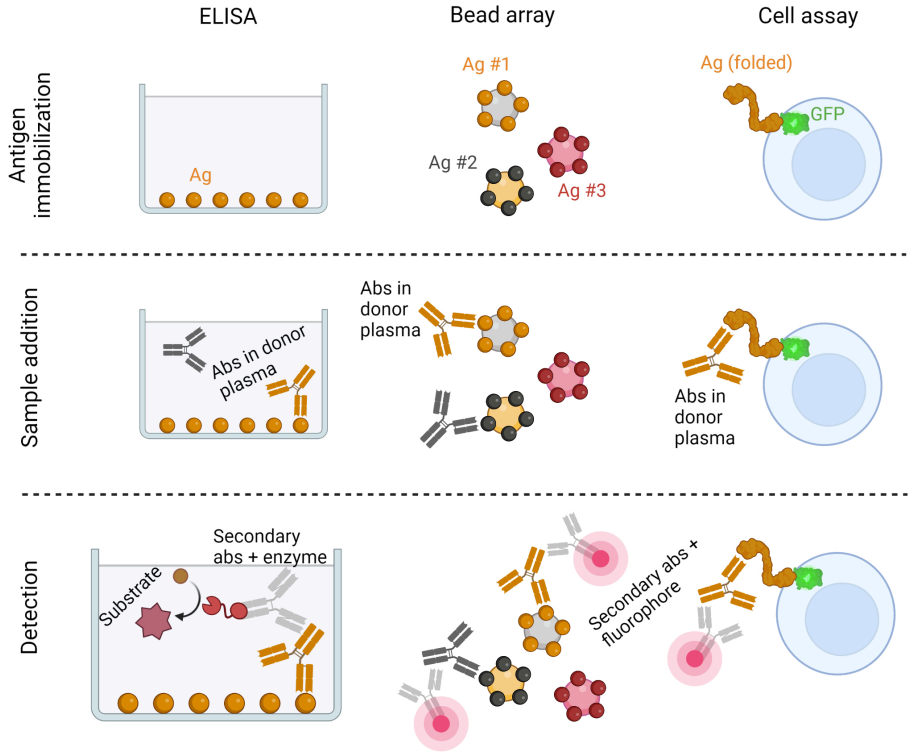

Figure 6. The principal differences of antibody detection methods. Ag: Antigen. Abs: Antibodies. GFP: Green Fluorescent Protein. Created with Biorender.com.

ELISA or bead-arrays do not. Here, a human cell line (HeLA) is transformed to express green fluorescent protein (GFP) and MOG on its surface, where the MOG molecule is then in its native state. After establishing a cell line, the following method and principle are similar to suspension bead array, where antibodies bind to the surface-MOG. After adding secondary antibodies, the cells are analyzed using flow cytometry. The GFP is used to evaluate the expression of MOG and normalize the secondary fluorescent antibody signal, and the data is presented as the ratio of secondary antibody / GFP.

4.4 ANIMAL MODEL – EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Rodent models have been widely used in immunology, and experimental autoimmune encephalomyelitis (EAE) is the most used model for MS. It has been the basis for much of our current understanding of the immunological processes in MS [63]. Standard protocols include immunization with myelin antigens to induce autoreactivity and an adjuvant to boost response, which leads to a monophasic inflammatory demyelinating encephalomyelitis with ascending flaccid paralysis as the primary symptom [156]. The symptoms are then scored based on severity. 0: No symptoms. 1: Tail weakness or paralysis. 2: Hind leg paresis or hemiparesis. 3: Hind leg paralysis or hemiparalysis. 4: Tetraplegia or moribund. 5: Death. While EAE is a much more homogenous disease than MS, induced by single autoantigens and monophasic, it is mainly driven by autoreactive T cells and, as such, a helpful model. Additionally, as the CNS compartment does not lend itself to sampling in pwMS, studies on CNS migrating T cells are complex in humans.

In **Paper III**, we utilized the EAE mouse model to study whether the discovered autoantigens were encephalitogenic, i.e., if an autoreactive immune response against these autoantigens led to autoimmune CNS disease. This would constitute indirect proof of their relevance in MS. As the encephalitogenic potential of autoantigens differs between strains due to differences in underlying immunogenetics [205-207], we used two different strains: SJL/J and DBA/1. The reasoning for this choice was that they are EAE susceptible to different autoantigens (PLP for SJL/J and MOG for DBA/1), reflecting their underlying MHC haplotype heterogeneity (H2s and H2q, respectively). Additionally, they require comparatively mild immunization adjuvants to induce disease. To detect subclinical neuroinflammatory disease, after the observation period of EAE, we performed *in vitro* T-cell autoreactivity analyses and immunohistochemical studies on the brain and spinal cord to study T-cell migration.

A mouse model was also used in **Paper IV** to study T-cell cross-reactivity. SJL/J mice were immunized with EBNA1, and draining lymph node T cells were harvested and examined for their antigen recall responses to EBNA1, CRYAB, and other CNS autoantigens. Here, we opted for pure

T-cell studies with milder immunization protocols, as CRYAB and EBNA1 have already been studied in the EAE context [160,161].

4.5 HUMAN MATERIAL AND SELECTION

The primary biological material we used for **Papers I-IV** was peripheral blood mononuclear cells (PBMCs), a fraction of mixed circulating immune cells isolated from venous blood. It contains T cells ($CD4^+$ and $CD8^+$), B cells, monocytes, and NK cells in approximately 70, 15, 5, and 10 % proportion, respectively [208]. Isolation of this subset from whole blood samples is standardized and uses centrifugation of whole blood over density gradient media [208].

Additionally, we opted for cryopreserved PBMCs over freshly isolated samples for two reasons. First, it vastly improves workflow and allows for more standardized handling. In sensitive analyses, factors like time from isolation to testing and even different FluoroSpot plates can have subtle influences. Using fresh PBMCs, keeping all factors consistent while running patients and controls simultaneously was practically impossible. As such, a common freezing protocol facilitated a standardized treatment of all samples and simultaneous testing of both disease and control groups to reduce experimental bias. Additionally, while cryopreservation can affect cell quality and results in follow-up assays, it has been evaluated for this exact context with little to no impact on results [209].

Cohort parameters like sex, age, and treatment were obtained during study enrollment to keep the study groups' age and sex adequately matched. Detailed clinical parameters were collected retrospectively, blinded to study results, from the Swedish MS-registry [210] (disease duration, treatment duration, and EDSS score) and digital healthcare records (presence of optical neuritis and confirmed MRI spinal lesions). Genetic data regarding HLA types were available for most of the recruited cohort due to inclusion in previous studies at our institution.

For antibody analyses in **Papers III** and **IV**, plasma from pwMS and controls from the Swedish nationwide incidence case-control EIMS (Epidemiologic Investigation of Multiple Sclerosis) cohort [211] were used. The controls were matched to cases according to geographical location, age, and sex. The plasma samples were randomized on plates and assigned ID codes for blinding regarding disease status. Unblinding was performed after all raw data had been collected and "locked in".

4.5.1 Cohorts

4.5.1.1 Paper I

In **Paper I**, we used model antigens and a PBMC cohort of 28 healthy donors to explore T-cell responses to two common antigens, one of viral and one of bacterial origin (Cytomegalovirus pp65

protein and a fusion of Diphtheria and Tetanus toxoid, respectively). As this paper evaluated the method, no disease group was included.

4.5.1.2 Paper II

In **Paper II**, we used a PBMC cohort of 52 natalizumab-treated pwMS (MS-Nat) and 24 age- and sex-matched healthy controls (HC) to study the responses to MOG. Here, as previous studies have demonstrated that responses to myelin antigens are quite heterogeneous [127,138], we opted for a larger patient group due to the expectation that responses in MS would be more heterogenous than in healthy controls.

4.5.1.3 Paper III

In **Paper III**, we utilized three separate PBMC cohorts. First, a smaller cohort of 16 MS-Nat and nine age- and sex-matched HC for the autoantigen screening panel. As the screening setup required a lot of cell material, we opted to run a smaller cohort while validating our findings in two larger cohorts.

The first validation cohort consisted of 61 MS-Nat and 28 matched HC and was used to confirm the initial screening hits. A second validation cohort was then used to explore the autoreactivity profiles and diagnostic potential in untreated MS. In this second validation cohort, 31 pwMS without ongoing DMT (MS-Un), 20 HC (which consisted of a representative sample of the 28 HC from the first validation) and 19 other neurological disease controls (OND) were used.

For antibody analyses, plasma samples from a cohort of 518 pwMS and 554 controls were used. Initially, an approximately 33% larger cohort was planned, but one of four assay plates failed. As patient and control samples were randomly distributed among the four plates, the exclusion did not bias the results.

4.5.1.4 Paper IV

In **Paper IV**, we analyzed one combined PBMC cohort consisting of 59 MS-Nat, 25 MS-Un, 19 HC, and 20 OND, partly overlapping with the cohort studied in **Paper III**.

For antibody analyses, plasma samples for a cohort of 713 pwMS and 722 controls (Con) were analyzed (An extension of the plasma-cohort in **Paper III**). A subgroup of 91 pwMS was further analyzed for cross-reactivity between CRYAB and EBNA1.

4.5.2 Cohort selection considerations

4.5.2.1 Disease-modifying treatments

For **Papers II-IV**, we studied natalizumab-treated pwMS. Treatment effects are generally a complex problem when studying T cells in MS. As T cells are disease-driving, most DMTs work by

downregulating or hindering T cells, which can affect T cell responses *ex vivo.* This has been even more pronounced recently, where modern high-efficacy treatments with high benefit-to-risk ratios are widely used, especially in Sweden, and few patients are left without DMT for a considerable time. For example, Fingolimod's mode of action is the modification of the sphingosine-1-phosphate receptor, resulting in sequestering of T cells in lymph nodes, making the study target inaccessible via blood sampling [212].

Natalizumab works by blocking the alpha-4-beta-1 integrin in leukocytes, an integrin necessary for the migration of T cells across the BBB, effectively blocking them from the target organ [213]. However, this means that, theoretically, otherwise migrating autoreactive T cells accumulate in the periphery, making them easily accessible via blood sampling. We hypothesized that natalizumab-treated persons with MS would be ideal for identifying otherwise rare autoreactive T cells. However, as with all treatment effects, it introduces some uncertainty in the results. We also included a cohort of pwMS without DMT in **Papers III** and **IV**.

4.5.2.2 Control groups

The main control group chosen were healthy donors, age- and sex-matched. Again, an overall strategy was to include approximately half of the patient-group size, as responses were hypothesized to be generally lower and more homogenous. We also included an OND control group in **Papers III** and **IV** to ensure that the autoantigens discovered were not ubiquitous to neurological disease. Here, we included persons with narcolepsy type 1, an autoimmune neurological disease with a shared HLA association with MS [214]. However, in the end, HLA had little influence on T-cell autoreactivity in our studies. It was ethically impossible to have the ideal control group of natalizumab-treated healthy controls. Therefore, alternatives were considered, like persons with another inflammatory disease for which natalizumab was regularly used. While rarely, natalizumab is used for treating inflammatory bowel diseases (IBD) in parts of the world. However, as it is not approved for IBD in Sweden, such a control group was unavailable.

4.6 ETHICAL CONSIDERATIONS

4.6.1 Human study participants

Most experiments in this thesis are based on materials obtained from healthy volunteers and persons with MS. The material consisted of venous blood samples and healthcare data from the electronic healthcare records and MS-registry. All participants gave their written informed consent before being enrolled in the studies, and all sample collections were approved by the Regional Ethics Board in Stockholm (2009/2107-3112, 2015/1161-31/4, and 04-252/1-4) and the Cantonal Ethics Committee of Zürich (no. 2013-0001).

Inclusion in the study conferred a minimal risk of physical harm for study participants, as blood sampling was the only procedure. Still, to minimize risks further, we opted to include patients and perform the blood sampling in conjunction with their regular treatment visits, where blood sampling was already performed as part of routine clinical care. To allow for the autoantigen screening and subsequent analyses, up to 64 ml of blood was sampled. While the eight consecutive blood sampling tubes might visually seem like much, it is only ~13% of a standard blood donation volume and is not harmful. A second potential risk of harm was collecting and handling sensitive personal data like health status and genetic data. The handling of personal data complied with the General Data Protection Regulation (GDPR) rules. All personal identification numbers were pseudonymized upon study enrollment, and the key linking study ID to personal identification numbers was stored on secure servers in encrypted files, to which only the principal researchers had access. All samples were handled according to the biobank law stating that study participants have autonomy over how their biological samples are handled. Thus, consent for study inclusion could be revoked at any time, and their samples would then be destroyed.

4.6.2 Animal models

For the experimental part of **Paper III**, we utilized an animal model of MS, namely EAE. A similar immunization model was used for **Paper IV** and was performed similarly but with milder immunization protocols. The animals were kept at the Karolinska animal facility in temperature-regulated rooms with a 12-hour light/dark cycle and housed in polystyrene cages with food and water access ad libitum. All animal experiments were performed according to previously approved ethical permission (Swedish National Board for Laboratory Animals, no: N138/14) and in accordance with the European Community Council Directive (86/609/EEC) and the "3R principle": Replace, Refine and Reduce.

As for the first R, replace, this thesis has mainly focused on humans with MS, for whom informed consent can be obtained, and participation conferred a minimal risk of harm. We also opted for this approach to ensure the autoantigens we studied were relevant to human disease first and foremost, as neuroinflammation in animals might not be translatable back into humans. However, more experimental studies cannot be performed in humans without causing significant harm, which has accidentally been the case previously [133]. The main problem with studies in humans with MS is that the causative relationship between T cells and disease is essentially impossible to prove within ethical bounds. Findings in human studies can be just an epiphenomenon of the disease, with little impact on the pathogenesis. Causal relationships can, however, be studied in animal models. The moral philosophy of using research animals is to use animals with lower cognitive ability to minimize the experienced harm. Mice fulfill this criterion while commonly used in this context and biologically similar enough, which allows for using already tested, established protocols to minimize suffering and the number of animals while obtaining meaningful results. The research

question itself, if T cells targeting these autoantigens cause CNS disease, is answerable, while there is no absolute *in vitro* replacement for the complex immune system-BBB-CNS interactions at play.

For refinement, we applied commonly used protocols for EAE-induction while replacing the specific autoantigen with the novel autoantigens of interest. Furthermore, we chose the specific strains SJL/J and DBA/1, two strains in which tolerance against myelin antigens is more easily broken, requiring milder immunization protocols and, thus, fewer immunization side effects and harm. However, classical EAE induces severe neurological symptoms, which cause suffering. To minimize harm, the animals were followed frequently with regular scoring to detect neurological symptoms and weight loss. If symptoms exceeded a pre-determined threshold (EAE score of 4 on two subsequent days), they were euthanized regardless of study duration. Additionally, a 40-day limit to the experiment was set beforehand to ensure non-detectable suffering did not continue needlessly.

For reduction, we estimated the number of animals needed per group based on previous experience. For the novel autoantigen immunizations, where the responses were difficult to predict as no existing literature or previous data existed, larger groups of animals were needed (n=6-7) to ensure meaningful comparisons could be made. However, for the negative and positive controls, where the result could be predicted better and expected to be more homogenous, we opted for fewer animals while still allowing for statistical analysis (n=3). Similarly, in **Paper IV,** we opted to keep the more predictable control group animals at a minimum (n=2) compared to the active immunization group (n=4).

5 RESULTS AND DISCUSSION

With the overarching goal of this thesis of identifying novel autoantigens in multiple sclerosis, a research field that has received much attention in the past decades, we first focused our attention on the methodology. A commonly cited problem in this field is the problem of standardized and sensitive assays to detect T-cell responses [74,167]. Another is the presence of rare, non-disease-causing autoreactive T cells in the healthy population [141,142], meaning just identifying an autoreactive T cell is insufficient. Instead, inflammatory potential and frequency of autoreactivity are more relevant. Also, as with any T-cell stimulation assay, particularly for autoreactive responses, which are likely rarer than T cells specific for common pathogens, the noise in assays due to unspecific activation of cells is of great concern.

One solution to the noise problem could be the utilization of synthetic peptides, either standalone or in mixed pools, but this introduces other concerns. As synthetic peptides in solution are directly loaded on surface-expressed HLA-molecules of APCs, it hinges on already expressed HLA and co-stimulatory molecules, leading to possibly weaker or even anergy-inducing T-cell activation. A screening approach would require a vast library of peptides to cover previously unknown and difficult-to-predict immunogenic ones, making it practically unfeasible. Additionally, standardized 15-20-mer peptides do not necessarily mimic the relevant epitopes presented *in vivo* [191], and small changes in peptide composition can vastly influence TCR recognition [192]. As such, longer oligomers or complete antigens, which are processed into peptides by the intracellular biological machinery, were preferred. However, these are rarely commercially available and introduce purity problems, as the source is often bacterial and might not be efficiently taken up and processed by APCs.

As such, this thesis's starting point was to solve these problems before a screening approach of autoreactivity could be used, leading to the method presented in **Paper I**, which made it possible to stimulate and detect antigen-specific T cells with high sensitivity utilizing longer recombinant proteins. As a further proof-of-concept, in **Paper II**, this method was applied in MS to investigate responses to the suggested autoantigen MOG, for which previous studies have presented conflicting results [127,138-144]. With this novel method allowing for the application of recombinant antigens, we performed a broad screening of T-cell autoreactivity in **Paper III**, identified four new T-cell autoantigens that were validated in independent cohorts, and demonstrated encephalitogenic potential in an animal model. Lastly, in **Paper IV**, we revisited the proposed but contentious autoantigen CRYAB, identified MS-associated autoreactive antibodies, and demonstrated cross-reactivity between CRYAB and EBNA1.

5.1 SENSITIVE DETECTION OF T CELLS USING MICROBEAD-COUPLED RECOMBINANT ANTIGEN

We hypothesized that a way to solve the antigen stimulation problem was to utilize microbeads as a vehicle (Figure 7). The idea came from the field of allergy, where microbeads have been investigated as a possible adjuvant for vaccination, as it has been demonstrated to induce $CD4^+$ T-cell responses in vivo effectively [215-217]. Additionally, it allows the covalent binding of proteins to beads via chemical coupling, effectively fixing otherwise insoluble or unstable antigens. In turn, this opens up for processing that would otherwise cause loss or destruction of the antigen. Lastly, APCs have been demonstrated to process particulate antigens effectively and present the epitopes with increased expression of co-stimulatory molecules [218], increase cross-presentation on MHC class I, and work efficiently through monocytes and not only specialized dendritic cells [217,219].

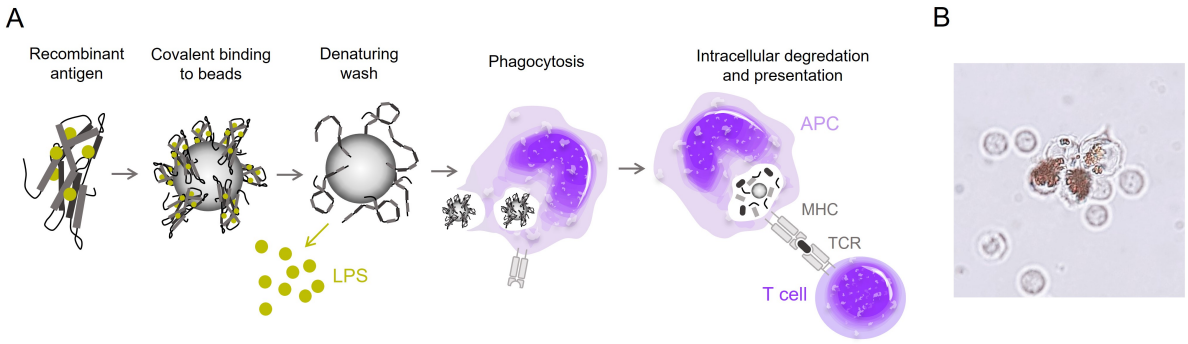

Figure 7. Schematic overview of the antigen-bead method. A) Graphical schematic of the antigen-processing steps. **B)** Light microscopy image of phagocytosed beads in a PBMC culture. LPS: Lipopolysaccharide. APC: Antigen-presenting cell. MHC: Major Histocompatibility Complex. TCR: T-cell Receptor. Adapted from **Paper I**.

In **Paper I**, we designed two model fusion antigens based on known T-cell epitopes from cytomegalovirus (CMV) protein PP65 [220] and a combination of tetanus and diphtheriatoxoid (T-D) [221], both fused to an albumin binding domain (ABD) protein [201] and a histidine tag. A protein containing the ABD and histidine tag alone was produced as a negative control. After histidine affinity purification, the antigens were coupled to 1 µm paramagnetic beads and washed in sodium hydroxide and detergents to remove remaining bacterial contaminants like LPS, which would not be possible without first covalently immobilizing the antigens. Here, we could confirm that it was possible to reduce the remaining LPS content to levels < 0.01 EU/ml, substantially below what is commonly contained in cell-culture grade media. However, for optimal signal-to-noise ratios, the exact washing condition needed to be titrated for each unique antigen. The reason is likely different properties like hydrophobicity and starting LPS contamination. At the same time, the harshest condition for all antigens was not optimal, as while a high amount of LPS can directly stimulate cells [222], a small amount of LPS enhances co-stimulation and antigen-specific responses, resulting in a more sensitive assay [223]. Additionally, exposure to high pH for extended periods can damage protein. By fluorescently staining the ABD (**Paper I** and **II**), the histidine tag (**Paper IV**), or directly measuring protein concentration (**Paper III**), we verified that the proteins remained coupled, even after performing the harsh denaturation washes (Figure 8).

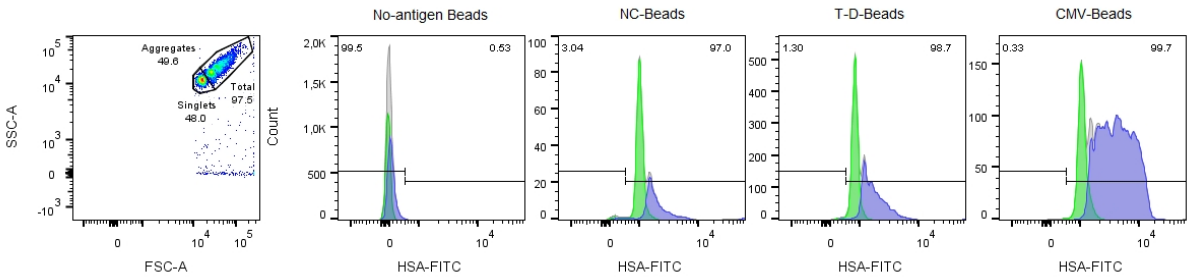

Figure 8. **Coupling quality control.** Flow cytometry analysis of antigen-beads after staining with fluorescein isothiocyanate conjugated human serum albumin (HAS-FITC). The left-hand panel shows representative forward and side-scatter plots for gating single beads (green) and aggregate beads (blue). Brackets denote the gating for negative and positive beads respectively, and numbers represent the % of beads in each gate. Adapted from **Paper I**.

As a proof of concept, in **Paper I,** we tested PBMCs from healthy donors for reactivity against the model antigens in an IFN-γ FluoroSpot assay. Here, increased IFN-γ responses were detected after stimulation with the antigen beads compared to beads with the ABD tag only (Figure 9). By comparing the responses of reactive and non-reactive individuals, the signal-to-noise ratios of antigen beads were as high as 29.5 and 9.5 for the CMV and T-D beads, respectively. Additionally, based on the background responses, the assay's sensitivity was 1 / 18 000, or 0.006 %, PBMCs. The responses to CMV and T-D did not meaningfully correlate, indicating that the responses seen were disconnected and antigen-specific. Altogether, **Paper I** demonstrated that the novel method allowed for the sensitive detection of antigen-specific responses using recombinant proteins.

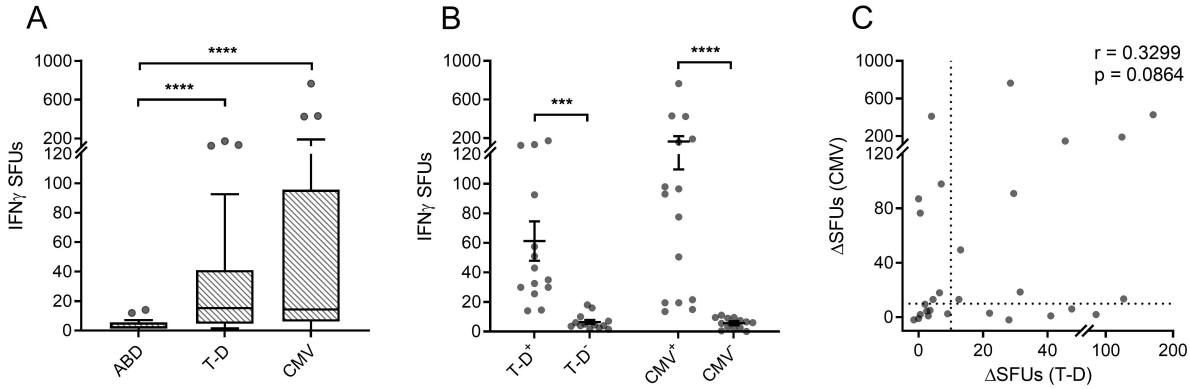

Figure 9. **IFN-γ responses to model-antigen-beads. A)** Raw IFN-γ SFUs after stimulation with albumin binding domain (ABD), tetanus-diphtheria- (T-D), or cytomegalovirus- (CMV) beads**. B)** Raw IFN-γ SFUs of T-D and CMV responsive and non-responsive individuals. The grouping of responsive and non-responsive individuals was based on background-adjusted SFUs (ΔSFUs), while raw was used for demonstrating signal-to-noise ratios. **C**) Correlation of ΔSFUs after CMV and T-D stimulation. The dotted lines represent the threshold for positivity. Adapted from **Paper I**.

The method was further evaluated in **Paper II**. By comparing the responses in unstimulated cells with ABD-beads and no-antigen-beads stimulated cells, it was demonstrated that the beads themselves, or unrelated, non-antigenic *E. coli*-produced protein coupled to beads do not induce responses above the general assay background. Additionally, by titrating the concentration of stimulating antigen, increased responses were detected at low antigenic concentrations, around ~0.1 µg/ml, or 2.5 beads per cell, with more optimal signal-to-noise ratios at 10 beads per cell.

5.2 DETECTION OF AUTOREACTIVE MOG-SPECIFIC T CELLS IN MS

As discussed in previous sections of this thesis, MOG is a well-established encephalitogenic autoantigen in mouse models. However, whether it is relevant in MS has been controversial due to studies investigating MOG-reactivity on the T-cell level reporting conflicting results [127,138-144], and MOG-autoantibodies have now been associated with other similar but distinct neuroinflammatory diseases (MOGAD) [224]. In **Paper II**, we revisited the question regarding MOG as an autoantigen in MS, utilizing the novel method developed in **Paper I**.

PBMCs from MS-Nat and HC were tested for MOG-reactivity in an IFN-γ/IL-17A/IL-22 FluoroSpot assay to detect primarily T_H1 and T_H17 responses. Increased MOG-reactivity was detected for all analyzed cytokines, with 46.2-59.6 % of MS-Nat displaying significant MOG-responses, compared to 0-12.5 % in HC (Figure 10A). Meanwhile, both polyclonal and background responses were similar between MS-Nat and HC. By depleting the PBMC population of cell types, i.e., $CD4^+$, $CD8^+$, monocytes ($CD14^+$), and B cells ($CD19^+$), it was demonstrated that MOG-autoreactivity was due to an increase of MOG-specific $CD4^+$ T cells, which were dependent on monocytes for antigen presentation restricted to HLA-DR (Figure 10B, C). This fits well with the

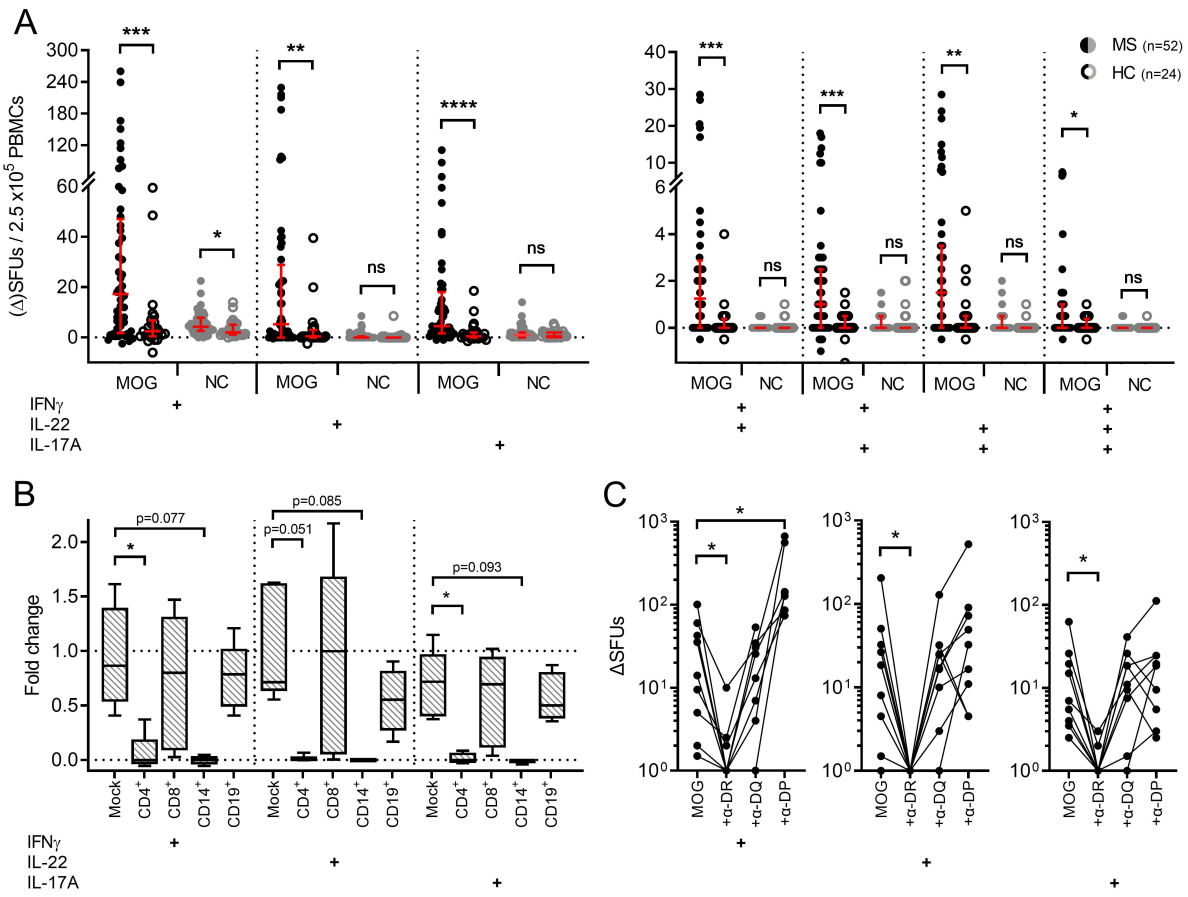

Figure 10. Increased MOG-autoreactivity in persons with MS. A) Cytokine responses in pwMS and HC after stimulation with MOG-beads or NC-beads. Background adjusted spot forming units (ΔSFUs) values depicted for MOG, raw SFUs depicted for NC. **B**) Fold-change of MOG-responses after depletion of specific cell types. The X-axis denotes the surface marker used for depletion (CD4 for T helper cells, CD8 for T cytotoxic cells, CD14 for monocytes, and CD19 for B cells. Boxes and staples represent the median, IQR, and range. Data based on 5 biological replicates. **C**) Effect HLA-blocking on MOG-induced cytokine responses. Each line represents one individual. Adapted from **Paper II**.

strong genetic HLA-DR and MS association [37] and observations that monocyte-derived conventional DCs can license autoreactive T cells in MS [68].

While one previous study found an association between MBP and MOG-reactivity, and location of lesions, particularly an association with IL-17A responses and spinal lesions [143], we could not correlate MOG responses to any distinct clinical phenotype in this study. However, we did not have as detailed MRI data to allow for the same comparison. Instead, a cruder comparison was made, where 95.8 % of IL-17A MOG-reactive pwMS had spinal lesions compared to 80.7 % of non-reactive. While not statistically significant, it followed the same pattern observed in the previous study. There was also a trend of longer natalizumab-treatment duration in MOG-unreactive pwMS, hinting that locking T cells out of the CNS over time decreases the number of autoreactive cells, although this association was weak and responses remained for several years despite treatment. Other clinical correlations were hampered as the patient group had been on natalizumab treatment for an average of many years, which essentially extinguishes disease activity.

We also examined the role of B cells in MS-associated MOG-reactivity. As expected, based on the clear associations of anti-MOG antibodies with other diseases than MS and previous results in MS cohorts [105,146,158,204,224,225], only one out of 29 tested pwMS were MOG-autoantibody positive as measured using a cell-based assay [159] (Figure 11), with similar results obtained using two ELISA-methods. Indeed, the role of B cells in MS pathogenesis and the observed effect of B-cell depletion treatment might lie in their role as APCs and interaction with T cells rather than autoantibody production [106,107,109]. However, no evidence for B cells as antigen presenters and activators of MOG-specific T cells was observed in this paper, as B-cell depletion did not affect responses. Instead, it was entirely dependent on $CD14^+$ monocytes. Nevertheless, this observation must be interpreted with caution, as the antigen-bead system used in the assays depends on phagocytosis of rather large particles, which could bias against B cells acting as antigen presenters. In summary, we demonstrate

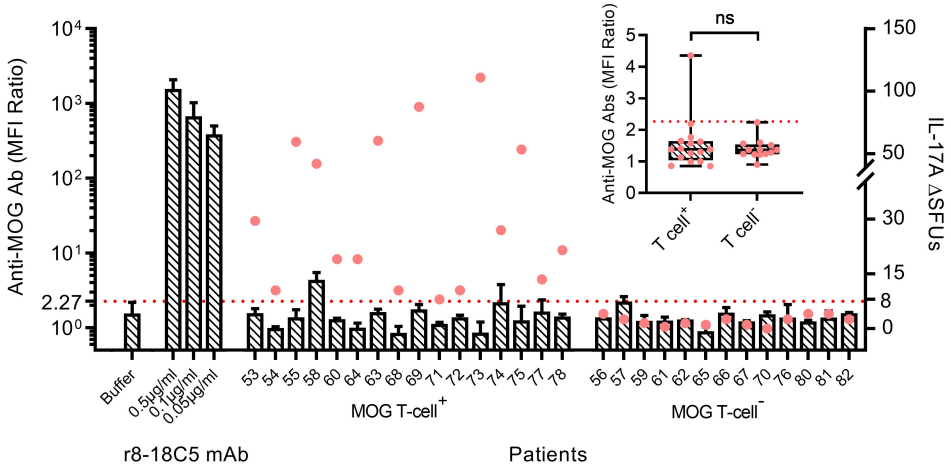

Figure 11. Anti-MOG antibodies in relation to MOG-specific T cells. Autoantibody responses (bars) and IL-17A T-cell responses (dots) in pwMS. Antibody data is plotted against the left Y-axis, and T-cell data is plotted against the right Y-axis. The red dotted line denotes the threshold for positivity for both assays. The inlaid graph shows the comparison at a group level. Adapted from **Paper II**.

that peripheral monocytes can drive MOG-specific proinflammatory $CD4^+$ T cells but can not exclude that B cells can do the same *in vivo*.

Interestingly, in previous studies investigating T-cell reactivity to MOG in MS, results have varied based on the source of the MOG used for stimulations. Older studies that first reported increased MOG-reactivity used MOG isolated from brain tissue [138,140]. However, follow-up studies using recombinant MOG reported either similar increased responses in both pwMS and the control groups [142-144] or no response in either [141]. As such, the LPS contamination inherent in proteins produced in bacteria might have masked responses in some studies, yielding false negative results. Therefore, stringent denaturing washing of bead-immobilized antigens could be important for detecting autoreactive T cells. An indication of this problem was observed in **Paper III**, as there were some positive correlations between LPS contamination and P-values, meaning LPS, if anything, masked the differences in autoreactivity. An alternative explanation could be that glycosylation is essential for T-cell recognition of MOG, which is not present in bacterial-expressed proteins, but would be in tissue-derived MOG. However, this is unlikely, as it would not explain the results of this paper and does not fit with the previous studies using peptides or recombinant MOG finding responses in both pwMS and controls.

Another solution is peptide-stimulations. Studies utilizing this method have reported mixed results [142,144,148], which could be due to insufficiently strong activation signals from peptide stimulations. Another possible explanation could be that the "perfect" peptides rarely were used. The generated peptide epitopes from intracellular degradation of full-length proteins might not be present in an overlapping peptide library or HLA-DRB1*15:01 *in silico*-predicted binding peptides or vice versa. This discordance between synthetic, possibly *in vitro* immunodominant epitopes and naturally processed *in vivo* encephalitogenic epitopes have been demonstrated for MBP [191].

Additionally, biologically relevant autoantigen-peptides might not have the highest HLA or even TCR affinity, as strong presentation should decrease the likelihood of escaping tolerance mechanisms. In that vein, one study found that lower-affinity autoreactive T cells were essential in maintaining autoimmune disease [226]. Altogether, the results of **Paper II** and previous studies make a case for using full-length antigens when investigating autoreactive T cells in MS.

5.3 IDENTIFICATION OF FABP7, PROK2, RTN3, AND SNAP91 AS AUTOANTIGENS IN MS

Next, we aimed to identify previously unknown T-cell autoantigens in MS. Previous studies identifying autoantigens have utilized more targeted approaches, like direct testing of single CNS proteins implicated by their expression or relevance in EAE [125,130,135,138,149,150,152], identification of CNS-infiltrating T-cell clones and then searching for the clones' cognate antigen [106,147], or results from autoantibody screenings [151,227]. However, broad screenings of bulk T-cell reactivity have been challenging due to limitations in methodology, as previously discussed. We aimed to perform an extensive, unbiased screening in **Paper III**, utilizing the novel method developed and presented in **Papers I** and **II**.

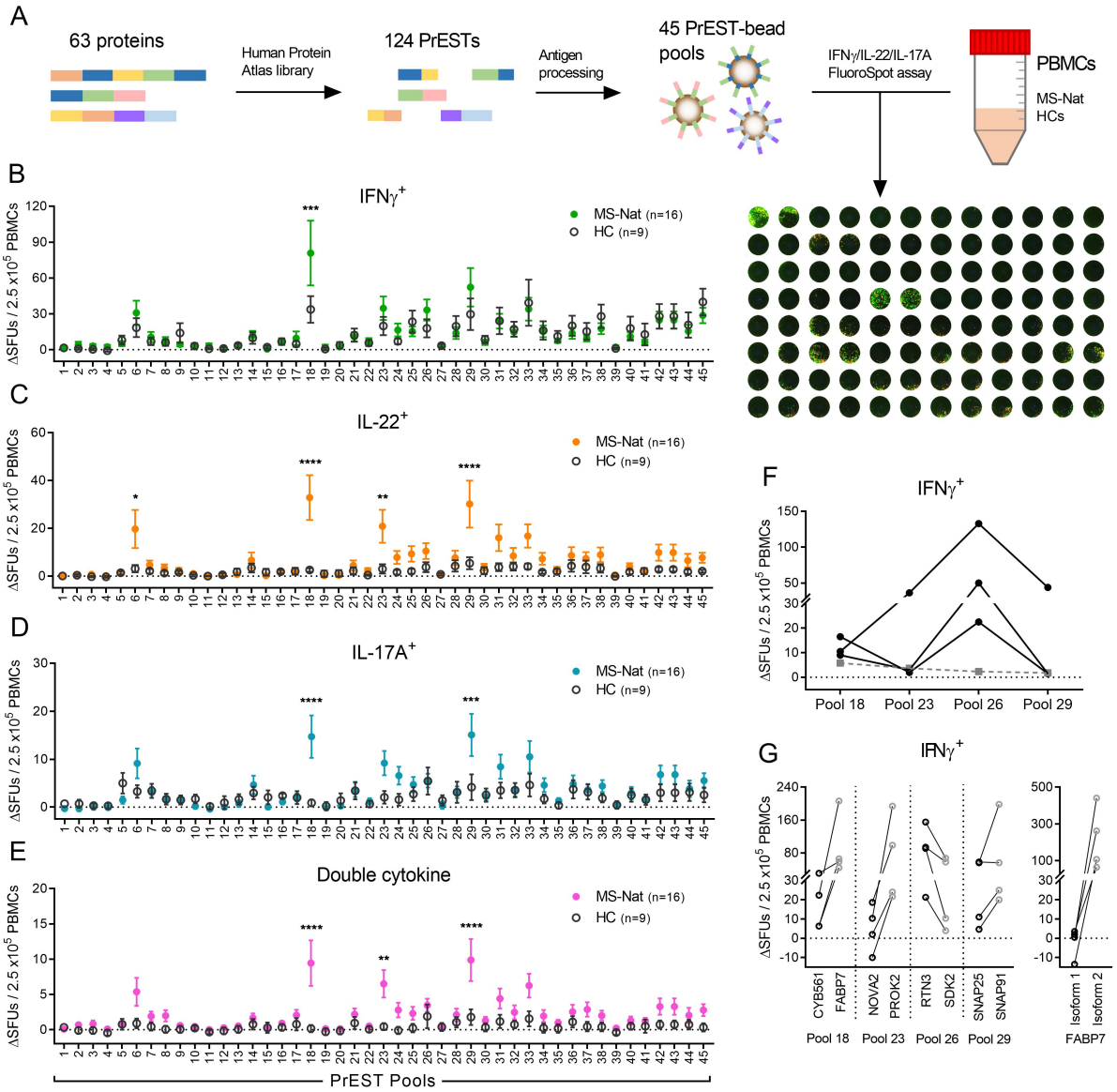

Figure 12. Autoantigen screening using a PrEST library. A) Schematic overview of the screening design, with a representative FluoroSpot plate shown. **B–E**) Cytokine responses against the 45 antigen pools in a cohort of MS-Nat and HC. Double cytokine (E) was calculated as cells producing any combination of the three analyzed cytokines, IFN-γ (B), IL-22 (C) and/or IL-17A (D). Plotted as mean and SEM. **F**) Depiction of a particular response pattern to PrEST pool 26. IFN-γ plotted (black) against mean IL-22 responses (Grey dashed line). **G**) Deconvolution of the identified PrEST pools (left-hand panel) and comparison of two different isoforms of FABP7 (right-hand panel). Adapted from **Paper III**.

Here, we collaborated with the Human Protein Atlas (www.proteinatlas.org) [202] to create a panel of 124 recombinant PrESTs covering 63 proteins with predominant CNS expression. The final panel included both previously investigated MS autoantigens but consisted of, in this context, primarily unstudied proteins. By processing the PrESTs with the bead method and investigating T-cell responses by FluoroSpot in a cohort of MS-Nat and HC, increased proinflammatory responses were detected against four novel candidate autoantigens: Fatty acid-binding protein 7 (FABP7), prokineticin 2 (PROK2), reticulon 3 (RTN3) and synaptosome associated protein 91 (SNAP91, also called clathrin coat assembly protein AP180). None of these have been implicated as MS autoantigens previously. Increased responses in pwMS to a MOG PrEST were similarly detected (Figure 12).

The screening findings were validated in two additional cohorts, exchanging the PrESTs for in-house produced full-length proteins. The first validation was performed in a larger independent MS-Nat and HC cohort, in which higher IFN-γ, IL-17A, IL-22, and dual cytokine responses against the four novel autoantigens were present in MS-Nat (Figure 13A). Similar results were seen for the included established autoantigens MOG, MBP, and PLP. However, increased responses were also detected for the CMV-antigen control in MS-Nat. While less of a difference than for the autoantigens, this finding prompted a second validation, using a cohort of MS-Un, HC, and OND-controls. MS-Un had increased IFN-γ responses towards all autoantigen tested, while there were no differences in the polyclonal or CMV control responses (Figure 13B). Increased T_H17 responses were, however, not detected in MS-Un.

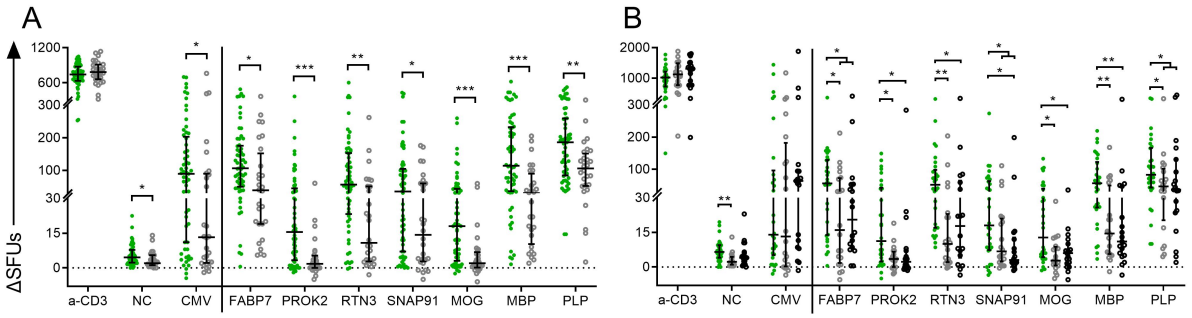

Figure 13. Validation of candidate autoantigens in additional cohorts. The four hits from the screening, as well as established autoantigens, were used to validate the findings of the screening. **A**). Results from a first validation cohort consisting of MS-nat (n=61, filled colored circles) and HC (n=28, open grey circles). **B**) Results from a second validation cohort consisting of MS-Un (n=31, filled colored circles), HC (n=20, open grey circles), and OND-controls (n=19, open black circles). Each dot represents one individual and staples denote the median and IQR. Adapted from **Paper III**.

As natalizumab blocks CNS (and partly gut) migration of T cells, autoreactive T cells would likely increase in frequency in peripheral blood, and CNS-homing autoreactive T cells have been identified in greater numbers in natalizumab pwMS [106]. As T_H17 cells are migratory [85], it is unsurprising that the IL-17A and IL-22 responses were more pronounced in MS-Nat, while non-detectable in MS-Un. As such, rather than an artifact of natalizumab treatment, it might represent a more pathological and disease-relevant response that is only detectable after blocking CNS

migration. One could resolve such a question by studying cells present in CSF. Unfortunately, the low number of cells in CSF, even during neuroinflammatory disease, does not lend itself to detecting rare autoantigen responses, especially in a screening fashion. Additionally, the most relevant T cells might be present in parenchyma rather than CSF. Another explanation altogether is a direct effect on cytokine expression by natalizumab. However, changes in expression are minor according to previous studies [228,229] and are unlikely to explain the results in this thesis. In accordance with this, all groups' polyclonal, i.e., antigen-agnostic, responses were similar. Additionally, background responses to CNS proteins in general, despite varying LPS contamination, were similar in both MS-Nat and HC (Figure 12B-E).

5.3.1 Characterization of autoreactivity

The increased autoreactive IFN-γ responses were further validated using flow cytometry analysis of autoantigen-stimulated PBMCs (Figure 14A, B). Autoreactive T cells were primarily $CD4^+$ and showed increased GM-CSF expression, hinting that a previously reported MS-associated T-cell population is autoreactive [81]. Additionally, the autoreactive $CD4^+/CD8^+$ ratio was higher in pwMS, implicating $CD4^+$ T cells as the more disease-associated T cell type. In line with the relative increase of autoreactive $CD4^+$ T cells, the autoantigen responses were all significantly HLA-DR restricted (Figure 14C), fitting with the MS-genetic associations [38].

While DR-restricted, there were similar responses in both DRB1*15:01 positive and negative individuals. This is not surprising, as while DRB1*15:01 confers the highest risk, it is not a pre-

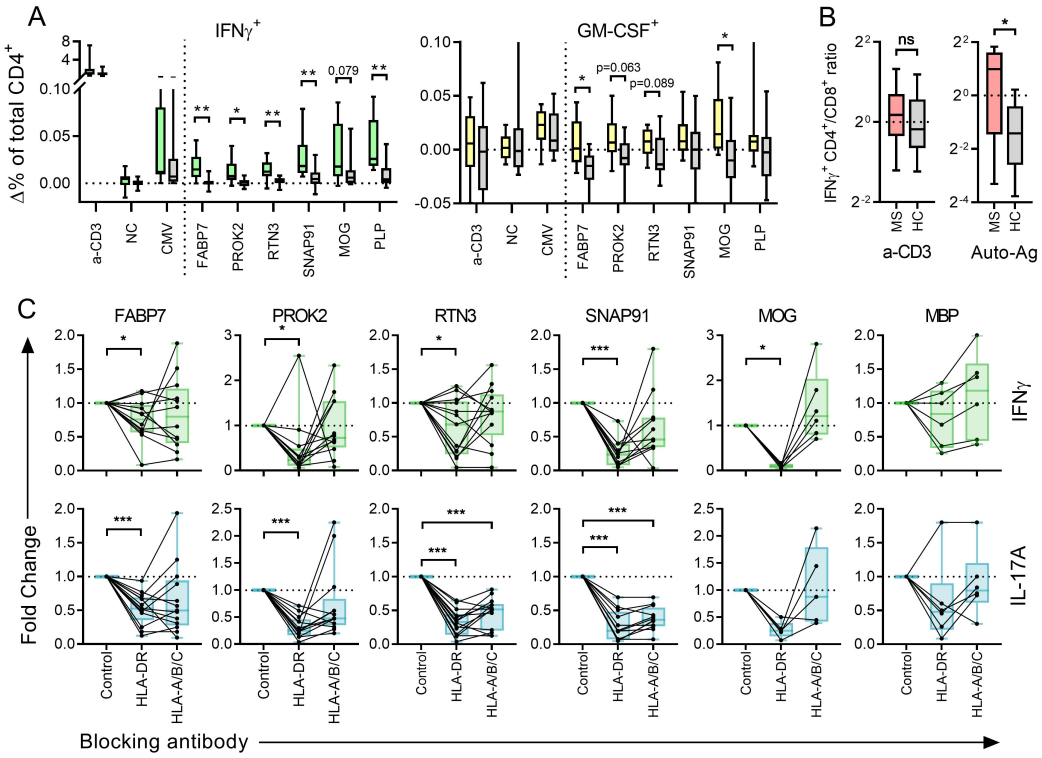

Figure 14. **Characterization of autoreactive cells. A**) Flow cytometry analysis of T cells with intracellular cytokine staining. **B**) Ratio of IFN-γ+CD4+ T cells versus IFN-γ+CD8+ T cells after polyclonal stimulation or autoantigen stimulation. For both A and B: PwMS in color, HC in grey. **C**) Influence of HLA-blocking on autoreactive T cells. Ag: Antigen. Adapted from **Paper III**.

requisite for MS, and the disease can develop on any HLA-DR background. As such, central disease-relevant autoantigens are likely not restricted to DRB1*15:01. Additionally, using full-length autoantigens instead of peptides minimizes the influence of specific HLA-haplotypes as the presented epitopes are naturally derived from processing by autologous APCs. This means peptides relevant to that particular HLA-haplotype *in vivo* are also represented *in vitro*.

Interestingly, there were generally higher autoreactive responses in males compared to females, fitting with the observation that males usually have a more aggressive disease course [230,231]. In contrast, there was no correlation to EDSS scores. However, the cross-sectional nature of **Paper III** naturally limits such correlations, and a prospective analysis would likely be more relevant and powerful. Remarkably, while the highest levels of autoreactivity were found in individuals early in their disease course, it persisted even in long-term disease and long-term natalizumab treatment. This could be explained by CNS autoreactivity being maintained in the periphery, suggesting a cross-reactive origin or frequent leakage of CNS-autoantigens [232].

Next, the presence of autoantibodies was investigated in a large cohort of pwMS and HCs. However, no significant differences were detected. Instead, apart from anti-RTN3, frequencies were similar to previous screens of autoantibodies in healthy persons [100]. However, autoantibodies targeting RTN3, specifically the N-terminal part of RTN3, were very frequent in both pwMS and HC, and the results were validated using an independent ELISA. While the frequency and magnitude of the response were suggestive of cross-reactivity to some common pathogen, no apparent homologies were detected using an *in silico* basic local alignment search.

Historically, analysis of T-cell autoreactivity has not been particularly effective at distinguishing between MS and non-MS. However, most previous studies have only analyzed reactivity against one or possibly a few autoantigens simultaneously. One study attempted a more extensive panel but did not detect any clear responses [103]. In **Paper III,** autoreactivity was tested against seven autoantigens simultaneously, providing a higher dimension of autoreactivity than previously reported. The autoreactive profiles were analyzed in the MS-Un cohort, demonstrating highly heterogeneous profiles with essentially unique patterns (Figure 15A). The heterogeneity also meant that each autoantigen in isolation performed poorly as a biomarker when analyzed using receiver operating characteristic (ROC) curves (Figure 15B) [233]. However, by creating a combined test factoring the number of different autoreactivities (from 0 to 4), a more powerful diagnostic tool was created, with a ROC area under the curve (AUC) of 0.88 (0.90 and 0.86 versus HC and OND, respectively), comparable to existing biomarkers [234]. The test was remarkably accurate in the extreme ends, with 4/4 positive reactivities resulting in 41 % sensitivity at 100 % specificity and 0/4 reactivities resulting in 97 % sensitivity at 50 % specificity. While not particularly useful as a broad diagnostic test, it could be valuable in confirming or ruling out MS with high accuracy in more challenging cases. However, this needs to be confirmed in a more clinically translatable cohort, i.e.,

recently debuted MS/CIS versus persons with common differential diagnoses. Importantly, these results indicate that MS pathogenesis does not hinge on one particular autoreactive response, like aquaporin-4 in NMOSD or acetylcholine receptors in myasthenia gravis, but rather the sum of MS-associated autoreactivities.

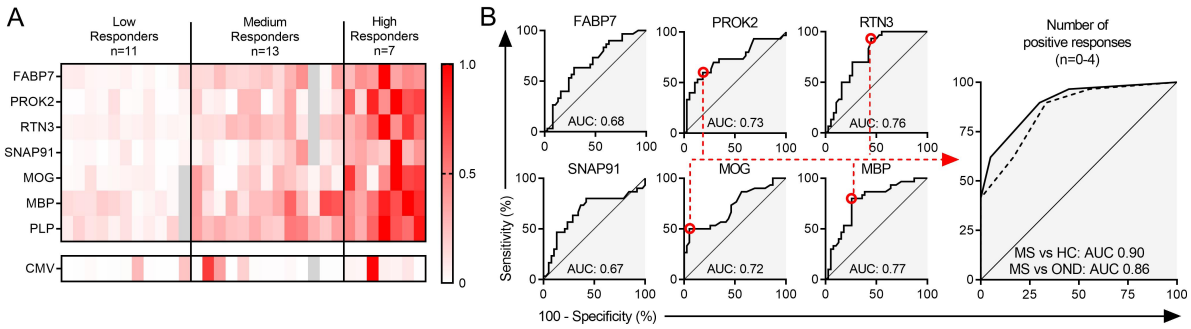

Figure 15. Autoreactive profiles and diagnostic potential. A). Autoreactive profiles of 31 MS-Un. Each column represents one individual. Plotted values are normalized against the highest recorded response for that particular autoantigen (0-1). **B**) Reciever operating characteristic curves for individual autoreactivities (small panels) and a composite test of the number of positive reactivities (large panel). The red circles mark the cut-off values for positivity used in the composite test. The solid line represents MS-Un versus HC and the dotted line represents MS-Un vs OND. Adapted from **Paper III**.

5.3.2 Demonstration of encephalitogenicity

Essentially, any immunological observation in MS could theoretically constitute an epiphenomenon, a disease-associated but ultimately non-pathogenic variation. This is a difficult problem to solve within ethical bounds, especially in MS, as the target organ is generally inaccessible. Further, due to processes like epitope spreading [134,161], detected autoreactivity could be a secondary effect while the initial insulting autoantigen remains elusive. There are, however, indirect routes of evidence that could strengthen findings. First, non-pathogenic epitope spreading as the disease progresses would mean a narrower autoreactive profile should be observed in early disease. However, in **Paper III,** similar responses were observed in those sampled within one year of first known symptoms and those sampled after a few years. Silent epitope spreading could occur during the pre-symptomatic prodromal phase of MS, but that would not preclude the autoantigens from being pathogenic. Rather, it supports the notion that several different autoreactivities must be present for the clinical disease to manifest.

Another way is using mouse models (i.e., EAE) to demonstrate the encephalitogenic potential of autoreactive T cells [63]. While not MS per se, it proves that autoreactive T cells can drive neuroinflammation in a biologically similar system. In **Paper III**, the EAE model was used to investigate the encephalitogenicity of the identified autoantigens. After immunization of SJL/J and DBA/1 mice with the novel autoantigens, the mice were observed for symptoms of EAE, and postmortem *ex vivo* studies of T-cell responses and immunofluorescent staining of CNS tissue were performed. The e*x vivo* analysis revealed that immunization induced autoreactive T-cell responses

for all autoantigens in SJL/J, and for PROK2 and SNAP91 in DBA/1. In the SJL/J strain, immunization induced lymphocyte migration to the brain, and migrating T cells were licensed to cross the BBB (Figure 16A, C). As such, autoreactive T cells targeting the four autoantigens were CNS-homing and gained pathological function. There were heterogenous T-cell reactivity and migration patterns where PROK2 stood out. It induced a proportionally larger IL-17 response and led to both brain and spinal cord infiltration. In contrast, in the DBA/1 strain, only SNAP91 induced CNS infiltration (Figure 16B, D). Despite moderate histological neuroinflammation, typical symptoms of EAE were not observed. The heterogeneous patterns of responses and lack of classical EAE are not surprising. As this was a first "blind" trial, it is likely that non-optimal strains were used, as EAE induction is highly dependent on the strain and autoantigen combination [205,206]. As such, more typical and severe symptoms could possibly develop in other strains.

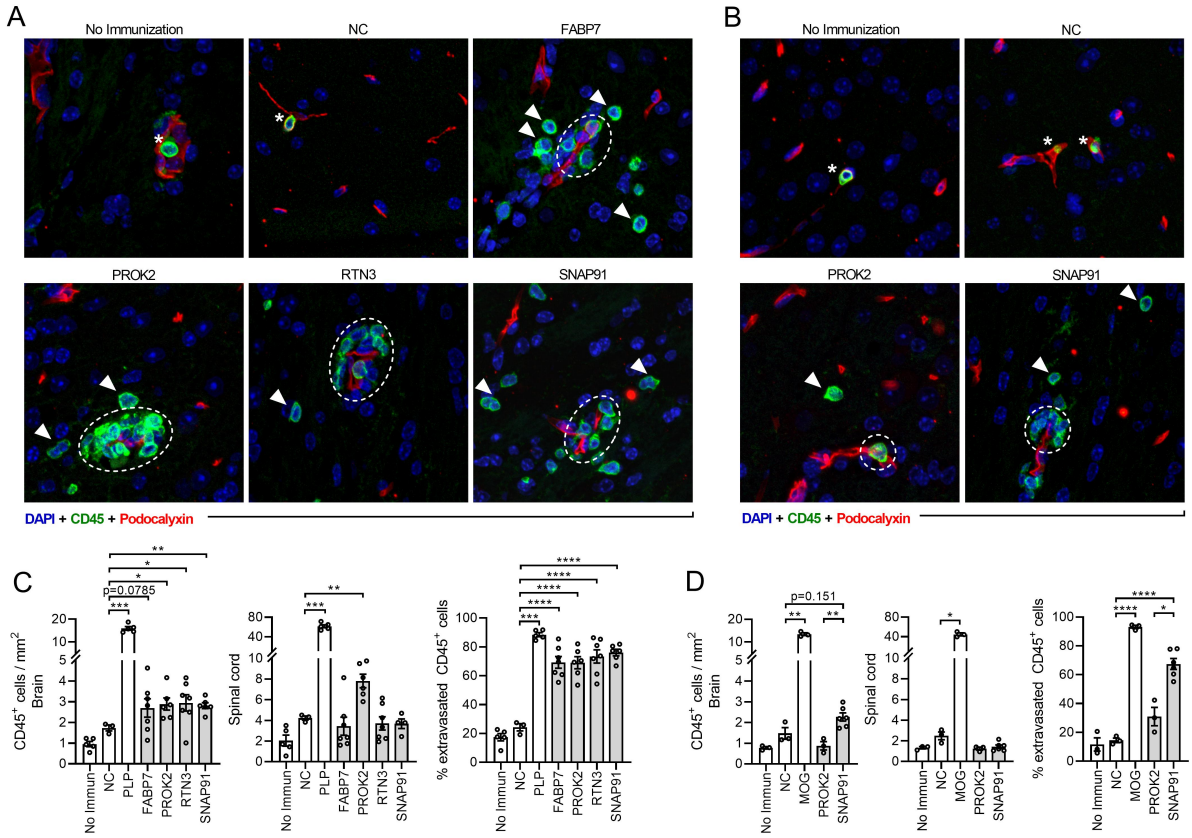

Figure 16. Encephalitogenic potential in mouse models. SJL/J (left-hand panels) and DBA/1 (right-hand panels) mice were immunized with the novel autoantigens. **A, B**) Representative immunofluorescence images used for analyzing leukocyte infiltration. Blue represents all cell nuclei (DAPI), green represents leukocytes (CD45) and red represents blood vessel endothelium (podocalyxin). Asterisks mark intravascular leukocytes, dashed lines mark perivascular leukocytes, and arrows mark intraparenchymal leukocytes. **C, D**) Enumeration and statistical analysis of brain and spinal cord infiltrating cells and proportion of cells that crossed the BBB. Adapted from **Paper III**.

5.3.3 The novel autoantigens

None of the four autoantigens are myelin components but are primarily associated with glial and neuronal cells. FABP7 (also called brain fatty acid binding protein) is an intracellular protein transporting hydrophobic molecules. Mainly expressed in glial cells throughout the CNS, with little detected expression in the periphery [235], it has previously been implicated in neuroinflammatory

disease, although not as an autoantigen. FABP7 is expressed in oligodendrocyte progenitor cells [236], and its expression is increased in demyelinating lesions in EAE [237]. Interestingly, it seems to have a neuroprotective effect where FABP7-knockout mice exhibit earlier EAE development and higher IFN-γ and IL-17A levels early on. Conversely, they develop a milder disease over time [237]. FABP7 has been implicated in remyelination, where decreased expression correlates with worse repair after injury and is generally decreased over time, especially in chronic lesions [238]. As such, the FABP7 expression pattern follows what would be expected of an MS-associated autoantigen. While speculative, a model where repeated CNS inflammation induces epitope spreading to FABP7, which leads to inhibited remyelination, development of chronic lesions, and accumulation of symptoms, is tempting. Such a model could explain why recovery during remission is heterogenous but often worsens over time.

PROK2 is a secreted protein mainly expressed in the CNS and lymphoid organs [239,240]. The prokineticin system involves various biological processes like angiogenesis, neurogenesis [241], neuroprotection [242], and circadian rhythm regulation [243]. Compared to the other three identified autoantigens, it is less CNS-specific in its expression pattern but the co-expression in CNS and lymphoid cells is reminiscent of other recently reported autoantigens [106]. Interestingly, impaired circadian rhythm and sleep disorders are increased in pwMS [244]. However, detailed symptomatologic data regarding sleep, mood, and fatigue were not available in this study, but its relationship with PROK2-autoimmunity could be an exciting study question in the future.

RTN3 is a membrane-bound protein associated with the endoplasmic reticulum and is involved in intracellular protein transportation [245]. It displays ubiquitous expression in the CNS but is most abundant in the neuropil and neuronal cell bodies [246]. Interestingly, it has been implicated in MS as a possible biomarker for treatment effect [232]. In that study, it was detectable in plasma from pwMS and decreased after treatment, suggesting that it leaks out from the CNS through a permeable BBB during inflammation. If that is the case, it could explain why autoreactive T cells are activated in the periphery and start migrating to the CNS and why head trauma increases the risk of MS.

SNAP91 is a neuronal-expressed protein mainly located in neuropil due to its synaptic association [247]. It is a clathrin assembly protein involved in the vesicle formation system in synapses for recycling neurotransmitters [248]. While myelin antigens induce classical EAE with ascending paralysis, it fails to represent the neurodegenerative features of MS. In contrast, neuronal-derived autoantigens more accurately mimic the degenerative properties and grey-matter-related disease in mouse models [149], which makes both SNAP91 and RTN3 exciting candidates. As imaging technology has advanced, a higher frequency of cortical grey-matter lesions in MS than previously thought has been reported [249], implicating neuronal autoantigens as relevant targets. The autoantigens' more neuronal expression could partly explain why classic EAE symptoms were not

observed in our mouse model. The experimental approach in **Paper III** did not address more atypical symptoms or degeneration and should be investigated in follow-up studies.

5.4 CROSS-REACTIVITY BETWEEN EBNA1 AND CRYAB

EBV infection constitutes one of the strongest environmental risk factors for MS, where molecular mimicry is one of the possible mechanistic explanations. In previous studies, antibodies against specific EBNA1 epitopes have been highly associated with MS [52,53,58], and a previous study delving deeper into one of these epitopes revealed a sequence homology and subsequent cross-reactivity with the autoantigen ANO2 [59]. Another MS-associated epitope (EBNA1 aa385-420) [52,53] contains a sequence homology to CRYAB and the recently reported GlialCAM [117] (Figure 17). CRYAB autoreactivity has been reported [153,154], although with some controversy [155], and no investigation into possible cross-reactivity has been reported. This prompted **Paper IV**, where we revisit CRYAB as an autoantigen in-depth and investigate the potential cross-reactivity with the MS-associated EBNA1 epitope.

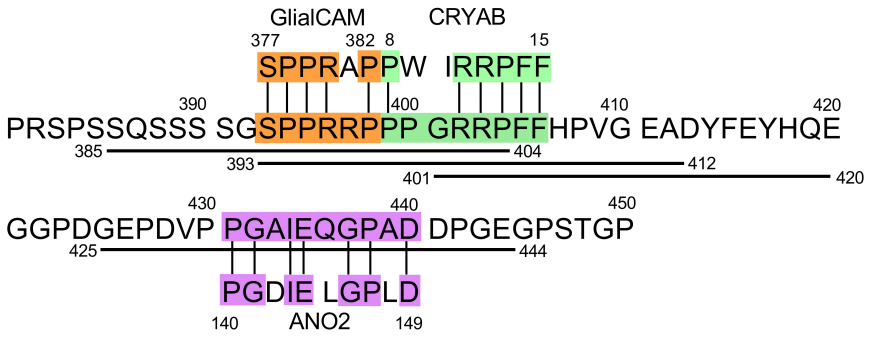

Figure 17. Previously reported sequence homologies in EBNA1. The amino acid sequence of EBNA1 (middle sequence) and the corresponding GlialCAM, CRYAB, and ANO2 sequences. The lines represent EBNA1 peptides which were included in **Paper IV**. The numbers denote the amino-acid position in the corresponding protein. Adapted from **Paper IV**.

First, a high-resolution antibody epitope mapping of CRYAB was performed using one aa-stepped overlapping CRYAB peptides and EBNA1 peptides covering known immunodominant and MS-associated regions (Figure 18A-C). Plasma from a large cohort of pwMS (n=713) and controls (n=722) was analyzed. There was a single autoantibody epitope in the N-terminus of CRYAB, which was associated with MS (OR 2.0). Depending on the exact peptide, positive responses were detected in 13.2-27.6 % of pwMS and 7.2-16.9 % of controls. A minimal epitope, CRYAB aa7-16 (HPWIRRPFFP), was identified, which closely correlated with the known EBNA1-homologous region (CRYAB aa8-15, PWIRRPFF). As reported in numerous previous studies [52,53,58], anti-EBNA1 antibodies were also associated with MS, particularly antibodies targeting EBNA1 aa393-412 (OR 2.9), coincidentally covering the CRYAB homology (EBNA1 aa399-406, PPGRRPFF). Antibodies targeting the two homology-containing peptides were highly correlated. Interestingly, exceptionally high anti-EBNA1 responses were even more associated with MS (OR 3.4), and a combination of high anti-EBNA1 and anti-CRYAB responses was even more so (OR 9.0).

These findings are not entirely novel, and antibodies targeting the N-terminus of CRYAB in MS have been reported previously, although only in small cohorts [250], which the results in **Paper IV** corroborate. However, studies in large cohorts showing strong MS association and precise mapping of the antibody epitope, as presented here, have been lacking. In contrast, antibody responses to whole CRYAB, and peptides derived from other parts of the protein have also been reported [163,251], of which there were none in this study. Whether or not earlier data regarding whole CRYAB is reliable is a contentious topic, as the chaperone ability of CRYAB has been reported to interact with antibodies in a specificity-independent manner, resulting in false-positive experiments [155]. Typically, a chaperone's ability depends on the complete protein and its tertiary or quaternary

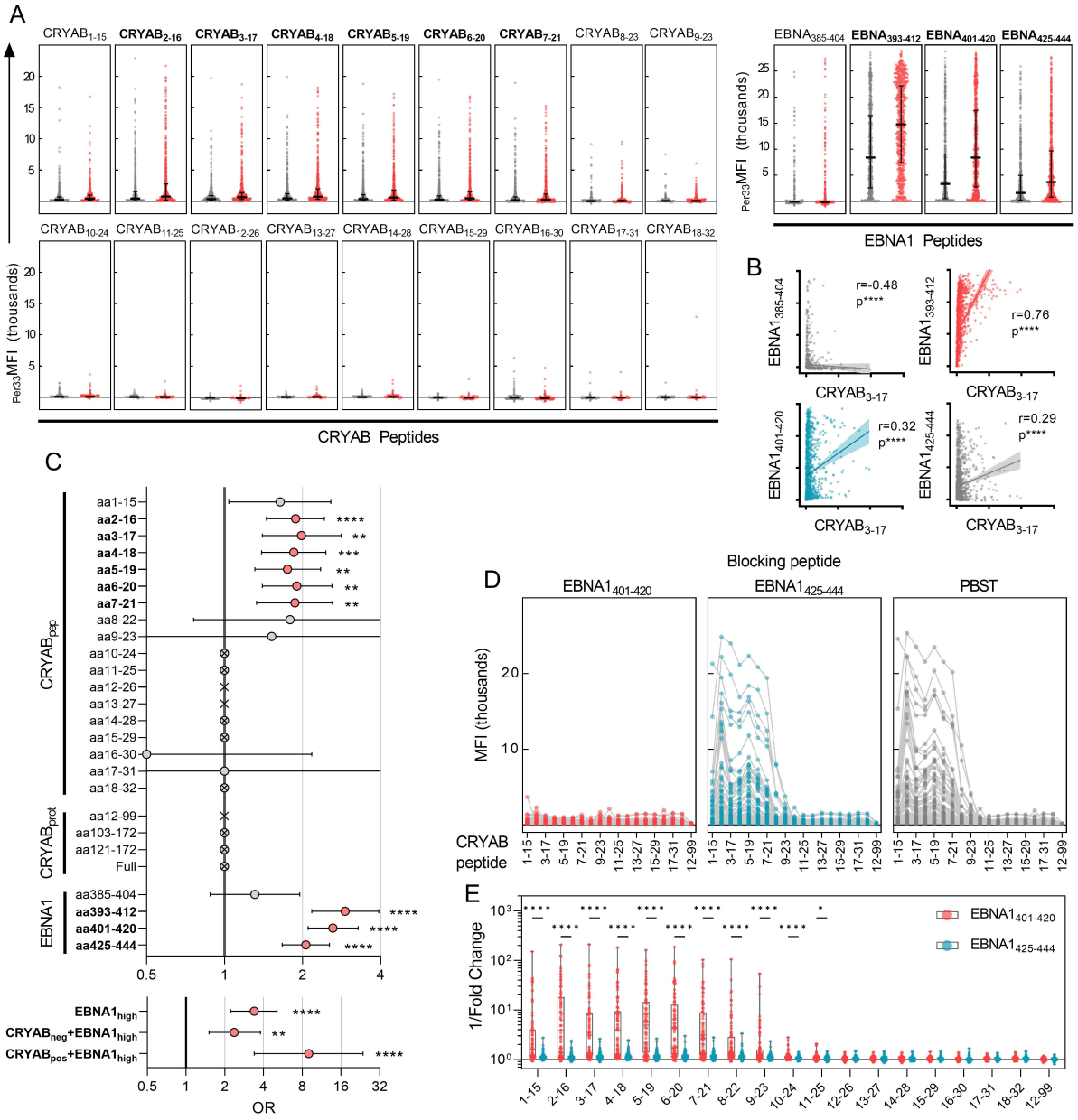

Figure 18. MS-association of cross-reactive anti-CRYAB and anti-EBNA1 antibodies. A) Anti-CRYAB and anti-EBNA1 antibodies in a cohort of pwMS and HC. **B**) Correlation between anti-CRYAB and different anti-EBNA1 antibodies. **C**) MS association of anti-CRYAB and anti-EBNA1 antibodies as well as combinations (bottom panel). Dots and staples denote ORs and 95% CI of OR. Uncalculatable ORs are marked as a cross, infinite ORs are marked as crossed circles. **D**) Anti-CRYAB reactivities after blocking with different EBNA1 peptides or buffer only (PBST). **E**) Fold-change of CRYAB-response after blocking with the two EBNA1 peptides. Higher value means more blocking effect. Adapted from **Paper IV**.

structure. For CRYAB, however, peptide-protein interactions have been demonstrated for smaller peptides [252,253], although peptide-antibody interactions specifically have not been demonstrated.

While these results were indicative of cross-reactivity due to molecular mimicry, as others have pointed out [250], direct evidence is missing. In **Paper IV**, it was obtained via antibody-blocking experiments (Figure 18D, E). Essentially all anti-CRYAB autoantibodies were depleted after blocking plasma with an EBNA1 aa401-420 peptide (which contains a core 5-aa homology, RRPFF), while responses were unaffected after blocking with an EBNA1 aa425-444 peptide. Significant blocking was detected from CRYAB peptides aa1-15 up to aa11-26, demonstrating that anti-EBNA1 antibodies cross-react with CRYAB via the shared homology RRPFF. This cross-reactivity against the homologous EBNA1 sequence, and not other immunodominant EBNA1 fragments, also demonstrate that the binding to CRYAB is antigen-specific and not due to specificity agnostic peptide-protein interactions.

While autoantibodies can be pathological in neurological autoimmune diseases [105], their relevance in MS pathogenesis remains more elusive than the T cells' role. However, high-affinity antibodies rely on T cells to help development. Additionally, the opposite relationship exists, as antigen-specific B cells can act as efficient APCs [254]. As such, we next investigated whether a similar cross-reactivity existed on the T cell side. In a mouse model, T cells were primed against EBNA1 via immunization and interrogated for their antigen-recall responses (Figure 19A). EBNA1-primed $CD4^+$ T cells responded to EBNA1 as well as CRYAB, which adjuvant-only primed $CD4^+$ T cells did not. In contrast, EBNA1 primed T cells did not respond to other MS-related autoantigens or CMV, indicative of a specific T-cell cross-reactivity between EBNA1 and CRYAB.

In MS-Nat, there were increased and highly correlated IFN-γ and IL-17A responses against both EBNA1 and CRYAB (Figure 19B, C). In contrast, polyclonal and CMV-responses were not increased and did not correlate. The increased EBNA1 and CRYAB responding T cells primarily consisted of central memory and effector memory $CD4^+$ T cells, meaning they were antigen-experienced (Figure 19D, E). In contrast, there were not increased EBNA1 or CRYAB responses in MS-Un. As discussed previously, natalizumab locks CNS-migrating T cells in the periphery. These cells may be particularly migratory and only present in frequencies below the assay detection limit in untreated pwMS. Interestingly, trends of lower tumor necrosis factor-alpha (TNF-α) responses against EBNA1 and CRYAB were observed in MS-Nat. TNF-α, while a proinflammatory cytokine, has been implicated as protective in MS [255].

Altogether, **Paper IV** supports the designation of CRYAB as an MS-related autoantigen, provides evidence that anti-CRYAB and anti-EBNA1 antibodies are cross-reactive, and makes a case for a similar cross-reactivity in the T-cell compartment as well. As such, it provides an additional piece explaining the EBV infection and MS link. The role of EBV as a driver of MS is supported by several

lines of evidence, like the striking epidemiological association [52] and dysregulated EBV immunity in pwMS [57]. Additionally, the fact that B cells constitute the main reservoir of EBV in chronic infection [45], B cells can activate brain-homing autoreactive T cells [106], EBV-infected B cells present CRYAB-derived peptides on HLA-DR [256], and the molecular mimicry between EBNA1 and CRYAB (and other autoantigens [59,117]) suggests that latent EBV infection might be an essential contributor to the B- and T-cell interaction in MS. In turn, this could be a central factor explaining the success of B-cell depletion therapies in recent years.

One reason for the controversy regarding the designation of CRYAB as an autoantigen is its evident therapeutic effect in neuroinflammation [162-164]. However, further studies have suggested a possibly dual role, where CRYAB in the presence of cytokines like IFN-γ is a proinflammatory mediator [165]. As such, the neuroprotective role under normal conditions and being an autoantigen are not necessarily exclusive phenomena and fits with previous observations.

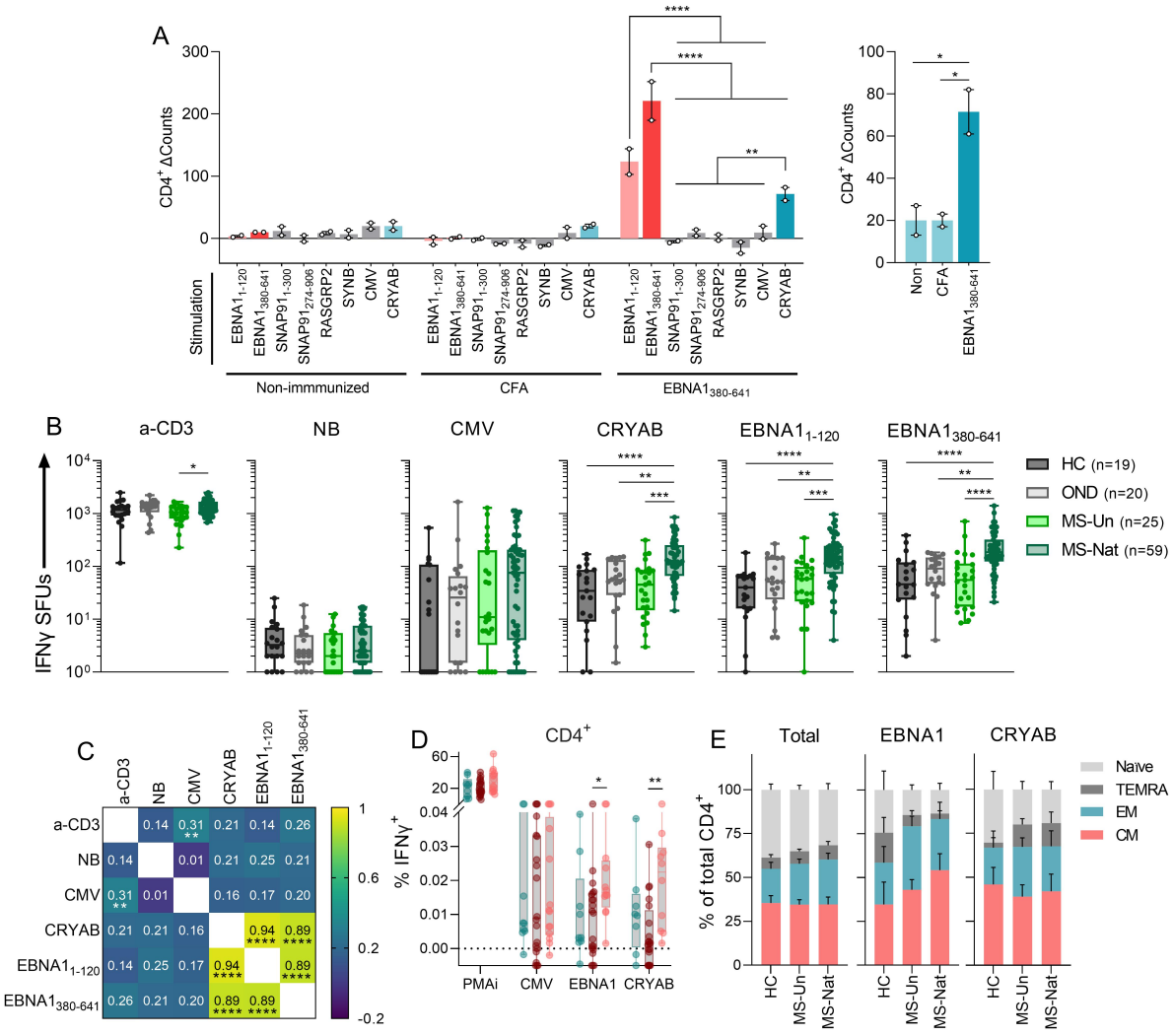

Figure 19. EBNA1 and CRYAB-reactive T cells. A) Recall stimulation of draining lymph node T cells from non-immunized, complete Freund's adjuvant or EBNA1 immunized mice. The right-hand panel shows the comparison of CRYAB responses. **B**) EBNA1- and CRYAB-reactivity in pwMS and controls **C**) Correlation matrix of the different responses in B. Numbers denote Spearman r and asterisks the p-value of the correlation. **D**) Intracellular cytokine staining of T cells after antigen stimulation in HC (n=9), MS-Un (n=20), and MS-Nat (n=14). **E**) Memory phenotype of bulk CD4⁺ T cells (left-hand panel) and antigen-stimulated IFN-γ⁺ CD4⁺ T cells (two right-hand panels). Adapted from **Paper IV**.

6 CONCLUSIONS

This thesis presents a novel methodology for detecting autoreactive T cells, identifies four new autoantigens, and strengthens the evidence regarding two controversial ones in MS. It suggests actionable targets which could be used for diagnostics and antigen-specific treatment strategies and highlights the immunological heterogeneity underpinning MS immunopathogenesis. Lastly, it demonstrates a mechanistic link between EBV infection and MS.

The key summarizing points from each respective paper are:

Paper I Antigen-coupled microbeads can stimulate and activate T cells with high sensitivity in follow-up assays.

Paper II HLA-DR restricted MOG-specific proinflammatory $CD4^+$ T cells are present in approximately half of persons with MS, strengthening MOG as an important autoantigen in MS. Antigen-coupled microbeads can be used to activate autoreactive T cells allowing for sensitive detection.

Paper III FABP7, PROK2, RTN3, and SNAP91 are encephalitogenic T-cell targeted autoantigens in MS. Autoreactive profiles in MS are highly heterogenous but can be utilized for diagnostics.

Paper IV Cross-reactive EBNA1 immunity targets CRYAB and is associated with MS, providing a mechanistic link between EBV infection and MS.

7 POINTS OF PERSPECTIVE

The presented novel method constitutes a powerful tool for identifying autoantigens and robust profiling of individual autoreactive profiles, which the autoimmunity research field has been lacking. As such, it is not necessarily limited to multiple sclerosis, but similar approaches could be used in other autoimmune diseases where autoreactive T cells play a role but where the target autoantigens are unknown, like amyotrophic lateral sclerosis.

As previously discussed, it also shows promise as a possible diagnostic tool, especially in confirming and ruling out MS. For clinical use, a method must be sensitive and specific but also pragmatic, i.e., cost- and time-efficient. The setup used in this thesis excels at these criteria. The workflow allows for the profiling of autoreactivity in 3 days, and the parallel analysis of several autoantigens and individuals means that costs are reduced. A rough calculation of associated material and staffing costs, albeit in optimal research conditions, lands the total sum for creating an autoreactive profile for one individual at around 2000 SEK, on par with commonly used diagnostic tools. However, a demonstration of real-world utility is needed, especially a prospective study of patient groups presenting at clinics where MS is a possible but not confirmed differential diagnosis.

Additionally, since this thesis' constituent papers were started, several additional autoantigens were reported, notably ANO2 [59,151], RASGRP2 [106], GDP-l-fucose-synthase [147], Beta-synuclein [149], and GlialCAM [117]. As a more high-dimension approach was superior in distinguishing between MS and non-MS, a more "complete" panel would likely perform even better. As such, the seven-autoantigens panel in **Paper III** should be expanded and re-evaluated. This project is now underway and will likely be completed in the coming year.

While diagnostics could be improved, good prognostic markers in MS lack even more. Better prognostics could help inform treatment decisions and be a step towards more personalized medicine. In this context, it would fit with the current paradigm of autoreactive T cells driving disease that persons with a higher degree of autoreactivity are at risk for a more aggressive disease course. Indicative of this, males, who on the average present with more aggressive disease, had relatively stronger autoreactive responses. However, no concrete conclusions could be drawn from this thesis due to the cross-sectional design of the studies. Nevertheless, the already performed high-dimension autoimmune profiling in 100 patients allows for exciting prospective follow-up studies investigating if autoreactivity could predict disease course. While not complete, this project has been initiated with some early indications of autoreactivity correlating with future disability progression and brain atrophy.

A possible avenue for antigen-specific treatment is using the autoantigen-bead panel to characterize individuals' autoreactive profiles. A tolerization panel could be tailor-made for that particular

individual's profile in a personalized fashion. While more laborious than off-the-shelf treatment, treating only the individually relevant autoantigens could potentially increase the benefit-to-risk ratio [74]. However, it could hypothetically be beneficial to induce tolerance to CNS-autoantigens in general, even if not an autoantigen (yet) for one particular individual, as a more tolerogenic milieu in the CNS and bystander tolerization could have an effect. Whether a general broad or personalized tolerization panel is superior should be evaluated in future trials.

An interesting and possibly troubling observation is that the screening identified four novel T-cell autoantigens, which was more than initially expected. Sixty-three proteins were included in the screening, with four identified candidates, which translates to a "hit rate" of ~6 %. While the screening-panel was extensive, it was not complete but based on the HPA data at the time. With their ongoing detailed protein expression mapping, HPA's new "brain atlas" lists as many as 202 brain-selective and 2685 brain-elevated genes at the time of writing. As our screening panel was mostly unbiased in composition, one can reasonably assume a similar "hit-rate" among other brain-selective proteins or possibly even brain-elevated. The actual MS autoantigen repertoire could, as such, contain anywhere from 10 to >100 additional targets. A second, even broader screening is now underway to investigate this further, which includes an additional 100 CNS proteins. However, non-CNS autoantigens have also been implicated in MS, complicating matters even more [106,147].

The troubling part is what this means for antigen-specific treatment. The main hypothesis for why such treatment strategies have been comparatively worse in MS compared to animal models is that too few disease-driving autoantigens have been targeted [74]. If there are >100 possible autoantigen targets, do treatments need to target all of them to have efficacy? If so, functional antigen-specific treatments seem practically impossible to achieve. Luckily, the answer is likely no for two reasons. First, a small population of autoreactive T cells is a normal part of the healthy immune system [132,136,140], and some rare cells are likely not enough to overcome tolerogenic barriers and drive disease. Supporting this is also the long subclinical prodromal phase in MS where, hypothetically, an initial immunological insult starts an immunological cascade involving some slow underlying processes, like epitope spreading [131,134], ultimately leading to MS. This is underscored by the 5–10-year gap from EBV-infection to first MS symptoms [52]. In **Paper III**, we present indications that the breadth of autoreactivity is pivotal, as single autoreactivities did not discriminate between MS and HC, but several different ones did. Likely, MS pathogenesis hinges on the sum of autoreactivities to overcome the tolerogenic threshold and targeting a large enough proportion could be sufficient. Secondly, there are indications that bystander tolerization is at play [257], where inducing tolerance against one autoantigen can also lead to tolerance to others. Still, more targets than have been used so far would likely result in higher efficacy [258], and the autoantigens reported in this thesis constitute promising targets.

The recent advances in our understanding of EBV infection and MS development, partly presented in this thesis, raise other implications in treating or even preventing MS development altogether. If EBV infection is a prerequisite for MS, an effective EBV vaccination could be a potential prophylactic treatment for MS (and other EBV-associated neoplastic diseases), like the human papillomavirus vaccine is for cervical cancer. However, it has not proven easy to develop a vaccine to prevent infection [259], although there are promising studies demonstrating it could be possible [260]. It is further complicated by the fact that a significant portion of those developing MS was infected in early childhood, meaning vaccination must occur at a young age. Also, the delay between vaccination and outcome in studies could be several decades, requiring enormous research investments [259]. Lastly, a lesson harshly learned by the swine-flu vaccination (Pandemrix®) and narcolepsy [261], molecular mimicry could pose a problem in vaccination [120], which is emphasized in MS by the known cross-reactivities and several other distinct MS-associated EBV-epitopes (which are not limited to EBNA1 [52]), where unknown cross-reactivities could potentially reside.

Altogether, the findings presented in this thesis have several exciting and promising implications and future lines of research, from pathogenesis to diagnostics, prognostics, and treatment.

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