Diploma Thesis

Effect of anti-CD20 therapy on cortical demyelination in a new rat model

submitted by

Jana Maria Dohrmann

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Assoz. Prof.in Priv.-Doz.in DDr.in Sonja Hochmeister

Dr.in BSc MSc Michaela Haindl

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Statutory declaration

I hereby declare that I have authored this thesis independently and without any support from third parties, that I have not used other than the explicitly marked sources, and that I have explicitly marked all material which has been quoted either literally or by content from the used sources.

Graz, 15.12.2020 Jana Maria Dohrmann eh

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Abbreviations

APC	antigen-presenting cell
BBB	blood-brain barrier
BCR	B-cell receptor
CAB	control antibody
CD	cluster of differentiation
CIS	clinically isolated syndrome
CNS	central nervous system
CSF	cerebrospinal fluid
DA	Dark Agouti
DGN	Deutsche Gesellschaft für Neurologie
DMT	disease-modifying therapy
DIS	dissemination in space
DIT	dissemination in time
EAE	experimental autoimmune encephalomyelitis
EBV	Epstein-Barr virus
EDSS	Expanded Disability Status Scale
FLAIR	fluid-attenuated inversion recovery
G1	group 1
G2	group 2
HC	healthy control
HLA	human leukocyte antigen
IFA	Incomplete Freund’s Adjuvant
IFN-γ	interferon-gamma
IHC	immunohistochemistry
IM	intramuscular
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
MS	multiple sclerosis
NFL	neurofilament light chain

PBS	phosphate buffered saline
PLP	proteolipid protein
PPMS	primary progressive multiple sclerosis
RAB	Roche antibody
RRMS	relapse-remitting multiple sclerosis
SC	subcutaneous
SPMS	secondary progressive multiple sclerosis
TCR	T-cell receptor
TNF-α	tumor necrosis factor-alpha

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Abstract

Background

Cortical demyelination is a remarkable feature of multiple sclerosis (MS), especially in the late progredient phase, and is believed to be a substrate for the diffuse cognitive disorder. In clinical trials, B-cell depleting anti-CD20 antibody therapy has proven to be effective in the relapse-remitting as well as the early progressive stage of MS. However, if this therapy can prevent the development of cortical pathology is yet unknown.

Objectives

We recently created a new rat model (Üçal et al, 2017) that is adequate for research on cortical demyelination and affiliated cellular signs in progressive MS. The aim of this study was to explore the impact of anti-CD20 therapy on the formation of cortical lesions in our new rat model and thus to increase the knowledge for the modus operandi of B-cells.

Methods

Rats were implanted with a catheter into the cerebral cortex, immunized with myelin oligodendrocyte glycoprotein (MOG) and injected with pro-inflammatory cytokines to stimulate cortical demyelination. We set up two experimental groups in which anti-CD20 therapy was applied either after or before MOG immunization. Animals were sacrificed at peak disease and brain matter was histologically analyzed.

Results

Histological analysis of the different cellular markers discovered that anti-CD20 therapy averted cortical pathology showing significant diminution in demyelination, microglial activation, apoptotic cells, astrocytic reactivity and neuronal loss in anti-CD20 treated animals in contrast to animals given an isotype-matched control antibody. Both experimental groups showed equal efficacy. While there is a significant difference between the healthy control (HC) and the control antibody groups, the difference between the HC and the anti-CD20 antibody groups did not

reach significance indicating similarity of the anti-CD20 treated animals to the healthy animals.

Conclusions

Our findings suggest a favorable impact of anti-CD20 therapy on conservation of the investigated structures indicating a contribution of B-cells in the development of cortical pathology, probably through various pathways. These promising results provide the basis for further research on the mechanism of tissue damage in the late phase of MS and might help to expand therapeutic schemes for progressive MS patients or even prevent progression in the first place.

Zusammenfassung

Hintergrund

Die kortikale Demyelinisierung ist ein bedeutendes Merkmal der Multiplen Sklerose (MS), insbesondere im späten progredienten Stadium. Dabei wird angenommen, dass sie eine der Hauptursachen für die diffuse kognitive Beeinträchtigung ist. In klinischen Studien hat sich die Therapie mit anti-CD20 Antikörpern sowohl in der schubhaften als auch in der frühen progressiven Phase der MS als wirksam erwiesen. Ob diese Therapie die Ausbildung von kortikaler Pathologie verhindern kann, ist jedoch noch nicht bekannt.

Ziele

Wir entwickelten kürzlich ein neues Rattenmodell (Üçal et al, 2017), das für die Erforschung der kortikalen Demyelinisierung und der damit verbundenen zellulären Merkmale der progressiven MS geeignet ist. Ziel dieser Studie war es, die Wirkung der anti-CD20 Therapie auf die Entwicklung kortikaler Läsionen in unserem neuen Rattenmodell zu untersuchen und somit das Verständnis für die Wirkungsweise von B-Zellen zu verbessern.

Methoden

Den Ratten wurde ein Katheter in die Großhirnrinde implantiert und nach erfolgter Immunisierung mit Myelin-Oligodendrozyten-Glykoprotein (MOG) wurden, um eine kortikale Entmarkung zu induzieren, proinflammatorische Zytokine injiziert. Wir erstellten zwei Versuchsgruppen, in denen die anti-CD20 Therapie entweder nach oder vor der MOG-Immunisierung verabreicht wurde. Die Tiere wurden zum Zeitpunkt der am meisten zu erwartenden kortikalen Demyelinisierung getötet und das Gehirngewebe histologisch aufgearbeitet.

Ergebnisse

Die histologische Analyse der verschiedenen zellulären Marker ergab eine signifikante Reduktion der kortikalen Demyelinisierung, der Mikroglia-Aktivierung, der apoptotischen Zellen, der Astrozytenreaktivität und des neuronalen Verlusts bei mit anti-CD20 Antikörpern behandelten Tieren verglichen mit Tieren, die mit einem

unspezifischen Kontrollantikörper behandelt wurden. Beide Versuchsgruppen zeigten die gleiche Wirksamkeit. Es gab einen signifikanten Unterschied zwischen der gesunden Kontrollgruppe (HC) und der Kontrollantikörpergruppe, nicht jedoch zwischen der HC und der anti-CD20 Antikörpergruppe, was auf eine Ähnlichkeit der mit anti-CD20 behandelten Tiere mit den gesunden Tieren hinweist.

Schlussfolgerungen

Unsere Ergebnisse deuten auf einen positiven Effekt der anti-CD20 Therapie auf die strukturelle Erhaltung der Großhirnrinde hin. Diese vielversprechenden Resultate ebnen den Weg für weitere Forschung zum gewebsschädigenden Mechanismus in der Spätphase der MS und könnten dazu beitragen, die therapeutischen Optionen für progressive MS-PatientInnen zu verbessern oder sogar das Fortschreiten der Erkrankung zu verhindern.

1 Introduction

1.1 Multiple sclerosis

Multiple sclerosis (MS), also termed as encephalomyelitis disseminata, is an immune-mediated disease of the central nervous system (CNS). It is characterized by inflammatory lesions disseminated throughout the brain and spinal cord, accompanied by demyelination and neurodegeneration.(1)
Its definite etiology remains yet to be unknown. Implicated in the development of the disease is a genetic predisposition for autoimmunity (2) as well as several environmental risk factors acting as triggers, e.g. low amounts of vitamin D, infection with Epstein-Barr virus (EBV), smoking and obesity.(3–6)

1.1.1 Clinical signs and symptoms

Depending on the location of inflammatory and degenerative processes in the CNS, a variety of neurological symptoms is possible. First clinical presentations are often alterations in sensibility and vision, then progressing to variable disorders in mobility, cognition and sphincter function.(1) The Uhthoff phenomenon describes temporary worsening of symptoms during heat or physical exertion which can be explained by higher sensitivity of signal transduction in demyelinated fibers at higher temperatures.(7) MS is one of the main reasons for non-traumatic irreversible neurological disablement in young to middle-aged adults when not being treated.

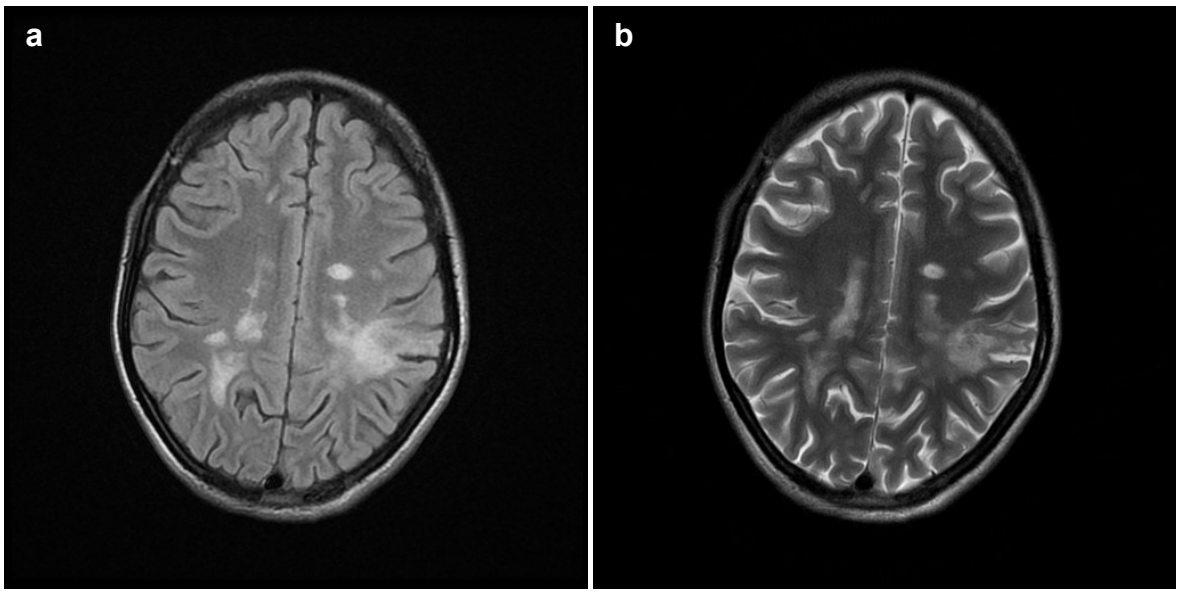


Fig. 1 MRI scans showing multiple hyperintense areas of the white matter in a MS brain. The same axial image is depicted as FLAIR (fluid-attenuated inversion recovery) in panel **a** and T2-weighted in panel **b**. Case courtesy of Dr. Kewal Arunkumar Mistry, Radiopaedia.org, rID: 34939.

1.1.2 Diagnostic tools

The diagnosis of MS is made in reference to clinical signs, characteristic results in cerebrospinal fluid (CSF), e.g. oligoclonal IgG-bands and raised protein, and blood testing, as well as evoked potentials and manifestation of typical focal MS lesions in the CNS detected by magnetic resonance imaging (MRI) of which an example can be seen in Fig. 1.(8,9) Imaging plays an important role in identifying acute disease activity, but also evaluating the course of the disease over time. Proof of spatial and temporal dissemination of the plaques is fundamental as intended to mean more than one clinical episode with more than one CNS region involved.(10) Biomarkers can provide supporting diagnostic references to neurodegenerative diseases such as MS. Neurofilament light chains (NFL) as early markers of neuronal damage in serum have been shown to be predictive for disease activity. They correlate with T1 and T2 lesions as well as volume alterations in MRI and could possibly be used for therapy planning to minimize irreversible destruction; however NFL is currently not yet included in clinical routine diagnostic workup.(11,12)
The revised 2017 McDonald diagnostic criteria, as seen in Table 1, allow standardized identification based on MRI findings and clinical syndromes. Unlike the 2010 Mc Donald diagnostic criteria, no differentiation between symptomatic and asymptomatic MRI lesions is demanded. The criteria guide towards early clinical diagnosis, hence early induction of therapy resulting in optimized long-term conditions for MS patients.(9,13)

Clinical appearance	**Further evidence required for MS diagnosis**
≥ 2 clinical attacks **and** ≥ 2 objective clinical lesions or ≥ 2 clinical attacks **and** 1 objective clinical lesion **and** historical data of a prior	None

clinical attack involving a lesion in another anatomic location	
≥ 2 clinical attacks **and** 1 objective clinical lesion	Dissemination in space (DIS), shown by: • an additional clinical attack implicating a different CNS site • or MRI
1 clinical attack **and** ≥ 2 objective clinical lesions	Dissemination in time (DIT), shown by: • a second clinical attack • or MRI • or presence of CSF-specific oligoclonal bands
1 clinical attack **and** 1 objective clinical lesion	DIS, shown by: • a second clinical attack implicating a different CNS site • or by MRI **and** DIT, shown by: • a second clinical attack • or by MRI • or presence of CSF-specific oligoclonal bands
Disease course defined by progression from onset (primary progressive MS)	One year of disability progression apart from clinical relapse **and** 2 out of 3 of the following criteria: • ≥ 1 T2-hyperintense lesions in ≥ 1 areas in the brain typical of MS (periventricular, cortical/juxtacortical, infratentorial) • ≥ 2 T2-hyperintense lesions in the spinal cord • presence of CSF-specific oligoclonal bands

Table 1 The revised 2017 McDonald criteria for MS diagnosis. DIS can be shown by ≥ 1 T2 lesions in ≥ 2 of the following areas of the CNS: periventricular, cortical/juxtacortical, infratentorial and spinal cord. DIT can be shown by concurrent appearance of gadolinium-enhancing and non-enhancing

lesions at any time or a new T2 and/or gadolinium-enhancing lesion on follow-up MRI, with respect to a baseline scan, regardless of the timing of the baseline MRI. Adapted from Thompson AJ et al, 2018.

1.1.3 Classification

Generally, the disease is classified into relapse-remitting (RRMS) and progressive forms. RRMS being the most common phenotype (85% of total cases) is defined by relapsing occurrence of acute neurological deficits remaining at least 24 hours which can recover completely or partially. After 5-15 years of disease, this type changes to a progressive form in around 50% of the patients, defined as secondary progressive MS (SPMS). Disease and disability progression may or may not be accompanied by clinical relapses. The primary progressive form (PPMS) in contrary is described as a gradually progredient accumulation of disability from the beginning without previous relapses and affects 15% of all MS patients (8), whereas the so-called clinically isolated syndrome (CIS) is characterized by a single attack conforming to relapse, e.g. optic neuritis or brainstem syndrome. MRI can then give information about the potential risk of developing MS and evidence of oligoclonal bands in CSF proof of temporal dissemination.(14,15)

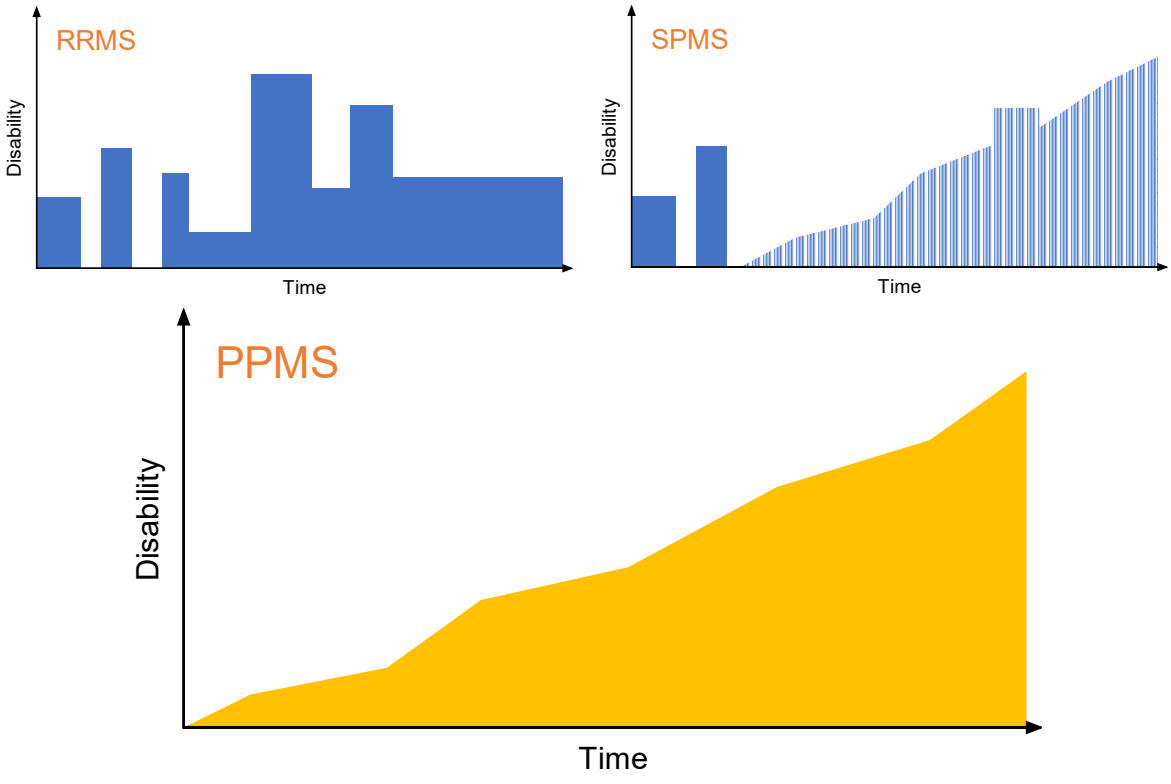


Fig. 2 Progression charts of the different types of MS. RRMS picturing unpredictable attacks followed by periods of complete or partial remission. SPMS representing initial relapse-remitting MS that suddenly starts to have decline without periods of remission. PPMS showing constant raise in disability over time without attacks. Adapted from Lublin FD et al, 2014.

Prognosis on an individual level at the beginning of the disease is hardly possible. The initial total number of T2 lesions correlates only moderately with risk of progression.(16) In patients with RRMS, time of transition to SPMS is the determining factor for long-term prognosis. On the contrary, spinal lesions at disease onset, symptomatic or not, increase the risk of developing PPMS.(17,18) Also the amount of cortical lesions is demonstrably associated with risk of progression and accumulating disability.(19)

1.1.4 Epidemiology

While RRMS is often diagnosed between the ages of 20 and 40 years with a female to male distribution being 3:1, PPMS has an equal sex ratio with an age peak at 40 years or older at onset.(20,21) Due to socioeconomic factors, incidence of MS is increasing globally with an estimated rate of 19.5/100,000/year in Austria. At least 2.3 million people worldwide are being affected by MS with North America and Europe having the highest prevalence, in Austria stating 158.9/100,000 persons.(22,23) MS can be seen as the most prevalent chronic-inflammatory disease of the CNS.

1.2 Pathogenesis

Since the first comprehensive description of the disease, the ‘Histologie de la sclérose en plaques’ by Jean-Martin Charcot from 1868, the process of inflammation and pathogenetic mechanisms of MS have become more understandable during the last 30 years. The original belief of a purely T-cell mediated autoimmune disease was expanded when B-cell targeted therapies showed beneficial effects.(24) It is now known that active demyelination and chronic neurodegeneration link to inflammation induced by T-cells, B-cells and plasma cells,

macrophages and activated microglia.(25) Lymphocytic infiltration in the CNS is often found in periventricular regions of the brain (26), but also along the optic nerve, brain stem, cerebellum and spinal cord and can be seen in MRI as T2-hyperintense lesions.(27) With oligodendrocytes and neurons being the main target cells, the autoimmune response results in damaged myelin and axons with glial scars presenting as typical demyelinating sclerotic plaques which in turn can inhibit the transmission of nerve impulses.(28) These lesions are mostly located in white matter, however gray matter lesions also exist. There are distinctive demyelinating plaques in cortical areas with contiguous leptomeningeal inflammation.(29) So-called ‘shadow plaques’ are eventually formed by proliferation of astrocytes (gliosis) and partial remyelination of surviving exposed axons by extant oligodendrocytes.(1)

1.2.1 Inflammation and neurodegeneration

MS shows a triphasic pathology.(30) The early phase driven by peripherally triggered inflammation is explicated by blood-brain barrier (BBB) disruption and new focal lesions in white and gray matter. With increasing disease chronicity, the inflammatory processes take place behind an already repaired BBB.(29,31) In the last stage, accumulated brain damage leads to age-related neurodegeneration and further progression even in the absence of inflammation.(25,30)

It can be discussed whether inflammation precedes neurodegeneration or neurodegeneration represents the actual pathophysiological process in MS.(1,32) Trapp et al. have shown in 1998 that axonal degeneration in chronic active MS lesions was associated with the extent of inflammation.(28) More recent publications in contrast primarily provide evidence on non-inflammatory causes of neurodegeneration, which means that axonal pathology is the surrogate for irreversible clinical disability and not necessarily for inflammation.(33–35)

The effects of demyelination, incomplete remyelination and gliosis seem to make surviving axons more vulnerable to further damage. Irreversible chronic neurodegeneration prevailed by microglial activation correlates to disease progression and leads to loss of function and structure.(36) Age modifies the response of the CNS to inflammation; from age 38-45 years, myelination slows down and the capacity of remyelination decreases.(37,38)

1.2.2 CD4+ and CD8+ T-cells

Peripheral T-cells are directed against endogenous components as part of molecular mimicry as well as misguided selection mechanisms.(39) T-cell receptors (TCR) in cell membranes then recognize endogenous antigens as extraneous molecules when they are tied to the MHC-peptide on the surface of other cells or presented as free antigens by antigen-presenting cells (APC), e.g. B-cells, dendritic cells and macrophages.(40) Autoreactive CD4+ T-cells are a central pathogenic element in MS. Not only having direct cytotoxic functions in inflammatory tissue, they are also of great importance in the adaptive immune system. In their reaction, they turn against myelin autoantigens by producing cytokines, such as IFN-γ, as part of the type 1 helper (Th1) mediated response. Other subtypes, e.g. Th22, Th17 and Treg also play an important role in the immunopathogenesis of MS.(39–41) Targets in the myelin are certain peptides of myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) leading to inflammation.(40) The distribution of cytokines causes activation of macrophages as well as recruitment of leukocytes which eventually results in demyelination. Myelin-specific CD8+ T-cells are even more plentiful in MS lesions, possibly being responsible for most of the tissue damage.(31,41) They are activated by oligodendrocytes and neurons expressing HLA I-molecules that bond the antigen to their specific TCR.(41) Those cytotoxic cells irrupt the CNS through the BBB causing a local inflammatory reaction which leads to BBB disruption and disorder of permeability, subsequently stimulating an invasion of the CNS with more inflammatory and humoral components such as complement factors and antibodies.(39)

1.2.3 B-cells

B-cells are an essential element of the adaptive immune system having manifold tasks concerning the immune response. They originally evolve from stem cells in the bone marrow and develop further to memory B-cells or plasma cells within follicles of secondary lymphatic tissue, e.g. spleen, lymph nodes and mucosa. Autoreactive B-cells are normally being stopped but in MS pathology, they manage to survive and disrupt the BBB.(42,43) The four mechanisms of B-cell

pathophysiology (Fig. 3) are antigen presentation, antibody production, cytokine regulation and formation of new lymphatic structures in the meninges which all show a great impact on MS pathogenesis.(24,44)

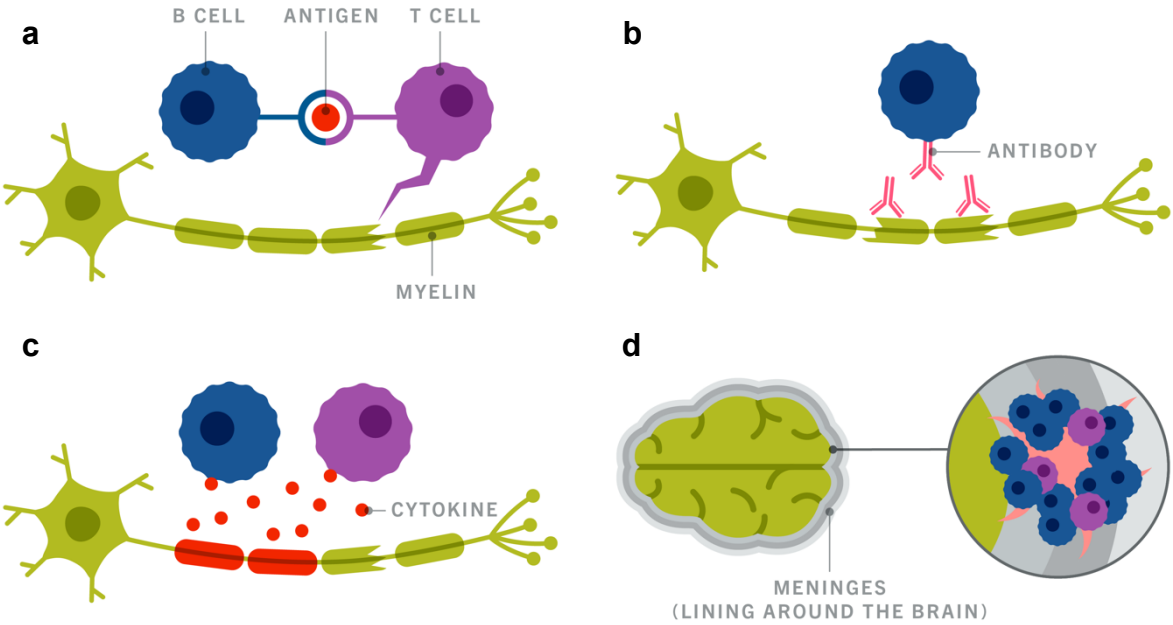


Fig. 3 The role of B-cells in MS pathogenesis. Activation of other immune cells, e.g. T-cells, by presenting antigens (**a**). Antibody production (**b**) to directly attack myelin structures. Release of cytokines (**c**) that stimulate inflammatory activity in the CNS. Formation of immune infiltrates in the meninges (**d**) to sustain the neuronal damage. © 2020 Genentech, USA Inc. Taken from https://www.gene.com/media/product-information/ocrevus.

After passing the BBB, B-cells are being stimulated, matured and clonally expanded in the CNS.(42) They are traceable especially in active lesions; in patients with chronic progredient forms of MS notably in ectopic lymphoid follicle-shaped structures in the meninges.(45) Besides these structures, activation of microglia, local inflammatory reaction and neuronal tissue damage in the proximate cortex can be found.(46)

Unlike other APC, B-cells selectively detect antigens by means of surface B-cell receptors (BCR) which are antibodies, specifically immunoglobulin M. The further activation, proliferation and somatic hypermutation of BCR result in memory B-cells and plasma cells producing antibodies.(42,47) A small number of these cells is able to conquer the BBB, resting in protected niches of what is seen a significant concept for progredient MS. Them producing antibodies further lead to neuronal tissue damage and activation of macrophages and natural killer cells.(48)

Autoreactive B-cells inside the CNS activate T-cells in presenting autoantigens (MOG, MBP) to them as well as producing pro-inflammatory cytokines, e.g. lymphotoxin, TNF-α and IFN-γ, which in turn results in an increased inflammatory response of more immune cells, e.g. T-cells.(47,49)
As an expression of this, in more than 90% of MS patients, proof of oligoclonal bands in CSF works as a diagnostic tool. These are produced by a small group of B-cell clones and accumulate in CSF.(50)

1.2.4 Evidence of CD20

The term ‘cluster of differentiation’ (CD) describes grouped immunophenotypic cell-surface marks that can be classified by biochemical or functional criteria. Such CD molecules consist of membrane-bound glycoproteins and take on receptor or signal function as well as intercellular communication. Specific CD are expressed at different times of B-cell maturation playing an important role in B-cell differentiation, signal transfer during antigen response and regulation of cytokine production.
CD20 is a special marker on the surface of various subgroups of B-cells. It is expressed in all maturation processes of B-cell development except for the first and last stage, e.g. stem cells, early B-cells and plasma cells, which is illustrated in Fig. 4.(51) CD20-positive B-cells in particular are a main element in MS pathogenesis since their targeted depletion has proven to be effective in suppressing disease activity. CD20 is eventually found on circulating T-lymphocytes including CD4+ Th1-cells, CD8+ cytotoxic cells, naive and memory T-cells. In comparison to CD20-positive B-cells, they own a lower surface density of CD20.(42,52,53)

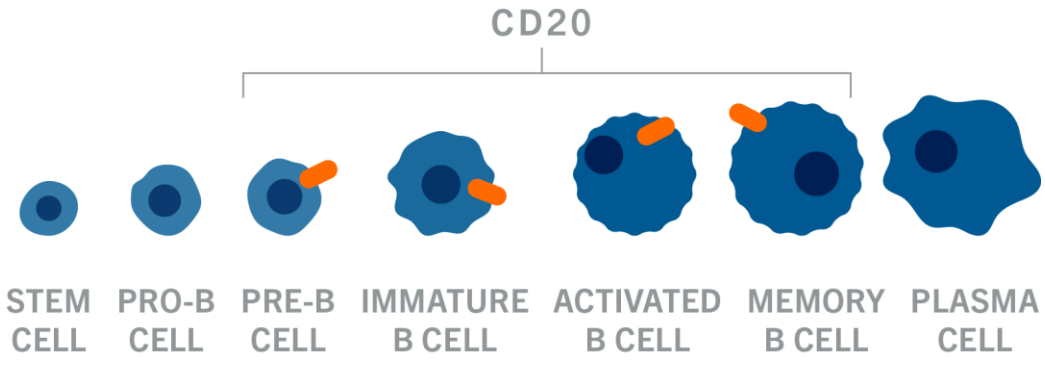


Fig. 4 Scheme of CD20-positive expression during B-cell development. CD20 molecules are located on the surface of pre-B-cells, immature B-cells, activated B-cells and memory B-cells. © 2020 Genentech, USA Inc. Taken from https://www.gene.com/media/product-information/ocrevus.

1.2.5 Cortical demyelination

Demyelination of the cortex is a remarkable feature in patients with MS, notably in the late progredient stage of the disease, contributing to progression and overall disability in the course of the disease. The specific pathological mechanism leading to such cortical lesions is yet not fully explained. They are located in cortico-subcortical, intracortical or most commonly subpial areas.(33,54) Based on current information, cortical demyelination is a substrate for diffuse cognitive impairment which is frequently found in the progressive form of MS.(55,56)

There is a histologically significant difference between cortical lesions and white matter lesions, with relatively sparse cellular infiltrates, broad microglial activation and apoptotic neurons in cortical pathology.(54,57) Whether the reason being different disease mechanisms or same basic mechanisms modified by local microenvironmental influences is currently under discussion. However, inflammatory cellular infiltrates containing lymphocytes and macrophages are seen around small or medium-sized blood vessels consequently forming typical white matter lesions. Cortical demyelination in turn underlies an infestation of antibodies, complement factors or other unknown inflammatory mediators.(58–60)

The theory of subpial demyelination comprises the subarachnoid space adjoining the cerebral meninges creating a microhabitat of low CSF circulation ergo restraining inflammatory cells and following lymphoid organization.(61–63) Their approach to produce pro-inflammatory cytokines, e.g. IFN-γ and TNF-α, creating a permanent inflammatory environment, eventually disturbs cortical tissue underneath and leads to subpial demyelination. Such increased IFN-γ and TNF-α levels can be detected in the CSF of MS patients, specifically at the time of an acute relapse.(64)

The main effect of demyelination is the interrupted electric transmission of nerve impulses that leap from one node of Ranvier to the next. This disconnection is possibly due to exposed potassium channels, usually covered by myelin, following

axonal hyperpolarization. Resulting in a redistribution of sodium channels, originally placed at the nodes of Ranvier, along the bare axon, the possibility of continuous forwarding of action potential within the demyelinated nerve segment is given.(1,46) When acute inflammation and edema in the area of the lesion and its surrounding tissue fade, remyelination and further on clinical remission can happen. Transduction however is mostly slow and repeated demyelination results in even slower signal conduction in the CNS over time.(46)

1.3 Treatment

According to the current state of knowledge, MS is yet an uncurable disease. Many patients first diagnosed with MS still fear to soon become dependent on a wheelchair. Such dramatic disease progress is nowadays quite rare. An increasing amount of different medication has become available over the past years and can delay, respectively favorably influence the progress of the disease. They can further reach improvement of quality of life by preventing strong restrictions. Because of the individually different course of the disease, a holistic approach to therapy that covers the whole patient and spectrum of symptoms is fundamental. Thus, the combination of physiotherapy, speech therapy, ergotherapy as well as psychotherapy and neuropsychological training fulfils the complex all-around treatment of symptoms. This requires a multidisciplinary and individualized approach. Including drug and non-drug therapy, the therapeutic goal is to preserve and improve the functional capacity of the patient and thus the quality of life.(65,66) Therapeutic concepts are in general distinguished between treatment of an acute relapse, disease-modifying therapy (DMT) and symptomatic therapy. Disease activity and number of relapses in RRMS can be reduced with long-term immunotherapy. There are various substances available to influence the immune system, extend symptom-free intervals in between relapses and reduce formation of new glial scars in the brain. They are individually applicable in its dose and frequency.(14)

Acute relapses are usually treated with highly dosed corticosteroids (cortisone pulse therapy), e.g. 1000 mg methylprednisolone, intravenously applied over 3-5 days.(67) They shorten the period of symptoms, decrease inflammation, seal the BBB, improve nerve conduction and cause changes to the immune system. In

patients with acute and severe neurological disorders that do not respond to corticosteroids, plasmapheresis as second choice can be useful.(14)

The group of disease-modifying therapies (DMT) shows anti-inflammatory, immune-modulating or immunosuppressive effects.(66) Treatment is also adjusted to the degree of severity, whether there is a mild/moderate form or highly active disease course. In addition to this, therapies are classified in first, second or third choice according to risk-benefit ratio. Currently authorized agents are shown in the following chart (Table 2).

Relapse treatment		
1st choice • Methylprednisolone (pulse therapy) *2nd choice* • Plasmapheresis		
Disease-modifying therapy		
	Mild/moderate form	Highly active form
CIS	• Glatiramer acetate • Interferon Beta-1a (IM) • Interferon Beta-1a (SC) • Interferon Beta-1b (SC)	
RRMS	• Dimethyl fumarate • Glatiramer acetate • Interferon Beta-1a (IM) • Interferon Beta-1a (SC) • Interferon Beta-1b (SC) • Teriflunomide	*1st choice* • Alemtuzumab • Cladribine • Fingolimod • Natalizumab • Ocrelizumab *2nd choice* • Mitoxantrone *3rd choice* • Experimental procedures, e.g. autologous stem cell therapy

SPMS		With relapses: • Interferon Beta-1a (SC) • Interferon Beta-1b (SC) • Mitoxantrone Without relapses: • Mitoxantrone
PPMS		• Ocrelizumab

Table 2 Therapeutic recommendations for MS. If DMT fails in mild/moderate forms of MS, patients are treated like highly active forms. Agents in alphabetical order; their presentation inside one box does not imply any superiority of one substance over another. Modified according to the DGN guideline 'Diagnosis and therapy of multiple sclerosis' from 2014. Safety notice: This current guideline is out of date due to safety problems when using Alemtuzumab and Natalizumab. An update is in progress and will be published by the end of 2020.

In RRMS, it is recommended to begin therapy as early as possible because chances of having a beneficial impact on the long-term course of the disease are then high. Amount and severity of relapses can be reduced in an early phase and transition into SPMS can efficiently be delayed or even prevented compared to starting therapy late. Highly active agents were found to be more effective than 'basic therapeutics'.(68)

1.3.1 Therapeutic options in progressive MS

The described treatment options above however do not work equally well in patients with (late) progressive forms of MS. Therapeutic options are still not sufficient in this field. B-cell depleting therapies, e.g. anti-CD20 antibodies, have shown positive effects in relapse-remitting forms as well as the early progressive phase of MS in clinical trials.(52,69) But if this kind of therapy is able to prevent the accumulation of cortical pathology is yet unknown.

MS pathogenesis is already in an early state characterized by concurrent and interactive inflammatory and neurodegenerative processes of the same progressive disease. Subclinical inflammatory disease activity consequently leads to fast accumulation of disability regardless of relapse activity.(1,25) Here, the latest

therapeutic strategy is with early use of highly active DMT to preserve neuroplasticity of the brain as far as possible to delay disability as long as possible. Current clinical trials show promising effects of various anti-CD20 depleting agents, such as Rituximab, Ocrelizumab and Ofatumumab. One of them is already recommended for PPMS: Ocrelizumab is used for short disease duration and active progression, interpreted by the Expanded Disability Status Scale (EDSS) and MRI findings. As a fully humanized anti-CD20 monoclonal antibody, it bonds with CD20 of B-cells and specifically depletes CD20-positive B-cells and their pro-inflammatory features. Long-term data is building confidence in Ocrelizumab being a fast and highly effective therapy for patients with PPMS and also RRMS.(53,70) Clinical evidence for its efficacy and reliability were published on the basis of long-term data of more than 5 years following the ORATORIO trial for treatment of PPMS with Ocrelizumab.(70) Current evaluation of extension studies of ORATORIO shows that the risk of confirmed disease progression over at least 48 weeks can be significantly reduced by an early treatment with Ocrelizumab throughout the entire observation period. Ocrelizumab thus favorably influences the late extent of impairment and consequently the quality of life in the long run.(71)

All substances presently used off-label in treating progredient MS have mainly anti-inflammatory effects. In fact, two big cohort studies throw no favorable light on anti-inflammatory therapies of the progressive form. These have not shown any substantial short or medium-term effect on disability progression in the course of PPMS or SPMS, with the exception of Ocrelizumab.(72,73) Proven therapeutic effects can be found in an overview of trials concerning the progredient form.(74)

1.3.2 Review and outlook

There is definitely more evaluation in the effectiveness of drugs, especially B-cell depleting therapies, needed. Research into medicines requires animal models for initial testing before venturing on clinical trials. Common animal models that have been used for decades, such as experimental autoimmune encephalomyelitis (EAE), are able to reproduce white matter lesions of RRMS quite well, which is why therapies could be tested in this regard. On the other hand, progredient MS showing more cortical lesions was hardly being represented. Therefore, we developed the following animal model.

1.4 Common animal models of MS

The most commonly used animal model in MS research is EAE which can be stimulated in rodents, often in mice and rats. Usually, active immunization with myelin protein components, such as MOG or MBP, leads to an ascending paresis starting from the tail and hind limbs and can, in severe protocols, even lead to a tetra paresis of the experimental animal. Histologically, this is shown by white matter lesions with heavy cellular infiltration, predominantly in the spinal cord. If there are lesions in the brain, they are mostly found in periventricular areas and along the brain stem. Cortical involvement is only seen in certain chronic experimental setups.(75,76) However, the changes are often subtle and of rather limited extent. These animal models are indeed very useful for studying the early relapse-remitting phase of MS but are not ideally suitable to model the progressive phase.(77) Furthermore, the severity of the animals suffering makes long-term observations ethically questionable.

1.4.1 A new rat model of cortical demyelination

Our research group recently published a new rat model which displays, to our knowledge for the first time, most of the histopathological changes seen in autopsy brain material of human progressive MS patients, such as widespread cortical demyelination, microglial activation and neuronal loss, and having the pathology persist for 30 days and longer.(78) The experimental setup includes the implantation of a tissue and MRI-compatible catheter into the frontal lobe of the rat. The catheter is allowed to heal in for 2 weeks to ensure complete restoration of the BBB after surgical trauma. The animals are then subcutaneously immunized with a low dose of 5 µg MOG, diluted in phosphate buffered saline (PBS), dissolved in Incomplete Freund's Adjuvant (IFA). This mild immunization leads to neither clinical nor histological abnormalities in the experimental animals, though they build a constant serum anti-MOG antibody titer. As a next step, the animals receive a high dose of the pro-inflammatory cytokines TNF-α and IFN-γ via the implanted catheter to selectively open the BBB and allow influx of the preformed anti-MOG antibodies. The injection is done as slow as the physiological turnover rate of brain interstitial fluid to avoid mechanical, injection-related injury. The following illustration in Fig. 5

shows the basic experimental setup leading to cortical demyelination in this rat model.

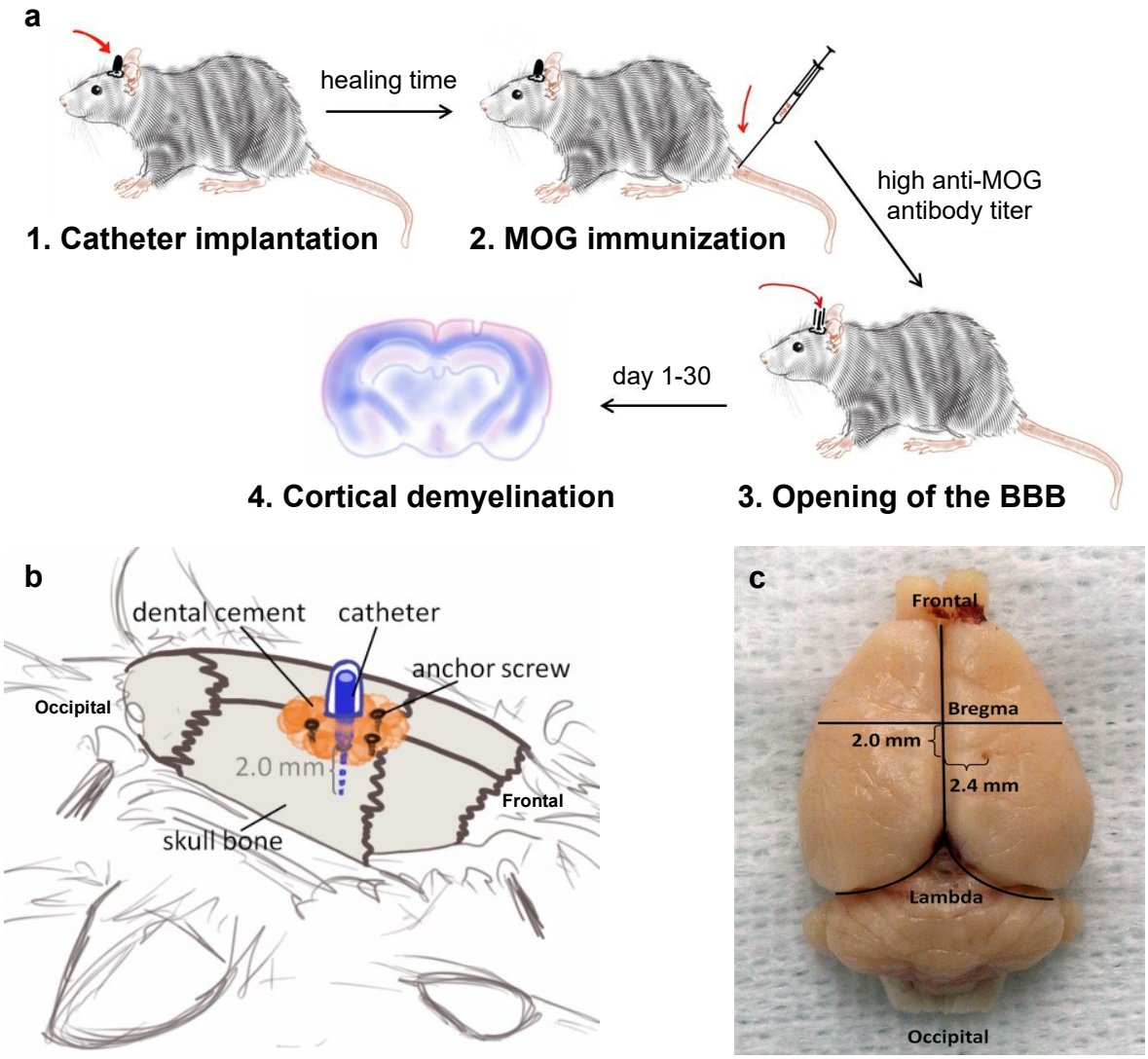


Fig. 5 Schematic illustration of the experimental setup leading to cortical demyelination in our new rat model (panel **a**). In the lower panels, the catheter implantation according to its coordinates is shown. Panel **b** depicts a representational drawing of the catheter insertion setup completed with three anchor screws and dental cement. The catheter was located in a depth of 2 mm. Its implantation coordinates are 2 mm posterior to the bregma and 2.4 mm to the right which can be seen in panel **c**. © Dr. Michaela Haindl.

From our previous experiments we know that cortical demyelination already starts in the injected hemisphere on day 1 after cytokine infusion, spreads further to the contralateral hemisphere by day 3 and reaches its maximum between days 9-15 with widespread cortical demyelination of both hemispheres (as shown as loss of

PLP immunoreactivity in Fig. 6) and is still detectable in small patches of demyelination and partial remyelination on day 30 after cytokine injection (Fig. 7e-i). Clinically, the animals show only a slightly slower appearing behavior between days 9-15 without any motor symptoms. They completely recover clinically after day 15. Cellular infiltrates are generally sparse and mostly found from day 1-3 with an obvious movement of the cells from the immediate surrounding of the incision channel towards the meninges where they remain detectable in low numbers up to day 30. Demyelination is accompanied by marked microglial activation which is another hallmark of cortical pathology in progressive MS. Regarding astrocytic reaction around the catheter insertion area, also systematically analyzed before, there was no evidence of increased astrogliosis even upon long-term implantation of 4 weeks or more. In this original publication, we also show that only the full experimental setup as described above leads to these extensive cortical changes, and whenever one component, either immunization or cytokine injection, is omitted, the cortical myelin remains intact. Inducing a second demyelination stage after day 30 is possible and leads to the same mild symptoms between days 9-15. Brains however appear macroscopically atrophic after a second cytokine injection, reminiscent of brain atrophy found on MRI scans of progressive MS patients (as shown in Fig. 7k; Fig. 7j shows a control brain for better comparison).

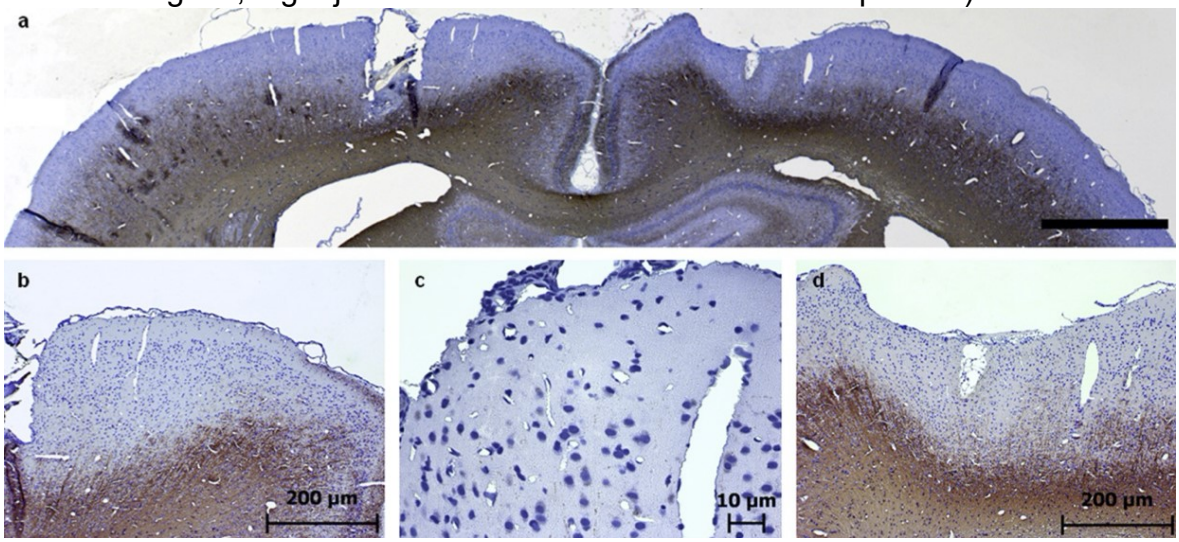


Fig. 6 Widespread cortical demyelination as shown as loss of PLP immunoreactivity. The panorama presented in panel **a** (scalebar = 500 µm) gives an overview of PLP loss expanding over both hemispheres of which panels **b-d** show certain parts for better clarification. Panel **b** depicts the catheter insertion area at an enlargement of 200x, and panel **c** at 630x with full PLP loss and no remaining traces detectable. Panel **d** shows the contralateral hemisphere located beneath an anchor screw at a magnification of 200x. Taken from Üçal et al, 2017.

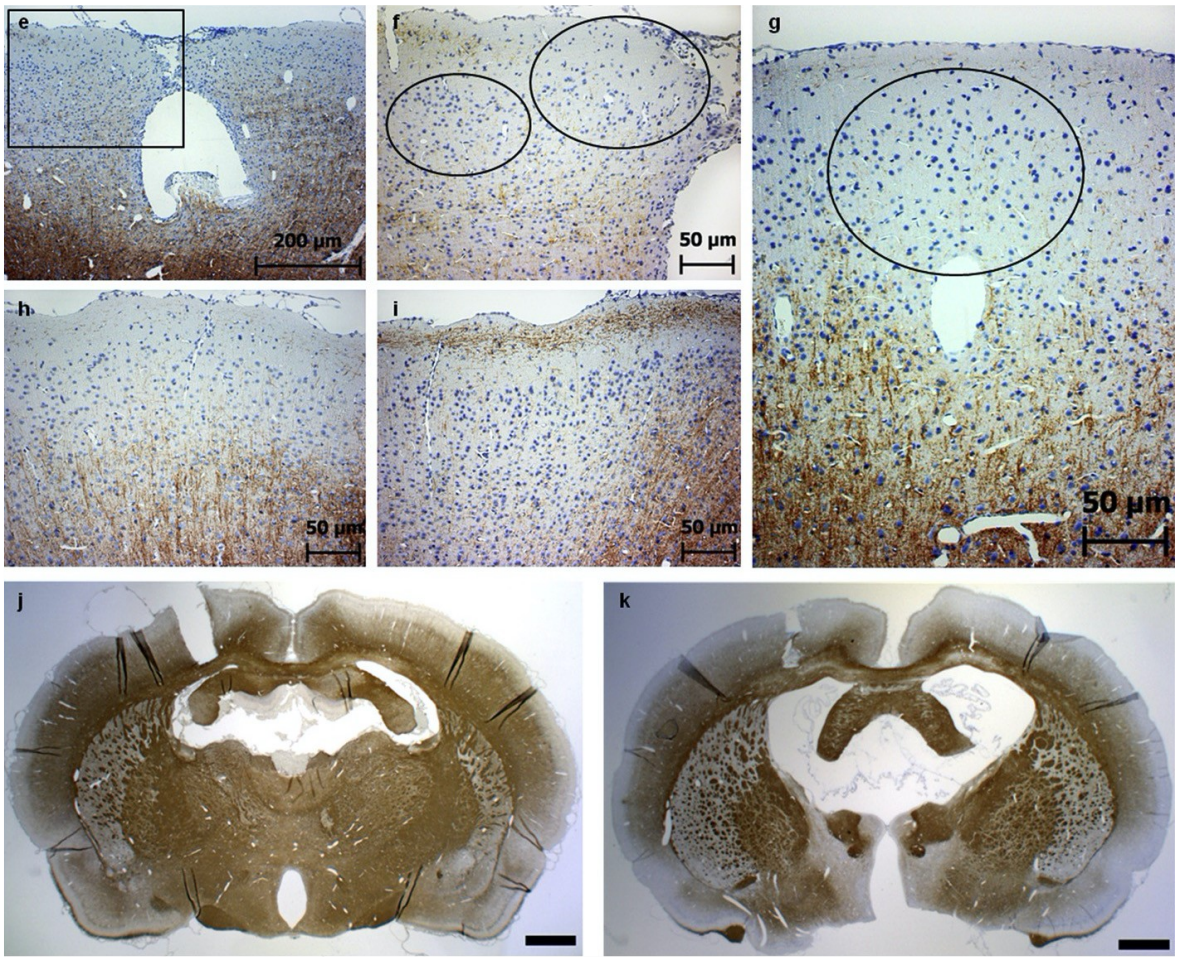


Fig. 7 Panels **e-i** show PLP immunoreactivity on day 30 after cytokine injection. Panel **e** displays the catheter insertion area at a magnification of 100x. The rectangular area is enlarged at 200x in panel **f** which includes circles presenting PLP loss intracortically (left circle) and subpially (right circle) alongside partially remyelinated areas below. Panels **g-i** show various cortical areas of the contralateral hemisphere with still missing PLP immunoreactivity subpially (panels **g-h**, 200x) and intracortically (panel **i**, 200x). Brain atrophy emerged in animals sacrificed on day 15 after a second cytokine injection (panel **k**, scalebar = 500 µm) in contrast to control brains of which an example is shown in panel **j** (scalebar = 500 µm). Taken from Üçal et al, 2017.

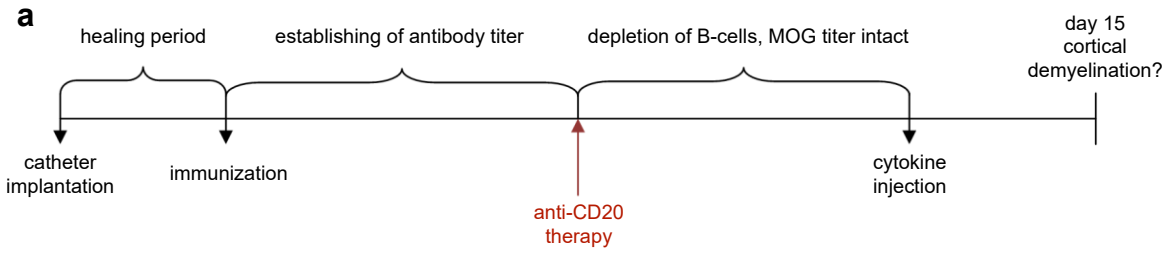
1.5 Aim of the study

Research efforts to elucidate pathogenesis and subsequently therapy for relapse-remitting MS have already led to a large number of therapeutic options available. Unfortunately, therapeutic options regarding progressive MS are currently very limited. Since B-cell depleting anti-CD20 antibody therapies were shown in clinical trials to be effective in both, relapse-remitting and (early) progressive MS, we now want to investigate in our project to what extent anti-CD20 therapy can avert the

development of cortical demyelination and atrophy in our rat model. We hope that this will give new insight into the pathological mechanisms of tissue damage in the late progredient phase of MS. The main goal of this study is to expand our comprehension of the mode of action of B-cells and therapeutic agents directed against B-cells in relation to cortical involvement in MS and subsequently improve therapeutic options of progressive MS. For this purpose, we want to carry out the following experiment.

1.6 Experimental setup

Initial setup of the experimental groups comprised of catheter insertion into the cerebral cortex, MOG immunization and cytokine injection via the catheter.
In the first experiment, B-cell depletion in rats (n=7) was induced by treatment with the anti-CD20 antibody after catheter implantation and MOG immunization which means that an antibody titer against MOG had already been established. The pro-inflammatory cytokines were then injected into the cortex through the implanted catheter. Since plasma cells remain unaffected by anti-CD20 treatment, the previously formed antibody levels in the blood and therefore also in the CSF of the animals were retained. This served as reenactment of the situation of a MS patient under treatment with anti-CD20 therapy with already existing intrathecal antibodies. We hypothesized a significant reduction in extent of cortical destruction in this trial setting.
In the second experiment which served as a preliminary test, rats (n=8) were treated with the anti-CD20 antibody after catheter implantation, but before immunization with MOG. This was to confirm the effectiveness of the antibody in the rat. The therapy should have, by eliminating B-cells, completely prevented the formation of both, an antibody titer against MOG as well as cortical lesions by showing less or no demyelination. Supplementary timelines of the two experiments are shown in Fig. 8 below.



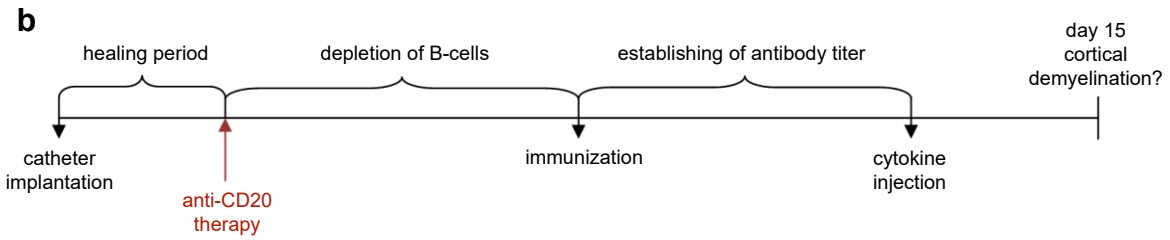


Fig. 8 Timelines according to the experimental setup. The agenda of the first experiment (**a**) with anti-CD20 therapy after MOG immunization. In the second experiment (**b**) anti-CD20 therapy is given before MOG immunization.

2 Materials and methods

2.1 Animals and experimental groups

The animal experiment was implemented with consent of the local authorities (BMBWF-66.010/0195-V/3b/2018).

Adult male Dark Agouti (DA) rats (n=29) were used in this experiment. Two groups (G1, G2) were formed with anti-CD20 antibody therapy, in particular a research antibody from Genentech/Roche (RAB), being applied by intravenous injection into the tail vein either after (G1) or before (G2) MOG immunization. Rats were sacrificed at peak disease on day 15 after cytokine injection.

The primary objective is the histologically detectable cortical demyelination, the extent of microglial activation, apoptosis rate, astrocytic reaction and neuronal preservation. This was statistically compared to the control antibody groups without anti-CD20 therapy, given an isotype-matched control antibody (CAB) instead, and to the healthy control (HC) group. The different animal groups are listed below in Table 3.

Experimental groups	
G1 MOG RAB	Injection of anti-CD20 antibody after MOG immunization
G2 RAB MOG	Injection of anti-CD20 antibody before MOG immunization
Control groups	
G1 MOG CAB	Injection of control antibody after MOG immunization
G2 CAB MOG	Injection of control antibody before MOG immunization
HC	Untreated animals (‘healthy control’)

Table 3 Summary of the various experimental and control animal groups. The experimental groups (G1 MOG RAB, G2 RAB MOG) differ in their order of anti-CD 20 therapy after (G1) and before (G2) MOG immunization. They are compared to the control antibody groups (G1 MOG CAB, G2 CAB MOG) and the healthy control (HC).

2.2 Immunohistochemistry

For immunohistochemistry (IHC), brains were dissected and routinely embedded in paraffin. They were then cut in sections, dewaxed in xylol (2x 10 min) and fixed in 96% ethanol (2x). Sections were further incubated in H_2O_2-methanol for 30 min and rehydrated in 70% ethanol, 50% ethanol and Aqua dest., respectively. After performing antigen retrieval in citric acid (pH 6.0) for 1 h using a commercial food steamer, slides were cooled at room temperature for 1 h and washed in PBS afterwards. Using Dako Pen, a circle was drawn around tissue sections and slides were placed in a staining tub containing PBS. Sections were incubated with 2.5% horse serum in diluent (2.5% PD) for 20 min prior to applying the primary antibody, also diluted in 2.5% PD, following overnight incubation at 4 °C.
After washing sections three times with PBS the next day, the secondary antibody was applied using VectorLab ImmPress Polymer System and further developed with DAB under microscopic control. Slides were stained by inserting them in hemalm for 1 min, followed by development in H_2O (2x), HCl-ethanol and Aqua dest. (2x). They were then put in bluing reagent for 3 min and dehydrated with Aqua dest., 50% ethanol, 70% ethanol, 96% ethanol (3x) and 100% ethanol. After finally clearing them in butyl acetate, they were covered with a cover slip. All individual steps of the immunohistochemical protocol are listed in the following Table 4.

Immunohistochemistry	
1	2x 10 min xylol
2	2x 96% ethanol
3	30 min H_2O_2-methanol
4	70% ethanol, 50% ethanol, Aqua dest.
5	1 h steaming in citric acid
6	1 h cooling at room temperature, washing in PBS
7	Circling tissue with Dako Pen, placing in staining tub with PBS
8	20 min 2.5% horse serum in diluent
9	Applying primary antibody diluted in 2.5% PD

Overnight incubation at 4 °C	
1	Washing 3x in PBS
2	Applying secondary antibody using VectorLab ImmPress Polymer System
3	Developing with DAB under microscopic control
4	1 min hemalm
5	2x H_2O
6	HCl-ethanol
7	2x Aqua dest.
8	3 min bluing reagent
9	Aqua dest., 50% ethanol, 70% ethanol, 3x 96% ethanol, 100% ethanol
10	Butyl acetate, cover slip

Table 4 Immunohistochemical protocol step by step.

In adjoining serial sections, IHC was carried out under light microscopy by using antibodies against the following objectives: proteolipid protein (PLP), activated microglia (Iba1), apoptotic cells (Caspase3), astrocytes (GFAP), neurons (NeuN) and neurofilament (Nf). The appendix contains detailed information about the primary and the respective secondary antibodies that were administered.

2.3 Histopathological evaluation

Tissue sections were analyzed thoroughly with respect to degree of cortical demyelination, microglial activation, apoptotic cells, astrocytic reactivity and neuronal cell loss, followed by photo documentation and statistical analysis.
For quantification of demyelination and cellular marks, adjacent serial sections of the catheter implantation area were used to ensure best comparability. Demyelination was assessed by extent of full cortical loss of PLP immunoreactivity for each hemisphere, using an optical raster at a magnification of 200x, and values were then converted to mm^2. Cellular components (Iba1, Caspase3, GFAP, NeuN)

were quantitatively evaluated in three optical grids in the cortex of each hemisphere at an enlargement of 200x. Mean values were consequently converted to cells per mm^2. All quantification was carried out in one representative slide per staining and animal. Neurofilament immunoreactivity was semi-quantitatively analyzed within the lesions and used for photo documentation in this experiment.

2.4 Statistical analysis

All obtained data was first structured in Microsoft Excel 2016. Statistical calculations were then performed using IBM SPSS Statistics 26. Further graphical depiction was illustrated and arranged in Microsoft Excel 2016.
Only data of the ipsilateral hemisphere speaking of the hemisphere in which the catheter was inserted is presented in this work. For quantitative variables, median and quartiles were reported as measure of central tendency. Statistical significance of the alterations observed in experimental groups compared to control groups was evaluated with Mann-Whitney U test, with a difference of the p-value < 0.05 considered statistically significant in a single test. To visualize assumed distinction between the groups, box plots were created, composed of median, lower (first) and upper (third) quartile, interquartile range, whiskers from minimum to maximum value and outliers as appropriate.

2.5 Photo documentation

Histological and immunohistochemical preparations were examined using a Zeiss light microscope. Glass slides were hereby digitized with the Zeiss ZEN imaging software to create representative pictures. All stained sections were screened to find one representative slide per group. With exception of the HC group, all images were taken next to the catheter insertion area to visualize comparable expression of cortical demyelination or remyelination and involved cells, respectively. Each photo was shot at a magnification of 200x to allow better comparison. Further image processing such as color and white balance adjustment was performed with Zeiss ZEN imaging software. Finally, letters and scale bars were inserted using Microsoft PowerPoint 16.

3 Results

3.1 Histopathological changes in the cortex

Histological evaluation indicates a significant diminution of cortical demyelination, microglial activation, apoptotic cells, astrocytic reaction as well as a reduced neuronal loss in anti-CD20 treated animals, in contrast to animals given the isotype-matched control antibody. While there was a significant difference detectable between the healthy control (HC) and the control antibody groups (G1 MOG CAB, G2 CAB MOG), the difference between HC and anti-CD20 antibody groups (G1 MOG RAB, G2 RAB MOG) did not reach significance in any of the quantified markers indicating a closer similarity of anti-CD20 antibody groups to HC regardless of the exact protocol of antibody administering. Furthermore, there was no significant difference exhibited between the two experimental setups (G1 MOG RAB vs. G2 RAB MOG, G1 MOG CAB vs. G2 CAB MOG). Below, detailed quantification of the selected cellular markers shows the anticipated pattern.

3.1.1 Loss of cortical PLP immunoreactivity

Considerably less PLP loss was found in anti-CD20 treated animals compared to control antibody groups, demonstrating higher resemblance of anti-CD20 antibody groups to HC. Plenty of remaining PLP immunoreactivity in the cortex is shown in Fig. 9a which also pictures the catheter implantation site on the right edge of the image. For comparison, Fig. 9b illustrates normal PLP immunoreactivity in an animal of the HC in contrast to Fig. 9c, depicting the brain of a fully diseased animal from previous experiments of Üçal et al, 2017 with missing or thinned out myelin indicated by pronounced PLP loss.

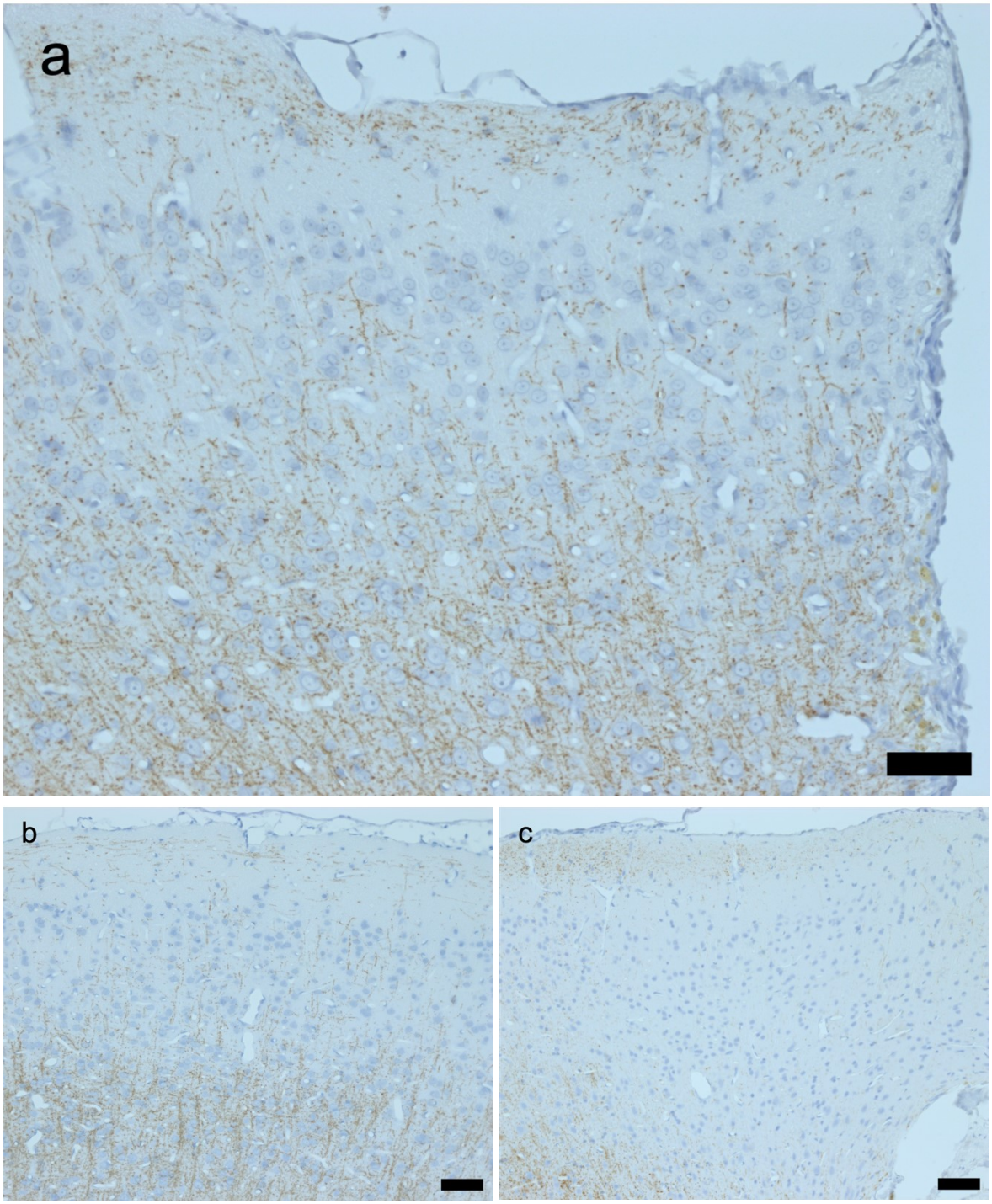


Fig. 9 PLP immunoreactivity at the catheter implantation site. Immunohistochemistry against proteolipid protein at a magnification of 200x. Scalebars indicate 50 µm. Panel **a** shows G1 MOG RAB at the left edge of the catheter insertion area. Panel **b** shows HC with normal PLP immunoreactivity. Panel **c** shows a diseased animal with pronounced PLP loss.

In this case, only one of the anti-CD20 antibody groups, G2 RAB MOG, exhibited a significant reduction of PLP loss compared to G1 MOG CAB ($p = 0.007$) and G2

CAB MOG (p = 0.014). A significant difference was also observed between HC and G1 MOG CAB (p = 0.020) as well as HC and G2 CAB MOG (p = 0.032). Quantitative evaluation of cortical PLP loss in mm^2 is given in the diagram in Fig. 10 in which the similarity of G2 RAB MOG and HC showing no or little PLP loss can be seen.

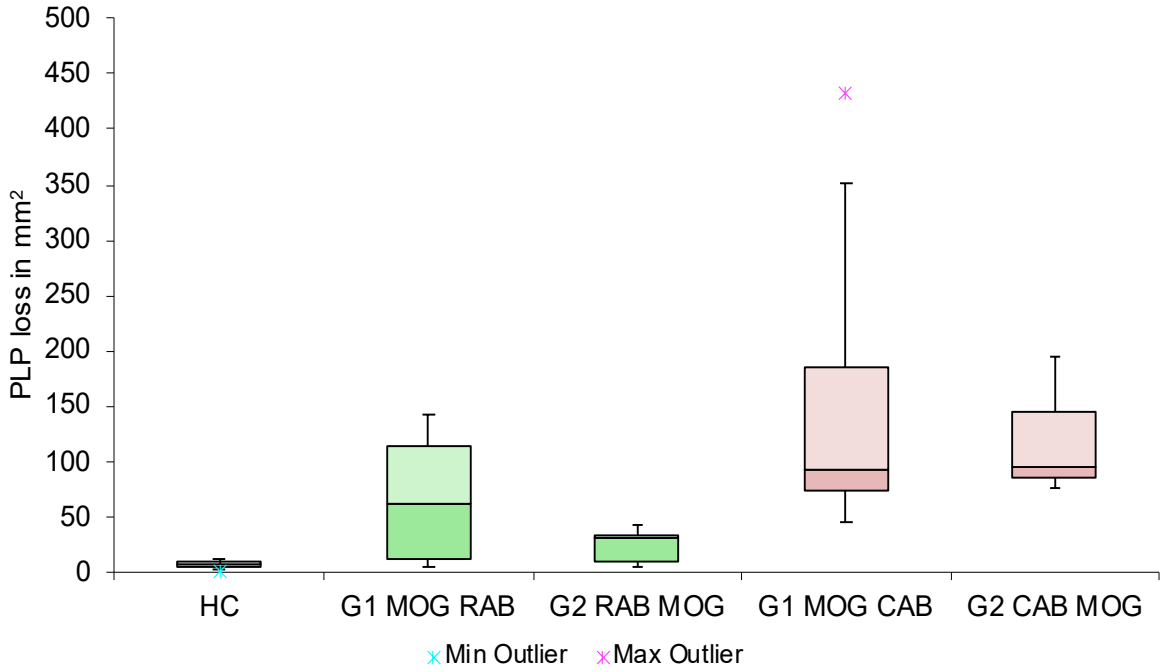
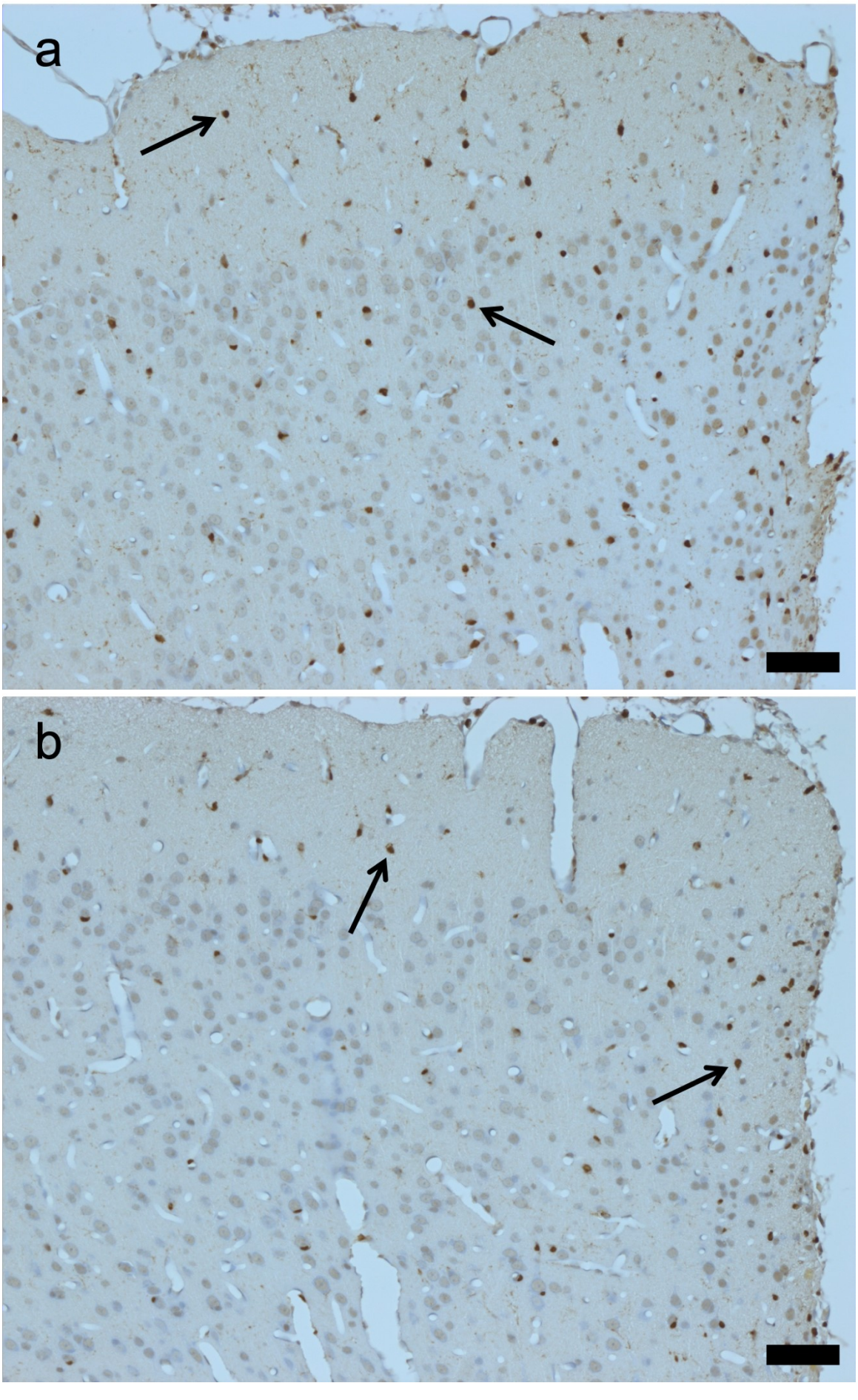


Fig. 10 Quantitative evaluation of PLP loss in mm^2.

3.1.2 Microglial activation

In our animal model as well as in the human MS disease, cortical PLP loss is accompanied by increased microglial activation, as demonstrated in Fig. 11a showing brain tissue of a rat of the control antibody group stained for activated microglia (Iba1). In contrast to that, in anti-CD20 antibody groups only minor traces of activated microglia can be found, as proven in Fig. 11b. The catheter insertion areas are again pictured on the right. To allow better comparison, an animal of the healthy control with almost no microglial activation is depicted in Fig. 11c.


a
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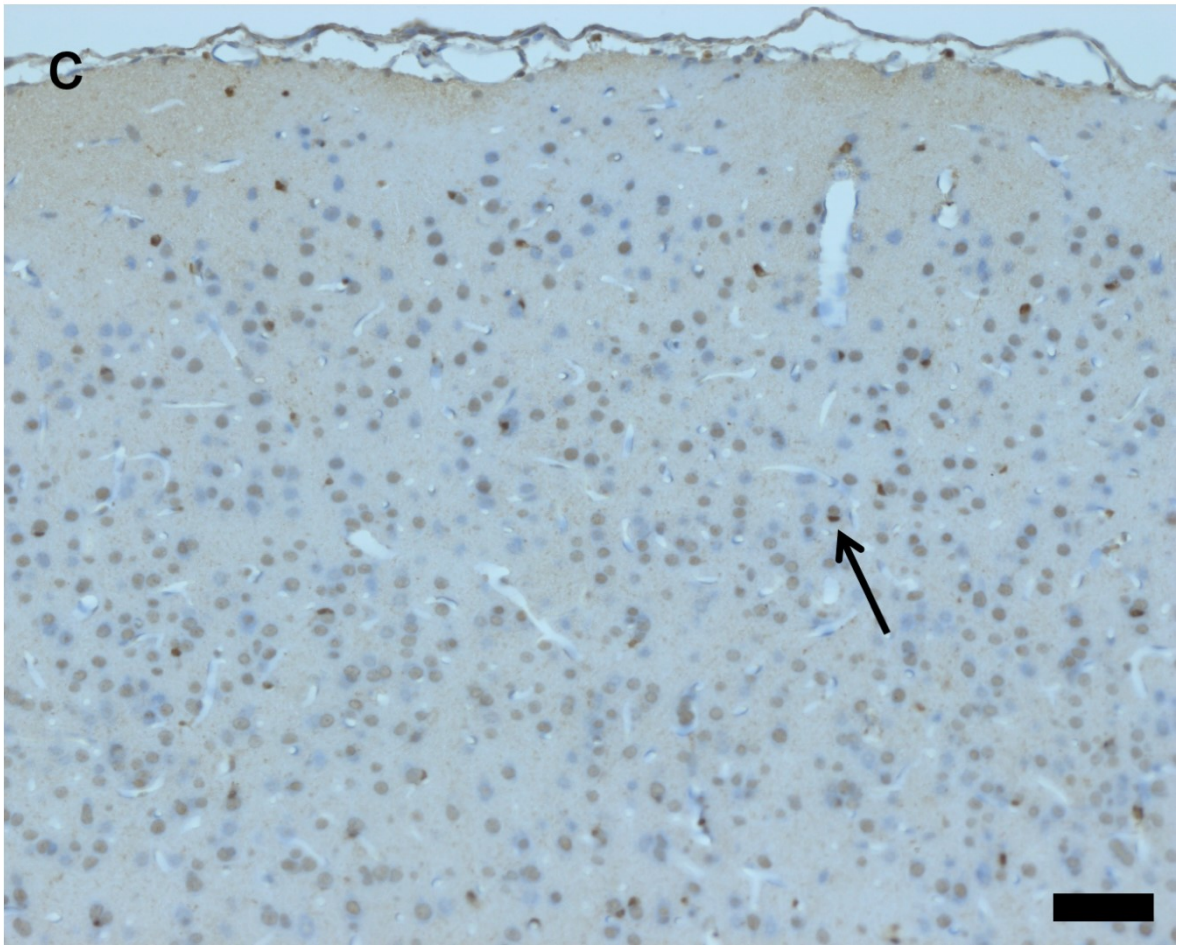


Fig. 11 Microglial activation around the catheter insertion area in different experimental setups. IHC for Iba1 at a magnification of 200x (scalebar = 50 µm). Examples of microglia, specifically stained for Iba1, are marked by arrows to clarify differentiation to the neuronal background. Panel **a** shows G2 CAB MOG with increased Iba1 immunoreactivity in the demyelinated area next to the catheter insertion site. The same region is shown in panel **b** with less immunoreactivity in G2 RAB MOG. Panel **c** shows HC with almost no microglial activation.

In contrast to control antibody groups, microglial activation was significantly decreased in anti-CD20 antibody groups with p-values ranging from 0.006 to 0.016. Again, there was a significant difference between HC and G1 MOG CAB ($p = 0.011$) as well as G2 CAB MOG ($p = 0.020$). Quantitative evaluation of Iba1 positive cells per mm^2 is depicted in the diagram in Fig. 12, also showing the approximation of anti-CD20 treated groups to HC.

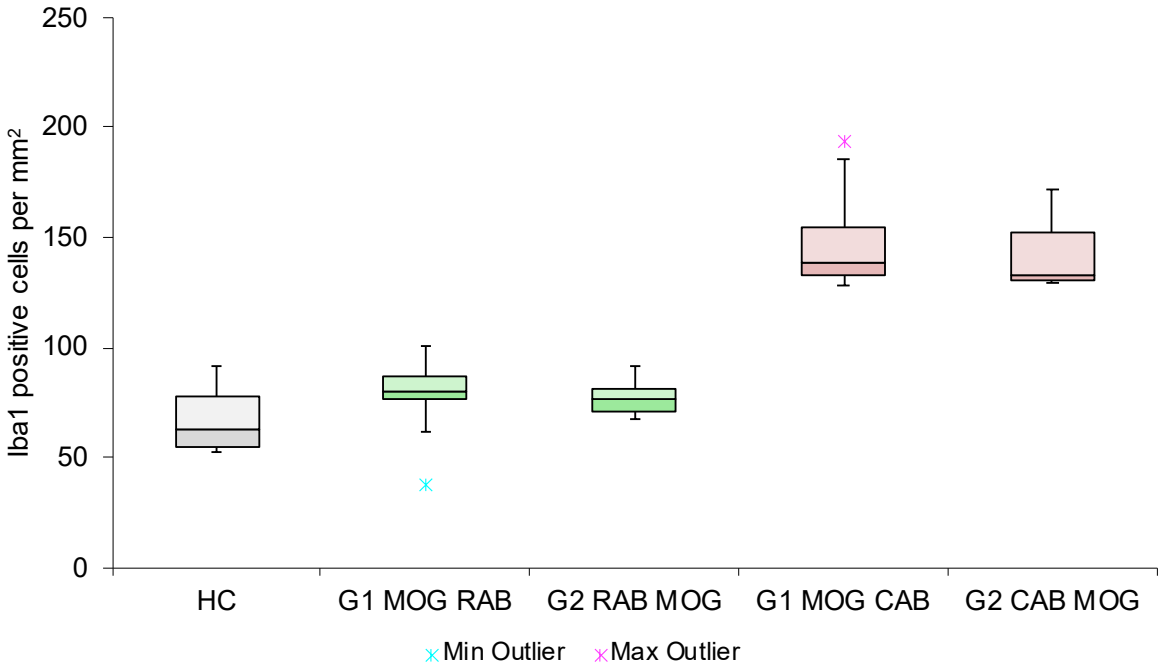


Fig. 12 Quantitative analysis of cells positive for Iba1 (activated microglia) per mm^2 in the respective groups.

3.1.3 Loss of neurofilament immunoreactivity

PLP loss in the cortex was further joined together with missing or at least reducing of neurofilament immunoreactivity in the same areas. In contrast, normal neurofilament immunoreactivity is depicted in Fig. 13a. Much less neurofilament immunoreactivity is found in diseased animals and expressively shown in Fig. 13b, depicting brain tissue of a rat from previous experiments of Üçal et al, 2017. In anti-CD20 antibody groups, only traces of absent neurofilament immunoreactivity are detectable immediately at the catheter insertion site which can be seen in Fig. 13c.

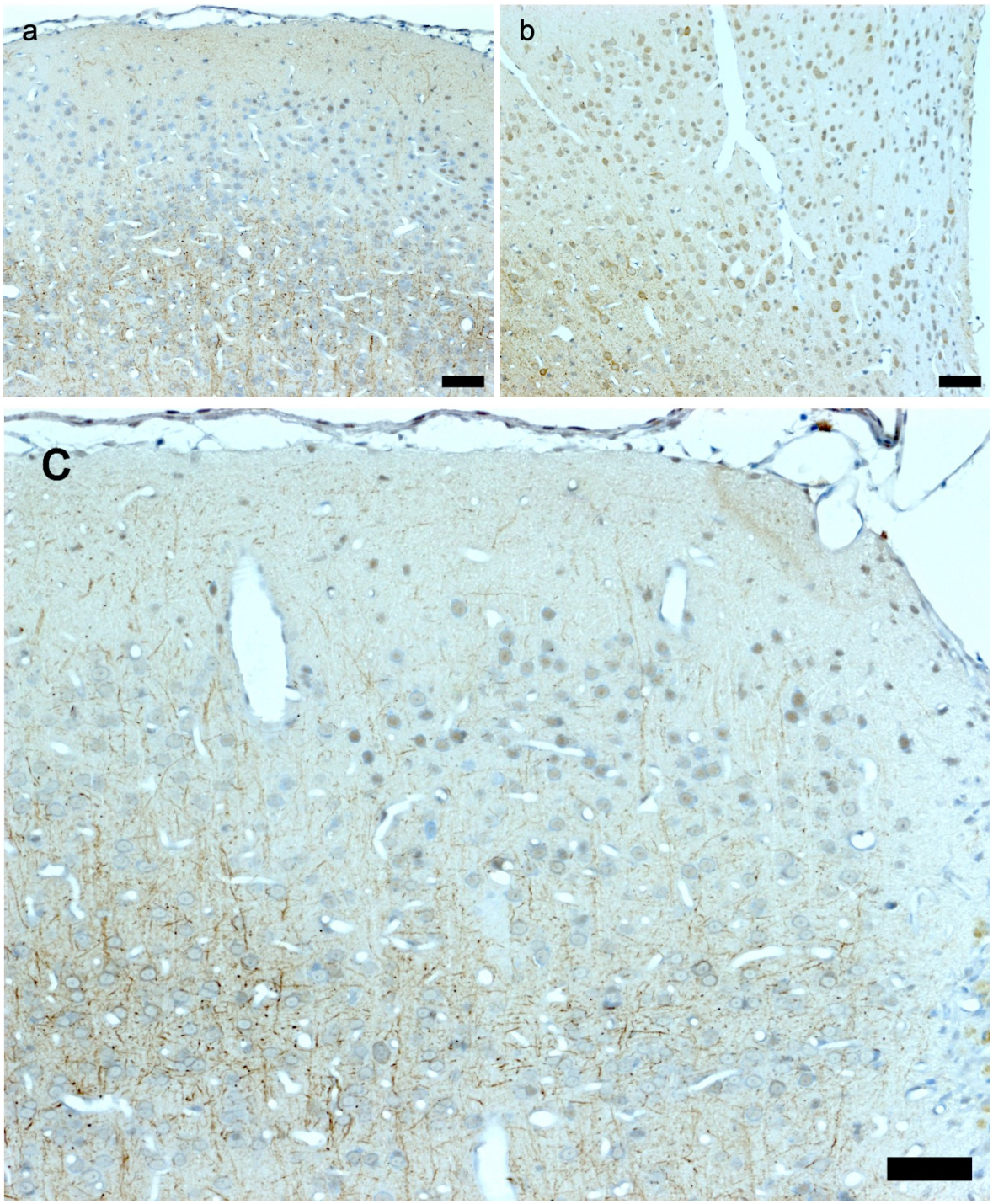


Fig. 13 Neurofilament immunoreactivity in different experimental setups. Immunohistochemistry against Nf at a magnification of 200x. Scalebars indicate 50 µm. Panel **a** shows HC with normal neurofilament immunoreactivity. Panel **b** shows brain tissue of a diseased animal with less immunoreactivity. Panel **c** shows G2 RAB MOG with intact immunoreactivity and only traces of thinned out neurofilament in the immediate catheter insertion area on the right edge.

3.1.4 Apoptotic cells

The number of apoptotic cells was evaluated through IHC against active Caspase3. A significant reduction of Caspase3 positive cells was observed in G1 MOG RAB compared to G1 MOG CAB ($p = 0.008$) and G2 CAB MOG ($p = 0.017$) as well as in G2 RAB MOG compared to G1 MOG CAB ($p = 0.007$) and G2 CAB MOG ($p = 0.014$) demonstrating most likely a direct effect of the anti-CD20 antibody treatment, resulting in better preservation of tissue structures. The low apoptotic cell count in HC was statistically opposed to control antibody groups with significant p-values being 0.011 in G1 MOG CAB and 0.020 in G2 CAB MOG. Quantitative evaluation of Caspase3 positive cells per mm^2 can be found in the diagram in Fig. 14, clearly representing similarity between anti-CD20 antibody groups and HC.

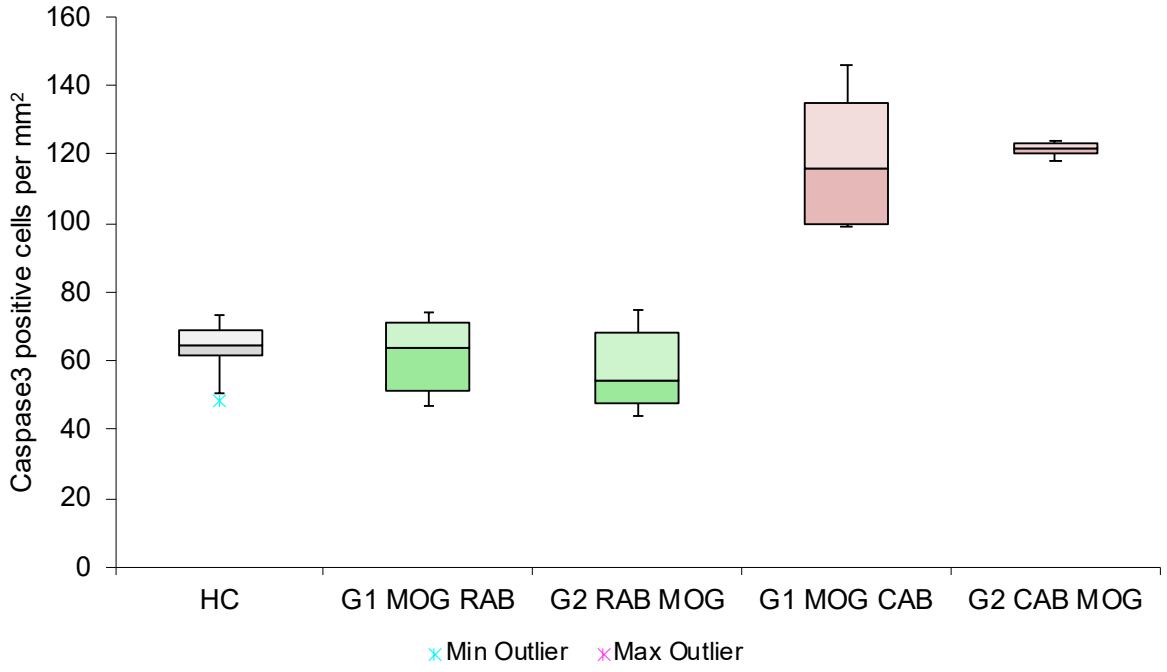
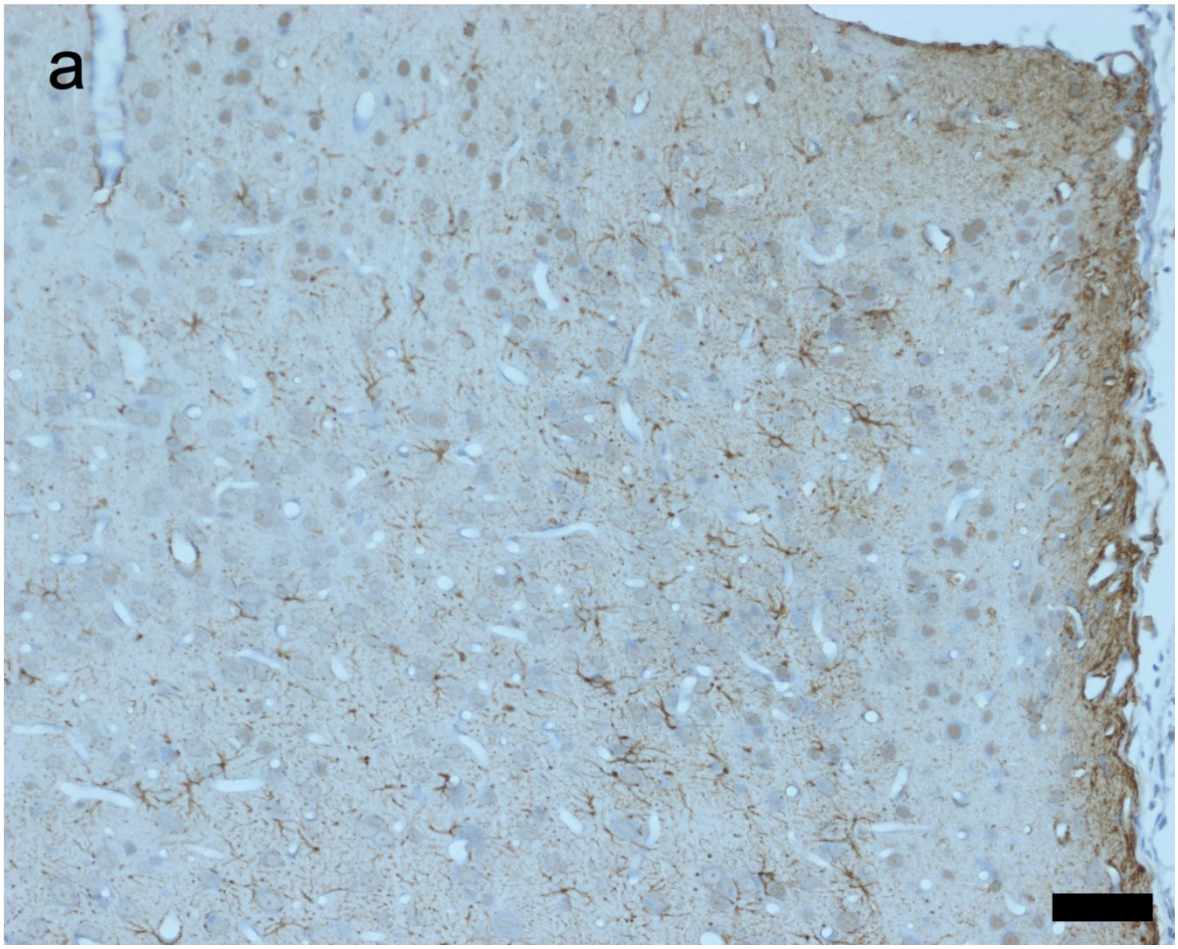


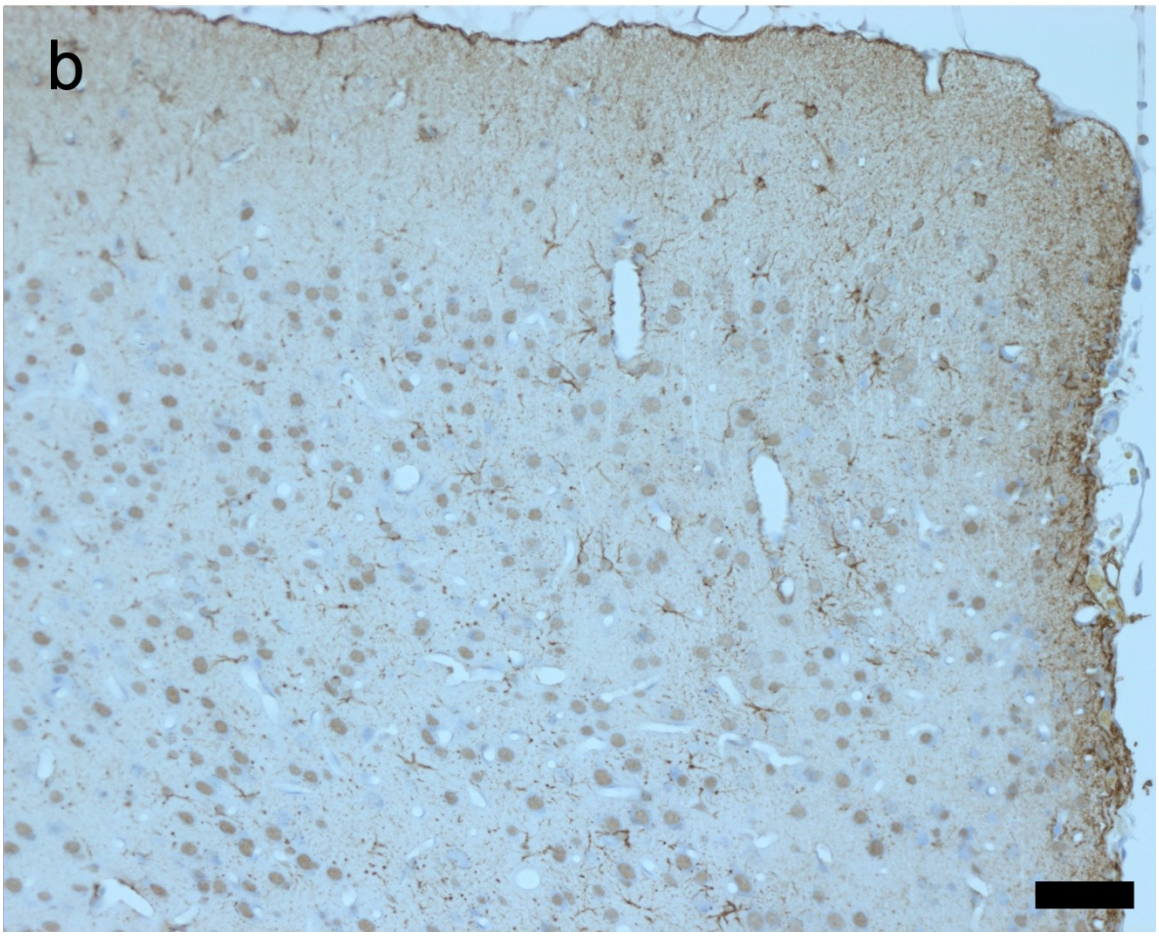
Fig. 14 Quantitative evaluation of apoptotic cells (positive for Caspase3) per mm^2.

3.1.5 Astrocytic reaction

Based on current knowledge, astrocytic reaction is increased in diseased animals as a rather unspecific reaction to any kind of insult or injury to the brain tissue. Astrocytes enlarge and extent their processes, a phenomenon that can be observed

immunohistochemically by staining astrocytes against GFAP. Whereas in control antibody groups a marked astrocytic reaction is still histologically detectable as seen in Fig. 15a, anti-CD20 antibody groups instead show much less astrocytic reaction in this area, depicted in Fig. 15b. Again, catheter insertion regions are pictured on the right. To allow better comparison, a slide of an animal of the HC is represented in Fig. 15c.

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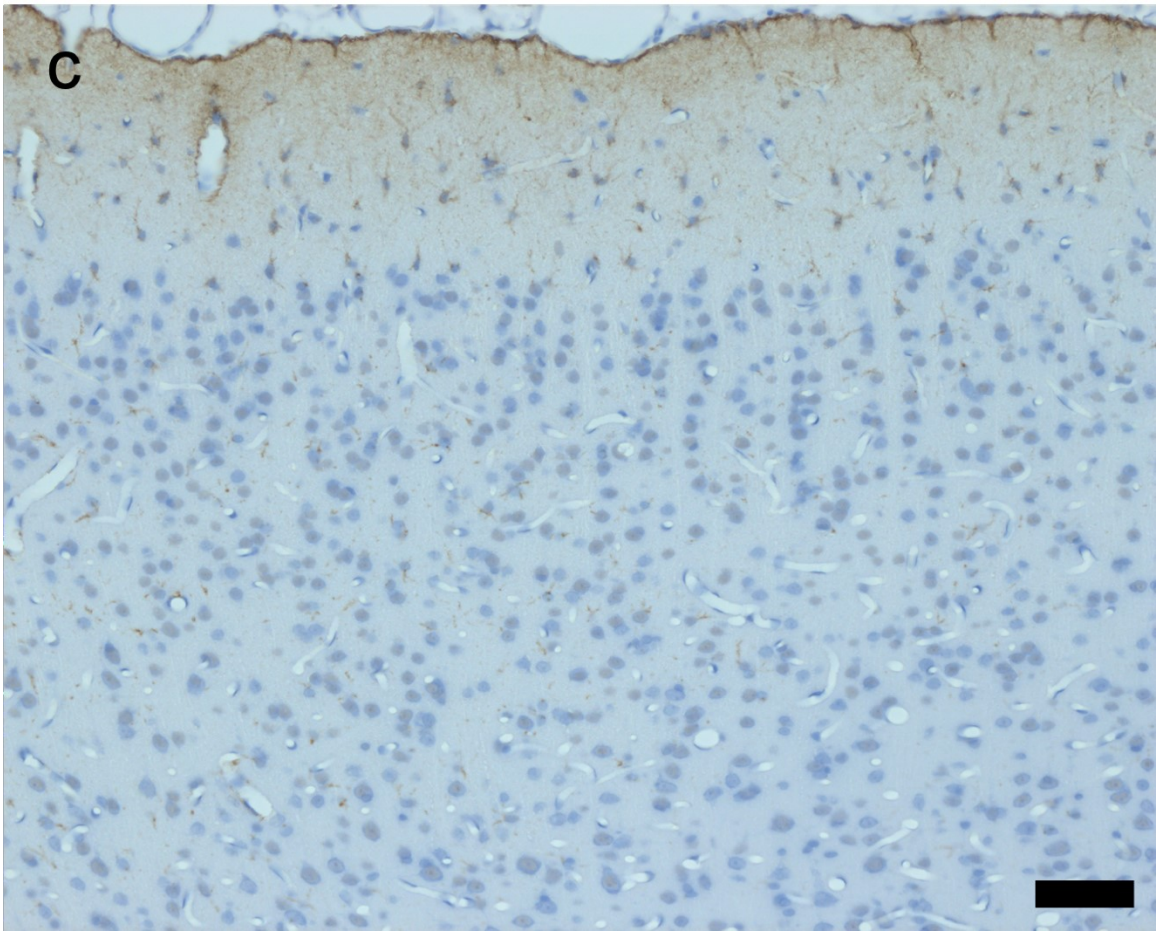
c

Fig. 15 Astrocytic response around the catheter insertion area in different experimental setups. IHC for GFAP at a magnification of 200x (scalebar = 50 µm). Panel **a** shows increased reaction in G2 CAB MOG. Panel **b** shows G2 RAB MOG with only minor GFAP immunoreactivity in the cortex. Panel **c** shows HC with almost absent (activated) astrocytes.

Similar to HC, only mild astrocytic reaction was observed in anti-CD20 antibody treated groups. A significant difference could be seen in all cases of anti-CD20 antibody groups compared to control antibody groups with p-values ranging from 0.006 to 0.046. Furthermore, HC reached significance compared to G1 MOG CAB ($p = 0.014$) and a marginal value in comparison to G2 CAB MOG ($p = 0.053$). Quantitative evaluation of GFAP positive cells per mm^2 is shown in the diagram in Fig. 16. The graph demonstrates the assumed astrocytic pattern in anti-CD20 antibody groups with approximation to HC.

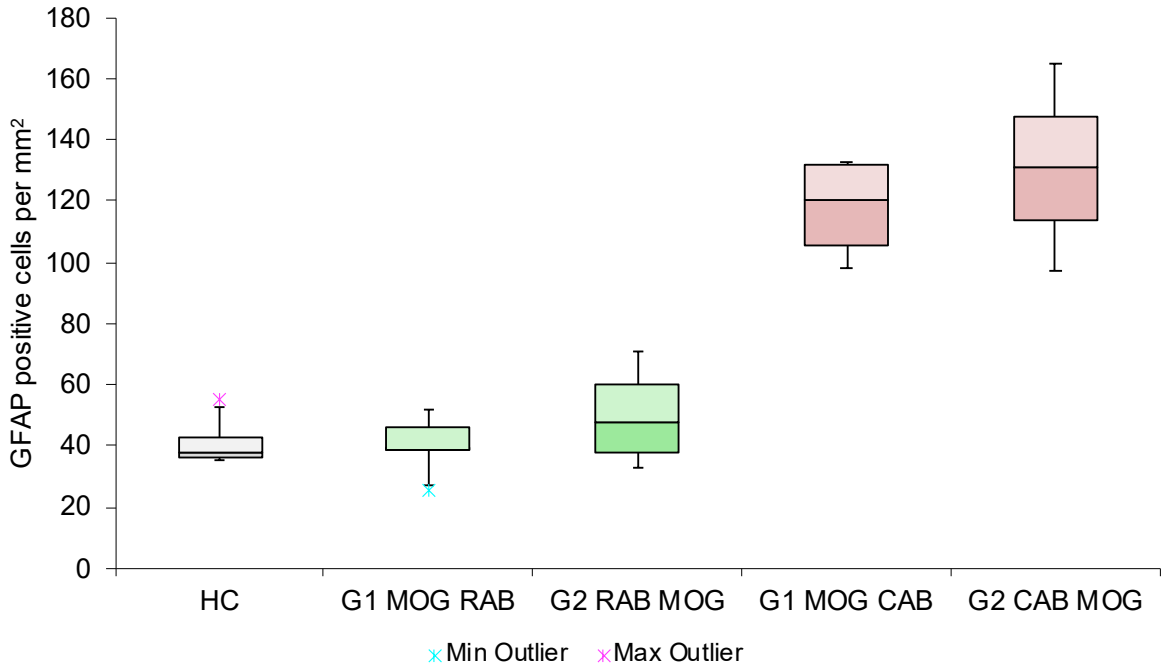

Fig. 16 Quantitative evaluation of GFAP positive astrocytes per mm^2.

3.1.6 Neuronal counts

Finally, the integrity of neurons was investigated through IHC against NeuN. Neuronal cell counts in anti-CD20 antibody groups remained at the level of HC or

slightly increased. In contrast to this, neuronal loss was observed in control antibody groups with dropping counts, the lowest being 390 cells per mm^2. There was a significant reduction of neurons in control antibody groups compared to anti-CD20 treated groups with p-values ranging from 0.011 to 0.014 except from G2 RAB MOG vs. G1 MOG CAB (p = 0.062). HC reached significance in comparison to G1 MOG CAB (p = 0.019) and G2 CAB MOG (p = 0.020). Quantitative evaluation of NeuN positive cells per mm^2 is given in the diagram in Fig. 17 showing that anti-CD20 treatment leads to a stabilization of the number of neurons in the cortical area as compared to the reduced neuronal count seen in control antibody groups.

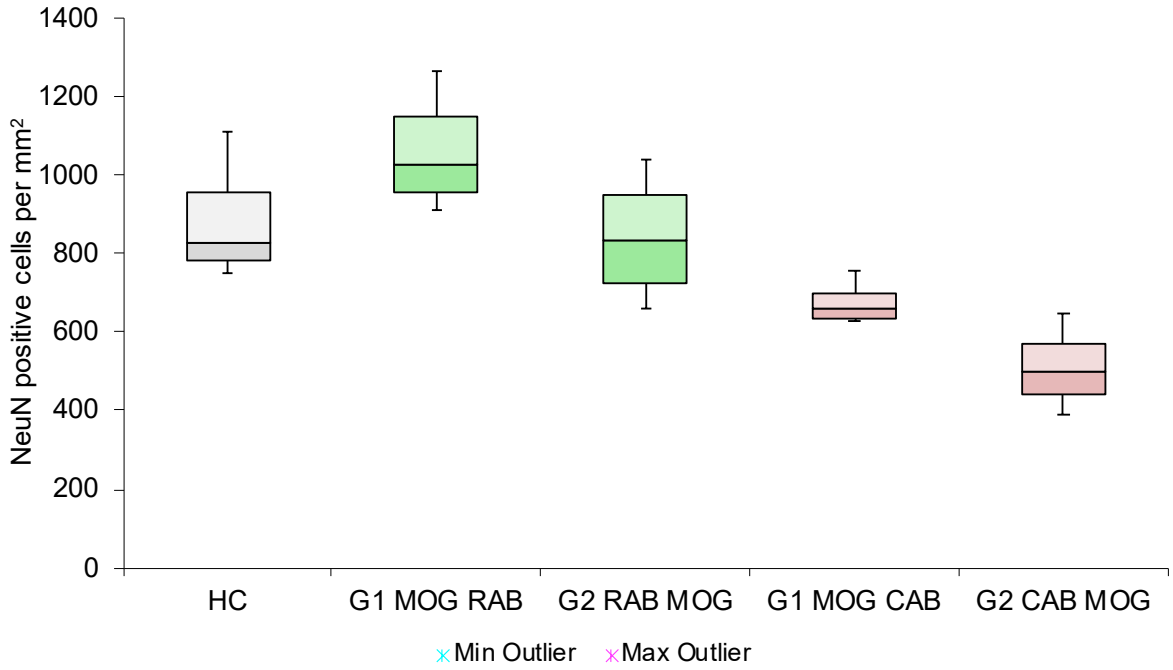

Fig. 17 Quantitative analysis of cells positive for NeuN (neurons) per mm^2.

3.2 Overall evaluation

Histologically, even after close detailed evaluation, cortical pathology is rather minor in anti-CD20 treated animals regardless of the exact protocol of administration. In general, less cortical demyelination and loss of neurofilament immunoreactivity was observed in anti-CD20 antibody groups compared to control antibody groups. Microglial activation was also significantly reduced in both anti-CD20 antibody groups compared to control antibody groups indicating approximation to HC. Furthermore, a significantly decreased number of apoptotic cells was exhibited in

both anti-CD20 antibody groups compared to control antibody groups, thus representing similarity to HC in this regard as well. Astrocytic reaction in the cortex was significantly higher in control antibody groups compared to both anti-CD20 antibody groups, also indicating less tissue damage in these experimental groups. There were significantly more neurons left in both anti-CD20 antibody groups compared to control antibody groups.

Both therapeutic approaches, G1 as well as G2, showed equal effectiveness. There was no significant difference neither in cortical demyelination nor cellular reaction between animals of the HC and anti-CD20 treated animals.

4 Discussion

Although the treatment of relapse-remitting MS has been tremendously improved in the past 10 years, the most substantial therapeutic challenge in MS still refers to the progressive phase which is marked by moderate to extensive cortical demyelination, accumulating slowly over decades of disease activity. To invent new potent therapies for MS patients, we need to elucidate central pathogenic factors and cellular mechanisms embroiled in disease development. Paradigm shifts in the comprehension of a disease typically arise at the junction of bedside and laboratory. Evolving clinical practice with B-cell targeted therapies joined with a more advanced comprehension of humoral immunity in MS patients has led to substantial process in deciphering the immunopathogenesis of the disease.

4.1 The role of B-cells

Understanding the role of B-cells in MS pathogenesis has grown enormously following clinical success of B-cell depleting therapies and increased experimental evidence for significant B-cell participation. Having been primarily contemplated as antibody-producing cells only, they are now seen as key actors in conjunction and interplay with T-cells, unfolding stimulatory and regulatory capacities and functioning as both pro- and anti-inflammatory mediators as well as antigen-presenting cells.(24,42) Indeed, B-cells have turned out to be a relevant target for many highly efficacious therapeutics. In particular, monoclonal antibodies against CD20-expressing B-cells revealed dramatic effects in recently reported clinical trials. This anti-CD20 antibody treatment showed to be favorable not only in relapse-remitting types of the disease as it is the first therapeutic approach proven to protect from disability worsening in the progredient stage.(52,53)

4.2 A suitable animal model

The evolution of an animal model called EAE, often applied in mice, has helped to comprehend pathological processes leading to MS. It is able to imitate inflammatory infiltrates, neurological symptoms and demyelination, all characteristic of the

disease. Last but not least, this model has played a significant role in construing the many functions of B-cells in regulating MS.(79) Because when antibodies and B-cells interacting with T-cells were found to be imperative for full disease manifestation, a new theoretical setting for the use of B-cell targeted therapeutics in MS was established.(80) Despite the promising effects of new therapeutic regimens, there are justifiable limitations concerning earlier versions of animal models according to EAE. Reproducing the late progredient stage of MS pathology which is marked by widespread cortical demyelination had not been sufficient, with only inconstantly and unpredictably appearing cortical demyelination or in other cases, rather short-lived cortical demyelination lasting only a few days until full remyelination.(81) Until 2017, when Üçal et al. (78) have proven to be successful in modeling key histological features of cortical lesions, being the pathological hallmarks of progressive MS, in a new rat model, subsequently paving the way for further research on adequate treatment.

In support of this, we were able to test anti-CD20 antibody therapy in this new rat model, reproducing widespread cortical demyelination caused by immunization with MOG following intracerebral injection of the pro-inflammatory cytokines TNF-α and IFN-γ via a previously implanted catheter. In two experimental groups, B-cell depletion was induced by treatment with the anti-CD20 antibody, given either after or before MOG immunization. In our study, the extent of cortical destruction was assessed by quantification and statistical analysis of loss of PLP immunoreactivity in histology combined with microglial activation and loss of neurofilament immunoreactivity as well as apoptosis rate, astrocytic reaction and neuronal survival. These parameters were compared to the two control groups injected with an unspecific isotype-matched antibody in the same setup, and to the healthy control group, in all selected markers.

4.3 Conclusions

Our findings suggest a favorable impact of anti-CD20 antibody treatment on conservation of observed cortical structures in this animal model. Furthermore, the distribution of cortical demyelination in animals treated with the control antibody is remarkably reminiscent of that in diseased and untreated animals. It is thus

reasonable to assume that early therapy with anti-CD20 antibodies by eliminating B-cells, could reduce or even completely avert the formation of cortical lesions in the long term. These promising results indicate a contribution of B-cells in the development of cortical pathology, probably through different pathways, which paves the way for further research on the modus operandi of B-cells and might finally help to improve our knowledge of cellular mechanisms behind the progressive stage of MS. This in turn may extend therapeutic options for progressive MS patients to stop further tissue damage of the brain or even prevent progression in the first place.

All selected markers are associated with cortical demyelination and its cellular mechanisms. Autoimmune-inflammatory response results in damaged myelin and axonal loss. The most abundant protein found in myelin is PLP of which, in our work, a loss is detectable in the lesions. Moreover, activated microglia causes the release of pro-inflammatory cytokines leading to tissue damage in the CNS. Its prevalence in chronic neurodegeneration correlates to disease progression. Functionally impaired neurons in turn are likely associated with microglial activation.(57,82) Axonal loss was found to explain the reduction in number and size of neurons in upper cortical layers, especially associated with early aggressive disease course.(83,84) However, rather than actually losing neurons, this could also be reflected as temporary impairment of neuronal marker expression referring to transient neuronal dysfunction, also presented in other conditions of brain injury, e.g. experimental apoplexy and trauma.(85) In addition, dendritic or axonal pathology is indicated by loss of neurofilament immunoreactivity. Astrocytes are known to practice several tasks, both pro- and anti-inflammatory depending on their microhabitat and activation state. Since they are involved in trophic assistance and perpetuation of tissue homeostasis, them being affected by apoptosis might be significant for a more vulnerable brain and subsequent tissue destruction.(86,87) Caspase, a commonly known protease, plays a role in mediating cellular death in chronic neurodegenerative conditions and presents apoptotic cells.(88)

Overall, the investigated cortex of animals treated with the anti-CD20 antibody seems to be approximate to the one of healthy animals.

4.4 Limitations

In spite of the many parallels between animal models and MS and the general importance of animal models in basic research and development of drugs, it is crucial to point out that animal models vary from MS in different manners. Several studies show that active EAE induction for example demands peripheral activation of T-cells by typically using the common antigen MOG, while the source of immune-mediated inflammatory activation in MS is more complex and currently not entirely known.(89,90) In addition to this, EAE is mediated by T-cells expressing CD4+, whereas MS pathology is attributed to T-cells expressing both CD4+ and CD8+.(91,92) Another aspect concerns the in EAE preferably used antigen MOG, for which B-cells serve as APC. Instead of models immunized with other myelin peptides, such as the short MOG_{35-55} fragment, where B-cells are not participating in capture and display of these antigens.(93,94) Remarkably, when EAE was stimulated with whole MOG, B-cell depletion was protective, but in EAE induced with MOG_{35-55} peptide, B-cell depletion aggravated disease severity.(95,96) While this is not at all an extensive list of the variations between EAE and MS, it still serves as a considerable reminder that, even though EAE is very similar to MS, it is not fully capable of replicating the disease in all respects. Even our new animal model is not entirely transferable to humans since the created scenario with a single opening of the BBB will never be able to reproduce long-term condition of progressive MS patients completely. Nevertheless, it is crucial to note that animal models simulating cellular schemes under controlled circumstances are inevitable and that therapeutic agents should be examined in such models prior to progressing to clinical trials.

Theoretically, there seem to be little limitations of anti-CD20 antibody treatment in many diseases. Still, B-cells resting in cervical and other lymph nodes are not completely depleted by anti-CD20 therapy which could provide a continuous source of disease activity. Even though therapies targeting humoral immunity more extensively than anti-CD20 antibodies could offer a higher rate of effectiveness, with the use of such highly potent immunosuppressive therapies led against all stages of B-cells in MS, side effects are expected to appear more often.(24) This may explain the relative safety of anti-CD20 antibody treatment. However, further studies are indispensable to recognize potential harmful side effects.

Another remaining limitation refers to treatment started at a late stage. Most likely, cortical pathology accumulates over many decades of disease activity. In clinically manifest progressive MS, the patient might have already undergone multiple transient insults to the cortex as a bystander phenomenon of RRMS, which at some point cannot remyelinate as efficient as before resulting in disability. As therapeutic options in treating the progredient stage of the disease expand, remyelinating chronically demyelinated axons and restoring function to disabled patients would be the main goal.

To not go beyond the scope of a diploma thesis, the work presented in my study only shows results of the ipsilateral hemisphere of rat brains, although the contralateral side displays rather similar values. Further research in this context could mean an extension of the overall experiment. It is possible to simulate additional relapses with renewed injection of pro-inflammatory cytokines on day 30 of our animal model. First signs of remyelination also begin on day 30. Concerning this, more knowledge could be gained in regard to long-term effects of anti-CD20 treatment, also in prevention of cortical pathology.

4.5 Outlook and future research

Further work is required to identify whether the benefits demonstrated in this pivotal study might be of the same effect in progressive MS patients. The degree to which these short-term impacts transpose into long-term conservation against disability and through which mechanisms, the optimal dose and duration of anti-CD20 therapeutic agents and when or if such treatment can be safely stopped and restarted; all these aspects should be investigated further. Surveilling and measuring the long-term safety of monoclonal anti-CD20 antibodies in MS will be important to aid positioning this treatment within the broader context of already available disease-modifying therapies. One can say that the combination of more sophisticated outcome measures and more competent trial designs will raise chances of discovering new therapeutic strategies for people suffering from progressive MS. Furthermore, because numerous mechanisms seem to trigger and perpetuate tissue destruction in progressive MS, combinatorial therapies may be necessary to interrupt the different pathways causing damage and to restore function. Moreover, reducing severe symptoms and improving quality of life has to

be included in an encompassing interventional approach which can be of additional benefit to patients with progressive MS.

Disclosures

This research project obtained a financial grant from Roche; the anti-CD20 antibody was provided by Genentech. The company had however no influence on experimental setup nor data interpretation.

Appendices

The subsequent antibodies have been used:

Antibody	Target	Host	Dilution	Company	Catalog number	AB ID
Caspase3	Anti-active Caspase3 antibody	Rabbit	1:500	Abcam	ab2302	AB_302962
GFAP	Anti-GFAP Ab-6 (Clone ASTRO6)	Mouse	1:100	Thermo Scientific	MS-1376	AB_10995984
Iba1	Anti-Iba1	Rabbit	1:1000	Wako	019-19741	AB_839504
NeuN	Anti-NeuN, Clone A60 (KC)	Mouse	1:100	Millipore	MAB377-KC	AB_2298772
Neurofilament	Anti-200kD Neuro-filament Heavy antibody	Rabbit	1:2000	Abcam	ab8135	AB_306298
PLP	Anti-myelin proteolipid protein	Mouse	1:500	AbD Serotec	MCA839G	AB_2237198
Biotinylated sec. AB rabbit	Anti-rabbit IgG	Donkey	1:200	GE Healthcare	RPN1004	AB_1062582
Biotinylated sec. AB mouse	Anti-mouse IgG	Sheep	1:200	GE Healthcare	RPN1001	AB_1062579

Biotinylated sec. AB sheep/goat	Anti-sheep/goat IgG	Donkey	1:200	GE Healthcare	RPN1025	AB_1082105
Anti-rabbit ImmPRESS reagent	Anti-rabbit IgG	Horse	Ready to use	Vector	MP-7401	AB_2336529
Anti-mouse ImmPRESS reagent	Anti-mouse IgG	Horse	Ready to use	Vector	MP-7422	AB_2336527
VectaFluor DyLight488	Anti-rabbit IgG	Horse	Ready to use	Vector	DI-1788	AB_2336781
VectaFluor DyLight594	Anti-mouse IgG	Horse	Ready to use	Vector	DI-2794	AB_2336783

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