Myelin oligodendrocyte glycoprotein (MOG) antibody associated disorders: Identification of MOG-specific B cells and analysis of the pathogenic potential of MOG antibodies

Dissertation

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Diese Dissertation wurde angefertigt unter der Leitung von Prof. Dr. Edgar Meinl
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ERKLÄRUNG

Ich versichere hiermit an Eides statt, dass meine Dissertation selbständig und ohne unerlaubte Hilfsmittel angefertigt worden ist. Die vorliegende Dissertation wurde weder ganz, noch teilweise bei einer anderen Prüfungskommission vorgelegt. Ich habe noch zu keinem früheren Zeitpunkt versucht, eine Dissertation einzureichen oder an einer Doktorprüfung teilzunehmen.

München, den 08.04.2021

Stephan Andreas Paul Erich Winklmeier

“When you've worked hard, and done well,
and walked through that doorway of opportunity,
you do not slam it shut behind you.
You reach back and you give other folks
the same chances that helped you succeed.”

Michelle Obama, 2013

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Abbreviations

AA	Amino acid(s)
Ab/Abs	Antibody/ antibodies
ADCC	Ab-dependent cell-mediated cytotoxicity
ADEM	Acute disseminated encephalomyelitis
Ala	Alanine
APC	Antigen presenting cells
AQP	Aquaporin
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
BBB	Blood brain barrier
BTN	Butyrophilin
C9neo	Complement component 9 neo-antigen
CBA	Cell- based assays
CD	Clusters of differentiation
CDC	Complement dependent cytotoxicity
cf.	From Latin *confer*
CHO	Chinese hamster ovary cell line
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
EAE	Experimental autoimmune encephalomyelitis
ED1	Antibody 1 from Ed Döpp against macrophages
EGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment crystallizable
GAD	Glutamic acid decarboxylase
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
H103A	His substituted by Ala at position 103
HEK293	Human embryonic kidney cell line

HeLa	Cervix carcinoma cell line from Henrietta Lacks
His	Histidine
IA	Immunoadsorption
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL-2	Interleukin-2
IVIG	Intravenous Ig
kDa	Kilodalton
Kv	Voltage-gated potassium channel
LETM	Longitudinally extensive transverse myelitis
LFB	Luxol fast blue
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MOGAD	MOG Ab associated disorders
MS	Multiple sclerosis
Nav	Voltage-gated sodium channel
NGF	Nerve growth factor
NK	Natural killer
NMDAR	N-methyl-D-aspartate receptor
NMO	Neuromyelitis optica
NMOSD	NMO spectrum disorders
OCB	Oligoclonal bands
ON	Optic neuritis
P42S	Pro substituted by Ser at position 42
PBMC	Peripheral blood mononuclear cells
PLEX	Plasma exchange
PN	Paranode
PPMS	Primary progressive MS
Pro	Proline
RIA	Radioimmunoprecipitation assays
RRMS	Relapsing-remitting MS
S104E	Ser substituted by Glu at position 104
Ser	Serine
SPMS	Secondary progressive MS

TLR	Toll-like receptor
TM	Transverse myelitis
TT	Tetanus toxoid
Tyr	Tyrosine

List of publications

1 Publications – included in the present thesis

The following two publications are the major part of the present cumulative thesis.

1.1 Spadaro M. et al. (2018) - Pathogenicity of human antibodies against myelin oligodendrocyte glycoprotein

Melania Spadaro, Stephan Winklmeier, Eduardo Beltrán, Caterina Macrini, Romana Höftberger, Elisabeth Schuh, Franziska S. Thaler, Lisa Ann Gerdes, Sarah Laurent, Ramona Gerhards, Simone Brändle, Klaus Dornmair, Constanze Breithaupt, Markus Krumbholz, Markus Moser, Gurumoorthy Krishnamoorthy, Frits Kamp, Dieter Jenne, Reinhard Hohlfeld, Tania Kümpfel, Hans Lassmann, Naoto Kawakami, Edgar Meinl.

This research article was first published in **Annals of Neurology** on July 16, 2018.

DOI: https://doi.org/10.1002/ana.25291

1.2 Winklmeier S. et al. (2019) - Identification of circulating MOG-specific B cells in patients with MOG antibodies

Stephan Winklmeier, Miriam Schlüter, Melania Spadaro, Franziska S. Thaler, Atay Vural, Ramona Gerhards, Caterina Macrini, Simone Mader, Aslı Kurne, Berin Inan, Rana Karabudak, Feyza Gul Ozbay, Gunes Esendagli, Reinhard Hohlfeld, Tania Kümpfel, Edgar Meinl.

This research article was first published in **Neurology: Neuroimmunology & Neuroinflammation** on October 14, 2019.

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2 Further publications – not specifically introduced in the present thesis

In addition, I contributed to the following publications, which are not part of the present thesis.

2.1 Thaler F. S. et al. (2019) – Abundant glutamic acid decarboxylase (GAD)-reactive B cells in gad-antibody–associated neurological disorders

Franziska S. Thaler, Anna L. Thaller, Michelle Biljecki, Elisabeth Schuh, Stephan Winklmeier, Christoph F. Mahler, Ramona Gerhards, Stefanie Völk, Frauke Schnorfeil, Marion Subklewe, Reinhard Hohlfeld, Tania Kümpfel, Edgar Meinl.

This research article was first published in **Annals of Neurology** on January 11, 2019.

DOI: https://doi.org/10.1002/ana.25414

2.2 Bronge M. et al. (2019) – Myelin oligodendrocyte glycoprotein revisited — sensitive detection of MOG-specific T-cells in multiple sclerosis

Mattias Bronge, Sabrina Ruhrmann, Claudia Carvalho-Queiroz, Ola B. Nilsson, Andreas Kaiser, Erik Holmgren, Caterina Macrini, Stephan Winklmeier, Edgar Meinl, Lou Brundin, Mohsen Khademi, Tomas Olsson, Guro Gafvelin, Hans Grönlund.

This research article was first published in **Journal of Autoimmunity** on May 1, 2019.

DOI: https://doi.org/10.1016/j.jaut.2019.04.013

2.3 Fernandez I. M. et al. (2019) – The Glycosylation Site of Myelin Oligodendrocyte Glycoprotein Affects Autoantibody Recognition in a Large Proportion of Patients

Iris Marti Fernandez, Caterina Macrini, Markus Krumbholz, Paul J Hensbergen, Agnes L Hipgrave Ederveen, Stephan Winklmeier, Atay Vural, Asli Kurne, Dieter Jenne, Frits Kamp, Lisa Ann Gerdes, Reinhard Hohlfeld, Manfred Wuhrer, Tania Kümpfel, Edgar Meinl.

This research article was first published in **Frontiers in Immunology** on June 11, 2019.

DOI: https://doi.org/10.3389/fimmu.2019.01189

2.4 Faissner S. et al. (2020) – Binding patterns and functional properties of human antibodies to AQP4 and MOG on murine optic nerve and retina

Simon Faissner, Florian Graz, Sabrina Reinehr, Laura Petrikowski, Steffen Haupeltshofer, Ulaş Ceylan, Gesa Stute, Stephan Winklmeier, Florence Pache, Friedemann Paul, Klemens Ruprecht, Edgar Meinl, H Burkhard Dick, Ralf Gold, Ingo Kleiter, Stephanie C Joachim.

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2.5 Gerhards R. et al. (2020) – Oligodendrocyte myelin glycoprotein as a novel target for pathogenic autoimmunity in the CNS

Ramona Gerhards, Lena Kristina Pfeffer, Jessica Lorenz, Laura Starost, Luise Nowack, Franziska S Thaler, Miriam Schlüter, Heike Rübsamen, Caterina Macrini, Stephan Winklmeier, Simone Mader, Mattias Bronge, Hans Grönlund, Regina Feederle, Hung-En Hsia, Stefan F Lichtenthaler, Juliane Merl-Pham, Stefanie M Hauck, Tanja Kuhlmann, Isabel J Bauer, Eduardo Beltrán, Lisa Ann Gerdes, Aleksandra Mezydlo, Amit Bar-Or, Brenda Banwell, Mohsen Khademi, Tomas Olsson, Reinhard Hohlfeld, Hans Lassmann, Tania Kümpfel, Naoto Kawakami, Edgar Meinl.

This research article was first published in **Acta Neuropathologica Communications** on November 30, 2020.

DOI: https://doi.org/10.1186/s40478-020-01086-2

2.6 Macrini C. et al. (2021) – Features of MOG required for recognition by patients with MOG antibody-associated disorders

Caterina Macrini, Ramona Gerhards, Stephan Winklmeier, Lena Bergmann, Simone Mader, Melania Spadaro, Atay Vural, Michaela Smolle, Reinhard Hohlfeld, Tania Kümpfel, Stefan F Lichtenthaler, Henri G Franquelim, Dieter Jenne, Edgar Meinl.

This research article was first published in **Brain** on March 11, 2021.

DOI: https://doi.org/10.1093/brain/awab105

2.7 Schlüter M. et al. (under revision) – Differential effects of long-term therapy with natalizumab on features of intrathecal IgG production indicates targeting of plasmablasts

Miriam Schlüter, Eva Oswald, Stephan Winklmeier, Ingrid Meinl, Joachim Havla, Peter Eichhorn, Edgar Meinl, Tania Kümpfel.

This research article is currently under revision in **Neurology: Neuroimmunology & Neuroinflammation**.

2.8 Winklmeier S. et al. (in preparation) – Persistence and function of memory B cells recognizing SARS-CoV-2 despite loss of specific IgG

Stephan Winklmeier, Katharina Eisenhut, Damla Taskin, Heike Rübsamen, Celine Schneider, Peter Eichhorn, Matthias Klein, Simone Mader, Tania Kümpfel, Edgar Meinl.

This research article is currently in preparation.

Declaration of contribution as a co-author

Spadaro M. et al. (2018) - Pathogenicity of human antibodies against myelin oligodendrocyte glycoprotein

Melania Spadaro, Stephan Winklmeier, Eduardo Beltrán, Caterina Macrini, Romana Höftberger, Elisabeth Schuh, Franziska S. Thaler, Lisa Ann Gerdes, Sarah Laurent, Ramona Gerhards, Simone Brändle, Klaus Dornmair, Constanze Breithaupt, Markus Krumbholz, Markus Moser, Gurumoorthy Krishnamoorthy, Frits Kamp, Dieter Jenne, Reinhard Hohlfeld, Tania Kümpfel, Hans Lassmann, Naoto Kawakami, Edgar Meinl.

This research article was first published in **Annals of Neurology** on July 16, 2018.

DOI: https://doi.org/10.1002/ana.25291

This publication **Spadaro M. et al. (2018)** is part of the present cumulative thesis.
I contributed to this publication as follows:

In this work, I performed the cell-based assay experiments on anti-human MOG reactivity in serum dilutions from the two patients, shown in Figure 1B, and on human and rat MOG recognition of the affinity-purified Abs from the two patients in comparison with the recombinant humanized Ab 8-18C5, shown in Figures 2F and 2G. During the revision, I performed the requested cell-based assay experiments on the MOG reactivity and on the MOG epitope recognition of the affinity-purified Abs from the two patients in comparison to the flow-through fraction of the purification process, mentioned in the text on page 4f., and I performed the ELISA experiments to show that the affinity-purified Abs did not bind to streptavidin alone, which is also mentioned in the text on page 4. In addition, I performed the cell-based assay experiments to confirm that the IgG isotype of the anti-MOG response of the affinity-purified Abs of both patients was IgG1, which is also mentioned in the text on page 4. Further, I extensively worked on the writing of the manuscript together with M. Spadaro, and contributed especially on the preparation of Figures 1, 2 and 4, and the completion of Table 1.

Prof. Dr. Edgar Meinl

Prof. Dr. Elisabeth Weiß

Summary

The myelin sheath is an essential protecting layer around the axons of the nerve cells in our brain. This insulation is necessary for a fast transmission of action potentials between cells and enables proper motor function, sensory function and cognition in our body. A proportion of patients with inflammatory diseases of the central nervous system harbour antibodies (Abs), that attack the myelin oligodendrocyte glycoprotein (MOG), which is expressed on the outermost surface of this insulating myelin layers. There is growing consensus that patients with Abs against MOG constitute the separate disease entity MOG Ab associated disorder (MOGAD).

In the first part of this thesis we aimed to elucidate the pathogenic mechanisms of MOG Abs in transfer experiments (in collaboration with PD. Dr. Naoto Kawakami and Prof. Dr. Hans Lassmann). Hereby, we selected two patients harbouring MOG Abs with a cross-reactivity to rodent MOG. We successfully affinity-purified MOG Abs from these patients, which recognized full-length MOG on transfected cells and showed a myelin staining on tissue sections. Further, we demonstrated that these patient-derived MOG Abs were pathogenic upon intrathecal injection in two different rat models. Together with cognate MOG-specific T cells, these Abs enhanced T-cell infiltration; together with myelin basic protein–specific T cells, which strongly breach the blood brain barrier, they induced demyelination associated with complement deposition. These pathogenic changes resembled a multiple sclerosis type II pathology, suggesting that these Abs are similarly pathogenic in patients.

Second, we set out to identify MOG-specific B cells in blood of patients with MOG Abs. Herby, we differentiated B cells from blood *ex vivo* into immunoglobulin-producing cells and quantified the MOG recognition of the produced Abs. Thereby, we detected in most, but not all patients MOG-specific B cells in the blood. These circulating MOG-specific B cells did not show a correlation with anti-MOG Ab levels in serum, suggesting other sources for MOG Abs in these patients. In addition, we were able to reveal an intraindividual heterogeneity of the anti-MOG autoimmunity by analyzing the epitope recognition of MOG Abs secreted from cultured blood cells.

Together, this thesis has two major findings. First, two pathogenic mechanisms of MOG Abs from MOGAD-patients are uncovered, namely demyelination and enhancement of cognate T cell activation. Second, patients with MOGAD differ in the presence of

circulating MOG-specific B cells; this could be useful in the future to stratify patients with MOGAD for therapy optimization.

Zusammenfassung

Die Axone der Nervenzellen in unserem Gehirn sind größtenteils von einer schützenden Myelinschicht umgeben. Diese Isolierung ist notwendig für eine schnelle Übertragung von Aktionspotentialen zwischen den Zellen und ermöglicht unserem Körper eine einwandfreie Funktion der Motorik, Sensorik und Wahrnehmung. Bei einem Teil der Erkrankten mit entzündlichen Krankheiten des zentralen Nervensystems kommen Antikörper vor, die das Myelin-Oligodendrozyten-Glykoprotein (MOG) angreifen. Dieses befindet sich auf der äußersten Oberfläche der isolierenden Myelinschicht. Seit den letzten Jahren gibt es eine wachsende Einigkeit darüber, dass Erkrankte mit Antikörpern gegen MOG die eigenständige Krankheitsentität MOGAD (aus dem Englischen für *MOG antibody associated disorder*) bilden.

Der erste Teil dieser Arbeit hatte zum Ziel, die pathogenen Mechanismen von MOG Antikörpern in Transferexperimenten zu untersuchen (in Zusammenarbeit mit PD. Dr. Naoto Kawakami und Prof. Dr. Hans Lassmann). Für diese Experimente haben wir zwei Erkrankte ausgewählt, die kreuzreaktive Antikörper gegen MOG aus Nagetieren aufweisen. Wir konnten erfolgreich affinitätsgereinigte MOG Antikörper von diesen Erkrankten isolieren, die das Volllängenprotein von MOG auf transfizierten Zellen erkannten und Myelin auf Gewebeschnitten färbten. Des Weiteren konnten wir zeigen, dass diese humanen MOG Antikörper nach intrathekaler Injektion in zwei verschiedenen Rattenmodellen pathogen waren. Zusammen mit kognitiven MOG-spezifischen T-Zellen verstärkten diese Antikörper die T-Zell-Infiltration. Gemeinsam mit Basischen Myelinprotein-spezifischen T-Zellen, die selbst in der Lage sind die Blut-Hirn-Schranke zu durchbrechen, lösten sie eine Demyelinisierung aus, die mit der Ablagerung von Komplement einherging. Diese pathogenen Veränderungen ähnelten einer Multiple-Sklerose-Typ-II-Pathologie, was darauf schließen lässt, dass diese Antikörper bei den Erkrankten ähnlich pathogen wirken.

Der zweite Teil dieser Arbeit hatte zum Ziel die MOG-spezifischen B-Zellen im Blut von Erkrankten mit MOG Antikörpern zu untersuchen. Dazu differenzierten wir B-Zellen aus Blut *ex vivo* zu Immunglobulin-produzierenden Zellen und quantifizierten die Reaktivität gegen MOG der freigesetzten Antikörper. Dabei konnten wir bei den meisten, aber nicht allen Erkrankten, MOG-spezifische B-Zellen im Blut nachweisen. Diese zirkulierenden MOG-spezifischen B-Zellen zeigten keine Korrelation mit dem Spiegel von MOG Antikörpern im Serum, was auf andere Quellen für MOG Antikörper bei diesen Erkrankten hindeutet. Darüber hinaus konnten wir eine intraindividuelle Heterogenität

der anti-MOG-Autoimmunität feststellen, indem wir die Epitoperkennung von MOG-Antikörpern der kultivierten Blutzellen analysierten.

Zusammengefasst trägt diese Arbeit zu zwei wesentlichen Erkenntnissen bei. Erstens werden zwei pathogene Mechanismen von MOG Antikörpern aus Erkrankten mit MOGAD aufgezeigt, nämlich Demyelinisierung und Verstärkung der kognitiven T-Zell-Aktivierung. Zweitens unterscheiden sich Erkrankte mit MOGAD durch das Vorhandensein von zirkulierenden MOG-spezifischen B-Zellen. Dies könnte in Zukunft nützlich sein, um Erkrankte mit MOGAD für die Therapieoptimierung zu stratifizieren.

Introduction

1 Demyelinating autoimmunity of the central nervous system (CNS)

1.1 Myelin loss in the CNS

The myelin sheath is the protective layer that wraps around axons of nerve fibers (**Figure 1**). In our brain, optic nerve and spinal cord it is formed and maintained by oligodendrocytes (Simons and Nave 2015). Myelin insulates the axon and increases the resistance of the membrane. Voltage-gated sodium and potassium channels are mainly located at the short unmyelinated parts of the axon, the nodes of Ranvier. This alternate structure of myelinated and unmyelinated parts enables action potentials to rapidly advance in the energy-efficient process of saltatory conduction. A damage of the myelin sheath results in slower transmission of action potentials, prolonged latency and can also lead to complete loss of nerve conduction (Cunniffe and Coles 2019, Lubetzki, Sol-Foulon et al. 2020).

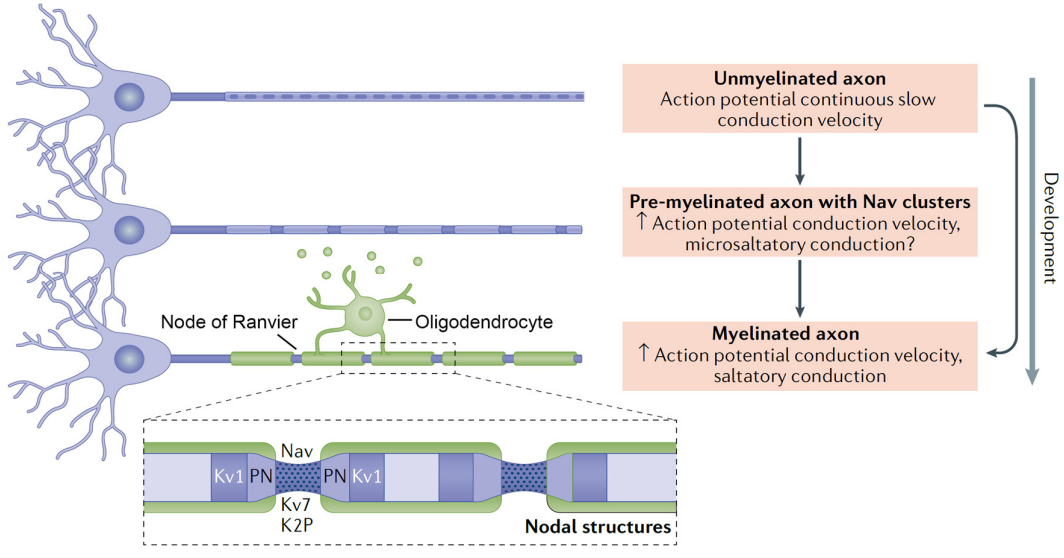

Figure 1| Influence of myelination on action potential conduction velocity. With increased myelination the conduction velocity is enhanced. Voltage-gated sodium (Nav) and potassium channels (Kv) are indicated in the enlarged nodal structure. The oligodendrocyte and myelin sheath are colored in green. PN: paranode. Modified and with permission from (Lubetzki, Sol-Foulon et al. 2020).

Demyelination occurs in several diseases and can have different causes, such as viral infection, loss of oxygen, toxic or metabolic reasons and inflammation (Love 2006). Thereby, autoimmunity can be an inflammatory trigger. Usually, our immune system protects us from invading pathogens. However, in about 7 – 9 % of the human population, it falsely recognizes self-antigens as foreign and attacks its own body (Theofilopoulos, Kono et al. 2017). If the autoimmune reaction is directed against the CNS, it may lead to

myelin loss. These inflammatory demyelinating disorders contain a broad spectrum of mechanistically and clinically heterogeneous diseases, which are explained in more detail under the introductory part 2.3. Amongst those, multiple sclerosis (MS) is the most common inflammatory demyelinating disease in young adults (Meinl, Derfuss et al. 2010, Mayer and Meinl 2012).

1.2 Multiple sclerosis and the suspected role of B cells in this disease

MS is a chronic neuroinflammatory disease, which can affect the brain, spinal cord and optic nerve. It is estimated that about 2.3 million people live with MS globally. The average age of clinical onset is at around 30 years of age and the disease occurs about three times more frequently in women than in men. Patients can suffer from sensory and visual disturbance, motor impairment, fatigue, pain and cognitive deficits (Thompson, Baranzini et al. 2018, Dobson and Giovannoni 2019). After a few decades of disease progression, about 25% of patients require assistance for mobility such as the use of a wheelchair (Sosnoff, Peterson et al. 2018). MS is a very heterogeneous disease that is triggered by genetic risk factors, including the human leukocyte antigen haplotype DRB1*15:01, and environmental risk factors, including low vitamin D levels, cigarette smoking and obesity (Thompson, Baranzini et al. 2018). Different disease courses have been described. The majority of patients with MS begin with a single neurological demyelinating episode, termed as clinically isolated syndrome (CIS), that typically affects the optic nerves, brainstem or spinal cord. Patients with CIS may recover over time from the presenting episode. However, about 30 - 70% of patients with CIS progress in disease course and develop MS with lesions that disseminate in space with multiple locations in the CNS, and in time with repeated episodes (Miller, Barkhof et al. 2005). This most common disease course of MS is kwon as relapsing-remitting MS (RRMS). Patients experience an alternation between temporary episodes of relapses followed by recovery phases of partial or complete remission. The majority of patients with RRMS develop later on secondary progressive MS (SPMS) with a continuous worsening of symptoms. A minority of MS patients are diagnosed with primary progressive MS (PPMS). This form features a progressive worsening from the onset with no relapses or remissions. Beside those three main disease courses, rare cases of other variants such as progressive-relapsing MS are described (Dendrou, Fugger et al. 2015, Thompson, Baranzini et al. 2018, Dobson and Giovannoni 2019).

The most characteristic pathological change in MS brains is the formation of multifocal lesions in the forms of large confluent demyelinated plaques in the white and grey matter (Lassmann 2013). However, the causes of inflammation and demyelination in MS are not yet fully understood. There are different possible forms and patterns discussed. A large cohort of pathological human tissue-samples of MS with actively demyelinating lesions was analyzed and from these samples, four fundamentally different patterns were found. Demyelination of MS type I and II patterns are proposed to be driven by autoimmune encephalomyelitis. This inflammation is dominated by infiltration of T-lymphocytes and macrophages. In addition, MS type II pathology features the involvement of antibodies (Abs) and activation of the complement system. Pattern III and IV are suggested to have a virus- or toxin-induced demyelination that leads to primary oligodendrocyte dystrophy (Lucchinetti, Bruck et al. 2000).

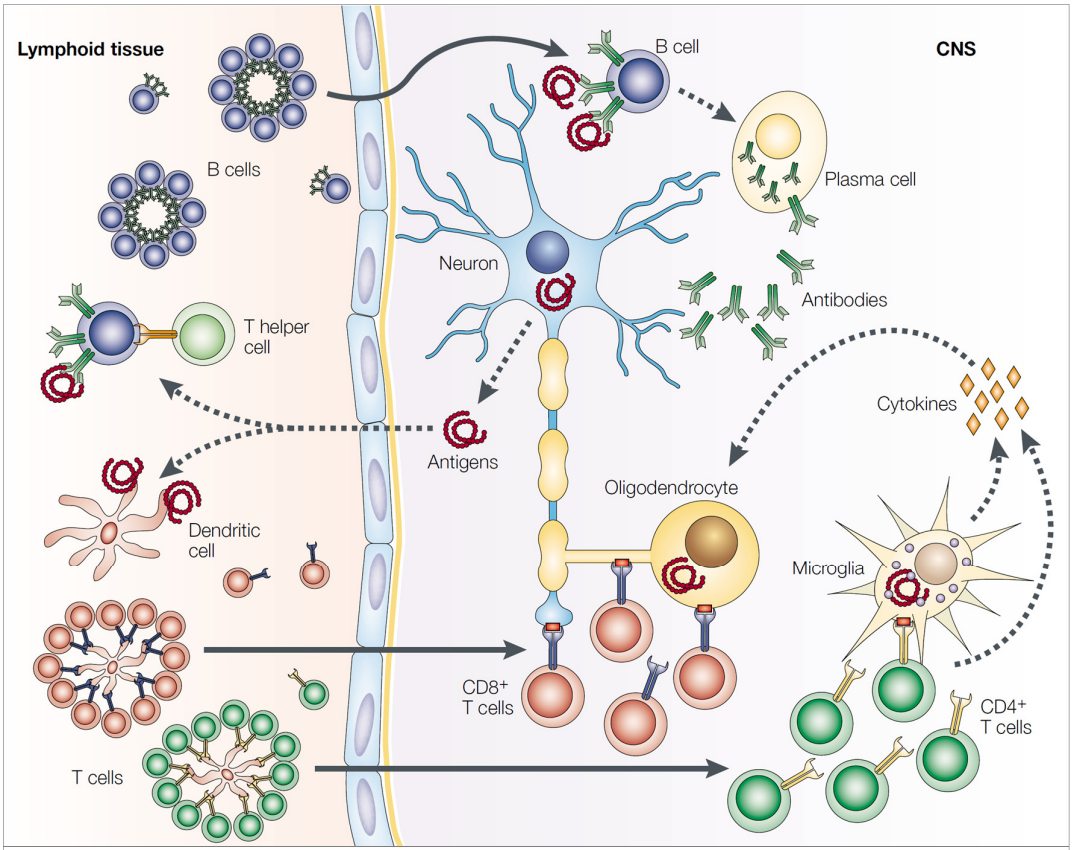

Figure 2| Proposed model of the immune responses in MS. T and B cells may get activated in the periphery by antigens released from the CNS and presented by dendritic cells, or may cross-react with foreign antigens. After expansion, the B and T cells may infiltrate to the CNS. B cells encounter their neural antigen and differentiate into plasma cells releasing antigen targeting Abs. Cytotoxic CD8+ T cells encounter and may target directly glial or neuronal cells presenting their specific peptide ligand. CD4+ T cells get reactivated by glial cells presenting their peptide ligand and attract other immune cells by releasing cytokines. Invading macrophages contribute to the inflammation and may attack the myelin sheath. With permission from (Hemmer, Archelos et al. 2002).

While the underlying immune-pathological mechanisms and targeted antigens are intensively studied in autoimmune animal models for MS, known as experimental autoimmune encephalomyelitis (EAE), their corresponding counterparts in humans remain largely undefined (Hohlfeld, Dornmair et al. 2016). Studies in these animal models identified T lymphocytes as the main drivers of the disease. However, several findings in human MS patients suggest that B cells and Abs also have a crucial role in the disease pathogenesis. Obermeier et al. and von Büdingen et al. could show that the characteristic feature of persisting oligoclonal immunoglobulin (Ig) G (IgG) in the cerebrospinal fluid (CSF) of MS patients is produced by intrathecal B cells. These plasma cells are clonally expanded, which indicates the existence of an antigen-driven B cell response within the CNS (Obermeier, Mentele et al. 2008, von Budingen, Gulati et al. 2010). Additionally, the pathological implication of B cells and Abs is supported by partial therapeutic benefits through Ig removal with plasma exchange or B cell depletion with monoclonal Abs against clusters of differentiation 20 (CD20) (Keegan, König et al. 2005, Hauser, Waubant et al. 2008, Greenfield and Hauser 2018). This leads to the proposed model (**Figure 2**) wherein both T and B cells contribute to the pathology of MS. Autoreactive T cells, that recognize components of the myelin sheath as foreign, get activated by antigen-presenting cells such as dendritic cells. Once activated, CNS-reactive T cells can disrupt the blood brain barrier (BBB), penetrate into the CNS and initiate a damaging and inflammatory response. After the BBB is breached, autoreactive B cells can infiltrate into the CNS and modify inflammation by secreting pro-inflammatory and regulatory cytokines. Those B cells are either activated in the periphery or after infiltration in the CNS compartment by self-antigens. Once activated, B cells proliferate and subsequently differentiate into plasmablasts and plasma cells that secrete auto-Abs. These Abs can

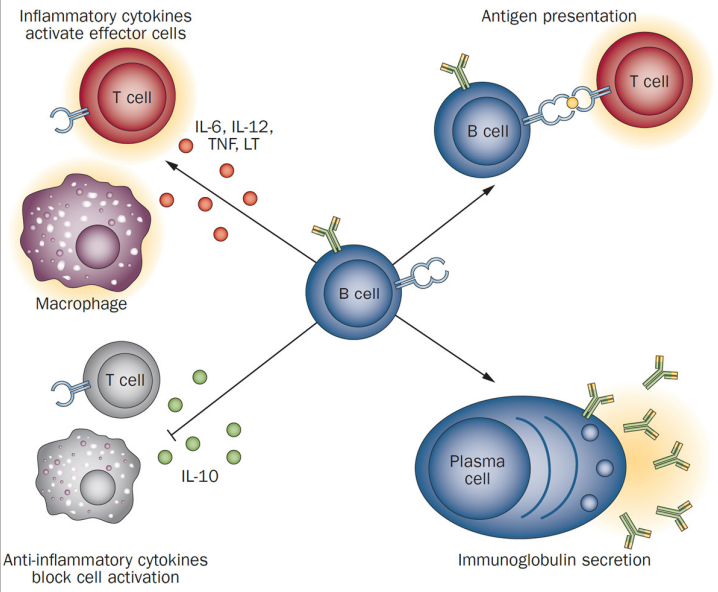

Figure 3| Proinflammatory (cytokine release, antigen presentation, immunoglobulin secretion) and anti-inflammatory (cytokine release) features of B cells. With permission from (Krumbholz, Derfuss et al. 2012).

target components of the myelin sheath and initiate a demyelination through the Ab-dependent cell-mediated cytotoxicity (ADCC), and through the complement dependent cytotoxicity (CDC). In addition, B cells are efficient antigen-presenting cells and can activate T lymphocytes by presenting their cognate CNS self-antigen via major histocompatibility complex (MHC) class II molecules (Krumbholz, Derfuss et al. 2012, Krumbholz and Meinl 2014, Hohlfeld, Dornmair et al. 2016, Hausser-Kinzel and Weber 2019). A summary of B cell function beyond Ig secretion is illustrated in **Figure 3**.

1.3 Specific autoantibodies as biomarkers

MS and associated diseases are part of a broad range of disorders, which often share overlapping features, particularly at the beginning of the disease. Therefore, specific biomarkers are required for a clear indication and treatment strategy. Classically, the presence of persisting oligoclonal IgG, known as oligoclonal bands (OCB), has been used as the prognostic biomarker. Those OCBs are only present in the CSF but not in the serum of the patients. Commonly, MS is diagnosed by magnetic resonance imaging. Thereby, disease activity can be visualized by gadolinium contrast agent to detect active lesions (Baecher-Allan, Kaskow et al. 2018). However, more defined biomarkers are needed to specify distinct disease-driving mechanisms. In the last years, several auto-Abs have drawn the attention as important candidates for diagnosis and stratification of patients.

A meaningful achievement was the discovery of auto-Abs against the water channel protein, aquaporin (AQP)4, on astrocytes (Lennon, Wingerchuk et al. 2004, Lennon, Kryzer et al. 2005). These Abs, which are detected in about 80% of patients, are highly disease-specific and enabled a clear separation of neuromyelitis optica (NMO) spectrum disorders (NMOSD) from the former “core disease” MS. However, a subgroup of patients with clinical features suggestive for NMOSD is seronegative for AQP4-IgG. About one third of them harbor Abs in their blood against another auto-antigen: the myelin oligodendrocyte glycoprotein (MOG). Patients with MOG-IgG are found in a broad clinical spectrum of autoimmune diseases of the CNS. There is growing consensus that these patients constitute a separate disease entity, recently termed as MOG Ab associated disorder (MOGAD), which is the main focus of the present study (Hausser-Kinzel and Weber 2019, Mader, Kumpfel et al. 2020).

2 Myelin oligodendrocyte glycoprotein antibody associated disorders

2.1 The MOG protein

MOG was identified 40 years ago by Lebar et. al. For their study, they used whole brain homogenate to induce EAE in guinea pigs and could show that MOG was a target of Abs, which mediated complement-dependent demyelination (Lebar, Boutry et al. 1976, Lebar, Lubetzki et al. 1986). In general, MOG is expressed exclusively in the CNS of mammals and is highly conserved between species (Delarasse, Della Gaspera et al. 2006). It is only a minor component of the myelin sheath, constituting less than 0.05% of all myelin proteins. However, MOG has an exposed position on the outermost surface of the myelin sheath expressed by oligodendrocyte processes (Brunner, Lassmann et al. 1989). This makes MOG easily accessible as a target for Abs compared to other more abundant myelin components, such as the myelin basic protein (MBP) (Mayer and Meinl 2012).

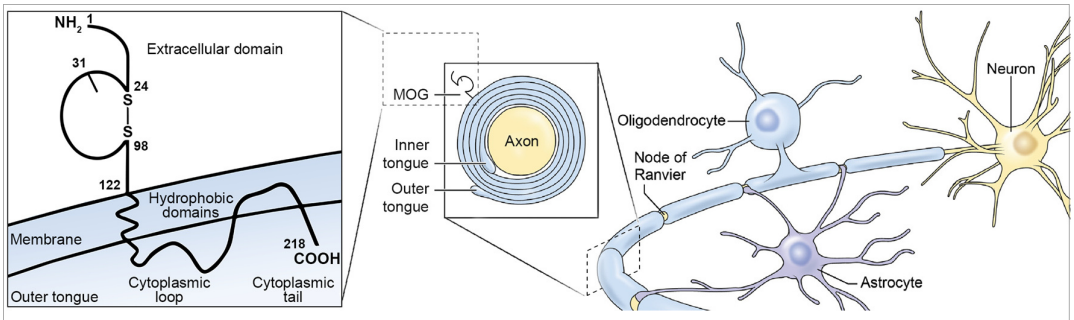

Figure 4| Detailed structure of MOG (left) and the myelin sheath (middle): MOG, with its extracellular domain, two hydrophobic domains, the cytoplasmic loop and tail. Oligodendrocytes wrap multiple layers of compacted cell membrane around the axons of neurons. MOG is expressed on the membrane of the outer tongue of the sheath. Right, astrocytes interact with the nodes of Ranvier, the unmyelinated parts of the axons. Modified and with permission from (Weissert, Kuhle et al. 2002, Fields and Dutta 2019).

MOG is a single-span transmembrane protein and belongs to the Ig superfamily (**Figure 4**). It contains one extracellular Ig variable domain at the N-terminus with a single glycosylation site at asparagine 31, a disulfide bond between the two cysteines at position 24 and 98, two hydrophobic domains (one transmembrane and one membrane-associated), one cytoplasmic loop, and a C-terminal cytoplasmic tail (della Gaspera, Pham-Dinh et al. 1998). In humans, 15 different alternatively spliced isoforms of MOG are described (Delarasse, Della Gaspera et al. 2006). Two of them are full-length variants: α1 (218 amino acids (AA); molecular mass 25.1 kilodalton (kDa)) and β1 (223 AA; molecular mass 25.6 kDa), which differ in their amino acids at the C-terminus. In the present study, the major investigated full-length variant α1 is used with arginine, asparagine, proline and phenylalanine as C-terminal amino acids from exon 10a (Boyle, Traherne et al. 2007, Reindl and Waters 2019).

So far, the biological role of MOG and its isoforms is not yet fully understood. Knockout mice with MOG deficiency revealed no clinical or histological abnormalities (Delarasse, Daubas et al. 2003). However, there are several studies that link MOG with different possible functional mechanisms. Hereby, MOG could act as an adhesion molecule by gluing neighboring CNS myelin fibers together and could be involved in microtubule stability of oligodendrocytes (Johns and Bernard 1999, Clements, Reid et al. 2003). In addition, the extracellular Ig domain of MOG has been shown to interact with different binding partners: with the complement component C1q and thereby potentially regulating the classical complement system (Johns and Bernard 1999); with the nerve growth factor (NGF) and thus potentially modulating central axon growth and survival (von Budingen, Mei et al. 2015); with the rubella virus as potential host cell receptor and thereby possibly making cells permissive for virus entry (Cong, Jiang et al. 2011). Furthermore, when MOG is correctly glycosylated, it may interact with the c-type lectin receptor DC-SIGN, a dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin that is expressed on the surface of dendritic cells and macrophages, and possibly keeps those antigen presenting cells (APC) in an immature and tolerogenic state; thus preventing autoimmunity (Garcia-Vallejo, Ilarregui et al. 2014). In contrast, autoimmunity could be caused by molecular mimicry. MOG is highly homologous to the milk protein butyrophilin (BTN) and shares sequential and structural similarities which may trigger a cross-reactive immunological response (Guggenmos, Schubart et al. 2004).

2.2 Detection methods of MOG antibodies

Immunoblotting and enzyme-linked immunosorbent assays (ELISA) were applied in early studies to detect Abs against MOG. These assays used the linear or refolded recombinantly produced extracellular domain of the MOG protein (usually AA 1–125) as substrate. However, these methods led to controversial results in initial investigations. Some studies showed a high proportion of Abs against MOG in MS, whereas others were not able to reproduce those findings. Further studies revealed that these assays detect MOG-specific Abs against non-native MOG, which do not cause disease and can occur frequently in the general population. Therefore, immunoblotting and ELISAs cannot be used as reliable detection methods for MOG Abs (Reindl and Waters 2019).

Radioimmunoprecipitation assays (RIA) were then developed and permitted the clinical association of MOG Abs with a demyelinating phenotype that was not MS. One of the pioneers in this field was Kevin O'Connor. For the RIA, he used self-assembling

radiolabeled tetramers, which show a high sensitivity and specificity. To provide the native folding environment for the extracellular domain of human MOG, an *in vitro* translation system is applied with endoplasmic reticulum microsomes isolated from a mouse hybridoma cell line. The extracellular domain of human MOG is connected to a monomer of streptavidin via a flexible linker, which allows a spontaneous assembly into a tetrameric structure. The RIA enables the detection of conformation-sensitive MOG Abs and can distinguish them from conformation-independent Abs (O'Connor, McLaughlin et al. 2007).

Cell- based assays (CBA) are now widely used for the detection of MOG Abs in research and clinic, and are considered as the current gold standard. Thereby, mammalian cells are transiently or stably transfected with plasmids that encode for full-length human MOG. These immortal cell lines, such as the cervix carcinoma cells from Henrietta Lacks (HeLa), human embryonic kidney cells (HEK293) or Chinese hamster ovary cells (CHO), transcribe MOG from the plasmid and express it in a native state on the cell surface. Auto-Abs against MOG from samples can bind to the expressed MOG protein and are usually detected via a specific secondary anti-human IgG Abs that is fluorescently labeled. The binding can be quantified by using visual scoring of a sample titration with a fluorescent microscope or by flow cytometry (Adey, Burton et al. 2013, Reindl and Waters 2019).

In the present study, a CBA is applied for detecting MOG Abs in serum, plasma, CSF or cell culture supernatants. HeLa cells are transiently transfected either with a plasmid encoding human full-length MOG C-terminally fused to an enhanced green fluorescent protein (EGFP), or with a plasmid encoding the EGFP alone as a control. The EGFP signal determines the level of transfected cells. Quantitative analysis is performed by flow cytometry as previously described (Spadaro and Meinl 2016). In addition, mouse or rat MOG and several mutated variants of human MOG are used to investigate different binding patterns of auto-Abs (**Figure 5 A** and **B**). In rodents, MOG-specific Abs, such as the from Linington et. al isolated mouse monoclonal Ab against MOG named 8-18C5, mainly recognize the FG loop of MOG (Linnington, Webb et al. 1984, Breithaupt, Schafer et al. 2008), whereas patients with Abs to MOG recognize different loops of MOG. Recently, a study has shown that over 75% of paediatric and adult MOG Abs target the CC′ loop around the amino acid proline 42 (Tea, Lopez et al. 2019). In addition, the majority of human Abs against MOG does not recognize rodent MOG (Mayer, Breithaupt et al. 2013).

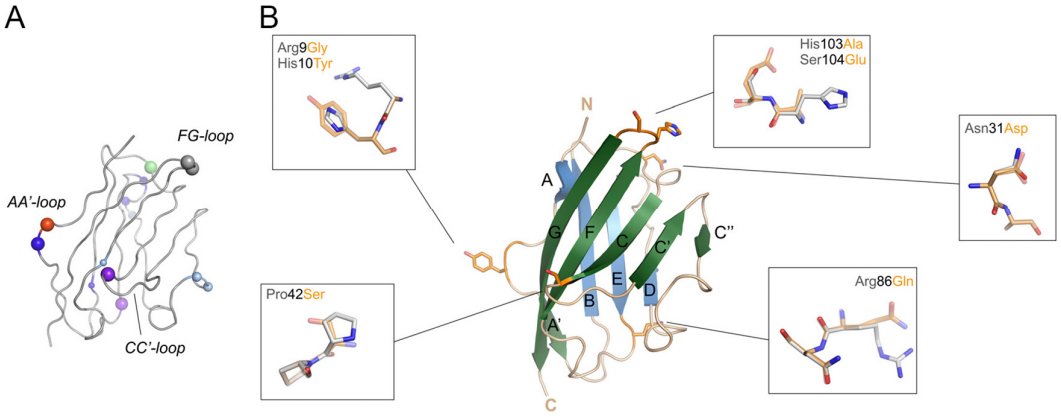

Figure 5| Three-dimensional structure of murine MOG, **A** shows the AA', FG and CC' loop. **B** displays the different epitopes with the mutated sites of variants of MOG that are recognized by MOG Abs and that were used in this study, the single mutations asparagine (Asn) at position 31 to aspartic acid (Asp), arginine (Arg) at position 86 to glutamine (Gln) and proline (Pro) at position 42 to serine (Ser), and the double mutations Arg at position 9 to glycine (Gly) / histidine (His) at position 10 to tyrosine (Tyr) and His at position 103 to alanine (Ala) / Ser at position 104 to glutamic acid (Glu). With permission from (Mayer, Breithaupt et al. 2013).

2.3 Clinical spectrum and pathology of MOG antibody associated disorders

As described in the section above, initial studies detected Abs against MOG with recombinantly produced domains or peptides of MOG. This led to controversial results regarding the value of those Abs as prognostic biomarkers, especially in MS. More recent studies that used conformationally intact MOG have enabled the investigation of MOG-IgG in a broad range of CNS diseases. While high levels of MOG-IgG are rarely found in patients with classical MS (more details on MS in the first part of the introduction), those Abs occur in a proportion of predominantly pediatric cases. Amongst those are patients with acute disseminated encephalomyelitis (ADEM), AQP4-IgG seronegative NMOSD, optic neuritis (ON), transverse myelitis (TM), or anti–N-methyl-D-aspartate receptor (NMDAR) encephalitis.

ADEM is characterized by multifocal demyelination due to inflammation in the brain and spinal cord. It often occurs after infection, such as measles, and is considered as a monophasic disease with highest incidence in early childhood. In rare occasions, multiphasic ADEM is described with two episodes, which are separated by at least three months. ADEM may be followed by ON, NMOSD or MS if further demyelinating events occur in the respective target areas. Since there is no prognostic biomarker defined for ADEM, a diagnosis depends greatly on exclusions of other diseases. Clinical features

may include fever, headache, somnolence, irritability, nausea and vomiting (Pohl, Alper et al. 2016).

NMOSD was formerly known as Devic disease or NMO. It is an inflammatory disorder of the CNS characterized by severe demyelination and can be monophasic or recurrent. When optic nerves are targeted, it leads to ON, when the spinal cord is targeted, it leads to myelitis, and if this extends over three vertebral segments, it is considered as longitudinally extensive transverse myelitis (LETM). As indicated in the first section of the introduction, the majority of patients with NMOSD can be stratified by serological tests for the presence of the pathogenic auto-Abs against the water channel protein AQP4, while a subgroup remains seronegative for AQP4-IgG. Clinical features may include those described below from ON or TM. In addition, with the development of an area postrema clinical syndrome, it can lead to intractable hiccups or nausea and vomiting (Wingerchuk, Banwell et al. 2015).

ON is an inflammation of the optic nerve and the most common optic neuropathy affecting young adults. It is divided into typical and atypical forms. In its typical form, ON is generally associated with MS or is considered as a demyelinating CIS at risk of conversion to MS. About 25% of MS cases present ON as the first symptom and about 70% develop ON during the disease progression, usually in the relapsing-remitting phase. The atypical form of ON is mainly associated with NMOSD. Clinical features of ON may include visual loss with diffuse blurring or fogging of vision, pain during eye movement and swelling of the optic disc (Toosy, Mason et al. 2014).

TM is an etiologically heterogeneous inflammation of the spinal cord. Half of the patients develop TM after an infection, while for up to 30% of patients the cause remains unknown and is termed as idiopathic. A distinct group of patients develop TM due to an acquired demyelinating disease such as MS or NMOSD. Hereby, the presence of brain lesions in patients with partial TM bears an 80% risk of transition to MS within the upcoming 5 years. An acute or subacute spinal cord dysfunction may result in clinical features such as pain, muscle weakness, paralysis, sensory problems, or bladder, bowel and sexual impairment (Beh, Greenberg et al. 2013).

Anti-NMDAR encephalitis is an inflammation of the brain associated with auto-Abs against the GluN1 subunit of the NMDAR. The disorder predominantly affects children and young adults, occurs with or without tumor association, and can relapse. The syndrome develops in several stages. About 70% of patients have early clinical features

consisting of headache, fever, nausea, vomiting, diarrhea, or upper respiratory-tract symptoms. Within less than two weeks, patients usually develop psychiatric symptoms with clinical features such as anxiety, insomnia, fear, mania, paranoia or short-term memory loss (Dalmau, Lancaster et al. 2011). In rare cases, anti-NMDAR encephalitis can coexist with an overlapping demyelinating syndrome (Titulaer, Hoftberger et al. 2014).

MOG-IgG are present in a larger proportion of pediatric patients compared to adults, and more females are affected than males with a 2 – 3 : 1 female to male ratio. Thereby, the clinical feature changes with age. Young children show an ADEM-like phenotype, whereas children older than nine years and adults have an opticospinal phenotype (Reindl and Waters 2019). About 20% of children with ADEM harbor Abs against MOG, while in pediatric or adult MS, it is rare with less than 5% of cases (Hohlfeld, Dornmair et al. 2016, Spadaro, Gerdes et al. 2016, Cobo-Calvo, d'Indy et al. 2020). Children diagnosed with ADEM tend to have transient MOG-IgG levels that disappear over time (Probstel, Dornmair et al. 2011). Those who have persistent levels are at high risk of relapses that often leads to ON or NMOSD (Reindl and Waters 2019). As initially mentioned, a subgroup of about 20% of patients with NMOSD are seronegative for AQP4-IgG and about one third of them harbor Abs against MOG (Hausser-Kinzel and Weber 2019, Mader, Kumpfel et al. 2020). Further, MOG-IgG occur in about 20% of patients with ON or myelitis such as TM or LETM (Weber, Derfuss et al. 2018). As a recent study shows, MOG-IgG may also exist concurrently with Abs against NMDAR. These cases are rare with a frequency of about 2% (Martinez-Hernandez, Guasp et al. 2020). Moreover, several reports describe the pathology of patients with MOG-IgG as MS pattern type II, characterized by active demyelination along with deposition of complement products in the brain (Di Pauli, Hoftberger et al. 2015, Spadaro, Gerdes et al. 2015, Jarius, Metz et al. 2016). Only in rare cases, intrathecal MOG-IgG production is observed (Kortvelyessy, Breu et al. 2017, Yanagida, Iizuka et al. 2017), whereas in the majority of patients, MOG-IgG is mainly present in the serum at high levels and not in the CSF, suggesting that these Abs have a peripheral origin (Mader, Kumpfel et al. 2020).

MOGAD – a separate disease entity: in the past few years, the above described broad spectrum of clinical features associated with MOG-IgG has evolved into a new inflammatory disease entity of the CNS. There is great consensus that MOGAD is distinct from classical MS and AQP4-IgG seropositive NMOSD. Autoimmune astrocytopathy is used to describe AQP4-IgG seropositive NMOSD since astrocytes are targeted by the

AQP4-IgG. On the other hand, autoimmune oligodendrocytopathy is used to describe MOGAD as it is the oligodendrocytes that are targeted by the MOG-IgG (**Figure 6**).

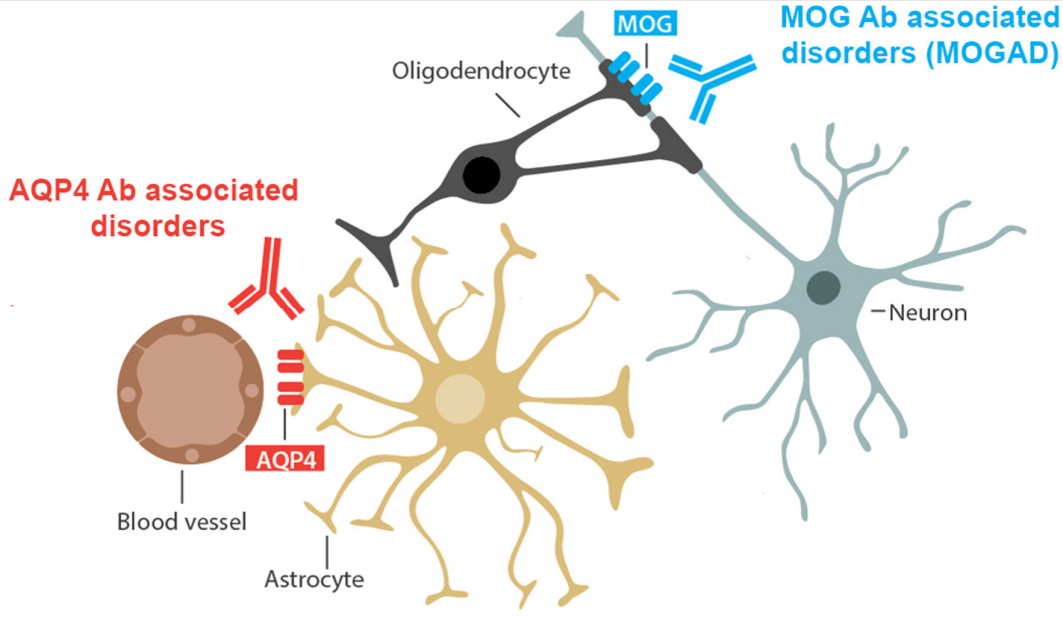

Figure 6| AQP4 Ab associated disorders (red) and MOG Ab associated disorders (MOGAD, blue). In the first disorder, Abs target AQP4, of which expression is focused on the endfeet of astrocytes surrounding blood vessels. In MOGAD, Abs target MOG, which is a component of the myelin sheath that is formed by oligodendrocytes. Modified and with kind permission from © Dr. Simone Mader, Else Kröner-Fresenius-Stiftung.

The prevalence of MOGAD is similar to AQP4-IgG seropositive NMOSD with 1 – 4 per 100,000 people but much lower than for MS, which has a prevalence of 80 – 300 per 100,000 people. The occurrence of new cases for MOGAD is 0.2 – 1.4 per 100,000 people per year, while the incidence of AQP4-IgG seropositive NMOSD is about 0.5 and for MS about 5 per 100,000 people per year (Zamvil and Slavin 2015, Jurynczyk, Jacob et al. 2019, Hegen and Reindl 2020, Mader, Kumpfel et al. 2020).

The pathology of MOGAD is characterized by an ADEM-like demyelination mostly around small venules, which can form confluent white matter lesions. In contrast to typical MS, intracortical rather than leukocortical demyelinated lesions predominate. Further, these lesions do not show a radially expansion of smoldering plaques in the white matter as seen in MS. Additionally, in MOGAD the inflammatory reaction is dominated by CD4 positive T cells with an infiltration of granulocytes, whereas in MS inflammatory infiltrates are mainly composed of CD8 positive T cells. Moreover, in MOGAD intrathecal MOG Ab production and OCBs are rarely observed, which suggests that pathogenic MOG Abs access the CNS from the blood as described for AQP4 Abs in AQP4-IgG seropositive NMOSD. However, in MOGAD less complement deposition

occurs than in AQP4-IgG seropositive NMOSD and the expression of AQP4 is preserved (Hoftberger, Guo et al. 2020, Takai, Misu et al. 2020). Overall, these pathological features of MOGAD enable a distinction from typical MS and from AQP4-IgG seropositive NMOSD.

2.4 Treatment options

As listed above, MOGAD comprises a broad phenotypic spectrum of disorders with different disease courses. This makes it difficult to conduct randomized trials to investigate treatment strategies (Mader, Kumpfel et al. 2020).

During relapses, high-dose steroids and plasma exchange/immunoadsorption (PLEX/IA) have been described to be effective (Mader, Kumpfel et al. 2020). Thereby, steroids decrease the inflammation and reduce the activity of the immune system (Coutinho and Chapman 2011). Furthermore, PLEX and IA constitute two possibilities to eliminate circulating Abs from patients' plasma. During PLEX, the treated plasma is replaced by a human albumin solution or fresh frozen plasma from healthy donors. Conversely, during IA, the patients' plasma is passed more selectively through an adsorber column to remove immunoglobulins and immune complexes, and the remaining part is re-infused into the patients' blood circuit (Heine, Ly et al. 2016).

For a long-term therapy, usually intravenous Ig (IVIG), classical immunosuppressants such as mycophenolate mofetil, azathioprine and methotrexate, or monoclonal Abs such as rituximab are used (Jurynczyk, Jacob et al. 2019, Mader, Kumpfel et al. 2020). IVIG is a pooled preparation of normal human immunoglobulins obtained from several thousand healthy donors. The mechanisms of an IVIG therapy are complex and not fully elucidated. Its therapeutic benefit might be due to the modulating effects on soluble mediators and cellular components of the immune system (Galeotti, Kaveri et al. 2017). Mycophenolate mofetil inhibits the enzyme needed for the *de novo* synthesis of guanosine nucleotides, which is required for DNA replication in lymphocytes more than in other cell types (Allison and Eugui 2000). Azathioprine also affects B and T cells by blocking the DNA replication. It becomes incorporated into replicating-DNA as a purine analog and can also inhibit the *de novo* pathway of purine synthesis (Maltzman and Koretzky 2003). Methotrexate, another immunosuppressive therapy, may have different mechanisms of function, including involvement in the inhibition of purine synthesis leading to cell cycle arrest (Friedman and Cronstein 2019). Further, monoclonal Abs are

used to target specific cell types of the immune system. Rituximab is a chimeric mouse/human monoclonal Ab and specifically targets cells expressing CD20. Thereby, mainly B cells but also a minor subset of T cells are depleted (Krumbholz and Meinl 2014, Schuh, Berer et al. 2016, Sabatino, Wilson et al. 2019).

The optimal therapy for patients with MOGAD is unknown. Different maintenance treatment options have been described to reduce relapse rates when compared to their baseline. Recent studies revealed that IVIG may have a favorable result on annual relapse rates while the treatment effects with rituximab appear to be heterogeneous. Here, only a proportion of patients with MOGAD benefited from B cell depleting therapies (Chen, Flanagan et al. 2020, Mader, Kumpfel et al. 2020, Whittam, Cobo-Calvo et al. 2020).

3 Pathogenicity of MOG antibodies

3.1 Lessons from animal models

In the past four decades, MOG was intensively studied in several autoimmune animal models such as EAE in guinea pigs, mice, rats or primates. These animal models enable investigation of chronic inflammatory demyelinating diseases and reflect the spectrum of MS and associated disorders. Initial experiments with chronic relapsing EAE in guinea pigs had shown that increased levels of Abs against MOG in the sera correlated with a demyelinating activity when injecting the sera of those guinea pigs into the subarachnoid space of normal rats (Linington and Lassmann 1987). Further studies elucidated that using MOG for active immunization together with Freund's adjuvant can trigger two different autoimmune responses: first, inflammation in the CNS due to induced encephalitogenic MOG-specific T cells, and second, demyelination due to production of Abs against MOG (Mayer and Meinl 2012). Importantly, the immune response depends on the species and the chosen strain. An immunization with rat MOG in Dark Agouti rats resulted in an acute/subacute disease with prominent T cell induced inflammation, but spared demyelination. In contrary, Brown Norway rats responded with a chronic disease featuring very pronounced Ab-mediated demyelination while remaining relatively resistant to induction of classical T-cell mediated EAE (Storch, Stefferl et al. 1998). In Lewis rats, immunization with the N-terminal extracellular domain (AA 1–125) of murine MOG led to an acute inflammatory demyelinating variant of EAE with a dominant MOG-specific Ab response, but poor induction of encephalitogenic T cells (Adelmann, Wood et al. 1995). An immunization with the extracellular domain of rat MOG in different mice

strains revealed an EAE in SJL/J mice with the formation of pathogenic auto-Abs against MOG but showed no production of those Abs in C57Bl/6 mice. Differences in the genes encoded within the major histocompatibility complex between the two strains might be the cause of this effect (Bourquin, Schubart et al. 2003). Further studies elucidated that using human MOG instead of rat MOG for immunization can also induce a pathogenic MOG-specific auto-Abs response in C57Bl/6 mice (Marta, Oliver et al. 2005). An immunization of Balb/c mice with rat cerebellar glycoproteins enabled the isolation and production of the widely used monoclonal mouse Ab against MOG named 8-18C5 (Linnington, Webb et al. 1984). The heavy chain of this pathogenic monoclonal Ab 8-18C5 was later used for generating transgenic knock-in C57Bl/6 mice. About 30% of all B cells were autoreactive against MOG and those animals harbored high levels of MOG-specific Abs. Interestingly, these mice exhibited benign phenotypes and did not develop spontaneous EAE. However, this changed when activated myelin-specific T lymphocytes were transferred to these animals by an intravenous injection. This led to an opening of the BBB and triggered an inflammatory cascade in the CNS. MOG-specific Abs were therefore able to access the brain and initiate demyelination (Litzenburger, Fassler et al. 1998). Moreover, another study revealed that only a proportion of monoclonal Abs against MOG were able to induce demyelination in a rodent EAE model. Hereby, the pathogenic potential is related to the ability of Abs to trigger CDC (**Figure 7**). Therefore, the Ab isotype can be suggestive for determining the Ab effector functions (Piddlesden, Lassmann et al. 1993). Further studies in marmoset monkeys also showed an EAE

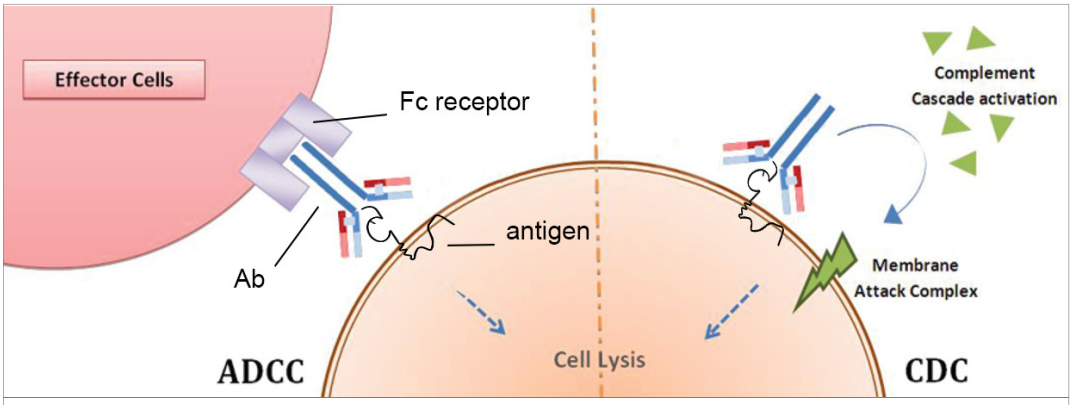

Figure 7| Scheme of ADCC and CDC. Left, Ab-dependent cell-mediated cytotoxicity (ADCC), including effector cells such as natural killer cells or phagocytic cells such as macrophages, recognizing the Fc part of the Ab via their Fc receptor. Targeted cells will be killed by the release of cytotoxic mediators or phagocytosis. Right, complement dependent cytotoxicity (CDC), including the components of the complement system. The bound Ab to its targeted antigen is recognized by the complement resulting in the formation of a membrane attack complex, that leads to cell lysis. Modified and with permission from (Kasi, Tawbi et al. 2012).

induced inflammation and complement dependent demyelination upon immunization with MOG for non-human-primate models (von Budingen, Hauser et al. 2004).

Taken together these findings from animal models show that the MOG protein is encephalitogenic in many different species, the correctly folded extracellular domain of MOG is responsible for the formation of demyelinating auto-Abs, and the proposed two-hit model is reflected by the associated pathology: breakdown of BBB due to encephalitogenic T cells (first hit) and access of complement-fixing MOG-specific Abs to the CNS to mediate demyelination (second hit) (Mayer and Meinl 2012, Reindl and Waters 2019).

3.2 Previous attempts to analyze pathogenicity of human MOG antibodies

Some former studies tried to elucidate the potential pathogenic activity of human Abs against MOG by using patient-derived materials. *In vitro* experiments with serum showed that these auto-Abs are primarily of the IgG1 subtype and can activate the complement cascade, resulting in the formation of the terminal complement complex on living human full length MOG transfected HEK-293A cells upon addition of human complement (Mader, Gredler et al. 2011). Furthermore, total IgG obtained from anti-MOG Ab positive patients can induce ADCC (**Figure 7**) by natural killer cell-mediated destruction of native MOG-expressing cells in culture (Brilot, Dale et al. 2009), and can produce complement-mediated myelin loss in *ex vivo* cultured organotypic brain slices from mice (Peschl, Schanda et al. 2017). When affinity-purified IgG against MOG is incubated with oligodendrocytes, this can lead to a loss of organization of the thin filaments and the microtubule cytoskeleton of those glial cells *in vitro* (Dale, Tantsis et al. 2014). In addition, purified total IgG from patients can mediate the uptake of human MOG protein by bone marrow-derived macrophages from mice. Thereby, the presence of MOG-specific Abs enables those myeloid cells to recognize, internalize, process and present the MOG antigen at very low concentrations in a fragment crystallizable (Fc)-dependent manner. When these APCs are co-cultured with naïve MOG-specific T cells, they can activate the T cells to differentiate in an encephalitogenic manner (Kinzel, Lehmann-Horn et al. 2016). *In vivo* experiments with peripheral injected concentrated serum from MS patients showed a slight enhanced demyelination in rats with EAE and axonal damage in the inflammatory lesions (Zhou, Srivastava et al. 2006). In another study, purified and pooled total IgG preparations from five MOG Abs seropositive NMO patients were injected in mouse brains intracerebrally. This caused myelin changes and altered the

expression of axonal proteins which was not associated with inflammation and largely independent of complement (Saadoun, Waters et al. 2014). Further, an investigation with an intrathecal injection of purified IgG from a patient with Abs against MOG accelerated EAE in mice but the auto-Abs did not induce demyelination. The authors proposed that MOG-specific Abs can instead foster T-cell activation by efficiently targeting and concentrating the MOG antigen to presentation-competent cells such as macrophages (Flach, Litke et al. 2016). Moreover, total IgG derived from MS patients with MOG-specific Abs was injected peripherally into a humanized mouse model that transgenically expresses human Fc-gamma receptors and could exacerbate EAE. This exacerbation was dependent on MOG recognition by the human-derived Abs (Khare, Challa et al. 2018).

Overall, there is evidence that human Abs against MOG are pathogenic. Some studies propose a demyelinating effect with complement involvement, whereas others consider more a T-cell activation due to APCs that were efficiently triggered by Ab-antigen complexes. Mainly serum or total IgG preparations are used for the *in vitro* studies or transfer experiments *in vivo*. These patient-derived materials may also comprise of additional auto-Abs or disease-modulating components, making a distinct interpretation difficult.

4 Potential sources of human antibodies against MOG

The production of Abs can last for a lifetime and different studies in animal models and human subjects have elaborated how long-lasting IgGs can be generated. First, Abs against MOG could be produced by circulating short-lived plasma cells and second, niched long-lived plasma cells may constantly release MOG Abs.

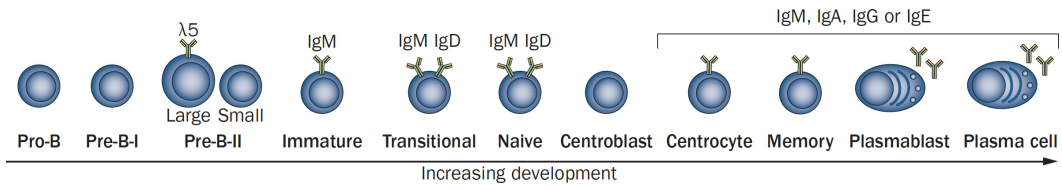

Figure 8| Overview of the different stages in B cell development. The initial phase from pro-B cells to immature B cells takes place in the bone marrow. During the transition, mature naïve B cells migrate via the bloodstream to secondary lymphoid organs and may undergo germinal-center reactions and class-switch recombination. They form memory B cells and antibody-secreting cells, which are plasmablasts or plasma cells. Modified and with permission from (Krumbholz, Derfuss et al. 2012)

Usually, antigen-specific naïve B lymphocytes are stimulated by antigen recognition via the B cell receptor followed by CD40 ligand signal from cognate T helper cells which

triggers the B cell proliferation and differentiation into memory B cells, plasmablasts and plasma cells (**Figure 8**). Plasmablasts are precursors from plasma cells, which still proliferate and already start to produce Abs at low levels; however, their lifespan is very short. In contrast to memory B cells and plasmablasts, plasma cells are terminally differentiated and do not divide but secrete Abs at high levels. Plasma cells and plasmablasts also differ in the expression of certain surface markers, cf. **Table 1**. During the primary immune response, memory B cells are formed within germinal centers and survive in secondary lymphoid organs in the absence of their cognate antigen. In the secondary immune response, persisting or cross-reactive antigens may continuously stimulate memory B cells. In this antigen-dependent manner, memory B cells undergo a massive expansion and differentiation towards circulating short-lived plasma cells. In addition, some long-lived plasma cells are generated that migrate to survival niches such as the bone marrow where they continuously release Abs at high rates without further stimulation. The lifespan of plasma cells has been proposed to range from several days (short-lived) to several months (long-lived). In contrast, an antigen-independent mode without the need for triggering the B cell receptor has also been described. Thereby, at a lower rate, all memory B cells are activated by cytokines or Toll-like receptor (TLR) ligands and undergo continuous proliferation and differentiation. In this way, a constant level of short-lived plasma cells and serum Abs could theoretically be kept throughout a human's life-span. Since this mechanism is non-selective, it maintains the broad spectrum of specific Abs by polyclonal activation of all memory B cells (Bernasconi, Traggiai et al. 2002, Traggiai, Puzone et al. 2003, Radbruch, Muehlinghaus et al. 2006).

Table 1| Comparison between plasma cell and plasmablast in terms of localization, survival duration, expression of different surface markers as the B cell markers CD19 and CD20, proliferation capacity, secretion of Ig, expression of surface bound Ig and of MHC class II. - = no, +/- = low, + = moderate, ++ = high (Bernasconi, Traggiai et al. 2002, Traggiai, Puzone et al. 2003, Radbruch, Muehlinghaus et al. 2006, Krumbholz, Derfuss et al. 2012, Kometani and Kurosaki 2015).

Features	Plasma cell	Plasmablast
Localization	In survival niches in bone marrow and spleen; in inflammatory tissue.	In lymphatic organs; also circulating in body fluids.
Survival	Years to decades	Days
CD19 expression	+/-	+
CD20 expression	-	+
Proliferation	-	+
Secretion of Ig	++	++
Surface Ig	+/-	++
MHC class II	+/-	++

Aims of the thesis

This thesis comprises three parts. First, the minor part, wherein we aimed to explore the pathogenic mechanism of human-derived Abs against MOG; second and third, the major part, wherein our aims were to investigate the source of these MOG-specific Abs and to analyze the antigen recognition:

- Pathogenic mechanisms of patient-derived MOG Abs
- Identification of circulating MOG-specific B cells
- Deeper insight into details of antigen recognition of MOG Abs

Results

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RESEARCH ARTICLE

Pathogenicity of Human Antibodies against Myelin Oligodendrocyte Glycoprotein

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Objective: Autoantibodies against myelin oligodendrocyte glycoprotein (MOG) occur in a proportion of patients with inflammatory demyelinating diseases of the central nervous system (CNS). We analyzed their pathogenic activity by affinity-purifying these antibodies (Abs) from patients and transferring them to experimental animals.
Methods: Patients with Abs to MOG were identified by cell-based assay. We determined the cross-reactivity to rodent MOG and the recognized MOG epitopes. We produced the correctly folded extracellular domain of MOG and affinity-purified MOG-specific Abs from the blood of patients. These purified Abs were used to stain CNS tissue and transferred in 2 models of experimental autoimmune encephalomyelitis. Animals were analyzed histopathologically.
Results: We identified 17 patients with MOG Abs from our outpatient clinic and selected 2 with a cross-reactivity to rodent MOG; both had recurrent optic neuritis. Affinity-purified Abs recognized MOG on transfected cells and stained myelin in tissue sections. The Abs from the 2 patients recognized different epitopes on MOG, the CC′ and the FG loop. In both patients, these Abs persisted during our observation period of 2 to 3 years. The anti-MOG Abs from both patients were pathogenic upon intrathecal injection in 2 different rat models. Together with cognate MOG-specific T cells, these Abs enhanced T-cell infiltration; together with myelin basic protein–specific T cells, they induced demyelination associated with deposition of C9neo, resembling a multiple sclerosis type II pathology.
Interpretation: MOG-specific Abs affinity purified from patients with inflammatory demyelinating disease induce pathological changes in vivo upon cotransfer with myelin-reactive T cells, suggesting that these Abs are similarly pathogenic in patients. ANN NEUROL 2018;00:000–000

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High levels of antibodies (Abs) to conformationally intact myelin oligodendrocyte glycoprotein (MOG) have initially been detected in pediatric patients,[1] then also in a proportion of patients with different

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demyelinating diseases such as optic neuritis, myelitis, encephalomyelitis, brainstem encephalitis, acute disseminated encephalomyelitis (ADEM), and anti–N-methyl-D-aspartate receptor (NMDAR) encephalitis, and in a few patients with multiple sclerosis (MS).[2–6] Patients with autoantibodies to MOG have distinct brain magnetic resonance imaging (MRI) characteristics.[7,8] It is debated whether anti-MOG disease constitutes a separate entity.[9]

In animal models, some monoclonal Abs (mAbs) to MOG induce demyelination provided the blood–brain barrier is breached giving the Abs access to the CNS (reviewed in Hohlfeld et al,[5] Mayer and Meinl[10]). Only a proportion of anti-MOG Abs are able to induce demyelination in vivo, related to complement activation[11] and recognition of conformationally correct MOG.[12,13] In rodents, pathogenic MOG-specific Abs mainly recognize the FG loop of MOG as the prototype mAb 8-18C5,[14] whereas patients with Abs to MOG recognize different loops of MOG, most frequently the CC′ loop around the amino acid P42.[15]

Previous experiments to test the potential pathogenic activity of human anti-MOG Abs in vitro reported that sera of patients with Abs to MOG activated complement,[16] stimulated natural killer cell mediated toxicity,[17] induced cytoskeletal changes in oligodendroglial cells,[18] mediated myelin destruction in slice cultures,[19] and facilitated MOG uptake by macrophages.[20] Peripheral injection of concentrated serum from MS patients in rats with experimental autoimmune encephalomyelitis (EAE) slightly enhanced demyelination and axonal loss.[21] Total IgG preparations pooled from 5 neuromyelitis optica (NMO) patients were injected intracerebrally and induced myelin changes independent of complement, but no inflammation.[22] Intrathecal injection of IgG from a patient with MOG Abs accelerated EAE in mice.[23] Peripheral injection of IgG from MS patients with Abs to MOG exacerbated EAE in mice.[24] Thus, there is evidence that human Abs to MOG are pathogenic, but one has to consider that patients with neuroinflammation may have multiple autoantibodies,[25–27] which complicates the interpretation of transfer experiments with whole IgG preparations. Transfer experiments with human affinity-purified Abs to MOG have not yet been done, and therefore detailed pathogenic mechanisms of human Abs to MOG remain to be elaborated.

Patients with Abs to MOG have a pathology described as MS pattern II,[28–31] characterized by active demyelination along with deposition of C9neo, suggesting an Ab-mediated demyelination.[32,33] Transfer experiments with autoantibodies to MOG from these patients were hampered because only a proportion of MOG Abs from patients cross-react with rodent MOG[15,28]; therefore, the linkage of human MOG Abs to a certain neuropathology is still speculative.

The aim of this study was to analyze which human Abs to MOG are pathogenic, to identify recognized epitopes of pathogenic autoantibodies, to test whether they can mediate MS type II pathology, and to explore their pathogenic mechanisms. To this end, we combined affinity purification of Abs that recognize cell-based MOG, epitope identification with mutants of MOG, staining of tissue sections, and transfer experiments in 2 EAE models. This showed that Abs to MOG were pathogenic by 2 mechanisms; in synergy with myelin basic protein (MBP)-specific T cells they mediate MS type II pathology, and together with MOG-specific T cells they enhance T-cell infiltration.

Patients and Methods

Standard Protocol Approvals, Registrations, and Patient Consents

We analyzed sera from 260 patients with inflammatory CNS diseases for anti-MOG reactivity. The clinical characteristics of patients who scored positive in our cell-based assay detecting Abs to MOG are summarized in the Table. All MOG Ab–positive patients were followed longitudinally. Informed consent was obtained from each donor according to the Declaration of Helsinki and the ethical committee of the medical faculty of Ludwig-Maximilians-Universität München approved this study.

Determination of anti-MOG Reactivity and Epitope Recognition

Patients positive for Abs to MOG were identified with a cell-based flow cytometry assay using viable cells and a serum dilution of 1:50, as described.[28,34] Isotype-specific secondary Abs were obtained from Southern Biotech (Birmingham, AL). To identify the recognized epitopes, mutant variants of MOG were applied and the percentage binding compared to human MOG was calculated as described.[15] In some experiments, we used a recombinant variant of the mAb 8-18C5 (designated r8-18C5), which has the same antigen recognition site, but a human IgG1 Fc part.[35]

Production and Validation of Recombinant Human MOG

We aimed to produce a recombinant version of the extracellular domain (ECD) of human MOG that comes as close as possible to the conformation of MOG displayed in transfected cells. To this end, we produced the ECD of human MOG in HEK293-EBNA cells and added at the C-terminus instead of the first transmembranous region a HisTag and an AviTag using the pTT5 vector.[36] MOG

was biotinylated by using the BirA biotin ligase Kit (Avidity, Aurora, CO). Folding of the purified protein (0.2mg/ml) was analyzed by circular dichroism using a Jasco J-810 spectropolarimeter (JASCO Corporation, Tokyo, Japan). To further validate the anti-MOG binding activity of our recombinant MOG, we tested whether this MOG was bound by B cells from mice with a knock-in of the heavy chain of the anti-MOG 8-18C5.[37] To this end, we formed MOG tetramers with our biotinylated MOG and fluorescently labeled streptavidin (Jackson ImmunoResearch, West Grove, PA).

Affinity Purification of Anti-MOG Abs

Biotinylated MOG was bound to a HiTrap Streptavidin HP column (GE Healthcare, Munich, Germany). Ig from plasma (obtained from ethylenediaminetetraacetic acid [EDTA]–blood) was first enriched by ammonium sulfate precipitation and then loaded on this column. Bound Ig was eluted (100mM glycin, 150mM NaCl, pH 2.5) and immediately neutralized with 1M Tris-HCl, pH 8.8. The eluates from both patients were separated by reducing and nonreducing sodium dodecyl sulfate gel electrophoresis and stained by Coomassie. The excised gel bands were in-gel digested essentially as described.[35] Peptides were analyzed by matrix-assisted laser desorption/ionization time of flight/time of flight using a 4800 Analyzer (Applied Biosystems, Foster City, CA). The eluates were tested by enzyme-linked immunosorbent assay (ELISA) for streptavidin reactivity using streptavidin-coated plates.

Staining of Tissue with Patient Abs

Rat brains were fixed in 4% paraformaldehyde (PFA) for 1 hour, cryoprotected with 40% sucrose, and snap frozen. Seven-micrometer-thick sagittal sections were incubated with 0.3% hydrogen peroxide for 20 minutes and with 10% donkey serum in phosphate-buffered saline (PBS) for 1 hour, and then labeled with the Abs at 4°C overnight. The next day, sections were labeled with a donkey–antihuman IgG (H+L) secondary Ab (Jackson ImmunoResearch) and visualized with an avidin–biotin–diaminobenzidine reaction.

Transfer EAE and Rat T-Cell Lines

Antigen specific T cells were established from Lewis rats immunized with antigen emulsified in complete Freund adjuvant as described previously.[38] The following antigens were used: recombinant MOG (amino acid 1–125), MBP purified from guinea pig brain, and ovalbumin (OVA) purchased from Sigma-Aldrich (St Louis, MO). To induce mild EAE, freshly restimulated 15×10^6 MOG-specific T cells or 1.2×10^6 MBP-specific T cells were injected intravenously in Lewis rats. Clinical scores were evaluated as follows: 0 = normal; 0.5 = loss of tail tonus; 1 = tail paralysis; 2 = gait disturbance; 3 = hindlimb paralysis. Two days after injection of T cells, 100 μg of the indicated Ab preparations was injected intrathecally into the cisterna magna to animals anesthetized by fentanyl/midazolam/medetomidine. For the monitoring of clinical score, animals were followed until full recovery and were then sacrificed. For histopathological analysis, 72 hours after Ab injection, animals were perfused with PBS and 4% PFA in PBS under terminal anesthesia with fentanyl/midazolam/medetomidine; the spinal cord and brain were then postfixed with 4% PFA in PBS at 4°C. The procedures are approved by the government of Upper Bavaria.

Histological Examination of the EAE Rats

Brain, spinal cord, and optic nerves were dissected and embedded in paraffin. Serial sections of all tissues were stained with hematoxylin/eosin, Luxol fast blue (LFB) myelin stain, and Bielschowsky silver impregnation for axons. Immunocytochemistry was performed on paraffin sections after antigen retrieval in a food steamer with EDTA buffer, pH 8.5. Primary Abs against the following targets were used in the following dilutions: CD3 (T cells; rabbit monoclonal; Neomarkers, Fremont, CA; RM-9107-5; 1:2,000), ED1 (phagocytic macrophages and microglia; mouse monoclonal; Serotec, Raleigh, NC; MCA341R, 1:10,000), Iba 1 (pan microglia and macrophages; rabbit polyclonal; Wako, Osaka, Japan; 019-19741; 1:3,000), cyclic nucleotide phosphodiesterase (oligodendrocytes; mouse monoclonal; Sternberger Monoclonals, Lutherville, MD/BioLegend, San Diego, CA; SMI 91; 1:2,000), glial fibrillary acidic protein (astrocytes; rabbit polyclonal; Dako, Santa Clara, CA; Z0334; 1:3,000), human Ig (biotinylated species specific antihuman Ig; donkey polyclonal, Jackson ImmunoResearch, 709-065-149; 1:1,000) and activated complement (C9neo antigen, rabbit polyclonal; 1:2,000).[11] Bound primary Abs were visualized with a biotin/avidin/peroxidase system. To quantify the inflammation, $CD3^+$ T cells/mm^2 were counted in a zone of 200 μm spanning from the ventral subpial surface into the tissue of the pons. To quantify demyelination, the distance of subpial demyelination from the ventral surface of the pons was measured. To this end, macrophages were stained with ED1 and the distance from the pial surface on which could be seen classical macrophages with degradation products was measured. This also represents the area of macrophages in LFB staining that contain myelin degradation products.

Results

Anti-MOG Reactivity in Patients with Inflammatory CNS Diseases and Cross-Reactivity to Rodent MOG

We tested sera from 260 patients with different inflammatory CNS diseases; 17 of them had autoantibodies to

MOG (clinical details in the Table). The highest anti-MOG reactivity was seen in patients with relapsing optic neuritis and NMO phenotype. The vast majority of patients with MS do not have Abs to MOG, but Abs to MOG are detected in special cases with MS.[34] The 5 patients with MOG Abs included in the Table fulfill the diagnostic criteria of MS, including MS-typical cerebrospinal fluid (CSF) and radiological features, but had a clinical phenotype that overlaps with NMO (severe myelitis, brainstem involvement, and optic neuritis). These patients did not have Abs to NMDAR or AQP4. Details of their clinical picture, their MRI, and their anti-MOG reactivity have been described in a previous paper.[34] We determined the cross-reactivity to rodent MOG of these patients. Further analysis of the pathogenic features of Abs to MOG was performed with Patients 7 and 5, who showed a high reactivity toward MOG and cross-reactivity to rodent MOG (Fig 1). Both patients had a recurrent optic neuritis, one of the diseases associated with MOG Abs.[39,40] These patients were followed for periods of about 26 and 35 months and kept recognizing MOG. Their anti-MOG reactivity was so high that a reactivity could still be detected at serum dilutions of 1:3,000 to 1:10,000. Both patients had anti-MOG of isotype IgG1. Patient 5 had in addition to IgG also persisting IgM to MOG.

The applications of mutant variants of MOG showed that the 2 patients recognized different epitopes on MOG (see Fig 1C, D). The binding to MOG of Patient 5 was reduced by the mutation P42S, indicating that this patient's Abs recognize the CC′ loop on MOG; the MOG Abs of IgG and IgM isotype showed similar reactivity to MOG mutants. Patient 7 showed a stronger reactivity to mouse MOG than to human MOG. Such a feature we had previously noted in 12 of 111 patients analyzed.[15] Consistent with the better recognition of mouse MOG, this patient also showed a stronger reactivity to the MOG mutant P42S, in which the serine present in murine MOG replaces the proline of human MOG. Another mutation at the EF loop (H103A, S104E) greatly reduces the MOG binding of this patient. MOG residues important for binding of Abs from Patients 5 and 7 are visualized in Figure 1E.

From Patient 5, we could also analyze CSF and this showed that anti-MOG IgG were present in this compartment, but there was no evidence that the anti-MOG IgG present in the CSF was produced intrathecally; after adjustment to equal IgG concentrations, similar anti-MOG reactivity was seen in CSF and serum (see Fig 1F).

Specificity of Affinity-Purified Abs to MOG

We produced the ECD of human MOG in HEK cells with an AviTag at the C-terminus replacing the transmembranous and intracellular part. Then MOG was enzymatically biotinylated at the AviTag and bound to a streptavidin column, which puts the extracellular part of MOG on the beads in the same orientation as in the membrane. The confirmation with beta-sheet formation was seen by circular dichroism (Fig 2A). To further validate this MOG preparation, we formed MOG tetramers and tested the binding to B cells from mice with a knock-in of the heavy chain of the anti-MOG mAb 8-18C5 and found that this stained about one-third of the B cells from these mice, which is in line with their published MOG-binding activity (data not shown).[37] With this protein, we could affinity purify MOG-specific Abs from both patients (see Fig 2). Starting from > 600ml blood, we eluted from the MOG-column 471 μg of IgG and 55 μg of IgM from Patient 5 and 571 μg IgG but no IgM from Patient 7. Mass spectrometry showed that the eluates from Patient 5 contained IgG, IgM, α-2 macroglobulin, fibrinogen, and albumin, and from Patient 7 IgG and fibrinogen. Importantly, no MOG was detected in the eluates. The eluates did not bind to streptavidin as seen by ELISA using streptavidin-coated plates. We could not obtain Abs that recognize MOG on transfected cells from donors who did not have a strong anti-MOG reactivity in their blood. This excludes that the anti-MOG reactivity we observed in the purified fraction is an artifact due to the purification procedure.

These affinity-purified Abs showed a highly enriched reactivity to human MOG in a cell-based assay; when plasma and affinity-purified Abs were adjusted to the same concentration of 12 μg/ml, we noted the following mean channel fluorescence (MCF) ratios, which were calculated as described above: Patient 5: plasma 14.9, purified 190.3, flow through 8.1; Patient 7: plasma 8.6, purified 207.5, flow through 3.5 (see Fig 2). We noted that in both patients the reactivity to our mutated variants was the same in the anti-MOG Abs from the starting material and the eluates. We also compared the affinity-purified MOG Abs from both patients with the prototype anti-MOG 8-18C5. For this comparison we used a recombinant variant of 8-18C5 with a human Fc-IgG1, so the same detection Ab could be used. Our dose responses show that these purified MOG Abs recognized MOG in a cell-based assay still in the ng/ml range and came quite close to the intensity of MOG binding of the 8-18C5. The isotype of the anti-MOG response of the affinity-purified Abs of both patients was IgG1. We also analyzed the cross-reactivity of the patient-derived MOG Abs to rat MOG, because their pathogenicity will be tested in a rat model (see below). We noted that Patient 7 recognized rat MOG more strongly than human MOG, which is consistent with our observation that this patient also recognizes mouse MOG more strongly than human MOG (see Fig 1D); mouse and rat MOG are very similar although not identical.

The flow through of the column used for affinity purification of MOG Abs from these 2 patients still contained

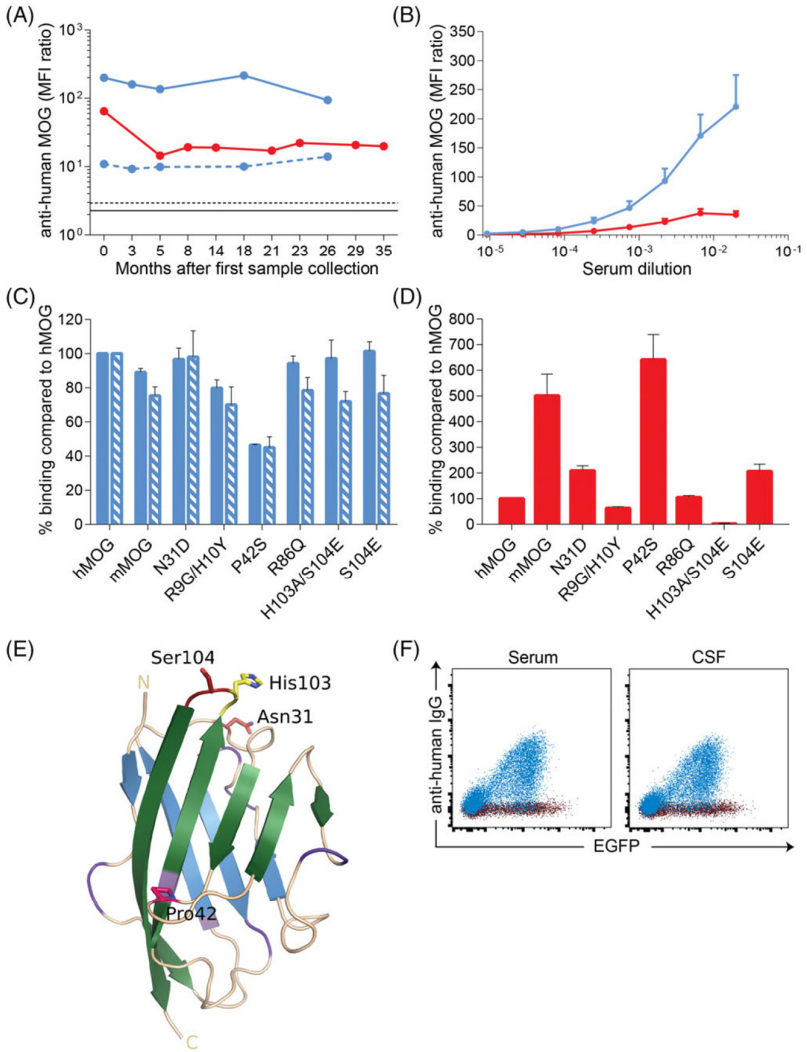

FIGURE 1: Anti–myelin oligodendrocyte glycoprotein (MOG) reactivity in the 2 patients selected for transfer experiments. The anti-MOG reactivity in serum and plasma of Patient 5 (blue) and Patient 7 (red) was determined with transfected cells as described in Patients and Methods. (A) Longitudinal analysis. Solid lines indicate anti-MOG IgG; the dotted bluish line shows persisting anti-MOG IgM in Patient 5. The solid black line shows the cutoff for anti-MOG IgG, the dotted black line the cutoff for anti-MOG IgM. (B) Anti-MOG reactivity in serum dilutions. (C, D) Reactivity to human MOG (hMOG), mouse MOG (mMOG), and the indicated mutations of MOG. The IgG responses are indicated in solid bars, the anti-MOG IgM response from Patient 5 in hatched bars. (E) The structure of the human MOG model[15] is shown as a ribbon representation with residues influencing antibody binding depicted as stick models. In addition, residues that differ between mouse and human MOG are colored pink (Pro 42), light violet (2 conservatively mutated interior 13-strand residues), and violet (remaining nonidentical residues). N and C indicate the N-terminal and C-terminal part of the extracellular domain of MOG. (F) Anti-MOG in cerebrospinal fluid (CSF) of Patient 5. CSF (IgG 0.022g/l) was used undiluted and serum was diluted 1:377 to obtain the same IgG concentration as in the CSF. The calculated mean fluorescence intensity (MFI) ratio (MOG-enhanced green fluorescent protein [EGFP]/EGFP) of the CSF was 72.44, whereas that of the serum sample was 86.34. Control EGFP transfectants are shown in gray, the MOG-EGFP transfectants in blue. Error bars indicate the standard error of the mean of 2 to 3 experiments.

anti-MOG reactivity as seen with MOG transfectants. This was not due to a limited capacity of the column, as it could still bind the mAb 8-18C5. Along this line, from another patient (Patient 14), we could obtain only a small amount of anti-MOG IgG with this column and the flow through still contained a similar reactivity to MOG as the starting

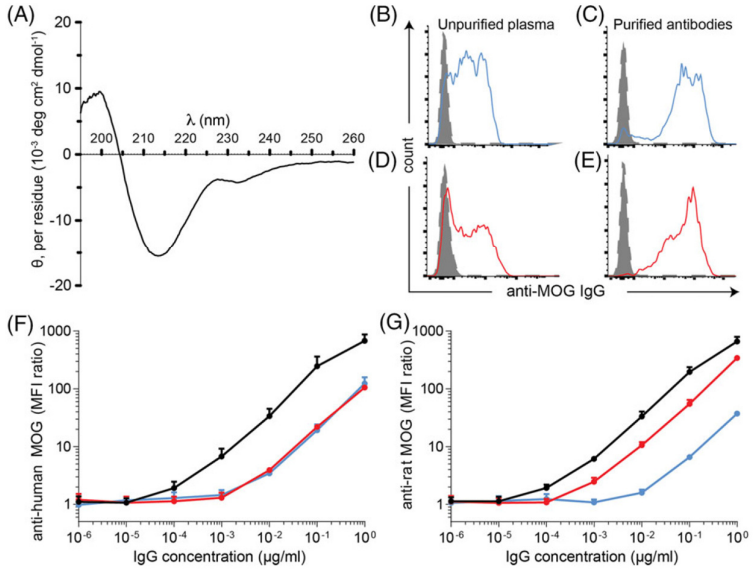

FIGURE 2: Affinity-purified antibodies (Abs) to myelin oligodendrocyte glycoprotein (MOG). (A) Circular dichroism spectrum of MOG (0.2mg/ml). The beta-sheet formation is indicated by the negative band at 213nm. (B–E) Comparative analysis of plasma and affinity-purified MOG Abs to cells transfected with MOG of Patient 5 (blue) and Patient 7 (red). Plasma and purified Abs were used at an IgG concentration of 12 µg/ml. Closed graphs indicate the recognition of enhanced green fluorescent protein (EGFP)-transfected cells, open graphs of MOG-EGFP transfectants. (F, G) MOG recognition of the affinity-purified Abs from Patients 5 (blue) and 7 (red) in comparison with the recombinant humanized mAb 8-18C5 (black) on transfected cells. Error bars indicate standard error of the mean of 2 to 3 experiments. MFI = mean fluorescence intensity.

material. Thus, the ECD of human MOG produced in HEK cells binds only a fraction of Abs to MOG.

Staining of Brain Tissue with Affinity-Purified Abs to MOG

The affinity-purified Abs from both patients bound to myelin in tissue sections from the rat; r8-18C5 was used as a positive control (Fig 3). We noted a stronger binding of the Abs from Patient 7 (see Fig 3C, D) than from Patient 5, which is consistent with the dose response of these preparations to rat MOG on the surface of rat transfectants (see Fig 2G). Because the MOG reactivity of these patients was established by using native cells, while the tissue was fixed with PFA, we compared the recognition of live and PFA-fixed cells after MOG transfection. This showed that Patients 5 and 7 recognized MOG also after PFA fixation of the transfected cells, but the background was much higher with fixed cells (data not shown).

Pathogenicity and Histopathological Changes Induced by Patient-Derived Abs to MOG

We analyzed the pathogenic potential of patient-derived MOG-specific Abs in 2 models of T-cell–mediated EAE in the Lewis rat. In both models, we injected the MOG Abs intrathecally 2 days after the injection of either MOG-specific T cells or MBP-specific T cells. Because the amount of purified Abs from patients was limited, we first established the details of the transfer models with 8-18C5 and the humanized r8-18C5. These experiments showed that EAE can be enhanced, when 8-18C5 or r8-18C5 were injected 2 days later than the T cells. Under these conditions, the peak of disease was reached at day 5; the animals recovered largely until day 10. Therefore, we sacrificed the EAE rats after injection with the patient-derived Abs at day 5.

The MOG-specific T cells alone did not induce a clinical effect in our Lewis rat model. However, when affinity-purified Abs from both Patients 5 and 7 were injected, a clinical disease was induced (Fig 4). As control, we used human ivIg and Ig obtained from a protein G column. This control human Ig did not induce disease, whereas the positive control 8-18C5 enhanced disease. In contrast to the MOG-specific T cells, the MBP-specific T cells induced a clinical disease on their own in the absence of any added Ab, consistent with previous observations with MBP-specific T cells in this rat model.[41] One day after injection of r8-18C5 and the Abs from Patient 7 the clinical disease was enhanced.

All animals shown in Figure 4 were perfused at day 5 and analyzed by histopathology. A quantitative analysis of the T-cell infiltration and of demyelination in all

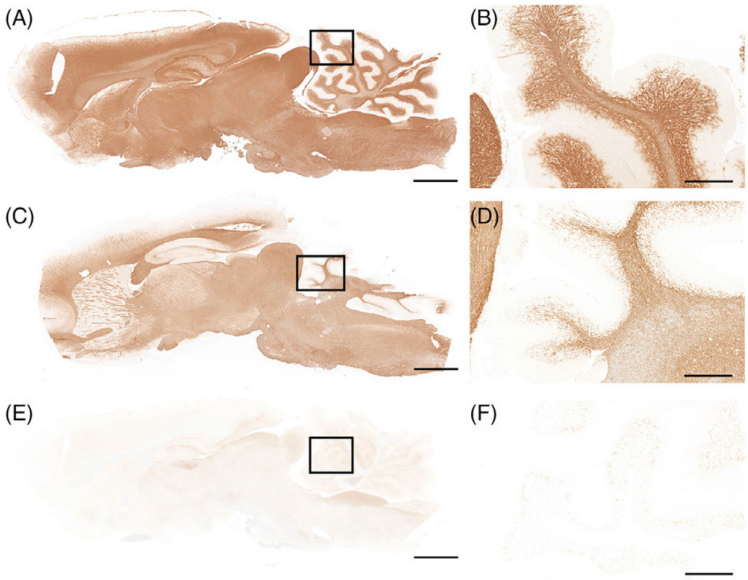

FIGURE 3: Myelin staining of affinity-purified myelin oligodendrocyte glycoprotein (MOG)-specific antibodies (Abs). Samples were stained on sagittal rat brain sections. The humanized r8-18C5 was used as positive control (A) and showed a specific myelin staining throughout the cerebrum and cerebellum (B; rectangle in A enlarged). The affinity-purified MOG-specific Ab from Patient 7 (C, D) showed a strong binding to myelin; a recombinant human IgG that does not bind MOG on transfected cells (r#7_D7) was negative (E, F). All Abs were used at a concentration of 3 µg/ml. Scale bars = 2mm (A, C, E), 300 µm (B, D, F).

17 animals revealed the following. The patient-derived MOG-specific Abs massively enhanced the T-cell infiltration in the subpial area of the pons when given together with cognate MOG-specific T cells, but not together with MBP-specific T cells (see Fig 4). Pathological analysis of animals injected with MOG-specific T cells alone or together with control Abs displayed a moderate inflammatory reaction in the spinal cord and less obviously in the brain and optic nerve, consisting of T-cell infiltrates in the meninges and CNS tissue and of $ED1^+$ macrophages, being restricted to the meninges (Fig 5, middle panels).

In combination with the injection of the MOG-specific Abs from Patients 5 and 7, a massively enhanced T-cell and macrophage infiltration in the meninges and the subpial CNS tissue was observed, and this was similar to the pathology observed after injection of the 8-18C5 Ab (see Figs 4 and 5). The enormous enhancement of the infiltration of T cells is already visible at a low magnification displaying cross sections of the whole spinal cord (see Fig 5, first and third rows). Human immunoglobulin reactivity was seen on subpial myelin, but only traces of activated complement (C9neo antigen) and a slight perivascular demyelination were present (data not shown).

Following transfer of MBP-specific T cells alone (which induced with the applied cell number a mild EAE on their own) or in combination with control Abs, a different pathology was seen. It consisted of mild to moderate T-cell infiltration together with the dispersion of $ED1^+$ macrophages throughout the tissue (Fig 6). In combination with patient-derived MOG-specific Abs, human Ig was also seen on subpial myelin, but this was associated with complement C9neo activation. This was accompanied by subpial demyelination (see Fig 4D), which was seen by LFB staining and by immunostaining for cyclic nucleotide phosphodiesterase. Demyelination and complement activation were massive with the Abs from Patient 7, less intense but detectable with the Abs from Patient 5, and absent after control Ab injection (see Figs 4D and 6). Due to injection into the cisterna cerebelli magna, the Abs hardly reached the optic nerve.

Thus, in this model, we see an impressive effect of the MOG Abs on the histopathology, but only a slight enhancement of the clinical disease. There are 2 reasons for this. First, the sensitivity to detect an enhanced clinical disease is lower if the control group is already sick (see Fig 4B) as compared to a model in which the control group is not sick at all (see Fig 4A). Second, the clinical score in this EAE model detects only motor functions. We have quantified the amount of lipopolysaccharide (LPS) in the samples used for in vivo experiments and found that the

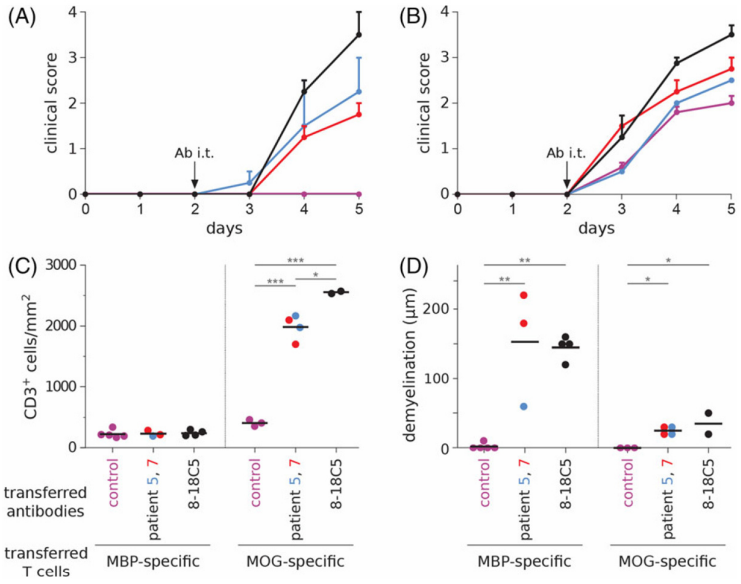

FIGURE 4: Pathogenicity of affinity-purified patient-derived myelin oligodendrocyte glycoprotein (MOG)-specific antibodies (Abs). Lewis rats were injected with MOG-specific (A) or myelin basic protein (MBP)-specific T cells (B). Two days later, 100 µg of affinity-purified MOG-specific Abs from Patient 5 (blue), Patient 7 (red), control IgG (purple), or 8-18C5 (black) were injected intrathecally (i.t.) into the spinal fluid (cisterna magna). (A) Three animals received human control IgG, 2 Abs from Patient 7 and 2 from Patient 5. Because the animals with the control IgG did not show any clinical disease, the induction of the clinical EAE with MOG-specific Abs from patients (data from the patients pooled) reached statistical significance at day 4 ($p < 0.05$) and day 5 ($p = 0.005$) using the unpaired 2-tailed *t* test. (B) Together with MBP-specific T cells, 1 animal received Abs from Patient 5, 2 animals Abs from Patient 7, 5 control IgG. As positive controls, r8-18C5 (A, B) and 8-18C5 (A) were used. Error bars indicate standard error of the mean. All animals were perfused at the end of the observation period and analyzed for histopathology. (C, D) Quantification of inflammation and demyelination of animals shown in A and B. (C) The T-cell infiltrates in the subpial region at the basis of the pons were counted with a 40 × objective, and the number of $CD3^+$ T cells/mm^2 was calculated. (D) The distance of subpial demyelination at the basis of the pons was measured. (C, D) We performed analysis of variance testing followed by Tukey honest significant difference test. * < 0.05; ** < 0.01; * < 0.001.**

contaminating amount of LPS was similar in control Ig and patient preparations; < 10ng were injected per animal. The same Ig preparations had different effects depending on the antigen specificity of the coinjected T cells; the patient Abs enhanced microglia activation and T-cell infiltration only together with MOG-specific T cells, but not in the context of MBP-specific T cells; a strong activation of terminal complement complex C9neo, conversely, was seen in the context of MBP-specific T cells, but little activation was seen in the context of MOG-specific T cells. We conclude from all this that the effects we describe were induced by the patient-derived Ig and not by LPS.

In this project, we had tested 3 different human Ig control preparations, namely ivIg, human IgG not specific for MOG obtained from a protein G column, and recombinant IgG with human Fc part. None of these human Ig variants recognized MOG, and none of them had any effect on enhancement of the disease. As a further control experiment, we injected OVA-specific T cells in the absence or presence of an intrathecal injection of r8-18C5. In this context, no induction of clinical disease and no demyelination or complement activation was present (data not shown).

Discussion

Our study shows that Abs to MOG affinity-purified from the blood of patients with inflammatory demyelination are pathogenic in transfer experiments to rodents. We found that these patient-derived MOG-specific Abs mediate damage to the CNS by different mechanisms. In synergy with T cells that induce clinical EAE, associated with profound blood–brain barrier damage and activation of macrophages (MBP-specific T cells in the Lewis rat in our model), human Abs to MOG mediate MS type II–like pathology, characterized by active demyelination (phagocytes containing myelin in the lesion) and local activation of the terminal complement complex, visible as deposition of C9neo.[32,33] We show here that these features are induced by the patient-derived MOG-specific Abs. This suggests that in patients with MOG Abs and MS type II pathology,[28–31] MOG Abs are responsible for this part of the pathology.

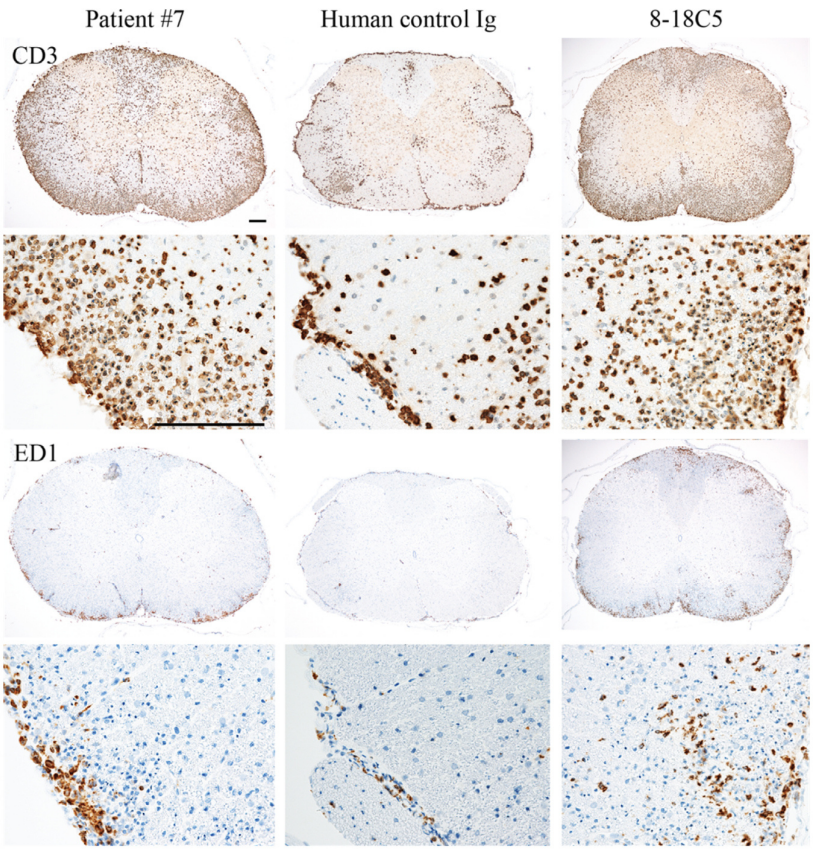

FIGURE 5: Affinity-purified antibodies (Abs) to myelin oligodendrocyte glycoprotein (MOG) enhance T-cell activation and promote microglia activation in the subpial parenchyma together with MOG-specific T cells. Spinal cord pathology is shown following passive cotransfer of MOG-specific T-cells with control IgG or anti-MOG Abs. Experimental autoimmune encephalomyelitis after injection of control Abs is characterized by T-cell infiltration in the meninges and diffusely in the spinal cord parenchyma, but ED1^{+} macrophages are largely restricted to the meningeal space (middle panels). After injection of Patient 7 Ab (left panels) or 8-18C5 (right panels), there is a massive enhancement of subpial T-cell infiltration and ED1^{+} macrophages pass the astrocytic glia limitans and infiltrate the central nervous system parenchyma. Scale bars = 100 μm.

Remarkably, most patients with MOG Abs and an MS type II pathology described so far do not have a typical MS,[42] but rather an encephalomyelitis overlapping with MS and NMO spectrum disorder. It is discussed whether this should be grouped as MOG Ab disease. Conversely, most patients with clinical MS and an MS type II pathology do not have Abs to MOG,[30,34] suggesting that these patients recognize other not yet identified autoantigens.

In our second model, in synergy with cognate MOG-specific T cells, which by themselves do not induce clinical disease, but only mild, predominantly meningeal inflammation in our rat model, the same affinity-purified Ab preparations induced clinical disease with other pathological features, namely a massively enhanced T-cell infiltration. An enhancement of T-cell activation by mAbs to MOG has been shown in 2 recent studies and suggested to be mediated by opsonization of the antigen.[20,23] We found that the patient-derived anti-MOG Abs not only enhanced T-cell infiltration induced by MOG-specific T cells, but also stimulated microglia/macrophage infiltration in the subpial gray matter. This indicates that human anti-MOG Abs in the CSF might also participate in the development of gray matter pathology together with MOG-specific T cells. MOG-specific T cells have been observed in patients with demyelination, and their recognized epitopes were identified.[43] Further studies are needed to analyze MOG-specific T cells in patients with Abs to MOG.

Our 2 EAE transfer models show that the human Abs to MOG mediate tissue destruction via 2 different

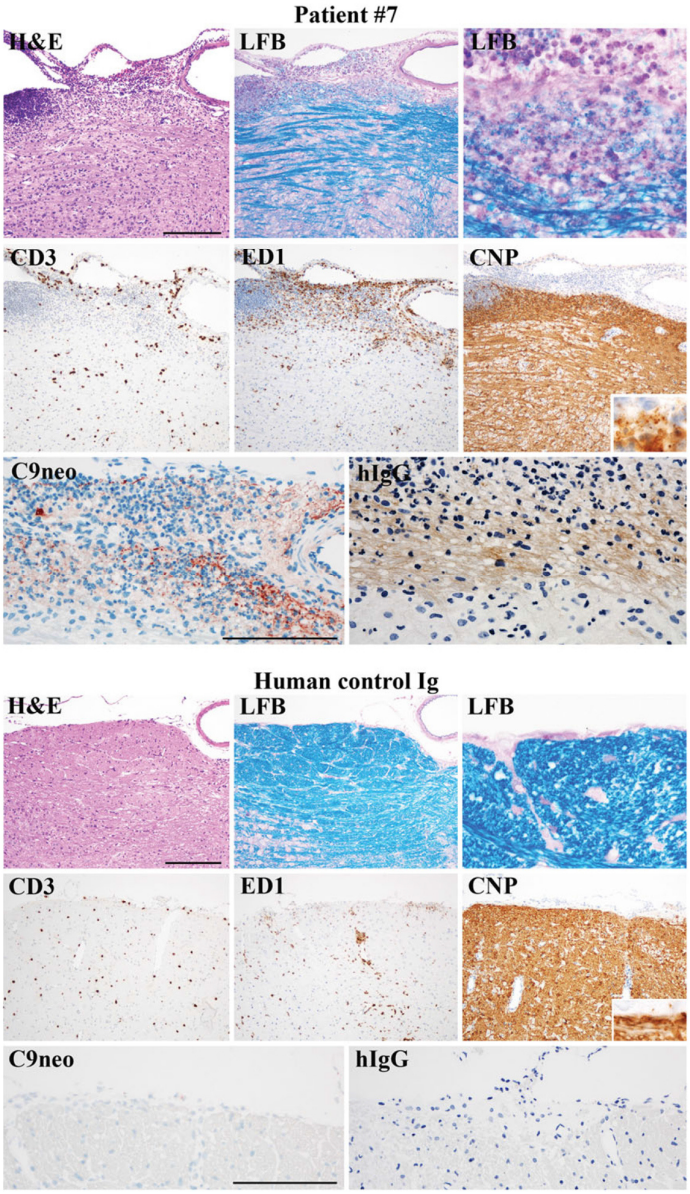

FIGURE 6: Affinity-purified antibodies (Abs) to myelin oligodendrocyte glycoprotein (MOG) induce complement activation and demyelination together with myelin basic protein (MBP)-specific T cells. Experimental autoimmune encephalomyelitis was induced with MBP–specific T cells. After 2 days, either MOG-specific affinity-purified Abs from Patient 7 (upper panels) or human control Ig (lower panels) was injected. When human control Ig was injected, there is a diffuse infiltration of the tissue by $CD3^+$ T cells and $ED1^+$ macrophages, but there is no deposition of human IgG on myelin or activation of complement (C9neo; lower panels). However, when anti-MOG Ig from Patient 7 was cotransferred, inflammation is massively enhanced and $ED1^+$ macrophages are concentrated at sites of active myelin destruction, associated with immunoglobulin deposition on myelin and complement activation (C9neo antigen deposition; lower left of upper panels). Scale bars = 100 μm. CNP, cyclic nucleotide phosphodiesterase; H&E = hematoxylin and eosin; LFB = Luxol fast blue.

TABLE 1. Features of Patients with Anti-MOG Reactivity

ID	Current Diagnosis	Gender	Age at First MOG$^+$ Sample, yr	Reactivity to Human MOG, MFI Ratio	Reactivity to Mouse MOG, MFI Ratio
5	Relapsing bilateral ON	F	42	220.7	212.9
14	Relapsing bilateral ON	M	54	44.9	20.6
8	NMOSD	M	37	38.3	3.0
7	Relapsing unilateral ON	M	46	34.7	216.8
16	NMOSD	M	30	18.6	5.6
17	Relapsing bilateral ON	F	31	18.2	2.1
6	Monophasic encephalitis	F	31	17.7	2.3
10	RRMS	F	37	11.9	8.3
13	Relapsing encephalomyelitis	M	34	8.6	5.5
1	NMOSD	M	40	6.1	1.7
3	Relapsing encephalomyelitis	M	26	5.4	1.8
4	RRMS	F	55	4.6	7.1
11	RRMS	F	50	4.1	1.5
2	Relapsing encephalomyelitis	F	66	4.0	0.9
9	RRMS	M	32	3.9	3.1
12	RRMS	F	23	2.9	3.9
21	NMOSD	F	33	2.7	1.8

Details about Patients 4, 9, 10, 11, and 12 are reported in Spadaro et al.[34] and about Patient 2 in Spadaro et al.[28] Patients with MOG antibodies might constitute a condition called MOG antibody disease. The cutoff for recognition of human MOG was 2.27 (mean + 3 standard deviation of controls). The MFI ratio was calculated as the mean of 2 to 5 experiments.

F = female; M = male; MFI = mean fluorescence intensity; MOG = myelin oligodendrocyte glycoprotein; NMOSD = neuromyelitis optica spectrum disorders; ON = optic neuritis; RRMS = relapsing–remitting multiple sclerosis.

mechanisms. This could be revealed because in our models the 2 different T-cell lines showed different intensities of T-cell reactivation in the CNS.[41,44] In the model with MBP-specific T cells, strong T-cell activation in the CNS was associated with blood–brain barrier disruption and the diffuse infiltration of the CNS tissue by recruited ED1$^+$ macrophages. Therefore, the incoming Abs find a good environment to mediate demyelination via Ab-dependent cell-mediated cytotoxicity and complement activation, which results in a pathology similar to MS type II. In the model with MOG-specific T cells, T-cell activation in the CNS is not optimal and recruitment of ED1$^+$ macrophages is sparse and largely restricted to the meninges. Here, the entering MOG-specific Abs massively enhance the T-cell recruitment and activation, because they recognize the same antigen; this then promotes infiltration of ED1$^+$ macrophages, which is associated with clinical disease but may be too low to effectively induce demyelination. Our observation that the patient-derived Abs perform tissue destruction by 2 different mechanisms, demyelination and enhanced inflammation, is consistent with a previous study transferring sera from immunized nonhuman primates.[45]

Our EAE experiments indicate further that the anti-MOG Abs are not pathogenic on their own, as together with irrelevant T cells no pathology was induced. This is consistent with previous observations in other EAE models[37] or after intrathecal injection of the 8-18C5 Ab[46] and supports the concept that the anti-MOG Abs perform a second hit to enhance pathology. Thus, human MOG Abs are pathogenic, but the precise pathological effects depend on their interactions with T cells; the human anti-MOG Abs can mediate MS type II pathology and gray matter injury upon transfer.

Experiences with mAbs in animals have shown that recognition of conformational MOG is required for

pathogenicity.[12,14] The secondary structure of MOG is characterized by 2 antiparallel beta-sheets that form an immunoglobulinlike beta-sandwich fold.[47] In rodents, pathogenic MOG-specific Abs mainly recognize the FG loop of MOG as the prototype mAb 8-18C5.[14] Although the epitope specificity of human anti-MOG Abs was previously dissected by ELISA[48] and transfection of mutated variants of MOG,[15] epitope specificity of pathogenic Abs from patients was unknown. The pathogenic MOG-specific autoantibodies from the 2 patients recognize different epitopes, and both are different from the one recognized by 8-18C5. Patient 5 recognized the CC′ loop, as its binding was reduced by the mutation P42S; this is the most frequently recognized part of human MOG.[15] This patient nevertheless strongly recognized mouse MOG, although the mouse MOG contains P42S. These 2 characteristics of MOG recognition (reduced reactivity to P42S, but strong recognition of mouse MOG) we had observed before in 5 of 111 patients.[15] Patient 7 recognized the FG loop of MOG, as its binding was completely abrogated by the mutation H103A+S104E. This resembles the recognition of 8-18C5, which is also abrogated by the double mutation H103A+S104E. A closer look at the reactivity of Patient 7 to other mutants of MOG points to epitopes that are discontinuous like the one recognized by the mAb 8-18C5,[47] but that differ from the 8-18C5 epitope as they are influenced by P42 positioned in the CC′ loop and/or the glycosylation site at N31 in addition to binding to the FG loop. The observed binding pattern of Patient 7 would therefore be consistent with the recognition of an ensemble of epitopes that include the FG loop and are located at the top, membrane-distal part and/or at the 5-stranded front β-sheet of MOG (see Fig 1E).

Together, this part of our analysis shows that pathogenic MOG Abs from patients recognize different loops on MOG.

The anti-MOG response of the patients with recurrent optic neuritis persisted for the observation periods of 26 and 35 months. This extends our knowledge of kinetic of MOG Abs. In children with ADEM, the Abs to MOG appeared only transiently and were rapidly lost, whereas in children with MS the MOG Abs persisted for years.[49] One of our analyzed patients had the unusual feature of having both an anti-MOG IgG and an anti-MOG IgM response. Both reactivities were directed against the same epitope of MOG. The co-occurrence of anti-IgG and anti-IgM to MOG is rare but was noted in a previous study in 3 of 19 children with ADEM and Abs to MOG.[17] The long-term persistence of an anti-MOG IgM response might be surprising, but it is consistent with recently described human IgM memory B cells that have passed the germinal center.[50] Our study shows that rarely an IgM response to MOG may also persist.

Our study has the following limitations. First, we injected the patient-derived Abs intrathecally, not systemically, although MOG Abs are typically detected in the blood. In pilot experiments with mAbs, we noted that EAE can be enhanced both by peripheral and by intrathecal injection, but that higher amounts of Abs were needed when the Abs were injected systemically. Because the amount of patient-derived Abs was limited, we chose intrathecal injection. We feel this is justified, as we found MOG Abs also in the CSF. From Patient 5, we could analyze CSF and found strong anti-MOG reactivity without evidence for intrathecal production of Abs to MOG. Second, we analyzed the pathology only at 1 time point after injection because we could inject only a limited number of animals with precious patient-derived Ig material. Compared to recombinant Abs, however, patient-derived Abs more closely reflect the human in vivo situation. This is important when evaluating the pathogenic potential of the MOG Abs present in the blood of patients, as the effector function of IgG is regulated by its glycosylation[51] and there is evidence that IgG glycosylation is altered in MS patients.[52] Third, we show that human MOG Abs identified in a cell-based assay include pathogenic Abs, but it remains unclear whether all of the MOG Abs are pathogenic and which features of the human Abs would allow predicting their pathogenicity. Our approach displaying the correctly folded extracellular part of MOG on a column purified only a proportion of MOG Abs. To affinity-purify and subsequently test the pathogenic activity of the other MOG Abs, MOG might have to be displayed in a membrane-bound environment. Our observation that the extracellular part of MOG purifies only part of the MOG Abs is consistent with the previous observation that in a cell-based assay a short construct of MOG lacking the intracellular part is less sensitive to detect anti-MOG Abs than full-length MOG.[53] Possible reasons for the differential reactivity to the 2 MOG variants with the same ECD include oligomerization or yet unidentified effects of the intracellular part of MOG on the conformation of the extracellular part. Furthermore, human MOG Abs are heterogeneous with respect to cross-reactivity to rodents. To address the pathogenicity of MOG Abs not cross-reactive with rodent MOG, mice with a knock-in of human MOG or even transfers to nonhuman primates might have to be used.

Together, we show here that Abs to MOG, which were affinity purified from the blood of patients and recognize different epitopes on MOG, synergize with T cells in transfer experiments to rodents; they induce MS type II pathology and trigger T-cell infiltration with microglia/macrophage activation in the subpial parenchyma. We conclude that MOG Abs contribute to the pathology of

patients with inflammatory demyelinating diseases by these mechanisms.

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Author Contributions

M.S., S.W., E.B., C.M., R.Hö., R.G., M.M., F.K., H.L., and N.K. conducted experiments, and acquired and analyzed data. E.S., F.S.T., L.-A.G., S.L., G.K., D.J., S.B., K.D., M.K., R.Ho., T.K., S.W., and C.B. analyzed data and contributed to manuscript preparation. M.S., H.L., T.K., R.Ho., N.K., and E.M. designed the study and wrote the manuscript.

Potential Conflicts of Interest

Nothing to report.

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2 Winklmeier S. et al. (2019) - Identification of circulating MOG-specific B cells in patients with MOG antibodies

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Identification of circulating MOG-specific B cells in patients with MOG antibodies

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Abstract

Objective

To identify circulating myelin oligodendrocyte glycoprotein (MOG)-specific B cells in the blood of patients with MOG antibodies (Abs) and to determine whether circulating MOG-specific B cells are linked to levels and epitope specificity of serum anti-MOG-Abs.

Methods

We compared peripheral blood from 21 patients with MOG-Abs and 26 controls for the presence of MOG-specific B cells. We differentiated blood-derived B cells in vitro in separate culture wells to Ab-producing cells via engagement of Toll-like receptors 7 and 8. We quantified the anti-MOG reactivity with a live cell–based assay by flow cytometry. We determined the recognition of MOG epitopes with a panel of mutated variants of MOG.

Results

MOG-Ab–positive patients had a higher frequency of MOG-specific B cells in blood than controls, but MOG-specific B cells were only detected in about 60% of these patients. MOG-specific B cells in blood showed no correlation with anti-MOG Ab levels in serum, neither in the whole group nor in the untreated patients. Epitope analysis of MOG-Abs secreted from MOG-specific B cells cultured in different wells revealed an intraindividual heterogeneity of the anti-MOG autoimmunity.

Conclusions

This study shows that patients with MOG-Abs greatly differ in the abundance of circulating MOG-specific B cells, which are not linked to levels of MOG-Abs in serum suggesting different sources of MOG-Abs. Identification of MOG-specific B cells in blood could be of future relevance for selecting patients with MOG-Abs for B cell–directed therapy.

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Glossary

Ab = antibody; **ADEM** = acute disseminated encephalomyelitis; **EGFP** = enhanced green fluorescent protein; **Ig** = immunoglobulin; **IL** = interleukin; **MFI** = mean fluorescence intensity; **MOG** = myelin oligodendrocyte glycoprotein; **PBMCs** = peripheral blood mononuclear cells; **TLR** = Toll-like receptor; **TT** = tetanus toxoid.

Antibodies (Abs) against myelin oligodendrocyte glycoprotein (MOG) are detected in a proportion of patients with inflammatory CNS diseases,[1–4] and there is growing consensus that these patients constitute a separate disease entity.[5–8] Abs against MOG are assumed to be pathogenic, based on in vitro experiments showing oligodendrocyte damage[9] and demyelination in slice cultures[10] and on in vivo transfer experiments with affinity-purified MOG-Abs from patients.[11]

The source of MOG-Abs is largely unexplored. Studies in animal models and human subjects have elaborated different ways to generate long-lasting immunoglobulin (Ig) G production. First, memory B cells could continuously generate short-lived plasma cells on antigen stimulation or via cytokines and Toll-like receptor (TLR) ligands.[12,13] Second, plasma cells might persist for many years in survival niches, e.g., in the bone marrow and continuously release Abs without further stimulation.[14] The optimal therapy for patients with anti-MOG disease is unknown. Current evidence indicates that only a proportion of anti–MOG-positive patients benefits from rituximab.[15–17] This might indicate different pathogenic mechanisms and different sources of MOG-Abs in these patients.

Here, we set out to identify MOG-specific B cells in blood of patients with MOG-Abs and controls by differentiating them ex vivo into Ig-producing cells and quantifying the MOG recognition of the produced IgG. Thereby, we aimed to analyze the abundance of circulating MOG-specific B cells in individual patients and to test whether there is a linkage to serum levels of MOG-Abs. Furthermore, our approach combining in vitro differentiation of B cells in separate wells with determination of epitope recognition allowed identifying intraindividual heterogeneity of anti-MOG autoimmunity.

Methods

Population

We analyzed 21 MOG-Ab–positive patients (52% female; mean age ±SD = 40 ± 12 years, range 15–60 years; table) and 26 age- and sex-matched healthy donors (62% female; mean age ±SD = 35 ± 13 years, range 20–61 years).

Differentiation of PBMCs into Ig-secreting cells

Briefly, 6×10^5 peripheral blood mononuclear cells (PBMCs) were seeded in 24-well plates in 1 mL/well RPMI medium containing 10% fetal bovine serum. TLR7/8 ligand R848 (2.5 μg/mL; Sigma-Aldrich, St Louis, MO) and interleukin (IL)-2 (1,000 IU/mL; R&D Systems, Minneapolis, MN) were added, and cells were cultured for 7–11 days. This combination of TLR7/8 ligation and IL-2 differentiates $CD19^+CD27^+$ memory B cells into Ig-producing cells, which have different requirements for activation and differentiation than naive B cells.[18] The in vitro stimulation we use in this study induces the production of IgG, IgA, and IgM.[18,19] For limiting dilution assays, PBMCs were distributed from 10^3 to 10^5 cells/well in 200 μL and stimulated for 11 days. The frequency of antigen-specific B cells was calculated according to the Poisson distribution.[18,19] Total B-cell frequency was determined by flow cytometry using the anti-human CD19-PerCP-Cy5.5 Ab (SJ25C1; eBioscience, San Diego, CA).

Flow cytometry for B-cell differentiation markers

Cells were stained using anti-human CD3-Alexa Fluor 700 (OKT3; eBioscience), CD19-APC/Fire 750 (HIB19; BioLegend, San Diego, CA), CD27-Brilliant Violet 605 (O323; BioLegend), CD38-eFluor 450 (HB7; eBioscience), CD138-PE (Mi15; STEMCELL Technologies, Vancouver, Canada), FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany), and TO-PRO-3 (Invitrogen, Eugene, OR).

Enzyme-linked immunosorbent assays

IgG was measured with the human IgG ELISA development kit (Mabtech, Nacka Strand, Sweden). Abs against tetanus toxoid (TT) were determined by coating TT (1 μg/mL; Merck Millipore, Burlington, MA) or bovine serum albumin (BSA, 1 μg/mL; Sigma-Aldrich) and detected by anti-human IgG horseradish peroxidase (Jackson ImmunoResearch, West Grove, PA; 109-036-003).

Detection of MOG-Abs

MOG-Abs were detected in a live cell assay, as described.[11,20] Briefly, HeLa cells were transiently transfected with human full-length MOG fused C-terminally to enhanced green fluorescent protein (EGFP)-N1 (Clontech Laboratories, Mountain View, CA) or with EGFP alone (control cells). As secondary reagents, biotin-SP-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) and Alexa Fluor 647–conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA) were applied. For the determination of anti-MOG reactivity, we gated on cells with an FITC fluorescence intensity above 500 and determined their mean fluorescence intensity (MFI) in the allophycocyanin channel. For serum (diluted 1:50), we calculated the MFI ratio between MOG-EGFP–transfected cells and cells transfected with EGFP alone. For cell culture supernatants

Table Features of patients with anti-MOG reactivity

ID	Initial diagnosis	Sex	Age at sampling (y)	Reactivity to MOG in serum (MFI ratio)[a]	Treatment at sampling (y)	Duration of disease (y)	Duration of last treatment (y)
4	MS	F	57	3.4	Glatiramer acetate	20	13
7a	CRION	M	47	47.4	Azathioprine	2.5	1
7b			50	32.9	Azathioprine	5.3	3.8
13	ON	M	38	20.5	None	4.8	—
14	Relapsing bilateral ON	M	54	45.4	Azathioprine	28	0.5
16	NMOSD	M	30	58.0	Cortisone	0.2	0.1
17	Relapsing bilateral ON	F	33	54.4	Azathioprine	6.1	0.8
22a	ON	M	37	7.4	Cortisone	0.1	0.1
22b			38	6.2	Azathioprine	1.2	0.3
23	Relapsing bilateral ON	M	15	111.0	None	6	—
24	ADEM	F	20	3.5	None	0.3	—
25	MS	F	59	4.5	Teriflunomide	4	0.8
26a	MS	F	47	66.3	Teriflunomide	16	5
26b			47	63.9	Rituximab	16.2	0
26c			47	62.0	Rituximab	16.5	0.3
26d			47	73.1	Rituximab	16.8	0.7
28a	ADEM	F	34	25.5	None	0.3	—
28b			34	19.4	None	0.6	—
31a	Autoimmune encephalitis	F	44	38.0	None	0.5	—
31b			44	40.1	None	0.7	—
37	ON	F	60	21.0	None	0.1	0.1
38	Relapsing ON	F	34	199.7	Rituximab	9	0.1
39	Relapsing ON	M	43	213.9	Rituximab	8	1.3
40	NMOSD	F	41	7.5	Eculizumab	4	3
41	ON	M	37	24.8	Azathioprine	3.3	3.2
42	NMOSD	M	35	27.1	Azathioprine	20	2.2
43	Bilateral ON	F	35	32.5	Azathioprine	3	0.7
44	NMOSD	M	32	26.1	Cyclophosphamide	0.1	0.1

Abbreviations: ADEM = acute disseminated encephalomyelitis; CRION = chronic relapsing inflammatory optic neuropathy; MFI = mean fluorescence intensity; MOG = myelin oligodendrocyte glycoprotein; NMOSD = neuromyelitis optica spectrum disorder; ON = optic neuritis.
[a] The cutoff for recognition of human MOG was 2.27 (mean +3 SD of controls).[11,20] The MFI ratio was calculated as the mean of 2–4 experiments.

(used undiluted), the MOG reactivity was determined as delta MFI (reactivity to MOG-transfected cells—reactivity to control transfected cells) because the reactivity to control cells of the cell culture supernatant was close to zero. Negative delta MFI was considered as zero. Threshold was set to mean +3 SD of the values from controls. Values beyond mean +5 SDs were not included in the threshold calculation. The recognition of epitopes on MOG was determined with a panel of mutated variants of MOG essentially as described.[21]

Statistical analysis

For Mann-Whitney *U* test, the nonparametric, unpaired, and 2-tailed test statistics were performed using GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA).

Data availability

Data presented in this study are available upon reasonable request.

Ethics statement

This study was approved by ethical committees of the Ludwig-Maximilians-Universität Munich and Hacettepe University Ankara. Informed consent was obtained from each donor according to the Declaration of Helsinki.

Results

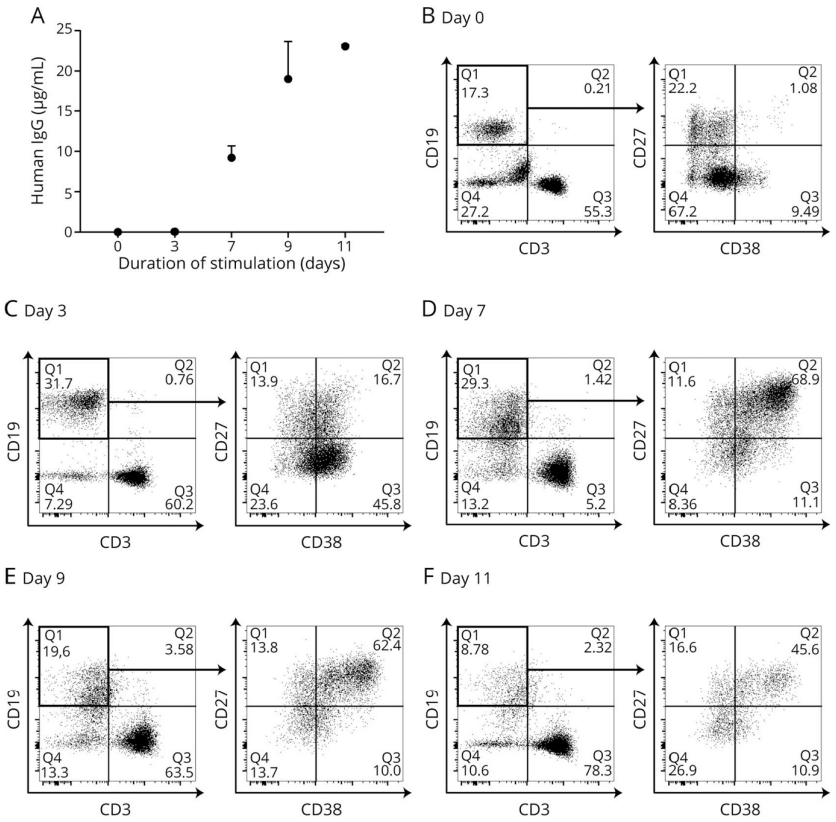
Differentiation of human B cells in vitro into Ig-secreting cells

We differentiated B cells into antibody-secreting cells and noted a strong IgG production at day 7, which further increased until day 11 (figure 1A), accompanied by development of plasmablasts ($CD3^-CD19^+CD27^{++}CD38^{++}$) (figure 1, B–F) that made up about 20% of all cells at day 7. At later time points, plasmablasts declined, whereas $CD3^+$ T cells prevailed (figure 1F and data not shown). About 10% of the plasmablasts (day 7) coexpressed CD138 (data not shown).

Identification of MOG-specific B cells in blood in a proportion of patients

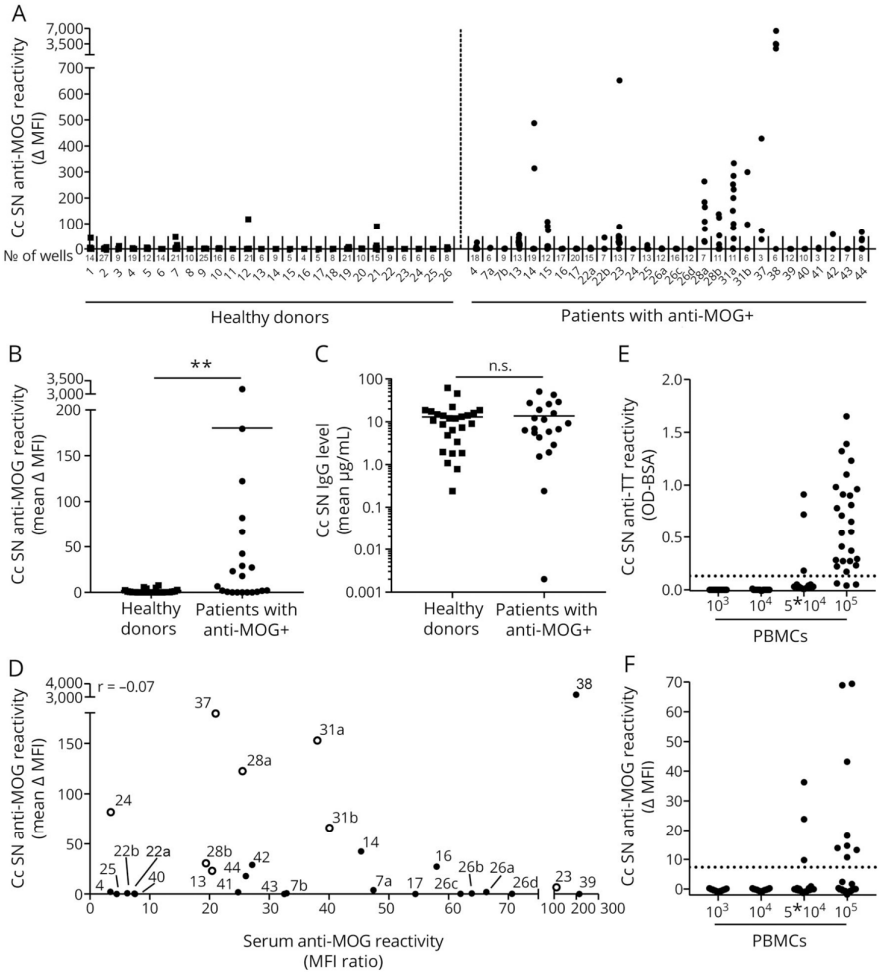
We determined the anti-MOG reactivity of IgG secreted from in vitro differentiated B cells and thereby obtained information on the presence and frequency of MOG-specific B cells in blood. We compared 21 anti–MOG-positive patients with 26 controls (figure 2, A–C). Patient versus control group showed a highly significant difference in anti-MOG reactivity of the in

Figure 1 Differentiation of human B cells in vitro into Ig-secreting cells



PBMCs of healthy controls were stimulated with IL-2 and R848 for the indicated periods. (A) IgG levels of cell culture supernatants were measured by ELISA. Each dot represents the mean of in total 2 stimulated wells from 2 different individuals. Error bars represent SEM. (B–F) Flow cytometry panels are displayed from 1 representative donor. For each time point, PBMCs were pregated on live and singlet cells. Gates Q1 ($CD3^-CD19^+$; black rectangles) of left panels were used for further gating on CD27 and CD38 in right panels. Plasmablast formation ($CD3^-CD19^+CD27^{++}CD38^{++}$) is shown in Q2 of right panels and peaked at day 7 (D). Ig = immunoglobulin; IL = interleukin; PBMCs = peripheral blood mononuclear cells.

Figure 2 Identification of MOG-specific B cells in blood of patients with MOG-Abs in serum



(A and B) PBMCs from MOG-Ab–positive patients (n = 21) and healthy donors (n = 26) were stimulated with IL-2 and R848. Anti-MOG reactivity in cc SNs was determined. Each dot represents 1 stimulated well. The number of stimulated wells is enclosed directly under the x-axis. (B and C) Each symbol represents the mean of all stimulated wells in 1 donor. Horizontal lines indicate the mean of all donors. (B) MOG-Ab production was significantly higher in patients than in controls (Mann-Whitney *U* test). (C) IgG levels of cc SNs were not significantly different between the 2 groups (Mann-Whitney *U* test). (D) Comparison of MOG-Ab levels in serum of patients and cc SNs of stimulated PBMCs. The mean anti-MOG reactivity of the stimulated PBMCs did not correlate with MOG-Ab serum levels in the respective patients (Spearman correlation; $r_{all} = -0.07$). Open circles indicate samples from patients with no treatment at time point of blood withdrawal ($r_{untreated} = -0.12$). (E–F) Limiting dilution analysis with PBMCs from anti–MOG-positive patient 24. PBMCs were seeded at concentrations of 10^3 (17 wells), 10^4 (17 wells), 5×10^4 (17 wells), and 10^5 (27 wells) cells/well and cultured for 11 days in the presence of IL-2 and R848. (E) TT production was assessed by ELISA. Dotted line indicates applied cutoff calculated as mean + 3 SD of negative wells. (F) MOG-Abs in cell culture supernatants were analyzed by flow cytometry with transfected cells. Dotted line indicates applied cutoff calculated as mean +4 SD of negative wells. According to the Poisson distribution in whole PBMCs, the frequency of MOG-specific B cells in patient 24 is calculated as 1:224,000 and for TT 1:68,000. **$p \leq 0.01$. Ab = antibody; BSA = bovine serum albumin; cc SN = cell culture supernatant; Ig = immunoglobulin; IL = interleukin; MFI = mean fluorescence intensity; MOG = myelin oligodendrocyte glycoprotein; ns = not significant; OD = optical density; PBMCs = peripheral blood mononuclear cells; TT = tetanus toxoid.

vitro differentiated B cells (figure 2B), while similar amounts of total IgG were produced (figure 2C).

A closer look at the patient group revealed a striking heterogeneity. In some patients, MOG-specific B cells were present in each well, in others in the majority of wells, and yet in others, no anti-MOG reactivity was detected in the secreted IgG. In 13/21 (about 60%) of anti–MOG-positive patients, we noted anti-MOG reactivity in at least 1 cultured well (figure 2A). The total amount of IgG produced

in vitro was similar in the samples from patients with (mean IgG = 6.82 μg/mL, n = 13) or without MOG-specific B cells (mean IgG = 8.82 μg/mL, n = 8) in their blood (data not shown).

From 5 patients, we could analyze samples obtained at different time points, and this showed the stability of our approach: From patient 7, 2 samples with a time interval of 3 years were negative. Likewise, both samples of patient 22 obtained with an interval of 1 year were negative. For patients 28a/b (interval of 4 months) and 31a/b (interval of 1 month), we could detect a positive signal for both time points. Patient 26 (no treatment for a/b; rituximab for c/d; all within 1 year) only showed a marginal positive signal in 1 well for the first blood sampling and was completely negative for samples b-d (figure 2A and table). We noted that in 4/26 healthy donors, a reactivity toward MOG was seen in at least 1 well (figure 2A).

We set out to determine the frequency of MOG-specific B cells in those patients where our first round of analysis indicated the presence of circulating MOG-specific B cells and where further samples were available. We performed a limiting dilution assay with samples from patients 24, 28, and 31. We calculated a frequency of about 1 MOG-specific B cell in 4.5×10^4 B cells and about 1 TT-specific B cell in 1.4×10^4 B cells for patient 24 (figure 2, E, F). Patient 28 had about 1 MOG-specific B cell in 1.4×10^5 B cells and about 1 TT-specific B cell in 8.3×10^4 B cells; patient 31 had about 1 MOG-specific B cell in 8.8×10^4 B cells and about 1 TT-specific B cell in 3.9×10^3 B cells.

MOG-specific B cells in blood and anti-MOG levels in serum did not correlate

Within the patient group, the amount of anti-MOG IgG produced after in vitro stimulation was not linked to the level of anti-MOG reactivity in serum ($r = -0.07$; figure 2D). We selectively analyzed the 8 samples we obtained from 6 patients who were untreated at the time of blood sampling. Also, in these samples, no correlation between circulating anti-MOG B cells and serum anti-MOG level was observed (open circles in figure 2D; $r = -0.12$).

Intraindividual heterogeneity of the anti-MOG response

We combined the B-cell differentiation in separate wells with the analysis of epitope reactivity. This was performed with samples from 6 patients. We show original data from selected wells of 2 patients (figure 3A) and the summary of all analyzed wells (figure 3B). The in vitro differentiated B-cell cultures reflected the fine specificity of the serum in 27/37 wells. Looking at individuals, this analysis revealed an intraindividual heterogeneity of the anti-MOG response in 4 of 6 patients that was not detectable when analyzing only serum.

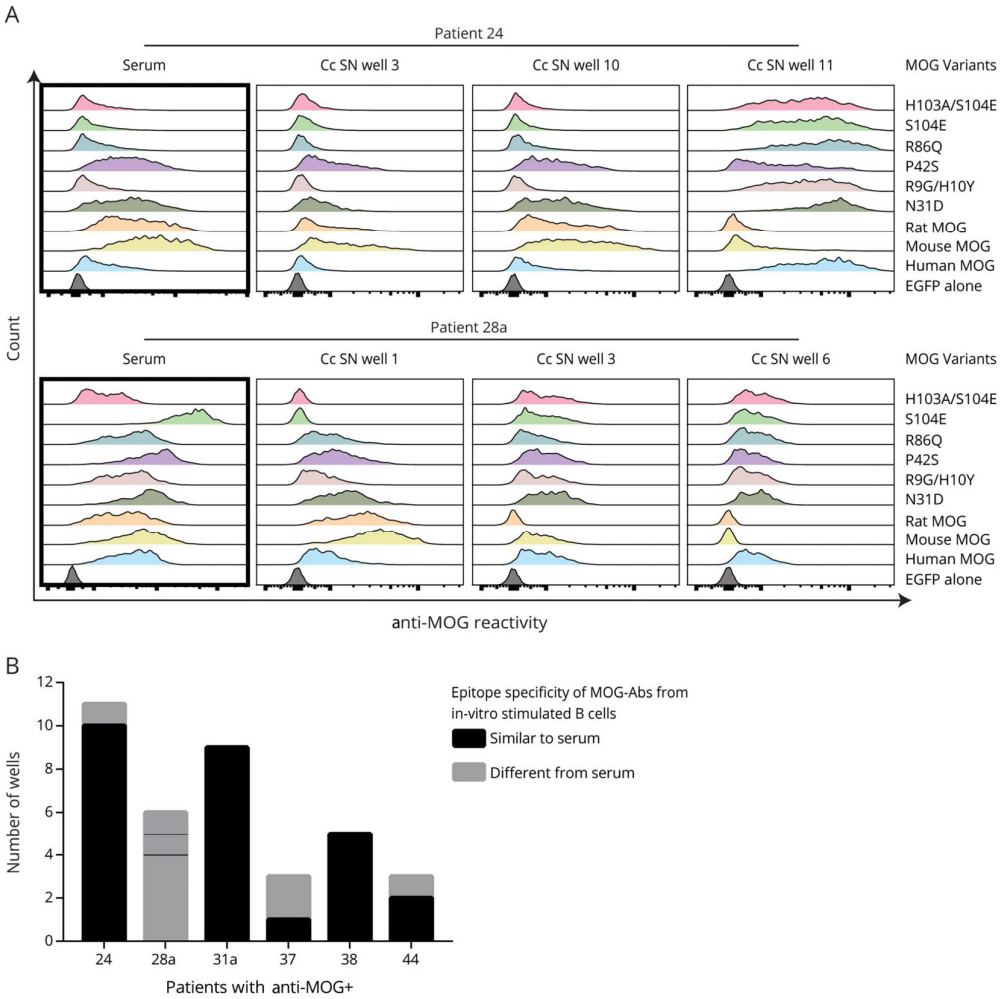
Discussion

Here, we describe circulating MOG-specific B cells in a proportion of patients with MOG-Abs. Although it is frequently a challenge to identify antigen-specific autoreactive T cells in patients with autoimmune diseases, the method we apply here is useful to quantify not only highly abundant antigen-specific B cells after infection and vaccination[18] but also autoreactive B cells such as MOG-specific B cells, which occur at much lower frequency. We have identified MOG-specific B cells by differentiating them into plasmablasts and then determining the in vitro development of MOG-Abs with a cell-based assay. An alternative method to enumerate antigen-specific B cells is the usage of a purified and labeled antigen.[22] The extracellular domain of recombinant MOG, however, does not completely mirror MOG in transfected cells.[11] We had used the recombinant extracellular part of MOG to form a tetramer, sorted B cells binding this MOG and produced their Ig in a recombinant way; we then found that these recombinant MOG-Abs bound MOG by ELISA, but did not bind to MOG on transfected cells (unpublished observation). Thus, the method we applied in this article is the first choice to identify and quantify MOG-specific B cells. The quantity of MOG-specific B cells was much lower than for the recall antigen TT. This reveals a difference to GAD65 autoimmunity, where GAD65-specific B cells were as abundant as B cells specific for recall antigens.[19]

The differentiation of B cells into antibody-secreting cells after TLR stimulation is a general feature of human memory B cells.[18] TLR7/8 stimulation, as applied in this study, induced MOG-Ab production provided the patient had preexisting MOG-specific B cells. TLR7/8 recognize single-stranded RNA viruses such as influenza virus; TLR9, which recognizes unmethylated CpG dinucleotide motifs located in bacterial and viral DNA, also mediates plasma cell differentiation.[18] Children with acute disseminated encephalomyelitis (ADEM)[1] and adult patients with optic neuritis and MOG-Abs frequently had an infectious prodrome.[23] The development of MOG-Abs after genital herpes has been described.[24] Attacks were preceded by infection in about 40% of anti–MOG-positive patients as seen in a multicentre study with 50 patients.[15] These clinical observations and our in vitro studies suggest that MOG-Abs can be induced on TLR stimulation. We noted that in 4/26 control donors, B cells could also be differentiated into MOG-Ab–producing cells in vitro. This is in line with the concept that autoreactive immune cells are part of the normal repertoire.[25] This is not necessarily linked to autoimmune pathology, but may reflect the susceptibility to develop autoantibodies, in the context of infections.

The extent of diversity of the individual anti-MOG response has been unknown. Our previous work with mutated variants of MOG has shown that individual patients respond to mutations at different loops of MOG; but this does not allow for conclusions about the heterogeneity of the anti-MOG response because MOG is so small that the maximal dimensions of a single Ab epitope (2.1×2.8 nm)[26] span a great area of the surface of MOG.[27] The approach we use here—differentiating B cells in separate wells and combining this with epitope analysis—allows identifying intraindividual heterogeneity of the anti-MOG autoimmunity.

Figure 3 Analysis of the intraindividual heterogeneity of the B-cell response to MOG



The cc SNs of individual wells with anti-MOG reactivity and the serum were further analyzed for recognition of mutants of MOG. (A) Flow cytometry histograms of selected cc SNs and serum from 2 patients. The MOG reactivities of the serum samples are framed. From patient 24, serum, cc SN well 3, and cc SN well 10 had the same pattern of reactivity to the MOG variants, whereas cc SN well 11 was different. From patient 28, serum and all cc SN samples showed a different reactivity to at least 1 MOG variant. (B) Summary of anti-MOG heterogeneity from 37 cc SNs from 6 patients. Cultured wells with the same reactivity pattern as found in serum of the respective patient are shown in black; those which differ from the pattern found in serum are shown in gray. In the blood sample 28a, 3 different patterns of anti-MOG reactivity could be dissected, indicated by the black lines, details in (A). cc SN = cell culture supernatant; MOG = myelin oligodendrocyte glycoprotein.

We found a highly significant difference in the frequency of MOG-specific B cells between patients and controls; but a closer look at the group with MOG-Abs revealed 2 subsets; in our study, about 60% of patients with MOG-Abs in serum had MOG-specific B cells in blood. This stratification of patients with MOG-Abs is not related to the intensity of the anti-MOG response in serum. In this respect, the autoimmunity against MOG is different to autoimmunity against AQP4 and NMDA-R, where a close correlation between serum levels of autoantibodies and circulating autoreactive B cells has been described.[28,29]

One limitation of our study is that some patients were under immunosuppressive treatment at the time of blood withdrawal; also, the number of patients with the same clinical phenotype and the same therapy is limited. However, despite

immunosuppressive treatment, patients had circulating MOG-Abs and also MOG-specific B cells in blood, consistent with other studies examining B cells of treated patients with other autoantibodies.[19,28,29] Furthermore, we had the chance to analyze blood cells from 6 patients with MOG-Abs before the onset of treatment, and these patients are very similar to the total cohort of patients in terms of abundance of MOG-specific B cells and lack of correlation between serum anti-MOG and circulating MOG-specific B cells.

The lack of linkage between autoantibodies to MOG and circulating MOG-specific B cells indicates different sources of the anti-MOG-Abs. Two sources have to be considered: long-lived plasma cells, which are negative for CD20, and $CD20^+$ memory B cells that are readily differentiated into anti–MOG-secreting cells.[12–14] MOG-Abs are transient in patients with an ADEM-like phenotype, whereas they persist for many years in others.[11,20,27,30]

The function of B cells extends beyond antibody production. B cells are extremely potent presenters of antigens that bind to their surface Igs; they selectively internalize their antigen and present it to T cells at concentrations 10^3- to 10^4-fold lower than required for presentation by nonspecific B cells or monocytes.[31] In animal models, MOG-specific B cells were essential as antigen-presenting cells to drive activation of MOG-specific T cells and encephalitis,[32] and in addition, MOG-specific Abs enhanced activation of cognate MOG-specific T cells.[11,33,34] Furthermore, B cells produce proinflammatory cytokines such as GM-CSF.[35]

The rationale for anti-CD20 therapy in patients with MOG-Abs is twofold: reduction of autoantibodies and elimination of B cells as central drivers of the immune response. The effect of rituximab on autoantibody levels is particularly strong in autoimmune diseases driven by IgG4 autoantibodies.[36] MOG-Abs are typically IgG1,[37] and previous results obtained with small cohorts showed that MOG-Abs may persist after rituximab,[27,38] but larger longitudinal studies are still pending. Clinically, only a proportion of patients with MOG-Abs respond to B-cell depletion,[15–17] and there is no biomarker for predicting the therapeutic response to anti-CD20: Treatment with the B cell–depleting Ab rituximab led to a decrease in the relapse rate in only 3/9 patients.[15] An international consortium analyzed the response to rituximab in 98 patients and reported that the overall response was weaker than in anti–AQP4-positive patients, and only a proportion of anti–MOG-positive patients benefited from rituximab.[16] In an Austral-Asian study, 1/6 patients failed to respond to rituximab.[17] The different responses to anti-CD20 might indicate different pathogenic mechanisms and different sources of MOG-Abs in these patients. Our study shows that MOG-Ab positive differ in the abundance of circulating MOG-specific B cells. Whether anti–MOG-positive patients with MOG-specific B cells in blood are preferred candidates for B cell depleting therapy needs to be assessed in future studies. Longitudinal observations from a decent number of patients are needed to analyze effects of therapies on circulating MOG-specific B cells. Our study shows that such examinations could be performed with frozen PBMCs, so a central analysis could be performed of PBMCs collected within a consortium.

Together, we show that circulating MOG-specific B cells are present in a proportion of patients with MOG-Abs and that their abundance is not linked to anti-MOG levels in serum. Our approach of differentiating B cells in separate wells and testing then the epitope specificity of the MOG-specific B cells gives insight into the intraindividual heterogeneity of the anti-MOG autoimmunity.

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Melania Spadaro, PhD	LMU, Munich, Germany	Author	Performed experiments and analyzed the data
Franziska S. Thaler, MD	LMU, Munich, Germany	Author	Involved in patient care, provided clinical samples, performed experiments, analyzed the data, and drafted the manuscript
Atay Vural, MD, PhD	LMU, Munich, Germany; Koç University, Istanbul, Turkey	Author	Provided clinical samples, performed experiments, and analyzed the data
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Reinhard Hohlfeld, MD	LMU, Munich, Germany; SyNergy, Munich, Germany	Author	Designed the study and drafted the manuscript
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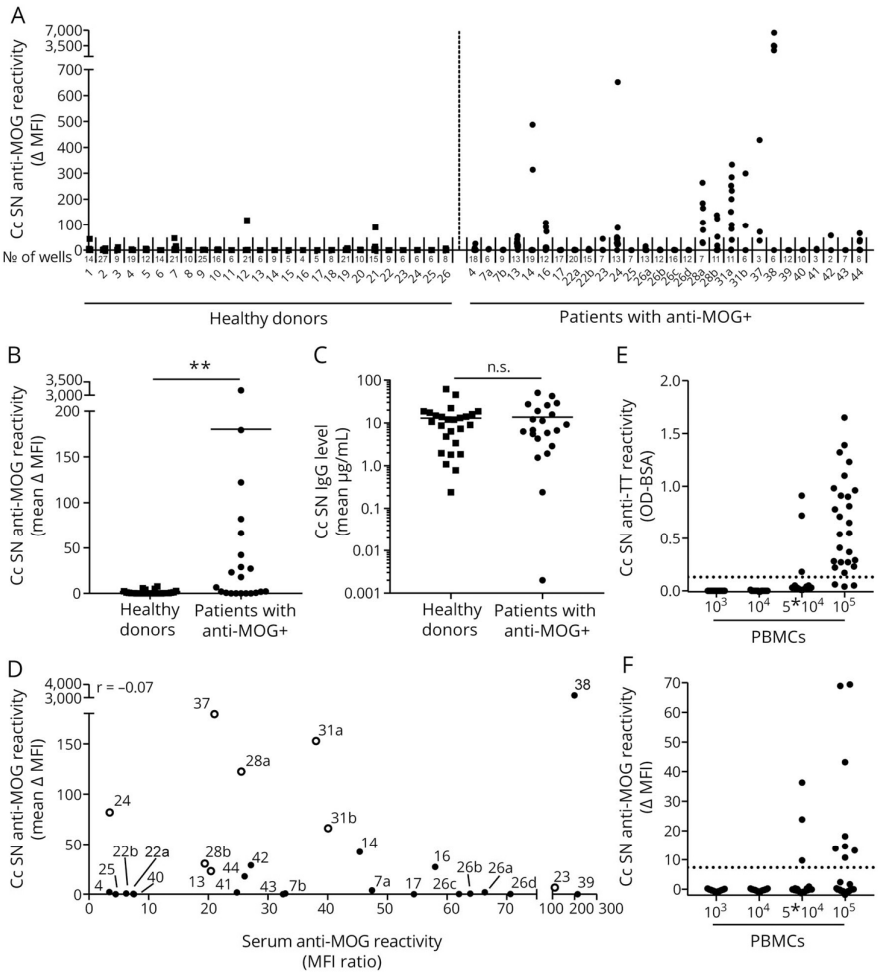
CORRECTION

Identification of circulating MOG-specific B cells in patients with MOG antibodies

Neurol Neuroimmunol Neuroinflamm 2020;7:e647. doi:10.1212/NXI.0000000000000647

In the article "Identification of circulating MOG-specific B cells in patients with MOG antibodies" by Winklmeier et al.,[1] first published online October 14, 2019, on the right side panel in figure 2a, the labelling of patient 15 should not have been included while the labelling of patient 26b should have been included. The corrected figure appears below. The publisher and the authors regret the error.

Figure



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1. Winklmeier S, Schlüter M, Spadaro M, et al. Identification of circulating MOG-specific B cells in patients with MOG antibodies. Neurol Neuroimmunol Neuroinflamm 2019;6:e625. doi: 10.1212/NXI.0000000000000625.

CORRECTION

Identification of circulating MOG-specific B cells in patients with MOG antibodies

Neurol Neuroimmunol Neuroinflamm 2021;8:e938. doi:10.1212/NXI.0000000000000938

In the article "Identification of circulating MOG-specific B cells in patients with MOG antibodies" by Winklmeier et al.,[1] there is an error in figure 1. The left Q3 panel in part D of figure 1 should read 56.2. The editorial staff regrets the error.

Reference

1. Winklmeier S, Schlüter M, Spadaro M, et al. Identification of circulating MOG-specific B cells in patients with MOG antibodies. Neurol Neuroimmunol Neuroinflamm 2019;6:e625. doi:10.1212/NXI.0000000000000625.

Discussion

This thesis contributed to the demonstration of the pathogenic activity of human MOG Abs in two pathomechanisms (Spadaro, Winklmeier et al. 2018). Further, in this thesis MOG-specific B cells could be identified, and a deeper insight into antigen recognition of MOG Abs could be obtained (Winklmeier, Schluter et al. 2019).

1 Pathogenic mechanism of MOG Abs

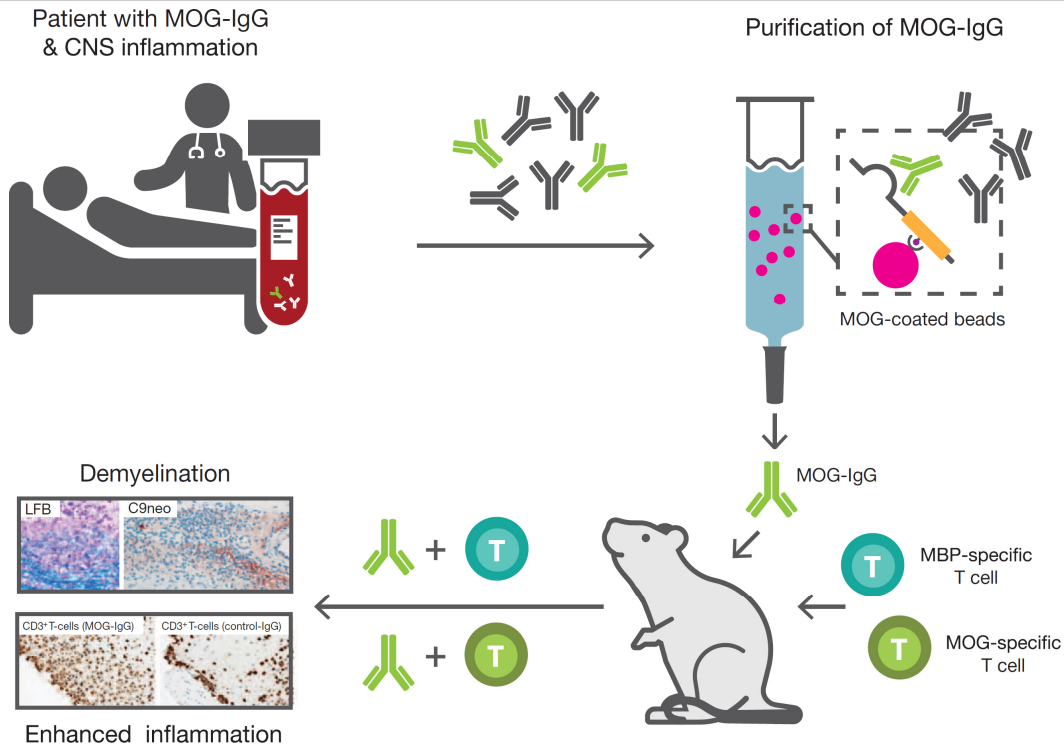

Figure 9| Strategy used to demonstrate the pathogenic mechanisms of patient-derived MOG Abs. Blood was withdrawn from patients with MOGAD, that showed a cross-reactivity to rodent MOG. MOG Abs were affinity-purified from patients' plasma and then intrathecally injected into the Lewis rats with EAE, where they enhanced the disorder by two different mechanisms. First, together with MBP-specific T cells, that were able to breach the BBB on their own, the patient-derived Abs resembled an MS type II pathology by inducing demyelination (luxol fast blue (LFB) staining) and complement activation (C9neo). Second, together with cognate MOG-specific T cells, the patient-derived Abs led to an enhanced inflammation by CD3 positive T cell recruitment and activation. With permission from (Mader, Kumpfel et al. 2020).

Abs against MOG define MOGAD, a clinical manifestation of a subset of patients with an inflammatory demyelinating disease of the CNS. Previous studies suggest that these Abs have a pathogenic potential. However, mainly serum or total IgG preparations were used in the *in vitro* and *in vivo* experiments. Thereby, patients may harbor multiple auto-Abs which makes an interpretation difficult. In addition, only a proportion of human-derived MOG-Ig cross-reacts with rodent MOG which limits the studies in these animals.

Therefore, we selected for the present study patients that have MOG Abs cross-reactive to rodent MOG. Further, we affinity-purified MOG-Ig from patients' plasma which was then injected into the Lewis rats intrathecally. In close collaboration with PD. Dr. Naoto Kawakami and Prof. Dr. Hans Lassmann, we elaborated the pathogenic mechanism of human MOG Abs in two different EAE models (**Figure 9**).

In our first model, the adoptive transfer of MBP-specific T cells induced a clinical EAE on its own, which is consistent with previous observations. These encephalitogenic T cells were strongly activated in the CNS which is associated with a BBB breach and recruitment of activated ED1 positive macrophages (Kawakami, Lassmann et al. 2004). Thereby, the injected human affinity-purified MOG Abs could act in synergy with the well-established inflammatory environment from the T cells. These patient-derived Abs induced demyelination via ADCC and complement activation. We revealed here that phagocytes contained myelin in the lesion area and that the terminal complement complex was formed, detected as deposition of the complement component 9 neo-antigen (C9neo). Both features are reflected by the MS type II pathology (Lucchinetti, Bruck et al. 2000) and suggest that these MOG Abs are responsible for this part of the pathology in those patients, which is in line with the reported phenotype of MOGAD in several studies (Di Pauli, Hoftberger et al. 2015, Spadaro, Gerdes et al. 2015, Jarius, Metz et al. 2016). Notably, MOG-IgG is detected in a proportion of patients with pattern II MS and the majority of these patients harbor auto-Abs that are not yet identified (Spadaro, Gerdes et al. 2016, Kortvelyessy, Breu et al. 2017).

In our second model, the adoptively transferred MOG-specific T cells were not able to induce an EAE in the Lewis rats on their own. The T cell activation in the CNS was not optimal. These T cells alone caused only mild inflammation and sparse recruitment of ED1 positive macrophages, which was predominantly restricted to the meninges. However, upon the additional injection of the human affinity-purified MOG Abs, the rats developed a clinical disease. Here, the MOG-specific Abs recognize the same antigen as the transferred cognate T cells. This leads to a massive enhancement of T cell recruitment and activation, which is in line with previous studies, that could show a boost of myelin-specific T cell activation and expansion due to myelin-specific Abs. Hereby, mouse MOG Abs, that recognized intact MOG, were internalized by APCs in an Fc-dependent manner, and stimulated the cognate T cells by presenting the antigen (Flach, Litke et al. 2016, Kinzel, Lehmann-Horn et al. 2016). Further, we found macrophages infiltrated into the subpial gray matter, indicating a potential synergistically participation of MOG-specific

Abs and T cells in the development of this pathology. Compared to the first model with MBP-specific T cells, we only observed little demyelination in the second model because there was only slight breaching of the BBB. While the epitope recognition was identified for AQP4-specific T cells in AQP4 Abs seropositive NMOSD (Hofer, Ramberger et al. 2020) and for MOG-specific T cells in MS patients (Varrin-Doyer, Shetty et al. 2014), so far, no disease-relevant MOG peptide could be found in patients with MOGAD (Hofer, Ramberger et al. 2020). Therefore, further studies with larger cohorts may be required. In addition, anti-MOG Abs did not act pathogenically on their own in our EAE transfer experiments if irrelevant T cells were injected. Previous studies using a transgenic knock-in mouse model of the monoclonal Ab 8-18C5 heavy chain or injecting the 8-18C5 intrathecally have revealed similar results (Vass, Heininger et al. 1992, Litzenburger, Fassler et al. 1998). In contrast, circulating anti-AQP4 Abs were able to induce pathogenicity in animal models on their own when given at high concentrations over a period of time (Hillebrand, Schanda et al. 2019). Taken together, we could show that human-derived MOG Abs are pathogenic by two mechanisms. They synergize with encephalitogenic T cells, performing a second hit. In addition, when the BBB is strongly breached, the MOG-Abs induce demyelination; together with cognate T cells, they enhance T cell infiltration. In the two animal models we used, the interaction of cognate T cells with human MOG Abs can induce MS type II pathology and grey matter injury as a second hit in both mechanisms.

2 Identification of circulating MOG-specific B cells

In several studies, it was reported that patients with MOGAD respond differently to B cell depleting therapies (Chen, Flanagan et al. 2020, Whittam, Cobo-Calvo et al. 2020). This raised the question, if patients with MOGAD also have different MOG-specific B cell frequencies and if we are able to detect them. The identification of antigen specific autoreactive T cells can be challenging. Similarly, B cells that require a native antigen structure for recognition, such as MOG-specific B cells, cannot be detected by simply using the extracellular part of the antigen. Therefore, we applied in the present study an alternative approach that has been described previously for quantification of highly abundant antigen-specific B cells after infection and vaccination (Pinna, Corti et al. 2009). Even though the frequency of autoreactive B cells is much lower, we were able to detect MOG-specific B cells in the peripheral blood. To this end, we differentiated human memory B cells in peripheral blood mononuclear cells (PBMCs) from MOGAD patients

and healthy controls *ex vivo* into Ig-secreting cells by stimulating them with resiquimod, a TLR 7/8 ligand, and the cytokine interleukin-2 (IL-2). Resiquimod stimulates directly memory B cells that express TLR 7. In addition, it binds to TLR 8 on myeloid cells and promotes cytokine secretion, which further supports B cell proliferation and differentiation. IL-2 is not only a growth factor for T cells and natural killer (NK) cells, but also for activated B cells (Pinna, Corti et al. 2009). The reactivity against MOG of the

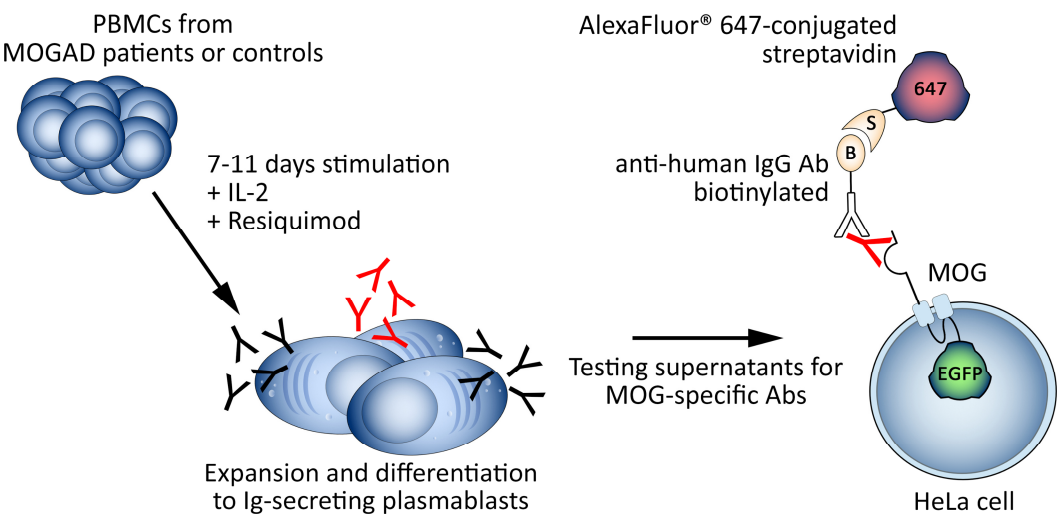

Figure 10| Strategy used to investigate MOG-specific B cells. PBMCs from MOGAD patients and healthy controls were differentiated to Ig-secreting cells by stimulating them for 7 to 11 days with the cytokine IL-2 and the TLR 7/8 ligand resiquimod (left). Secreted Abs in the cell culture supernatants were tested for reactivity against MOG by using a cell-based assay with full-length MOG transfected HeLa cells (right). Bound MOG Abs were detected with a biotinylated secondary Ab against human IgG and a fluorochrome-conjugated streptavidin in flow cytometry. Transfection efficiency of MOG was verified by the expression level of the fusion protein EGFP at the C-terminus of MOG. More details about this assay can be found in the introductory chapter 2.2.

secreted Abs was then quantified with our cell-based assay (**Figure 10**).

Here, we found that the anti-MOG reactivity from stimulated PBMCs was significantly higher from patients with MOGAD compared to healthy donors, while the total IgG production was similar between the two groups. A closer look revealed two subsets in our patient cohort; mainly, subjects with and without MOG-specific B cells. In the majority of MOG Abs seropositive patients, about 60%, we were able to detect MOG-specific B cells in their peripheral blood. To determine the frequency of antigen-specific B cells, we performed limiting dilution assays and calculated the frequency according to the Poisson distribution (Pinna, Corti et al. 2009). Thereby, we could show that the abundance of circulating MOG-specific B cells was much lower than for the recall antigen tetanus toxoid (TT). In contrast, other autoreactive B cells are described to be more frequent such as for the intracellular target-antigen glutamic acid decarboxylase (GAD)65 in GAD65

Abs-associated neurological disorders. There, GAD65-specific B cells occurred with a comparable frequency as the recall antigen TT (Thaler, Thaller et al. 2019).

To our experience, the *in vitro* differentiation assay, used in the present study, is the first choice to detect conformationally dependent autoreactive B cells. In general, the stimulation of TLR 7/8 in this assay induces proliferation of all human memory B cells independent from their B-cell receptor signaling. This was also the case for MOG-specific B cells in the PBMCs of our patients with MOGAD. It is described that the genetic information of viruses and bacteria serve as a danger signal associated with infection for our immune system and are recognized by different TLRs on immune cells. Viral single-stranded RNA can bind to TLR 7/8, whereas DNA from invading pathogens is a potential ligand of TLR 9 (Diebold, Kaisho et al. 2004, Heil, Hemmi et al. 2004). Different studies suggest that autoimmunity can be induced by cross-reactive Abs that occur after an infection (Rojas, Restrepo-Jimenez et al. 2018). In terms of patients with MOGAD, *Jarius et al.* described in a multicenter study that attacks were preceded by an infection in at least 40% of 37 patients with MOG Abs (Jarius, Ruprecht et al. 2016). Especially, patients with ADEM or ON were frequently observed to have an infectious prodrome (Ramanathan, Reddel et al. 2014, Koelman and Mateen 2015). Additionally, further case reports connect the occurrence of MOG Abs with several bacterial or viral infections, such as *Mycoplasma pneumonia* (Bonagiri, Park et al. 2020), the Epstein-Barr virus (Kakalacheva, Regenass et al. 2016, Nakamura, Nakajima et al. 2017), the Zika virus (Neri, Xavier et al. 2018), the influenza A virus (Amano, Miyamoto et al. 2014), or the genital herpes simplex virus (Nakamura, Iwasaki et al. 2017). Aside from the patient cohort, 4/26 donors in our control group also developed MOG Abs after *in vitro* stimulation. Autoreactive immune cells do not lead necessarily to autoimmunity. They may occur as part of our healthy immune repertoire, and can also be related to a previous infection (Cohen 2014). Taken together, the clinical observations and our results indicate that MOG Abs can arise after TLR stimulation.

There are different sources possible, that may produce MOG-Ig in patients with MOGAD. Long-lasting levels of MOG Abs could be generated in different ways. First, memory B cells that recognize MOG could be stimulated by their cognate antigen to proliferate and differentiate into short-lived plasma cells, or by cytokines and TLR ligands in an antigen-independent manner. Second, long-lived plasma cells may constantly release MOG Abs in survival niches such as the bone marrow. Since the main source of Abs against MOG is largely unexplored, we compared the amount of MOG Abs secreted in our *in vitro* assay

with the MOG-IgG level in corresponding serum samples within the patient cohort. Here, we could not recognize a linkage, assuming that circulating memory B cells are not the main source for MOG Abs in the sera from our patient group. To exclude that an immunosuppressive treatment may alter our interpretation, we selectively repeated the analysis with 8 samples from 6 donors, who were untreated at the time point of blood withdrawal. These results were similar and confirmed our previous observation that MOG-specific B cells do not correlate with MOG Ab titers in serum. This is comparable to the autoimmunity against GAD65, where the source of GAD Abs is also not linked to circulating GAD-specific memory B cells. Further, *Thaler et al.* could show that plasma cells niched in the bone marrow were responsible for the production of GAD Abs (Thaler, Thaller et al. 2019). In terms of MOGAD, we suggest that long-lived plasma cells may also be present in survival niches such as the bone marrow. In contrast, serum levels of Abs against AQP4 or NMDAR are directly linked to the amount of Abs produced by stimulated B cells in peripheral blood (Makuch, Wilson et al. 2018, Wilson, Makuch et al. 2018).

3 Deeper insight into details of antigen recognition

Previous studies showed that a correctly folded native structure of MOG is essential to detect pathogenic Abs, that can induce demyelination in animal models, and to distinguish them from non-demyelinating ones. Methods that used the extracellular domain of MOG or a non-native version were only able to measure non-pathogenic Abs (Brehm, Piddlesden et al. 1999). The immunoglobulin-like fold of the MOG protein forms a sandwich structure consisting of two antiparallel beta-sheets (Breithaupt, Schubart et al. 2003). While the binding specificity of demyelinating Abs against MOG derived from animals were studied intensively, little is known for epitope recognition of pathogenic human Abs. In our first study, we were able to affinity-purify MOG-specific Ig from two patients' plasma which induced the previously described pathogenic phenotype in our animal models. Here, patient 5 required the CC' epitope, as the binding signal was reduced, if the amino acid proline was substituted by serine at position 42 (P42S). The CC' epitope of MOG is the most frequently recognized loop from patients with Abs to MOG (Mayer, Breithaupt et al. 2013). Further, patient 5 still recognized mouse MOG, even though it contains the P42S substitution. This binding characteristic was seen in about 5% of analyzed sera from patients with MOGAD in a previous study (Mayer, Breithaupt et al. 2013). In contrast, the recognition pattern for patient 7 was completely

different. The affinity-purified MOG Abs from this patient required the FG epitope for binding. Here, the double mutation of MOG at position 103 by substituting histidine with alanine (H103A) and at position 104 by substituting serine with glutamic acid (S104E) reduced the recognition completely. This resembles the typical recognition pattern of pathogenic MOG Abs in rodents, which recognize mainly the FG loop of MOG, as it is for the monoclonal Ab 8-18C5 (Breithaupt, Schubart et al. 2003). A more detailed analysis however revealed that the recognition pattern of patient 7 was distinct from the binding pattern of 8-18C5. Apart from the FG epitope the affinity-purified MOG Abs from patient 7 had an enhanced binding to MOG variants with an amino acid substitution at position 42 and 31.

Besides MOG-IgG patient 5 also harbored MOG-IgM, which is rarely found in MOGAD. The epitope specificity did not differ between the two isotypes. It has been reported that MOG-IgM can co-occur with MOG-IgG in children with ADEM or adults with ON in less than 16 % (Brilot, Dale et al. 2009, Pedreno, Sepulveda et al. 2019). Further, we have observed a persisting MOG-IgM level which can be explained by the presence of IgM memory B cells (Weill and Reynaud 2020). So far, there is no clinical relevance described for MOG-IgM in the pathogenesis of MOGAD (Pedreno, Sepulveda et al. 2019). MOG-IgG also persisted on a long-term basis in our two patients during the observation period of two to three years.

In our second study, we were able to investigate how diverse the anti-MOG response appears in individual patients. The common analysis of serum for epitope specificity does not allow to display the heterogeneity of the containing MOG Abs. Our strategy, as described earlier, of stimulating PBMCs from MOGAD patients overcomes this limitation of the serum. The secreted MOG Abs in the *in vitro* differentiated cell culture supernatants from different wells revealed an intraindividual diversity of the anti-MOG autoimmune response. Taken together, these findings of the different epitope specificity of our two pathogenic affinity-purified MOG-Ig, the persisting reactivity of MOG-IgG and MOG-IgM, and the deeper insight into the intraindividual heterogeneity of MOG-IgG extends our understanding of the MOG Abs repertoire in humans.

4 Conclusions and implications

In the first part of this thesis, the pathogenic mechanism of patient-derived MOG Abs was investigated with two different animal models. We could demonstrate that human

MOG Abs recognize different epitopes on MOG and that these Abs act synergistically together with T cells in Lewis rats initiating an MS type II pathology when the BBB was breached and enhancing an infiltration of cognate MOG-specific T cells. Our first study was limited in the amount of patient-derived affinity-purified MOG Abs. Therefore, we injected the MOG Abs intrathecally instead of intravenously, which enabled us to reduce the needed Ab amount per animal. Further, the used number of animals was restricted due to the limited amount of affinity-purified MOG Abs. Previous experiments with the monoclonal MOG Ab 8-18C5 have given similar results for peripheral or intrathecal injection. Our decision for an intrathecal injection was also supported by the circumstance that MOG Abs were present in the CSF of patient 5. Additionally, patient-derived Abs reflect more the *in vivo* situation in humans than monoclonal Abs. Since we investigated the pathogenicity in Lewis rats our observations were limited to cross-reactive human MOG Abs towards the rodent variant of MOG. Future studies are required to address this concern. It may be needed to use knock-in animals with human MOG or even transfer experiments with primates.

In the second part, we extend the understanding of the anti-MOG immune response by studying the MOG-specific B cell compartment. Here, we could reveal that the majority of patients with MOGAD have circulating MOG-specific memory B cells in their blood and that the serum MOG-IgG level is not linked to these circulating B cells. In addition, we showed that patients with MOGAD may harbor an intraindividal heterogeneity of MOG Abs. Our second study was limited in the number of participating patients in terms of their clinical phenotype or treatment. Further, some patients of our cohort were under immunosuppressive therapy when blood was withdrawn. However, we were also able to investigate a proportion of samples taken at a time point before a treatment was started. The results of these samples reflect and support our overall findings regarding MOG-specific B cell abundance and source of MOG-IgG serum levels.

The optimal therapy for patients with MOGAD is unknown. Recent findings revealed that only a proportion of these patients benefit from B cell depleting therapies such as rituximab. Our data indicate that there are mainly two groups of patients with MOGAD. One group with and one group without circulating MOG-specific B cells. Although no correlation between serum MOG-IgG levels and circulating MOG-specific B cells could be detected, it still may be beneficial for the subset of patients with MOG-specific B cells to be treated with a B cell depleting therapy. Besides the role of Abs production, B cells are highly potent antigen-presenting cells for activating T cells and important drivers of

the immune response (Lanzavecchia 1985). In addition, independent to their humoral function, MOG-specific B cells have been shown to play a critical role as antigen presenters for T cell response in an EAE mouse model (Molnarfi, Schulze-Topphoff et al. 2013). Therefore, multicenter studies with larger cohorts are required to assess the question of whether MOG-specific B cells could serve as a biomarker to stratify patients with MOGAD for a B cell depleting therapy.

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Curriculum Vitae

Education

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2015 – 2016	**Scientific assistant**, Walter Schottky Institute, Center for Nanotechnology and Nanomaterials, Technical University of Munich (TUM) Supervised by Prof. Dr. Jose A. Garrido
01 – 10/2013	**Master thesis research stay at Harvard Medical School**, Children's Hospital Boston, Stem Cell Institute, Boston, MA, USA Supervised by Prof. Dr. Richard Gregory
2010 – 2013	**Master of Science in Biochemistry**, University of Munich (LMU)
2007 – 2010	**Bachelor of Science in Biology**, University of Munich (LMU) Bachelor thesis in the department of Plant Biochemistry and Physiology supervised by Prof. Dr. Jürgen Soll

Selected extracurricular activities

10/2020	**Leading teams & groups**, online workshop, Graduate Center, LMU Munich
10/2020	**Scientific writing**, workshop, Graduate Center, LMU Munich
2018 – 2019	**SEA:certificate**, Social Entrepreneurship Academy, Strascheg Center for Entrepreneurship, Munich
10/2018	**Change maker training**, InnovationsGeist, LMU Munich
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Selected stipends and conferences

2019	**Stipend** of Netzwerk für Stipendiaten mit besonderem unternehmerischen und gesellschaftlichen Engagement e.V.
2019	**Poster presentation** at DGFI, Munich, Germany
2018	**Poster presentation** at Keystone Meeting, B Cells: Mechanisms in Immunity and Autoimmunity, Dresden, Germany
2018	**Oral presentation** at Interact 10. Symposium, Munich, Germany
2018	**Oral presentation** at XXVI. Symposium on Research in Neurology, Munich, Germany
2013	**Trainee Program Stipend**, Children's Hospital Boston, Boston, MA, USA
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