DISSERTATION

Identification and characterization of human monoclonal antibody target structures in the brain – Blood vessel antibodies in autoimmune encephalitis

Identifizierung und Charakterisierung von Zielstrukturen humaner monoklonaler Antikörper im Gehirn – Gefäßantikörper bei Autoimmunenzephalitiden

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Table of Contents

List of Figures

List of Tables

Abbreviations

ADEM	Acute demyelinating encephalomyelitis
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor
AQP4	Aquaporin 4
BBB	Blood-brain barrier
CASPR2	Contactin-associated protein-like 2
Cb	Cerebellum
CNS	Central nervous system
CSF	Cerebrospinal fluid
Ctx	Cortex
DG	Dentate Gyrus
ECM	Endothelial cell growth medium
FCS	Fetal calf serum
GABAR	γ-aminobutyric acid receptors
GAD	Glutamic acid decarboxylase
GFAP	Glial fibrillary acidic protein
GRP78	Glucose-regulated protein 78
hCMEC/D3	Human cerebral microvascular endothelial cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
LEMS	Lambert-Eaton myasthenic syndrome
LGI1	Leucine-rich glioma-inactivated protein 1
mAb	Monoclonal antibody
mcl	Monoclonal
mGluR5	Metabotropic glutamate receptor 5
MOG	Myelin oligodendrocyte glycoprotein
MOGAD	Myelin oligodendrocyte glycoprotein autoantibody-associated disease
MS	Multiple sclerosis
NMDAR	N-methyl-D-aspartate receptor
NMOSD	Neuromyelitis optica spectrum diseases
PCD	Paraneoplastic cerebellar degeneration

pcl	Polyclonal
SMA	Smooth muscle actin
TEER	Transendothelial electrical resistance
VGCC	Voltage-gated calcium channel

Abstract

Autoimmune encephalitis is a rare group of inflammatory brain diseases associated with autoantibodies against neuronal surface proteins, which play a key role in the pathogenesis of the disease. Exploration of the antibody repertoire in patients with autoimmune encephalitis has revealed that cerebral spinal fluid and serum samples regularly contain additional non-disease-defining antibodies with unresolved role and relevancy in disease pathomechanism. Therefore, our study aimed to explore the autoantibody repertoire by systematically screening recombinant human monoclonal antibodies generated from the CSF of patients with autoimmune encephalitis.

Additionally, the question of target accessibility for CNS targets in autoimmune encephalitis and other autoantibody-associated diseases is yet to be resolved. Recent studies suggest antibody-mediated barrier disruption of brain blood vessels as a possible mechanism of action. Therefore, in this work, we investigate the presence of brain vessel reactive autoantibodies by indirect immunofluorescent staining, aim to identify targets by immunoprecipitation and mass spectrometry and explore potential functional effects *in vitro* and *in vivo*.

Our findings show a wide array of target structures of non-disease-defining antibodies and confirm the presence of autoantibodies targeted against brain blood vessels. For monoclonal antibody 011-138 with reactivity against both brain blood vessels and cerebellar Purkinje cells, we identify Myosin-X as a target antigen. Further, our results suggest that the treatment of hCMEC/D3 cells in our *in vitro* blood-brain barrier model with monoclonal antibody 011-138 leads to impaired barrier properties and decreased expression of tight junction protein Occludin. Moreover, Occludin downregulation was observed after intraventricular injection and post-mortem tissue analysis.

In summary, our findings indicate that autoantibodies against brain blood vessels occur in autoimmune encephalitis patients and possibly contribute to a disruption of the blood-brain barrier. We show vessel reactive antibodies with differential patterns, identify a corresponding antigenic structure, and demonstrate the potential mechanism behind the observed barrier disruption. Hence, our findings contribute to solving the question regarding the role of non-disease-defining antibodies in the human autoantibody repertoire and provide evidence for the concept of antibody-mediated disruption of the blood-brain barrier in inflammatory neurological diseases.

Abstract (German)

Autoimmun-Enzephalitiden sind eine Gruppe seltener entzündlicher Erkrankungen des Gehirns, die mit Autoantikörpern gegen neuronale Oberflächenproteine assoziiert sind. Diese spielen eine Schlüsselrolle in der Pathogenese der Erkrankung. Untersuchungen des Antikörper-Repertoires bei Patienten mit Autoimmunenzephalitis haben ergeben, dass Liquor und Serumproben der Patienten regelhaft weitere bisher nicht krankheitsdefinierende Antikörper enthalten, deren Rolle und Relevanz im Krankheitsmechanismus noch ungeklärt sind. Unsere Studie hatte daher zum Ziel, das Autoantikörper-Repertoire durch systematisches Screening rekombinanter humaner monoklonaler Antikörper, die aus dem Liquor von Patienten mit Autoimmunenzephalitis gewonnen wurden, weiter zu erforschen.
Eine weitere bisher ungeklärte Frage ist die Zugänglichkeit von ZNS-Zielantigenen für Autoantikörper - bei Autoimmunenzephalitis und anderen mit Autoantikörpern assoziierten Krankheiten. Aktuelle Studien weisen darauf hin, dass eine durch Antikörper vermittelte Barriere-Störung der Blutgefäße im Gehirn einen möglichen Mechanismus darstellt. Daher untersuchen wir in dieser Arbeit das Vorhandensein von gehirngefäßreaktiven Autoantikörpern durch indirekte Immunfluoreszenzfärbung, ermitteln Zielstrukturen durch Immunpräzipitation und Massenspektrometrie und erforschen potenzielle funktionelle Effekte *in vitro* und *in vivo*.

Unsere Ergebnisse zeigen, dass die bisher nicht krankheitsdefinierenden Antikörper eine Vielzahl von Zielstrukturen im Gehirn binden und bestätigen das Vorhandensein von Autoantikörpern, die sich gegen Blutgefäße im Gehirn richten. Für den monoklonalen Antikörper 011-138, der eine Reaktivität gegen Blutgefäße im Gehirn sowie gegen Purkinje-Zellen im Kleinhirn zeigt, identifizieren wir Myosin-X als Zielantigen. Darüber hinaus legen unsere Ergebnisse nahe, dass die Behandlung von hCMEC/D3-Zellen in unserem *in vitro* Blut-Hirn-Schranken-Modell mit dem monoklonalen Antikörper 011-138 zu einer Beeinträchtigung der Barriere-Eigenschaften sowie zu einer verminderten Expression des Tight-Junction-Proteins Occludin führt. Darüber hinaus konnten wir eine Occludin-Downregulation nach intraventrikulärer Injektion und post-mortem Gewebeanalyse beobachten.

Zusammenfassend deuten unsere Ergebnisse darauf hin, dass Autoantikörper gegen Gehirnblutgefäße bei Patienten mit Autoimmunenzephalitis auftreten und zu einer Beeinträchtigung der Blut-Hirn-Schranke beitragen könnten. Wir zeigen gefäßreaktive Antikörper mit unterschiedlichen Mustern, identifizieren eine Zielstruktur in Hirnblutgefäßen und legen den potenziellen Mechanismus hinter der beobachteten Barriere-Störung dar. Unsere Ergebnisse tragen somit dazu bei, die Rolle der nicht krankheitsdefinierenden Antikörpern des menschlichen Autoantikörper-Repertoires zu explorieren und liefern Evidenz für das Konzept der durch Antikörper vermittelten Beeinträchtigung der Blut-Hirn-Schranke bei entzündlichen neurologischen Erkrankungen.

1 Introduction

1.1 Autoimmune encephalitis and the impact of autoantibodies in neurology

Autoimmune encephalitis is a rare but treatable disease and represents the third most common cause of encephalitis (Granerod et al., 2010). Autoimmune encephalitis typically refers to encephalitis syndromes associated with autoantibodies against surface-expressed central nervous system (CNS) targets. The discovery of autoantibodies and their respective antigens has led to the distinction of entirely new neurological disease entities as exemplified by anti-N-methyl-D-aspartate receptor (NMDAR)-encephalitis in which case pathogenic autoantibodies target NMDA receptors. Over the past decade, a multitude of novel antineuronal antibodies have emerged (Prüss, 2021) - targeting ion channels such as γ-aminobutyric acid receptors (GABAR) (Kreye et al., 2021; Petit-Pedrol et al., 2014), metabotropic glutamate receptor 5 (mGluR5) (Lancaster et al., 2011) and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (Lai et al., 2009) or crucial synaptic proteins like leucine-rich glioma-inactivated protein 1 (LGI1), and contactin-associated protein-like 2 (CASPR2) (Irani et al., 2010). Common clinical features in autoimmune encephalitis syndromes involve neuropsychiatric symptoms such as changes in behavior, cognitive deficits, and reduced level of consciousness.

Major advances in unraveling the pathophysiological mechanisms are largely due to the utilization of patient-derived recombinant monoclonal antibodies (mAbs). Unlike investigations of patient serum or cerebrospinal fluid (CSF), in studies with recombinant mAbs, pathogenic effects can be attributed to defined autoantibodies and corresponding targets (Duong and Prüss, 2023; Kreye et al., 2016; Kreye et al., 2021). Detailed studies *in vitro* and *in vivo* revealed that in NMDAR-encephalitis, which represents the most common form of autoimmune encephalitis, NMDAR mAbs promote receptor internalization and, thereby, changes in synaptic transmission (Kreye et al., 2016). Functional investigations of $GABA_AR$-encephalitis showed internalization-independent reduction of GABAergic inhibitory currents (Kreye et al., 2021; Noviello et al., 2022). This underlines the importance of investigating recombinant monoclonal antibodies to provide further insight into antibody-target interactions and to connect clinical syndromes with underlying pathomechanisms.

Autoantibodies are reshaping the clinical landscape in neurology beyond autoimmune encephalitis. One of the most prominent examples is the discovery of anti-aquaporin 4 (AQP4) antibodies in patients with inflammatory demyelinating disease who experience optic neuritis and myelitis (Jarius et al., 2010). This clinical presentation was believed to be a severe variant of multiple sclerosis (MS). However, investigations of the antibody repertoire of this distinctive patient cohort led to the discovery of anti-AQP4 antibodies and the distinction of neuromyelitis optica spectrum diseases (NMOSD) patients from patients with multiple sclerosis (MS) (Bennett et al., 2009; Jarius et al., 2010; Lennon et al., 2005). Understanding the underlying pathomechanistic differences resulted in a distinction in diagnosis and treatment. Similarly, myelin oligodendrocyte glycoprotein (MOG) was suspected to be a potential demyelination autoimmune target in MS. Later, studies investigating serum and CSF MOG-antibodies revealed that antibodies against conformationally correct MOG did not occur in MS patients but rather in patients diagnosed with acute demyelinating encephalomyelitis (ADEM) or AQP4-IgG-negative neuromyelitis optica spectrum disorder (NMOSD) patients (Brilot et al., 2009; Hamid et al., 2017), thereby identifying the MOG autoantibody-associated disease (MOGAD) as a separate entity. Potentially pathogenic autoantibodies are being discovered continuously, and the examples of NMOSD and MOGAD underline the potential impact of newly discovered antibody targets. This dynamic has led to the deliberation of potential autoantibody contribution to a multitude of further neurological diseases, including dementia (Barthel et al., 2023).

In autoimmune encephalitis, the generation and investigation of patient-derived recombinant monoclonal antibodies have revealed that the CSF and serum samples of patients are regularly comprised of further "non-disease-defining antibodies", in addition to the established disease-defining mAbs. This has been a recurrent observation in our lab and was demonstrated in a study of NMDAR-encephalitis samples in which the majority of antibodies strongly reacted with further brain epitopes (Kreye et al., 2016). Reactivity of autoimmune encephalitis patient samples to non-defined antigens has also been regularly observed in diagnostic laboratory assays. Taken together, recent developments in neurology and neuroscience have shown the impact that newly discovered antibodies may exert and thereby fuel the investigation of currently "non-disease-defining mAbs" in the antibody repertoire of autoimmune encephalitis patients. These investigations will provide insight into their potential for diagnostics, prognostics, and possible involvement in disease pathomechanism.

1.2 CNS autoimmunity and target accessibility

Although the interaction of antibody and central nervous system (CNS) target is the key feature of autoimmune encephalitis, profound knowledge of how antibodies gain access to their CNS targets is still lacking. The two generally discussed mechanisms of autoantibody entry into the CNS are a) via diffusion of IgG or b) via B-cell trafficking from the periphery. Across the different types of autoimmune encephalitis, the detected absolute antibody titers are almost always higher in serum than in CSF (Sun et al., 2020). Very high titers are suggestive of diffusion of antibodies from serum to CSF. In contrast, successful generation of patient-derived monoclonal antibodies from CSF is direct evidence for antigen-specific B-cells in the CSF. In both cases, the tightly regulated blood-brain barrier (BBB) must be overcome, and the question of how the BBB is crossed remains elusive.

BBB disruption has been demonstrated to be a key component in disease development and disease severity for inflammatory neurological diseases such as NMOSD (Tomizawa et al., 2012). In NMOSD, anti-AQP4-IgG binding to CNS AQP4 leads to complement- and antibody-dependent cellular toxicity of astrocytes (Bennett et al., 2009). Although CSF plasma cells produce anti-AQP4-antibodies, high levels of anti-AQP4-IgG in CSF are associated with high serum levels (Jarius et al., 2010). Taking this into account, a study by Shimizu et al. (2017) investigated how AQP4-IgGs penetrate the BBB. Interestingly, they discovered a monoclonal antibody against Glucose-regulated protein 78 (GRP78) in patients with NMOSD, which strongly binds to brain microvascular endothelial cells and, after repeated administration, causes extravasation of serum albumin, IgG, and fibrinogen in mouse brains. Therefore, this study demonstrates that a non-disease-defining mAb potentially propagates the entry of AQP4-antibodies and may contribute to NMO attacks. Furthermore, the same group investigated anti-GRP78-antibodies in patients with Lambert-Eaton myasthenic syndrome (LEMS) with and without paraneoplastic cerebellar degeneration (LEMS-PCD) (Shimizu et al., 2019). For LEMS-PCD, the presumed pathomechanism is based on the entry of anti-voltage-gated calcium channel- (VGCC) antibodies, previously present in the serum, to the CNS and subsequent antibody-mediated cerebellar symptoms. This study hypothesized that the resulting differential phenotype derives from BBB dysfunction facilitated by anti-GRP78-antibodies. Indeed, their ex-

periments show that anti-GRP78-antibodies in patients with LEMS-PCD disrupt endothelial barrier functions, whereas IgG in patients with only LEMS did not alter endothelial cell functions. This led us to the question of whether currently non-disease-defining antibodies in patients with autoimmune encephalitis also bear the potential to influence barrier function and contribute to disease mechanisms.

1.3. Research question

To investigate the role of non-disease-defining antibodies in patients with autoimmune encephalitis, we decided to perform a screening of monoclonal patient antibodies and thereby identify reactive antibodies. We then aimed to explore the potential influence of tissue reactive non-disease-defining antibodies on barrier properties of brain blood vessel. Therefore, we worked with the following leading hypotheses:

I. Recombinant monoclonal antibodies targeting brain blood vessels can be found in the autoantibody repertoire of autoimmune encephalitis patients.
II. Antibodies bind to specific detectable antigenic targets on brain blood vessels.
III. Blood-vessel reactive antibodies exert specific functional effects.

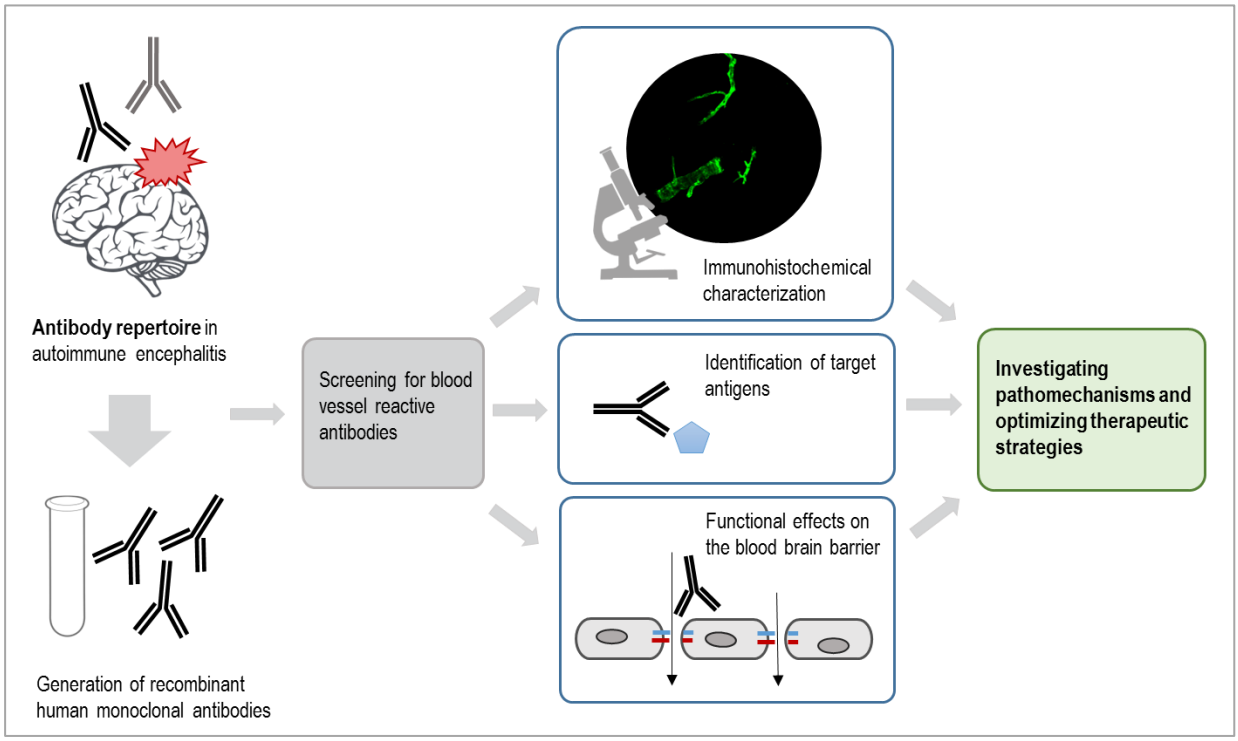

Figure 1: Graphical abstract. Our study aims to investigate the antibody repertoire of patients with autoimmune encephalitis. We use recombinant human monoclonal antibodies to screen the repertoire for brain blood vessel reactive antibodies with specific targets and functions. (Own representation: Lucie Y. Li)

2 Material and Methods

2.1 Key Resources

2.1.1 Commercial antibodies

Table 1: Commercial Antibodies

Antibody	Clonality	Host	Catalog Number	Manufacturer
Anti-CD31	mcl	Rat	#553708	BD Biosciences
Anti-CD34	mcl	Mouse	#ARG52756	Arigo Biolaboratories
Anti-Claudin5	pcl	Rabbit	#34-1600	Thermo Fisher Scientific
Anti-Collagen IV	pcl	Rabbit	#ab6586	Abcam
Anti-Myosin X (C-1)	mcl	Mouse	#sc-166720	Santa Cruz Biotechnology
Anti-Smooth muscle actin	mcl	Mouse	#M0851	Agilent Dako
Anti-Occludin	pcl	Rabbit	#71-1500	Thermo Fisher Scientific
Anti-Vascular endothelial-cadherin	mcl	Rabbit	#2500	Cell Signaling
Anti-Zonula occludens-1	mcl	Mouse	#33-9100	Thermo Fisher Scientific
Alexa Fluor 488 anti-hIgG (H+L)	pcl	Goat	#109-545-003	Jackson ImmunoResearch
Alexa Fluor 594 anti-hIgG (H+L)	pcl	Goat	#109-585-003	Jackson ImmunoResearch
Alexa Fluor 594 anti-rabbit IgG (H+L)	pcl	Goat	#A-11037	Thermo Fisher Scientific
Oregon Green 488 anti-rabbit IgG (H+L)	pcl	Goat	#O-11038	Thermo Fisher Scientific
Alexa Fluor 488 anti-rat IgG (H+L)	pcl	Goat	#ab150157	Abcam
Alexa Fluor 594 anti-mouse IgG (H+L)	pcl	Goat	#A-11032	Thermo Fisher Scientific
Oregon Green 488 anti-Mouse IgG (H+L)	pcl	Goat	#O-6380	Thermo Fisher Scientific
Anti-Glyceraldehyde-3-Phosphate Dehydrogenase	mcl	Mouse	#MAB374	Millipore
Anti-alpha Tubulin	Mcl	Mouse	#T9026	Sigma Aldrich
Peroxidase anti-hIgG (H+L)	pcl	Goat	#PI-3000-1	Vector Laboratories
Peroxidase anti-mouse IgG (H+L)	pcl	Horse	# PI-2000-1	Vector Laboratories

(Own representation: Lucie Y. Li)

2.1.2 Tables of chemicals and materials

Table 2: Chemicals

Chemical	**Manufacturer**	**Identifier**
Acrylamide	Roth, Karlsruhe, Germany	#3030.1
Ammonium persulfate	Sigma-Aldrich, St. Louis, USA	#A3678
Tetramethyl ethylenediamine (TEMED)	Roth, Karlsruhe, Germany	#2367.3
Tris(hydroxymethyl)aminomethane	Roth, Karlsruhe, Germany	#4855.2
Glycine	Roth, Karlsruhe, Germany	#3908.2
Methanol	Roth, Karlsruhe, Germany	#7342.1
Nonfat dried milk powder	AppliChem, Darmstadt, Germany	#A0830.5000
Tween 20	Roth, Karlsruhe, Deutschland	#9127.1
Urea	Sigma-Aldrich, St. Louis, USA	#U1250
Isopropanol	Merck-Millipore, Darmstadt, Germany	8.187.661.000
Chemiluminescence detection reagent	Sigma-Aldrich, St. Louis, USA	RPN2106V1 RPN2106V2
Developer G153	AGFA, Mortsel, Belgium	G152
Rapid fixer G354	AGFA, Mortsel, Belgium	G354
Sodium dodecyl sulfate (SDS)	Serva, Boehringer Ingelheim, Ingelheim am Rhein, Germany	#20783.01
2-mercaptoethanol	Serva, Boehringer Ingelheim, Ingelheim am Rhein, Germany	#28625
Bromophenol blue	Sigma-Aldrich, St. Louis, USA	B8026
Dithiothreitol (DTT)	Roth, Karlsruhe, Germany	#6908
PageRuler Prestained Protein Ladder	ThermoFisher Scientific, Waltham, USA	#26616
Ponceau S	Roth, Karlsruhe, Germany	#5938.1
Copper sulfate	Roth, Karlsruhe, Germany	#P025.1
BCA Protein Assay Solution A	Thermo Fisher Scientific, Waltham, USA	#23222
BCA Protein Assay Solution B	Thermo Fisher Scientific, Waltham, USA	#23224
Protein G Dynabeads	ThermoFisher Scientific, Dreieich, Germany	#10007D
2-Methylbutan	Roth, Karlsruhe, Germany	#3926.1
Bovines Serum Albumin Fraktion V (BSA)	Roth, Karlsruhe, Germany	#T844.4
Sodium azide NaN3	Serva, Boehringer Ingelheim	#30175
Normal Goat Serum (NGS)	Biotrend, Köln, Germany	#88-NG225
Immu-Mount	Epredia, Thermo-Fisher Scientific, Waltham, Germany	9990402
4',6-diamidino-2-phenylindole (DAPI)	Sigma-Aldrich, St. Louis, USA	#D9542
Phenylmethylsulfonylfluoride (PMSF)	Roth, Karlsruhe, Germany	#6367.4
Protease inhibitor	Sigma-Aldrich, St. Louis, USA	#P8340
Triton X-100	Roth, Karlsruhe, Germany	#3051.2
Dextran 70kDa	Roth, Karlsruhe, Germany	#9228.2

Hanks' Balanced Salts Solution (HBSS)	Sigma-Aldrich, St. Louis, USA	#H6648
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich, St. Louis, USA	#H3375
Collagen-I	Sigma-Aldrich, St. Louis, USA	#C7661
Matrigel Matrix Coating	Corning, New York, USA	#356234
Cellovations Microvascular Endothelial cell growth medium Kit enhanced	PELOBiotech, Martinsried, Germany	#PB-MH-100-4099
EBM-2 Endothelial basal Medium	Lonza, Basel, Switzerland	#CC-3156
Fetal bovine serum	Lonza, Basel, Switzerland	#CC-4147
Hydrocortisone	Lonza, Basel, Switzerland	#CC-4147
Ascorbic acid	Lonza, Basel, Switzerland	#CC-4147
Fibroblastic growth factor (FGF)	Lonza, Basel, Switzerland	#CC-4147
Penicillin/Streptomycin	Merck-Millipore, Darmstadt, Germany	#A2212
Chemically defined lipid concentrate	Thermo Fisher Scientific, Waltham, USA	# 11905-031
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, St. Louis, USA	200-449-4
Trypsin	Sigma-Aldrich, St. Louis, USA	T4174
Piperazine-N,N′-bis(2-ethanesulfonic acid)(PIPES)	Sigma-Aldrich, St. Louis, USA	#P6757
Magnesium chloride (MgCl2)	Roth, Karlsruhe, Germany	#2189.2
Sucrose	Roth, Karlsruhe, Germany	#4661.1
Potassium chloride (KCl)	Merck-Millipore, Darmstadt, Germany	#4930
Ethylene glycol bis(2-aminoethyl)tetra acetic acid (EGTA)	Sigma-Aldrich, St. Louis, USA	E-3889
Sodium chloride (NaCl)	Roth, Karlsruhe, Germany	#3957.1
Sodium dihydrogen phosphate (Na2HPO4)	Roth, Karlsruhe, Germany	#P030.1
Calcium chloride (CaCl2)	Roth, Karlsruhe, Germany	#5239.1
Fluorescein-sodium salt	Sigma-Aldrich, St. Louis, USA	#F6377
Ringer's solution	Fresenius Kabi, Bad Homburg, Germany	B231553
Polyethyleneimine (PEI) branched	Sigma-Aldrich, St. Louis, USA	408727
Paraformaldehyde (PFA)	Roth, Karlsruhe, Germany	#0335.3

(Own representation Lucie Y. Li)

Table 3: Apparatuses and Materials

Apparatus/Material	**Manufacturer**	**Identifier**
Amersham Hybond ECL Nitrocellulose Membrane	GE Healthcare, Chicago, USA	9004-70-0
Chromatography paper Whatman 3MM	GE Healthcare, Chicago, USA	#3030-861
Glass plates	Bio Rad, Hercules, USA	N/A
Casting chamber	Bio Rad, Hercules, USA	N/A
10- and 15-Well-Combs	Bio Rad, Hercules, USA	N/A
Power Pack HC	Bio Rad, Hercules, USA	043BR
Transblot SD	Bio Rad, Hercules, USA	#1703940
Electrophoresis Cell (Mini-PROTEAN II)	Bio Rad, Hercules, USA	N/A
Hypercassette 24x30cm	Amersham Bioscience, Little Chalfont, United Kingdom	RPN11643
Amersham Hyperfilm ECL	GE Healthcare, Chicago, USA	#28-9068
Fluorescence microscope DMLB	Leica Microsystems, Wetzlar, Germany	DMLB
Medical film processor Curix 60	AGFA, Mortsel, Belgium	Curix 60
DynaMag™-2 Magnet	Thermo Fisher Scientific, Waltham, USA	#12321D
Superfrost Plus adhesion microscope slides	Epredia, Thermo-Fisher Scientific, Waltham, Germany	J1800AMNZ
Confocal Laser microscope Leica SL	Leica Microsystems, Wetzlar, Germany	SL
Sonicator Sonoplus	Bandelin electronic, Berlin, Germany	GM70/ UW 70
Homogenizer with PVC	Heidolph Instruments, Schwabach, Germany	RZR2021
Centrifuge	Eppendorf, Hamburg, Germany	5804 R
Centrifuge	Eppendorf, Hamburg, Germany	5417C
Nylon Net filters 100µM NY1H	Merck Millipore, Darmstadt, Germany	NYH1H04700
Nylon Net filters 30µM NY30	Merck Millipore, Darmstadt, Germany	NYH1H04700
Photometer reader HT2	Anthos Mikrosysteme, Krefeld, Germany	12 500
Trans-well inserts 0.4µm pore size	Corning, New York, USA	#3401
Epithelial-Volt/Ohm-Meter EVOM3	WPI, Sarasota, Fl, USA	EVOM3
Chopstick electrodes STX-PLUS	WPI, Sarasota, Fl, USA	STX-PLUS
Human cerebral microvascular endothelial cells hCMEC/D3	CELLutions Biosystems inc., Burlington, Ontario, Canada	#CLU512
Cryostat JUNG frigocut 2800N	Leica Microsystems, Wetzlar, Germany	2800
Cryostat Ag protect	Leica Microsystems, Wetzlar, Germany	CM 1860

(Own representation: Lucie Y. Li)

2.2 Key Methods

Table 4: Synopsis of methods

	Hypothesis	Method in vitro	Methods in and ex vivo
SCREENING	I	Immunohistochemical characterization • Screening on murine brain section and purified mouse brain vessels • Systematic characterization • Identification of candidates for further investigation	
FURTHER CHARACTERIZATION			Intraventricular injection and binding in murine brain • Pattern recapitulation
	II	Identification of target antigen • Co-Staining • Immunoprecipitation • Mass Spectrometry • Cell-based Assay	Recognition of target antigen • Co-Staining
	III	Barrier function • Transendothelial electrical resistance • Permeability	
		Cell function • Protein expression by Western Blot • mRNA expression (Real-Time-PCR)	Cell function • Protein expression by Western Blot

Overview of the in vitro and in vivo methods used to investigate each hypothesis. (Own representation: Lucie Y. Li)

2.2.1 Immunohistochemical characterization

Recombinant human monoclonal antibodies were screened using indirect immunofluorescent staining on murine brain sections (for a detailed description please refer to the publication Li et al. 2023).
Antibody signals were screened and captured using a Leica DMLB epifluorescence microscope. The signal intensities were assigned to one of five possible intensities.

Table 5: Scoring table

Score	Score description
0	No reactivity/equal to negative control
1	Stronger intensity than negative control, but rather weak; possibly background/unspecific binding
>1	Stronger than 1, less than 2
2	Clearly positive and specific tissue reactivity, has anatomical correlate
>2	Stronger than 2, less than 3
3	Very intense and highly specific binding pattern on tissue, comparable to commercial antibody

Semi-quantitative evaluation of reactivity of monoclonal recombinant antibodies to murine brain structures. (Own representation: Lucie Y. Li)

2.2.2 Methods to investigate the effects of blood vessel reactive monoclonal antibodies on barrier function

The blood-brain barrier can be recapitulated *in vitro* using different models. To investigate the influence of vessel reactive monoclonal antibodies on the barrier function, we utilized immortalized human cerebral microvascular endothelial cells (hCMEC/D3) in an *in vitro* BBB model with trans-well experiments.

Measurement of TEER (transendothelial electrical resistance)

Transendothelial electrical resistance (TEER) is a measure to evaluate the ion resistance of an endothelial monolayer. A stable TEER is held up by an intact endothelial layer, whereas a decrease in TEER is associated with impacted integrity of the barrier.

For our experimental set-up (Figure 2), trans-well inserts (0.4µm pore size, Corning) were coated with a reconstituted basement membrane (Matrigel®, Corning), and endothelial cells were seeded at a density of 4 x 10^4 in Cellovations™ Microvascular Endothelial Cell Growth Medium (ECM) Kit enhanced (PELOBiotech, Martinsried, Germany, #PB-MH-100-4099) supplemented with 5% fetal calf serum (FCS) and additional supplements as well as growth factors according to manufacturer's instructions. Coated trans-well inserts without cells served as blank filters. The trans-well inserts were situated in a 12-well cell culture plate filled with ECM to provide medium for the cell layer from abluminal. The cells were grown to achieve a confluent monolayer. After five days, the medium was changed to ECM depleted of growth factors and with a reduced concentration of 0.5% FCS for differentiation. Next, for an additional 24 or 48h, endothelial cells were treated with patient antibodies at a 5µg/ml or 10µg/ml concentration or left untreated. TEER measurements across each trans-well were conducted using chopstick electrodes (STX-PLUS, WPI, Sarasota, Fl, USA) and an Epithelial-Volt/Ohm-Meter (EVOM3, WPI). Values were measured in triplicate and the TEER values of blank filters were subtracted from the measured values. In BBB models with immortalized endothelial cells, contributing components to the TEER involve R_{para} (resistance associated with paracellular ion transport) and R_m (resistance associated with ion transport across the cell membrane), where tight-junctions effectively block paracellular transport in an intact barrier (DeStefano et al., 2018).

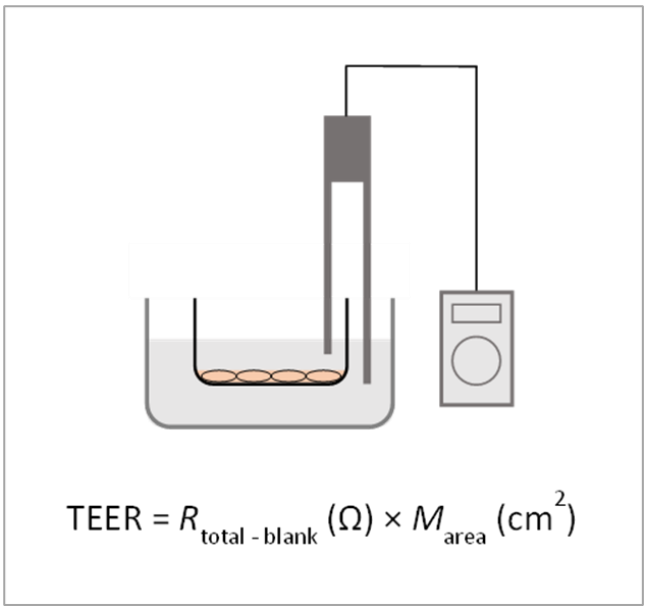

Figure 2: Trans-well experiments. HCMEC/D3 cells were seeded into a Matrigel coated trans-well insert. The insert was placed into a 12-well plate and resistance (R in Ω) was measured across the endothelial monolayer using chopstix electrodes with a Voltohmmeter. The transendothelial resistance (TEER) was calculated by subtracting the resistance of the blank trans-well insert and normalized to the trans-well insert area (M in cm^2). (Own representation: Lucie Y. Li)

Transport experiments across the *in vitro* Blood-Brain Barrier

To further investigate the possible implication of monoclonal antibodies on the barrier functions, we measured the permeability of hCMEC/D3 cells in the trans-well experimental set-up. Downregulation of tight junctions leads to disruption of the BBB and increased paracellular transport and, thereby, permeability.

HCMEC/D3 were grown as described above and assessed for paracellular transport of fluorescein-sodium (Sigma). HCMEC/D3-covered trans-well inserts, as well as blank filters, were placed into a 12-well plate filled with 1,5ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered Ringer´s solution (pH 7.4) (lower/receiver compartment). 500µl of Ringer-HEPES containing 1µM fluorescein-sodium was added into the cavity (upper compartment) of each trans-well. The plates were kept at 37°C and 5% CO_2 during the experiment. The trans-well insert was transferred to a fresh Ringer-HEPES containing receiver compartment every 20 minutes to allow the passing of fluorescein-sodium from the donor solution (upper compartment) to the receiver compartment through the monolayer of endothelial cells. Aliquots were taken from the donor solution at the beginning and from the receiver compartment at the end of the experiment. To assess the amount of fluorescein-sodium, the fluorescence of the collected samples was subsequently measured at 485/535nm (excitation/emission) wavelengths using a microplate reader (Thermo Scientific). All conditions were measured at least in triplets. The permeability coefficients were calculated according to the method of van Bree et al. (1988). The clearance through the monolayer for each condition was calculated compared to blank filters. The slope of the clearance fitted by linear regression was then used to determine the Pe (permeability coefficient). The results are shown as Pe in %of control (untreated cells).

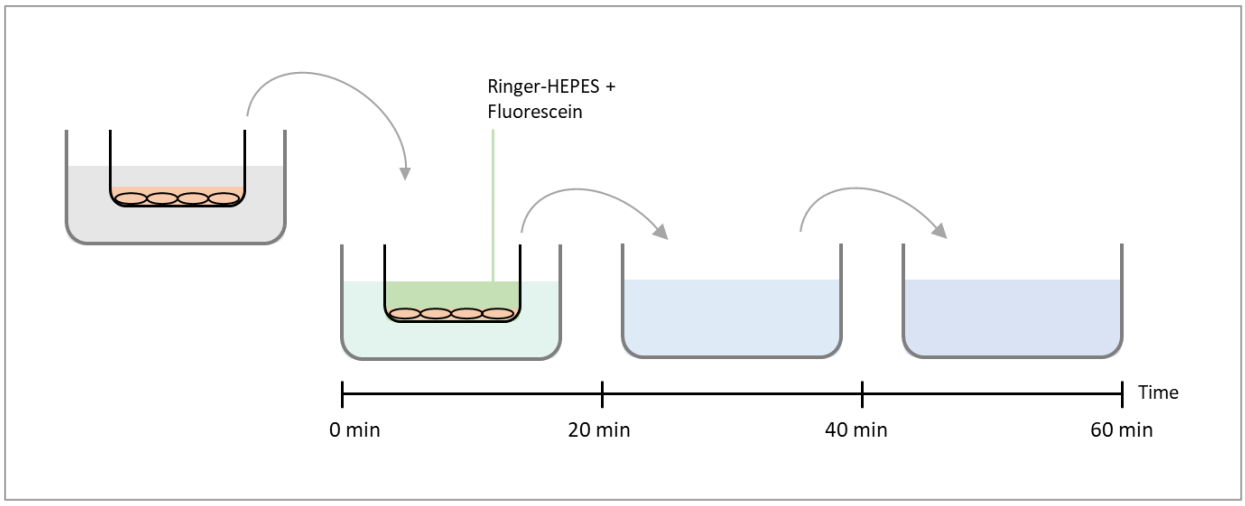

Figure 3: Experimental set-up of permeability measurements. After the formation of the monolayer, trans -well medium was changed into Ringer-HEPES containing 1µM of fluorescein-sodium. The trans-well was placed into a Ringer-HEPES containing 12-well plate for 20min before transferring to the next well. (Own representation: Lucie Y. Li)

3 Results

For a detailed report of the results of this study, please refer to our publication (Li et al., 2023). The following provides supplementary information and figures not included in the corresponding publication.

3.1 Autoimmunity to brain blood vessels and Purkinje cells

Screening for reactivity against brain antigen structures was conducted on all available human recombinant monoclonal antibodies from six patients by indirect immunofluorescence staining on murine brain sections. This resulted in a screening of 147 CSF-derived human recombinant monoclonal antibodies. Table 6 provides an overview of the six patients and their diagnosis, as well as their gender and age of diagnosis.

Table 6: Patients

Patient	Diagnosis	Age (at diagnosis)	Gender	Publication
003	NMDAR-Encephalitis	18	f	Kreye et al., 2016
011	NMDAR-Encephalitis	34	f	Kreye et al., 2016
080	NMDAR-Encephalitis	22	f	N/A
113	$GABA_AR$-Encephalitis	8	f	Kreye et al., 2021; Nikolaus et al., 2018
136	mGluR5-Encephalitis	15	m	Schnell et al., 2023
187	Autoimmune Encephalitis	66	m	Van Hoof et al., in preparation

Recombinant human monoclonal antibodies were isolated from six patients with autoimmune encephalitis. If available, patient details can be obtained from the stated publications. If not available, patients are part of ongoing investigations. (Own representation: Lucie Y. Li)

For the reactive antibodies, the staining signal of each antibody was categorized by intensity (method section table 5) and localization. The localization was assigned to a defined brain region (e.g., hippocampus, cerebellum), cell type (e.g., astrocyte), or, when

possible, subcellular region. Figures 4 and 5 provide a comprehensive overview of signals detected in each patient's antibody repertoire. Figure 4 displays the repertoires of patients with diagnosed NMDAR-encephalitis, and Figure 5 of those with other types of autoimmune encephalitis. The screening and categorization allowed for the exploration of pattern distribution in each patient and across patients. For example, patient 011 (Figure 4B) shows a remarkable clustering of antibodies with reactivity against astrocytes, of which for mAb 011-116, the target antigen could later be identified by our group as glial fibrillary acidic protein (GFAP), an astrocytic intermediate filament protein. Interestingly, cerebellum reactive antibodies had a high frequency throughout all patient repertoires. For example, 23/28 reactive mAbs from patient 080 showed reactivity against cerebellum structures. Another brain region frequently targeted in this cohort of mAbs is the hippocampus. As both represent regions of characteristic functions, further investigations of these subgroups would be highly interesting.

This study further focused on the mAbs with reactivity against brain blood vessels. The frequency of vessel reactive antibodies in this cohort was 10.2% (15/147) for any reactivity and 4.1% (6/147) for strongly reactive antibodies (intensity score ≥2). Looking closely at the binding characteristics of the vessel reactive antibodies, we were able to distinguish between antibodies binding to vessels of all sizes up until the capillary level, as well as antibodies binding preferably to mid- to large-size vessels (Li et al., 2023). Among the strongly reactive antibodies, mAbs 113-111, 113-126 and 011-138 showed simultaneous reactivity in murine cerebellum. In the case of mAb 113-111 and 113-126 the reactivity was against cerebellar cortex molecular cell layer. Whereas antibody 011-138 reacted strongly (intensity score 2) to cerebellar Purkinje cells in addition to strong reactivity to brain blood vessels. Figure 6A additionally depicts a wide-field microscope image of a 011-138 stained unfixed murine brain section. As exemplarily shown in Figure 6B reactive antibodies were co-stained with blood vessel markers Collagen IV and CD31 to further confirm vessels as the targeted structure. The overlap in signal with smooth muscle actin (SMA) staining shows that 011-138 binds preferably to mid- to large-size vessels. The binding of mAb 011-138 to both brain blood vessels and a defined group of neuronal cells (cerebellar Purkinje cells) made this particular antibody a highly interesting candidate for target identification and functional testing.

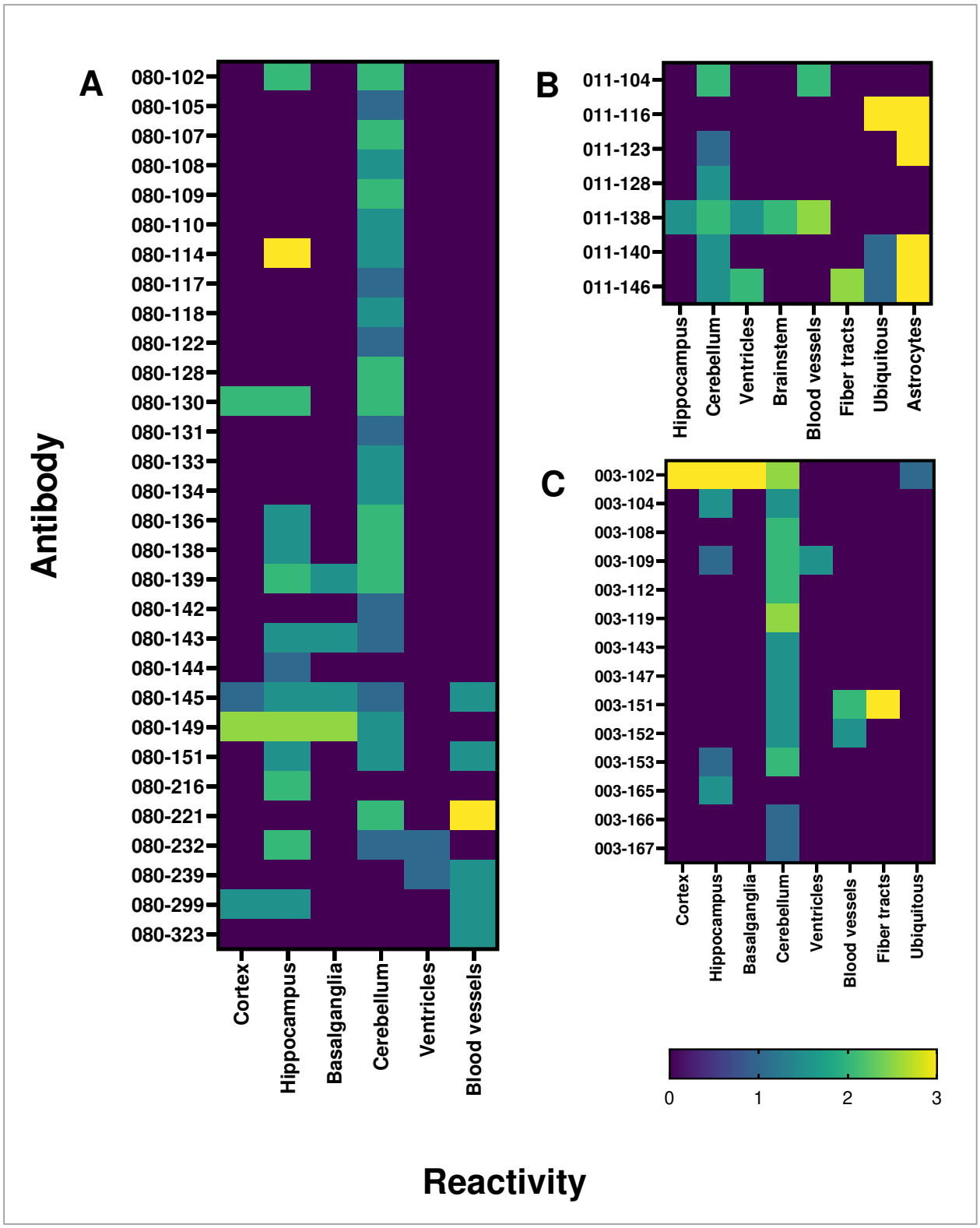

Figure 4**:** Immunofluorescence reactivity overview (1). Heatmap depicting the signal intensity of reactive human recombinant monoclonal antibodies in defined signal localizations. Each row represents a monoclonal antibody, and each column corresponds to a defined signal localization. The color represents signal intensity. Antibodies corresponding to **A)** Patient 080, **B)** Patient 011 and **C)** Patient 003 diagnosed with NMDAR-encephalitis. (Own representation: Lucie Y. Li)

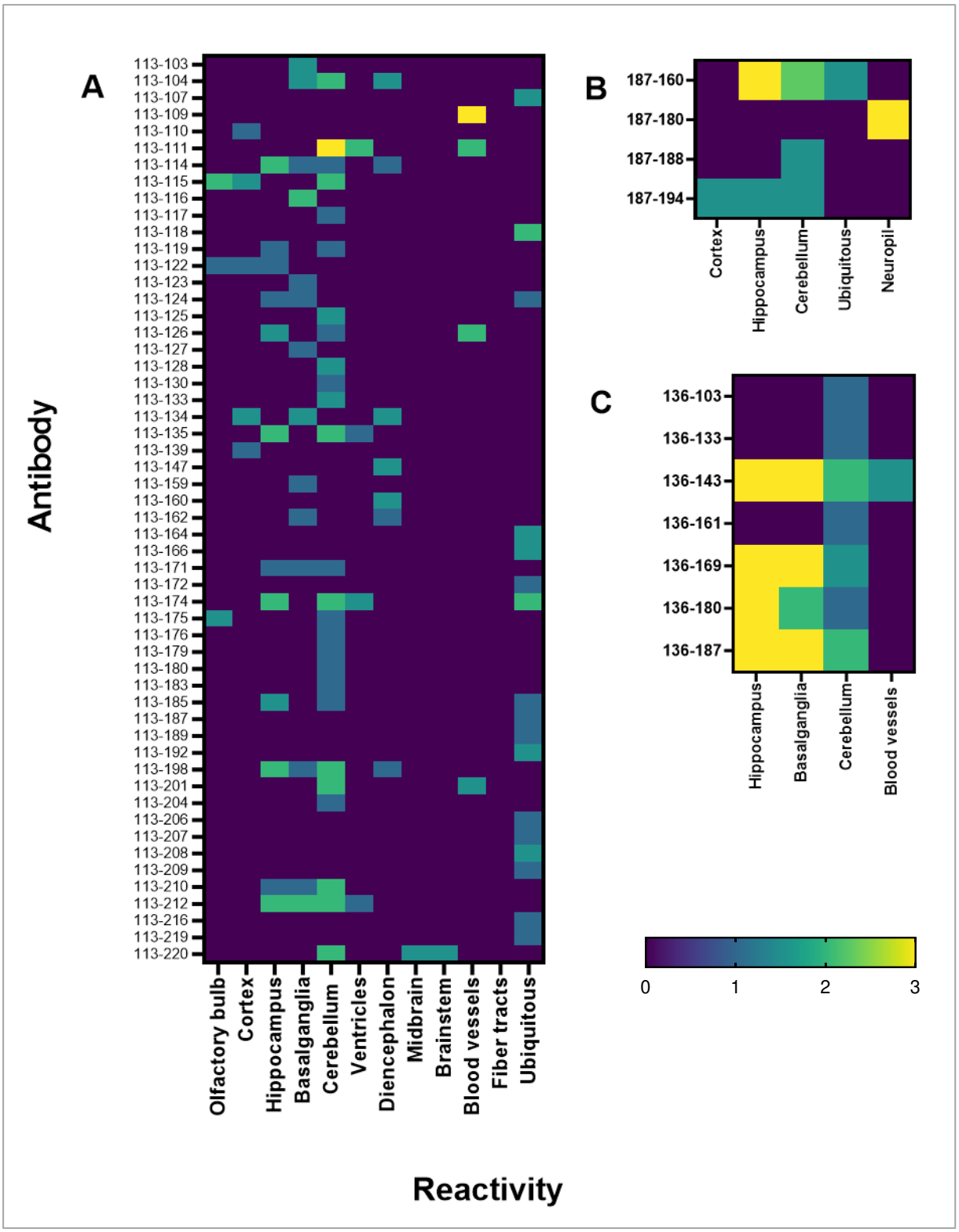

Figure 5: Immunofluorescence reactivity overview (2). Heatmap depicting the signal intensity of reactive human recombinant monoclonal antibodies in defined signal localizations. Each row represents a monoclonal antibody, and each column corresponds to a defined signal localization. The color represents signal intensity. Antibodies corresponding to A) Patient 113, B) Patient 187 and C) Patient 136 diagnosed with autoimmune encephalitis. (Own representation: Lucie Y. Li)

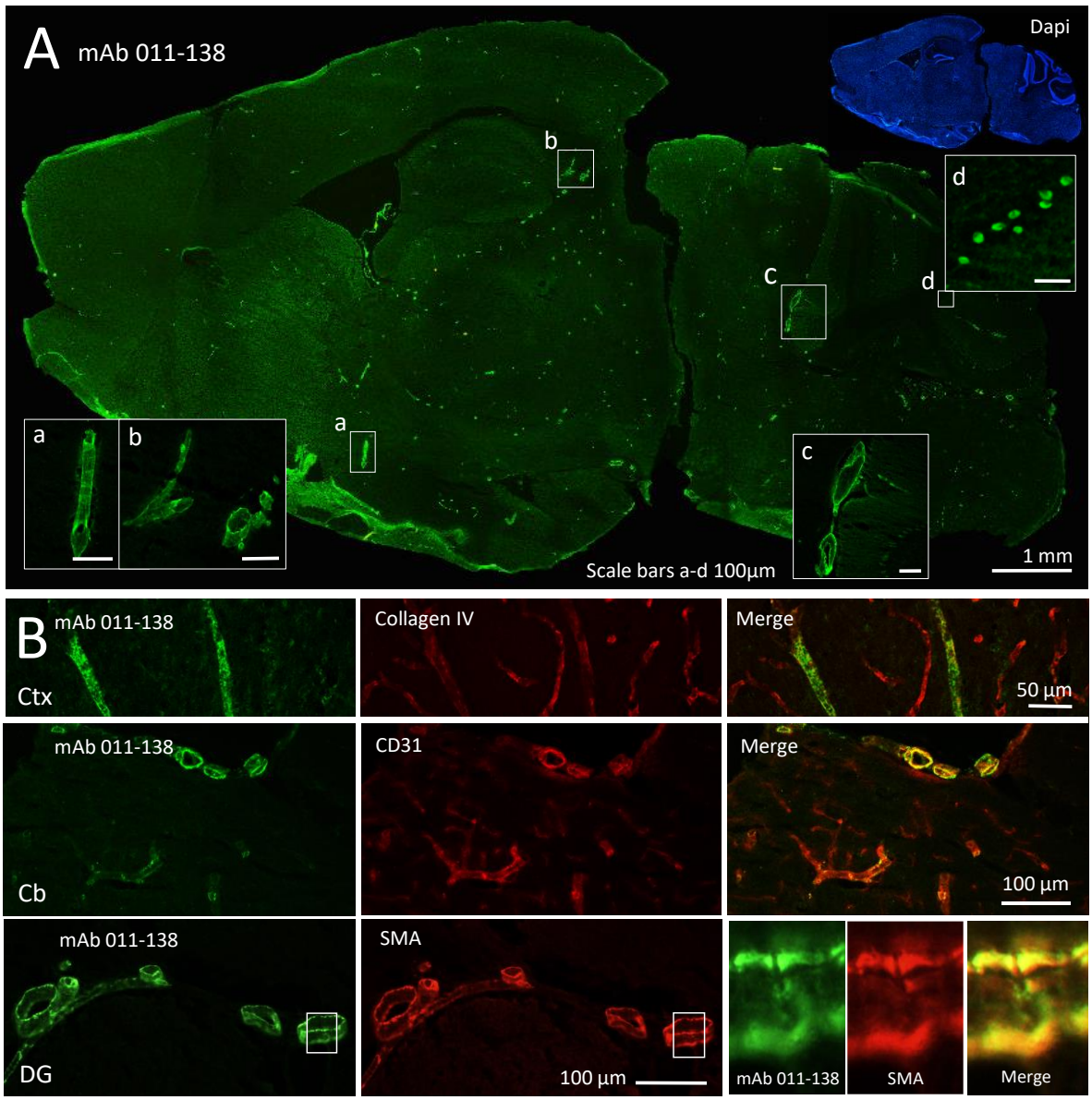

Figure 6: MAb 011-138 binds to brain blood vessels and Purkinje cells. Unfixed sagittal murine brain sections were incubated with 5µg/ml of human recombinant monoclonal antibody (mAb) 011-138 and secondary anti-human IgG. **A)** Mab 011-138 shows strong reactivity to mid-size and large-size blood vessels across brain regions (insets a, b, and c). Additional binding to cerebellar Purkinje cells with a marked somatic pattern was observed (see inset d). **B**) Double stainings with commercial antibodies against Collagen IV (upper panel) and CD31 (middle panel) confirm the immunoreactivity of mAb 011-138 to blood vessels. Signal-overlap of human IgG and SMA (smooth muscle actin) is shown in a brain sector adjacent to the dentate gyrus (DG) (lower panel). The human IgG signal was mainly found within the SMA-positive muscle layer of vessels. **Ctx** Cortex, **Cb** Cerebellum, **DG** Dentate Gyrus. (Modified from Li et al., 2023)

3.2 Functional effects of mAb 011-138

To address whether brain blood vessel reactive antibodies exert specific functional effects, we conducted *in vitro* experiments and an *ex vivo* expression analysis of barrier proteins. As described in our publication (Li et al. 2023), human cerebral microvascular endothelial cells (hCMEC/D3) mimic the phenotype of the *in vivo* BBB and were utilized in our experimental set-up to assess the effect of blood vessel reactive antibodies on barrier properties.

Detailed results from the transendothelial electrical resistance (TEER) experiments and the expression analysis of barrier markers can be obtained from the publication (Li et al. 2023). In summary, we were able to show a significant reduction of TEER when hCMEC/D3 cells were treated with 011-138 for 48h compared to untreated cells and control antibody mGo53 treated cells. As a potential cause for this disruption in barrier function, we identified a significant decrease in Occludin expression in mAb 011-138 treated endothelial cells. This reduction in expression of Occludin was also observable in *ex vivo* Western blot experiments with the brain tissue of animals intrathecally infused with mAb 011-138 compared to the control antibody.

Permeability represents another important property of barrier function and was investigated by assessing the clearance of fluorescein-sodium across an hCMEC/D3 monolayer in a trans-well system to obtain the Pe (Permeability coefficient). Figure 7 shows the results as permeability in %of control, where the mean permeability of an untreated monolayer serves as a control. Overall, measurements across several conditions showed mixed results. Despite a significant reduction of TEER in the 48h condition, permeability remained substantially unchanged after treatment with mAb 011-138 for 48h in the 5µg/ml as well as the 10mg/ml condition. Similarly, multiple replicates of the 24h 5µg/ml condition did not cause a significant change in permeability. Interestingly, the high-dose condition and exposure for 24h led to changes in permeability compared to untreated cells. However, for this result, the standard deviation values are high and the increase in permeability was not significant.

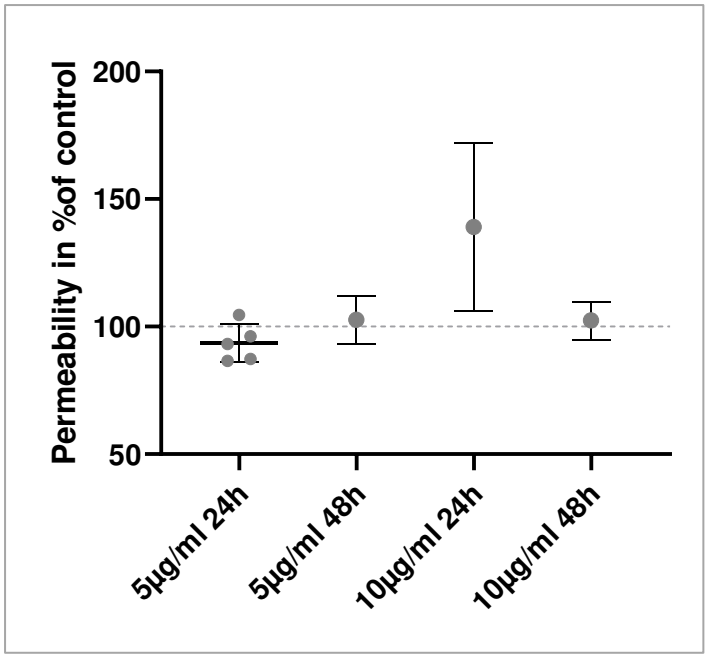

Figure 7: Permeability in %of control after treatment with mAb 011-138. The clearance of fluorescein-sodium across an endothelial cell monolayer in a trans-well set-up was obtained to calculate the Permeability coefficients of cell layers under different treatment conditions. Human cerebral microvascular endothelial cells (hCMEC/D3) were treated for 24 or 48h with 5µg/ml or 10µg/ml of monoclonal antibody 011-138. Values are shown as percent of control (control = 100%, indicated on the Y-axis). For the 5µg/ml 24h condition, five replicates were performed, each dot representing the mean of n = 3 measurements. The mean and SD of all values are shown. For all other conditions, the mean of n = 3 measurements is shown. (Own representation: Lucie Y. Li)

4 Discussion

The following section is meant to complement the discussion in the publication (Li et al., 2023) according to the hypotheses stated in the introduction.

Hypothesis I: Recombinant monoclonal antibodies targeting brain blood vessels can be found in the autoantibody repertoire of autoimmune encephalitis patients.

Our systematic, semi-quantitative analysis of each monoclonal binding pattern provides an overview of individual patient repertoires, including disease-defining and non-disease-defining mAbs. Through this analysis, antibodies with reactivity against a broad spectrum of brain antigens, including brain blood vessels, can be found. Through our characterization we observed distinct clusters within one repertoire but also subgroups across different patients. This served as a starting point for a more in-depth exploration.

As proposed, recombinant monoclonal antibodies targeting brain blood vessels were identified in this cohort of patients with autoimmune encephalitis. The frequency of strongly vessel reactive antibodies was 4.1 % (6/147). Other subgroups, such as cerebellum or hippocampus reactive antibodies, comprise larger portions of the repertoire. Since each recombinant monoclonal antibody was generated from a patient B-cell, the frequency in this context can be viewed as the prevalence of specific B-cells in the patient CSF. However, a high prevalence does not indicate a high relevance. For comparison, the frequency of NR1-subunit reactive antibodies in the repertoire of NMDAR-encephalitis patients was 4.2% (2/47), 10.5% (6/57) and 7.2% (1/14) for patients 003, 007, and 008 respectively (Kreye et al., 2016). Moreover, Kreye et al. (2016) provide evidence that one pathogenic antibody clone is sufficient to cause disease pathogenesis. This rather demonstrates the importance of individual, structurally relevant targets. It would therefore be to highly interesting to investigate the frequency of these relevant targets across different patient repertoires. A more accurate estimate of the frequency of vessel reactive antibodies and antibodies against specific vessel target structures in patients with autoimmune encephalitis will have to be assessed with a larger sample size.

Through the gateway of our screening, vessel reactive antibodies underwent *in vitro* and *in vivo* investigation to unveil Myosin-X as one target antigen of vessel reactive mAb 011-

138 (Li et al., 2023). Our work underlines, that immunofluorescent staining patterns can provide initial evidence for possible target antigens. Certain patterns and pattern combinations provide evidence pointing towards certain antigens. Such as in the case of mAb 011-138 the combination of reactivity to vessels and cerebellar Purkinje cells narrowed down the number of plausible candidates from immunoprecipitation mass spectrometry analysis. We used a semi-quantitative scoring system to efficiently categorize a larger number of samples with the main goal of identifying candidates for in-depth characterizations. Nonetheless, this is also a limitation of this study. The score may vary depending on the investigator; only relative differences between the signal intensities can be captured, and antibodies with lower signal intensity in our assay may be prematurely excluded.

Hypothesis II: Antibodies bind to specific detectable antigenic targets on brain blood vessels.

As discussed in our publication (Li et al., 2023), vessel reactive antibodies binding either mid- to large-sized vessels or vessels of all calibers could be identified in patients with autoimmune encephalitis. Naturally, the binding pattern will result from the specific antigen and its distribution within the vasculature. Therefore, immunofluorescent staining patterns and co-staining with commercial antibodies of known targets provide important evidence in the search for target epitopes. In our study, the combinatory approach with data from immunofluorescent staining and mass spectrometry data led to the identification of Myosin-X as a target of mAb 011-138. Monoclonal 011-138 served as a capture antibody in immunoprecipitation with subsequent mass spectrometry analysis, and target confirmation was performed using Myosin-X transfected HEK-cells (Li et al., 2023). Thereby, as proposed, we identified a specific antigenic target of a currently non-disease-defining antibody on brain blood vessels.

Antibody 011-138 was generated from an antibody-secreting cell from patient CSF, indicating the presence of anti-Myosin-X antibody-producing B-cells in patient CSF. Staining of the patient CSF on unfixed murine brain sections resulted in the same characteristic staining pattern as monoclonal antibody 011-138 (Li et al. 2023), further supporting the presence of anti-Myosin-X producing B-cells in the CSF. Taken together, mAb 011-138 has most likely gained access to its CNS target by B-cell trafficking into the CNS.

The question arises as to whether and how CSF anti-Myosin-X antibody 011-138 reaches the vessel structures. To address the first question, our results from *in vivo* intraventricular injection provide evidence that the presence of mAb 011-138 in the CSF does allow the antibody to reach its target structures in murine brain vessels. Firstly, the vessel staining pattern observed on unfixed brain sections was recapitulated in our *ex vivo* post-mortem staining analysis of the infused mice. Secondly, the *ex vivo* analysis of the murine brain tissue after intraventricular injection with antibody 011-138 revealed a decrease in expression of junction protein Occludin, resembling our previous *in vitro* findings on the cellular level. To address the question of how 011-138 reaches the vessel structure, one possible pathway for the intraventricularly injected antibody could be through the blood-CSF barrier at the Choroid plexus. The blood-CSF barrier has been mainly studied under the aspect of blood contents passing into the CSF and, therefore, blood components such as pathogens gaining access to the brain. From the blood side, the barrier consists of the vessel endothelium followed by the Choroid plexus epithelium, both of which contain Occludin as an important tight junction component and regulator (Furuse et al., 1993). Changing perspectives to the CSF side, one conceivable explanation could be that antibodies from the CSF first damage the Choroid plexus barrier and subsequently diffuse to the vessel structures. It is possible that the measured decrease in Occludin expression results from a combinatory effect of a decreased expression in the vasculature and also in the Choroid plexus epithelium since whole brain homogenates were used for Western blot analysis. This explanation assumes that the Choroid plexus epithelium expresses Myosin-X, which is indeed described as being expressed although at low levels (Berg et al., 2000; Sousa et al., 2006), and without a definite description of expression in the choroid plexus epithelium. Furthermore, CSF is in constant contact with the surface of the brain, along the basement membrane shared by the pia mater and the glia limitans, and therefore also in close proximity to the arteries entering the brain parenchyma, which could also be considered a possible pathway for antibodies from the CSF. However, evidence for bulk flow through this route is currently lacking (Bedussi et al., 2015). Lastly, the possibility of traumatic injury through the injection of the intraventricular pump and diffusion through the parenchyma and interstitial fluid cannot be fully ruled out. Ultimately, the mechanisms of entry and target accessibility cannot be answered by the present study and require further, careful investigation.

There have been ongoing discussions about the functionality and relevancy of intracellular targets**.** Based on our current knowledge about immune-mediated neurological diseases, it is to be expected that many of the newly discovered intracellular targets might only serve as biomarkers of disease and are not per se pathogenic (Prüss, 2021). However, there are neurological diseases associated with antibodies against intracellular targets, such as glutamic acid decarboxylase (GAD) and Synapsin, where studies show evidence for the pathogenicity of the associated antibodies. In the case of Stiff-Person syndrome associated with GAD-antibodies, *in vitro* testing showed blocking of GAD enzymatic activity by patient sera (Raju et al., 2005). Anti-Synapsin antibodies have been identified in patients with various neurological symptoms, including limbic encephalitis (Holtje et al., 2017; Piepgras et al., 2015). In another study investigating the possible mechanism of action, CSF of patients with high titers of IgG and IgA autoantibodies against Synapsin led to impaired transmission in hippocampal neurons compared to control CSF. Further, anti-Synapsin autoantibodies were shown to be internalized by Fcγ II/III receptors and to escape the endocytic compartment to reach their cytosolic target (Rocchi et al., 2019). The role and relevancy of intracellular targets are highly controversial, and every newly discovered target will require detailed clinical and experimental evaluation.

Hypothesis III: Blood vessel reactive antibodies exert specific functional effects.

The effects of vessel reactive antibodies on barrier function and properties were studied in our work using various assays. The results of our study show a decrease of transendothelial electrical resistance (TEER) in an *in vitro* BBB model, which was associated with a decreased expression of junction protein Occludin in hCMEC/D3 endothelial cells as well as in murine brain after intraventricular injection (Li et al., 2023).
Permeability measurements, on the other hand, led to mixed results. A clear limitation of the *in vitro* functional assays conducted lies within the comparably low intrinsic TEER established by the hCMEC/D3 monolayer. HCMEC/D3 cells are established and commonly used to investigate barrier functions *in vitro* (Helms et al., 2016) since they can be efficiently grown in cell culture. Ideally, TEER values inversely correlate with permeability, with decreased TEER values and increased permeability indicating impaired barrier function. However, with lower intrinsic TEER values, no clear correlation to permeability can be drawn (Gaillard and Boer, 2000; Mantle et al., 2016). Additionally, in models with low

TEER, permeability might not only result from paracellular but also transcellular transport, making clear-cut analyses difficult.

To ensure that the observed effects are specific to mAb 011-138 and not due to unspecific effects through the addition of any antibody, we utilized mAb mGo53 as a control antibody in our *in vitro* as well as in our *in vivo* experiments. mGo53 is an established negative control antibody in our group of the IgG1 subclass with no known reactivity to brain tissue. MAb 011-138 is an IgG2 antibody and is therefore almost completely resembled by control antibody mGo53, although IgG2 antibodies have a shorter hinge domain (Vidarsson et al., 2014).

Although the concept of barrier disruption in inflammatory neurological diseases has been extensively investigated, only recently has the role of autoantibodies in this context been explored. Studies around GRP78 autoantibodies have shown that non-disease-defining GRP78 antibodies potentially propagate the entry of AQP4-IgG in NMOSD and VGCC antibodies in LEMS-PCD (Shimizu et al., 2017; Shimizu et al., 2019). To demonstrate the specific effect of GRP78 antibodies, GRP78 antibodies were depleted from the pooled IgG of 50 patients using the GRP78 protein. This successfully reversed the previously observed effects. Since in our experiments the concentration of monoclonal antibody used (5µg/ml or 10µg/ml) is many-fold higher than found in real-life patients, it would be highly interesting to investigate whether patient CSF from patients has a comparable effect *in vitro* and *in vivo* and if this effect is reversible after pre-absorption.
In the GRP78 antibody studies, the material for obtaining IgG originates from serum and plasma exchange material, and the monoclonals were generated from CSF B-cells (Shimizu et al., 2017; Shimizu et al., 2019). Both components caused properties of barrier dysfunction, leaving the question of the access point of the antibodies open. As stated before, considering the source B-cells were obtained from CSF, it would be likely that antibody-producing B-cells were trafficked into the CNS from the periphery. In the case of anti-Myosin-X antibodies, however, so far, no serum reactivity against Myosin-X transfected HEK cells could be detected (data not shown).

The target protein Myosin-X is a ubiquitously expressed unconventional myosin that acts as an actin-based molecular motor (Berg et al., 2000). In the context of cell-cell junctions, Myosin-X plays a role in the barrier formation in polarized epithelial cells (Liu et al., 2012)

and mediates the transport of VE-cadherin to cell edges in endothelial cells (Almagro et al., 2010). Our results indicate that the interaction between antibody 011-138 and its target Myosin-X is associated with decreased Occludin expression and barrier disruption. The exact molecular link between antibody binding and decrease in occludin expression, however, remains to be clarified.

Further screening for vessel reactive antibodies and identification of targets will be needed to understand the mechanism of action in this subgroup of antibodies. Investigating patients with known vascular pathologies and/or barrier implications would be especially interesting. This could, for example, be assessed through CSF/Serum albumin and IgG ratios (Reiber, 2004) or from imaging diagnostics.

While our study provides initial evidence for possible functional effects of vessel reactive autoantibodies in patients with autoimmune encephalitis, additional research is needed to verify and further explore the mechanisms of mAb 011-138 and other vessel reactive antibodies.

Outlook

The involvement of BBB disruption in autoimmune encephalitis has been proposed previously (Dalmau et al., 2007). The prognosis of NMDAR-encephalitis has been shown to be associated with BBB disruption (Yu et al., 2021). As stated in the introduction, most patients have higher titers of disease-defining neurologlial surface autoantibodies in serum than in CSF (Sun et al., 2020), with high titers suggesting passage of antibodies into the CNS. Similarly, disease-causing VGCC antibodies in patients with LEMS-PCD also gain passage from the periphery to the CNS. For those patients, Shimizu et al. (2019) have shown that anti-GRP78 antibodies act as a gate opener for anti-VGCC-antibodies. This leads to the question of whether a similar mechanism of action is applicable for the passage of NMDAR antibodies and other antibodies in autoimmune encephalitis to the CNS. Our study provides a first step to exploring this possibility. Ultimately, the question emerges as to whether the principle of gate-opening autoantibodies could apply to other forms of autoimmune neurological diseases as well. Vessel reactive antibodies have been found regularly in our lab, across different types of encephalitis, and in a recent study, also in patients after COVID-19 infection (Franke et al., 2023).

Our work followed a highly exploratory approach and provided starting points for following investigations. Future studies will likely identify more targets, reveal new functional effects, and will provide answers as well as provoke more questions. As mentioned in the introduction, large parts of the human autoantibody repertoire are still unknown. Therefore, not only vessel reactive antibodies but also other subcategories need to be further explored. Autoantibodies against other groups of brain cells with well-characterized functions, such as cerebellar neurons and astrocytes, are especially interesting candidates for investigation. Major advances in the field of antibody-mediated neurological diseases have been achieved in recent years, and with each newly unraveled antibody-target mechanism, opportunities for improved diagnosis, prognosis, and treatment arise.

5 Conclusion

In our study, we explore the autoantibody repertoire of patients with autoimmune encephalitis. We investigate currently non-disease-defining autoantibody with a focus on the subgroup of vessel reactive autoantibodies.

Based on previous findings, we proposed and confirmed the presence of brain blood vessel reactive autoantibodies in our autoimmune encephalitis patient cohort. Using indirect immunofluorescent staining on murine brain sections in our screening, we identified (6/147) (4,1%) strongly reactive vessel targeting antibodies. For monoclonal antibody 011-138, we have identified an antigenic target structure and investigated the potential functional effects of antibody-target interaction on barrier disruption. Monoclonal antibody 011-138 binds to Myosin-X and disrupts barrier function in our *in vitro* blood-brain barrier model. Our results indicate tight-junction protein Occludin downregulation as a possible underlying mechanism. In this way, our study explores the concept of antibody-mediated blood-brain barrier disruption as a mechanism of action in autoimmune encephalitis.

In many cases of central nervous system autoimmunity, it is still unclear how pathogenic antibodies reach the other side of the blood-brain barrier. The exact mechanisms of IgG diffusion and/or B cell trafficking are still a matter of ongoing debate. This work provides a first step to elucidate the possible involvement of vessel reactive antibodies in autoimmune encephalitis in barrier disruption and as a gate opener to facilitate the entry of other autoantibodies.

Human recombinant monoclonal antibodies provide a valuable tool for discovery and in-depth investigations. Not only can disease-defining antibodies be studied in more detail, but monoclonal antibodies also aid the investigation of the role and relevancy of currently non-disease-defining autoantibodies. Discoveries in the field of autoimmune neurological diseases keep shaping and changing the clinal and scientific landscape.

References

Almagro, S., Durmort, C., Chervin-Petinot, A., Heyraud, S., Dubois, M., Lambert, O., Maillefaud, C., Hewat, E., Schaal, J. P., Huber, P. and Gulino-Debrac, D. (2010) 'The motor protein myosin-X transports VE-cadherin along filopodia to allow the formation of early endothelial cell-cell contacts', *Mol Cell Biol*, vol. 30, no. 7, pp. 1703–1717.

Barthel, P. C., Staabs, F., Li, L. Y., Buthut, M., Otto, C., Ruprecht, K., Prüss, H. and Höltje, M. (2023) 'Immunoreactivity to astrocytes in different forms of dementia: High prevalence of autoantibodies to GFAP', *Brain, behavior, & immunity - health*, vol. 29, p. 100609.

Bedussi, B., van Lier, M. G. J. T. B., Bartstra, J. W., Vos, J. de, Siebes, M., VanBavel, E. and Bakker, E. N. T. P. (2015) 'Clearance from the mouse brain by convection of interstitial fluid towards the ventricular system', *Fluids and barriers of the CNS*, vol. 12, p. 23.

Bennett, J. L., Lam, C., Kalluri, S. R., Saikali, P., Bautista, K., Dupree, C., Glogowska, M., Case, D., Antel, J. P., Owens, G. P., Gilden, D., Nessler, S., Stadelmann, C. and Hemmer, B. (2009) 'Intrathecal pathogenic anti-aquaporin-4 antibodies in early neuromyelitis optica', *Annals of neurology*, vol. 66, no. 5, pp. 617–629.

Berg, J. S., Derfler, B. H., Pennisi, C. M., Corey, D. P. and Cheney, R. E. (2000) 'Myosin-X, a novel myosin with pleckstrin homology domains, associates with regions of dynamic actin', *Journal of cell science*, 113 Pt 19, pp. 3439–3451.

Brilot, F., Dale, R. C., Selter, R. C., Grummel, V., Kalluri, S. R., Aslam, M., Busch, V., Zhou, D., Cepok, S. and Hemmer, B. (2009) 'Antibodies to native myelin oligodendrocyte glycoprotein in children with inflammatory demyelinating central nervous system disease', *Annals of neurology*, vol. 66, no. 6, pp. 833–842.

Dalmau, J., Tüzün, E., Wu, H., Masjuan, J., Rossi, J. E., Voloschin, A., Baehring, J. M., Shimazaki, H., Koide, R., King, D., Mason, W., Sansing, L. H., Dichter, M. A., Rosenfeld, M. R. and Lynch, D. R. (2007) 'Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma', *Annals of neurology*, vol. 61, no. 1, pp. 25–36.

DeStefano, J. G., Jamieson, J. J., Linville, R. M. and Searson, P. C. (2018) 'Benchmarking in vitro tissue-engineered blood-brain barrier models', *Fluids and barriers of the CNS*, vol. 15, no. 1, p. 32.

Duong, S. L. and Prüss, H. (2023) ‘Molecular disease mechanisms of human antineuronal monoclonal autoantibodies’, *Trends in molecular medicine*, vol. 29, no. 1, pp. 20–34.

Franke, C., Boesl, F., Goereci, Y., Gerhard, A., Schweitzer, F., Schroeder, M., Foverskov-Rasmussen, H., Heine, J., Quitschau, A., Kandil, F. I., Schild, A.-K., Finke, C., Audebert, H. J., Endres, M., Warnke, C. and Prüss, H. (2023) ‘Association of cerebrospinal fluid brain-binding autoantibodies with cognitive impairment in post-COVID-19 syndrome’, *Brain Behav Immun*, vol. 109, pp. 139–143.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S. and Tsukita, S. (1993) ‘Occludin: a novel integral membrane protein localizing at tight junctions’, *The Journal of cell biology*, vol. 123, 6 Pt 2, pp. 1777–1788.

Gaillard, P. J. and Boer, A. G. de (2000) ‘Relationship between permeability status of the blood-brain barrier and in vitro permeability coefficient of a drug’, *European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences*, vol. 12, no. 2, pp. 95–102.

Granerod, J., Ambrose, H. E., Davies, N. W. S., Clewley, J. P., Walsh, A. L., Morgan, D., Cunningham, R., Zuckerman, M., Mutton, K. J., Solomon, T., Ward, K. N., Lunn, M. P. T., Irani, S. R., Vincent, A., Brown, D. W. G. and Crowcroft, N. S. (2010) ‘Causes of encephalitis and differences in their clinical presentations in England: a multicentre, population-based prospective study’, *The Lancet Infectious Diseases*, vol. 10, no. 12, pp. 835–844.

Hamid, S. H. M., Whittam, D., Mutch, K., Linaker, S., Solomon, T., Das, K., Bhojak, M. and Jacob, A. (2017) ‘What proportion of AQP4-IgG-negative NMO spectrum disorder patients are MOG-IgG positive? A cross sectional study of 132 patients’, *Journal of neurology*, vol. 264, no. 10, pp. 2088–2094.

Helms, H. C., Abbott, N. J., Burek, M., Cecchelli, R., Couraud, P. O., Deli, M. A., Forster, C., Galla, H. J., Romero, I. A., Shusta, E. V., Stebbins, M. J., Vandenhaute, E., Weksler, B. and Brodin, B. (2016) ‘In vitro models of the blood-brain barrier: An overview of commonly used brain endothelial cell culture models and guidelines for their use’, *J Cereb Blood Flow Metab*, vol. 36, no. 5, pp. 862–890.

Holtje, M., Mertens, R., Schou, M. B., Saether, S. G., Kochova, E., Jarius, S., Pruss, H., Komorowski, L., Probst, C., Paul, F., Bellmann-Strobl, J., Gitler, D., Benfenati, F.,

Piepgras, J., Ahnert-Hilger, G. and Ruprecht, K. (2017) ‘Synapsin-antibodies in psychiatric and neurological disorders: Prevalence and clinical findings’, *Brain Behav Immun*, vol. 66, pp. 125–134.

Irani, S. R., Alexander, S., Waters, P., Kleopa, K. A., Pettingill, P., Zuliani, L., Peles, E., Buckley, C., Lang, B. and Vincent, A. (2010) ‘Antibodies to Kv1 potassium channel-complex proteins leucine-rich, glioma inactivated 1 protein and contactin-associated protein-2 in limbic encephalitis, Morvan's syndrome and acquired neuromyotonia’, *Brain*, vol. 133, no. 9, pp. 2734–2748.

Jarius, S., Franciotta, D., Paul, F., Ruprecht, K., Bergamaschi, R., Rommer, P. S., Reuss, R., Probst, C., Kristoferitsch, W., Wandinger, K. P. and Wildemann, B. (2010) ‘Cerebrospinal fluid antibodies to aquaporin-4 in neuromyelitis optica and related disorders: frequency, origin, and diagnostic relevance’, *Journal of neuroinflammation*, vol. 7, p. 52.

Kreye, J., Wenke, N. K., Chayka, M., Leubner, J., Murugan, R., Maier, N., Jurek, B., Ly, L. T., Brandl, D., Rost, B. R., Stumpf, A., Schulz, P., Radbruch, H., Hauser, A. E., Pache, F., Meisel, A., Harms, L., Paul, F., Dirnagl, U., Garner, C., Schmitz, D., Wardemann, H. and Pruss, H. (2016) ‘Human cerebrospinal fluid monoclonal N-methyl-D-aspartate receptor autoantibodies are sufficient for encephalitis pathogenesis’, *Brain*, vol. 139, Pt 10, pp. 2641–2652.

Kreye, J., Wright, S. K., van Casteren, A., Stöffler, L., Machule, M.-L., Reincke, S. M., Nikolaus, M., van Hoof, S., Sanchez-Sendin, E., Homeyer, M. A., Cordero Gómez, C., Kornau, H.-C., Schmitz, D., Kaindl, A. M., Boehm-Sturm, P., Mueller, S., Wilson, M. A., Upadhya, M. A., Dhangar, D. R., Greenhill, S., Woodhall, G., Turko, P., Vida, I., Garner, C. C., Wickel, J., Geis, C., Fukata, Y., Fukata, M. and Prüss, H. (2021) ‘Encephalitis patient-derived monoclonal GABAA receptor antibodies cause epileptic seizures’, *The Journal of experimental medicine*, vol. 218, no. 11.

Lai, M., Hughes, E. G., Peng, X., Zhou, L., Gleichman, A. J., Shu, H., Matà, S., Kremens, D., Vitaliani, R., Geschwind, M. D., Bataller, L., Kalb, R. G., Davis, R., Graus, F., Lynch, D. R., Balice-Gordon, R. and Dalmau, J. (2009) ‘AMPA receptor antibodies in limbic encephalitis alter synaptic receptor location’, *Annals of neurology*, vol. 65, no. 4, pp. 424–434.

Lancaster, E., Martinez-Hernandez, E., Titulaer, M. J., Boulos, M., Weaver, S., Antoine, J.-C., Liebers, E., Kornblum, C., Bien, C. G., Honnorat, J., Wong, S., Xu, J., Contractor,

A., Balice-Gordon, R. and Dalmau, J. (2011) ‘Antibodies to metabotropic glutamate receptor 5 in the Ophelia syndrome’, *Neurology*, vol. 77, no. 18, pp. 1698–1701.

Lennon, V. A., Kryzer, T. J., Pittock, S. J., Verkman, A. S. and Hinson, S. R. (2005) ‘IgG marker of optic-spinal multiple sclerosis binds to the aquaporin-4 water channel’, *The Journal of experimental medicine*, vol. 202, no. 4, pp. 473–477.

Li, L. Y., Kreye, J., Burek, M., Cordero-Gomez, C., Barthel, P. C., Sánchez-Sendín, E., Kornau, H.-C., Schmitz, D., Scharf, M., Meybohm, P., Reincke, S. M., Prüss, H. and Höltje, M. (2023) ‘Brain blood vessel autoantibodies in patients with NMDA and GABAA receptor encephalitis: identification of unconventional Myosin-X as target antigen’, *Frontiers in cellular neuroscience*, vol. 17, p. 1077204.

Liu, K. C., Jacobs, D. T., Dunn, B. D., Fanning, A. S. and Cheney, R. E. (2012) ‘Myosin-X functions in polarized epithelial cells’, *Molecular Biology of the Cell*, vol. 23, no. 9, pp. 1675–1687.

Mantle, J. L., Min, L. and Lee, K. H. (2016) ‘Minimum Transendothelial Electrical Resistance Thresholds for the Study of Small and Large Molecule Drug Transport in a Human in Vitro Blood-Brain Barrier Model’, *Molecular pharmaceutics*, vol. 13, no. 12, pp. 4191–4198.

Nikolaus, M., Knierim, E., Meisel, C., Kreye, J., Prüss, H., Schnabel, D. and Kallinich, T. (2018) ‘Severe GABAA receptor encephalitis without seizures: A paediatric case successfully treated with early immunomodulation’, *European journal of paediatric neurology : EJPN : official journal of the European Paediatric Neurology Society*, vol. 22, no. 3, pp. 558–562.

Noviello, C. M., Kreye, J., Teng, J., Prüss, H. and Hibbs, R. E. (2022) ‘Structural mechanisms of GABAA receptor autoimmune encephalitis’, *Cell*, vol. 185, no. 14, 2469-2477.e13.

Petit-Pedrol, M., Armangue, T., Peng, X., Bataller, L., Cellucci, T., Davis, R., McCracken, L., Martinez-Hernandez, E., Mason, W. P., Kruer, M. C., Ritacco, D. G., Grisold, W., Meaney, B. F., Alcalá, C., Sillevis-Smitt, P., Titulaer, M. J., Balice-Gordon, R., Graus, F. and Dalmau, J. (2014) ‘Encephalitis with refractory seizures, status epilepticus, and antibodies to the GABAA receptor: a case series, characterisation of the antigen, and analysis of the effects of antibodies’, *The Lancet Neurology*, vol. 13, no. 3, pp. 276–286.

Piepgras, J., Holtje, M., Otto, C., Harms, H., Satapathy, A., Cesca, F., Benfenati, F., Gitler, D., Pich, A., Zander, J. F., Ahnert-Hilger, G. and Ruprecht, K. (2015) 'Intrathecal immunoglobulin A and G antibodies to synapsin in a patient with limbic encephalitis', *Neurol Neuroimmunol Neuroinflamm*, vol. 2, no. 6, e169.

Prüss, H. (2021) 'Autoantibodies in neurological disease', *Nature Reviews. Immunology*, pp. 1–16.

Raju, R., Foote, J., Banga, J. P., Hall, T. R., Padoa, C. J., Dalakas, M. C., Ortqvist, E. and Hampe, C. S. (2005) 'Analysis of GAD65 autoantibodies in Stiff-Person syndrome patients', *J Immunol*, vol. 175, no. 11, pp. 7755–7762.

Rocchi, A., Sacchetti, S., Fusco, A. de, Giovedi, S., Parisi, B., Cesca, F., Höltje, M., Ruprecht, K., Ahnert-Hilger, G. and Benfenati, F. (2019) 'Autoantibodies to synapsin I sequestrate synapsin I and alter synaptic function', *Cell death & disease*, vol. 10, no. 11, p. 864.

Schnell, S., Knierim, E., Bittigau, P., Kreye, J., Hauptmann, K., Hundsdoerfer, P., Morales-Gonzalez, S., Schuelke, M. and Nikolaus, M. (2023) 'Hodgkin Lymphoma Cell Lines and Tissues Express mGluR5: A Potential Link to Ophelia Syndrome and Paraneoplastic Neurological Disease', *Cells*, vol. 12, no. 4.

Shimizu, F., Schaller, K. L., Owens, G. P., Cotleur, A. C., Kellner, D., Takeshita, Y., Obermeier, B., Kryzer, T. J., Sano, Y., Kanda, T., Lennon, V. A., Ransohoff, R. M. and Bennett, J. L. (2017) 'Glucose-regulated protein 78 autoantibody associates with blood-brain barrier disruption in neuromyelitis optica', *Sci Transl Med*, vol. 9, no. 397.

Shimizu, F., Takeshita, Y., Sano, Y., Hamamoto, Y., Shiraishi, H., Sato, T., Yoshimura, S., Maeda, T., Fujikawa, S., Nishihara, H., Kitanosono, H., Tsujino, A., Motomura, M. and Kanda, T. (2019) 'GRP78 antibodies damage the blood-brain barrier and relate to cerebellar degeneration in Lambert-Eaton myasthenic syndrome', *Brain*, vol. 142, no. 8, pp. 2253–2264.

Sousa, A. D., Berg, J. S., Robertson, B. W., Meeker, R. B. and Cheney, R. E. (2006) 'Myo10 in brain: developmental regulation, identification of a headless isoform and dynamics in neurons', *J Cell Sci*, vol. 119, Pt 1, pp. 184–194.

Sun, B., Ramberger, M., O'Connor, K. C., Bashford-Rogers, R. J. M. and Irani, S. R. (2020) 'The B cell immunobiology that underlies CNS autoantibody-mediated diseases', *Nat Rev Neurol*, vol. 16, no. 9, pp. 481–492.

Tomizawa, Y., Yokoyama, K., Saiki, S., Takahashi, T., Matsuoka, J. and Hattori, N. (2012) ‘Blood-brain barrier disruption is more severe in neuromyelitis optica than in multiple sclerosis and correlates with clinical disability’, *The Journal of international medical research*, vol. 40, no. 4, pp. 1483–1491.

van Bree, J. B., Boer, A. G. de, Danhof, M., Ginsel, L. A. and Breimer, D. D. (1988) ‘Characterization of an "in vitro" blood-brain barrier: effects of molecular size and lipophilicity on cerebrovascular endothelial transport rates of drugs’, *J Pharmacol Exp Ther*, vol. 247, no. 3, pp. 1233–1239.

Vidarsson, G., Dekkers, G. and Rispens, T. (2014) ‘IgG Subclasses and Allotypes: From Structure to Effector Functions’, *Front Immunol*, vol. 5.

Yu, Y., Wu, Y., Cao, X., Li, J., Liao, X., Wei, J. and Huang, W. (2021) ‘The Clinical Features and Prognosis of Anti-NMDAR Encephalitis Depends on Blood Brain Barrier Integrity’, *Multiple sclerosis and related disorders*, vol. 47, p. 102604.

Statutory Declaration

"I, Lucie Yuanting Li, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Identification and characterization of human monoclonal antibody target structures in the brain – Blood vessel antibodies in autoimmune encephalitis" (Deutsch: Identifizierung und Charakterisierung von Zielstrukturen humaner monoklonaler Antikörper im Gehirn – Gefäßantikörper bei Autoimmunenzephalitiden) independently and without the support of third parties, and that I used no other sources and aids than those stated. All parts that are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, and statistical processing) and results (in particular regarding figures, charts, and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons and that I have correctly marked my contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to the publication of this dissertation correspond to those stated in the below joint declaration made together with the supervisor. The publication created within the scope of the dissertation complies with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

Detailed declaration of contribution to the publication

Lucie Y. Li contributed the following to the below-listed publication:

Publication:

Li, L. Y., Kreye, J., Burek, M., Cordero-Gomez, C., Barthel, P. C., Sánchez-Sendín, E., Kornau, H.-C., Schmitz, D., Scharf, M., Meybohm, P., Reincke, S. M., Prüss, H. and Höltje, M. (2023)
'Brain blood vessel autoantibodies in patients with NMDA and GABAA receptor encephalitis: identification of unconventional Myosin-X as target antigen', Frontiers in cellular neuroscience, vol. 17, p. 1077204.

Contribution in detail:

Conception:
This study was conceptualized by Prof. Dr. Harald Prüß, PD. Dr. rer. nat. Markus Höltje, Dr. Jakob Kreye and Lucie Y. Li.

Methods:

Lucie Y. Li established the methods using the hCMEC/D3 cell-based *in vitro* blood-brain barrier model in our laboratories under the scientific and methodological guidance of M. Burek.

Experiments:

Lucie Y. Li performed all lab work for the immunofluorescence screening, including cutting, staining, and imaging of the murine brains as well as the vessel purification, staining, and imaging. Lucie Y. Li conducted the experiments required for target identification, including tissue preparation and immunoprecipitation. Lucie Y. Li conducted all experimental procedures for the investigation of functional implications in the *in vitro* BBB model, including set-up and measurement of the Transendothelial electrical resistance and Permeability parameters. Lucie Y. Li executed Western blot experiments as well as all postmortem ex vivo experiments.

Analysis, Visualization, Manuscript:

Imaging analysis and quantification, as well as protein expression analysis, was done by Lucie Y. Li and PD Dr. rer. nat. Markus Höltje. All functional data of the in vitro BBB model was analyzed by Lucie Y. Li. All figures and figure legends were created by PD Dr. rer. nat. Markus Höltje in close collaboration with Lucie Y. Li. Lucie Y. Immunohistochemical stainings performed by Lucie Y. Li resulted in figures 1; 2; 3A, B, F-H, 4C-E and 6A-C, as well as intensity measurements in figure 4F+G. Functional assays performed by Lucie Y. Li resulted in figures 3C-E. Immunoblots performed by Lucie Y. Li resulted in figures 3I+J and 6D. Li wrote the first draft of the paper (apart from the paragraphs in the method section regarding the Real-Time-PCR, Indirect immunofluorescence assay on monkey and rat brain sections, and the generation of human monoclonal antibodies). All co-authors contributed to the data, the figures, and the manuscript and have contributed to the review process of the manuscript.

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Signature, date, and stamp MD/PhD supervisor

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Signature, date MD/PhD candidate

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18	SLEEP MEDICINE REVIEWS	12,620	11.401	0.01356
19	Neurology-Neuroimmunology & Neuroinflammation	5,161	11.360	0.01049
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Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfaktor
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33	PAIN	46,662	7.926	0.02957
34	Annual Review of Vision Science	1,345	7.745	0.00387
35	Current Neuropharmacology	7,580	7.708	0.00713
36	BRAIN PATHOLOGY	7,083	7.611	0.00689
37	Acta Neuropathologica Communications	8,201	7.578	0.01628
38	NEUROIMAGE	131,266	7.400	0.10055
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43	NEUROBIOLOGY OF DISEASE	23,538	7.046	0.01982
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Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfaktor
45	Fluids and Barriers of the CNS	2,427	6.961	0.00258
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58	Neurotherapeutics	7,998	6.088	0.00899
59	CEPHALALGIA	13,467	6.075	0.01470
60	Neural Regeneration Research	9,531	6.058	0.00956

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Brain blood vessel autoantibodies in patients with NMDA and $GABA_A$ receptor encephalitis: identification of unconventional Myosin-X as target antigen

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Introduction: The antibody repertoire from CSF-derived antibody-secreting cells and memory B-cells in patients with encephalitis contains a considerable number of antibodies that do not target the disease-defining autoantigen such as the GABA or NMDA receptors. This study focuses on the functional relevance of autoantibodies to brain blood vessels in patients with $GABA_A$ and NMDA receptor encephalitis.

Methods: We tested 149 human monoclonal IgG antibodies from the cerebrospinal fluid of six patients with different forms of autoimmune encephalitis on murine brain sections for reactivity to blood vessels using immunohistochemistry. Positive candidates were tested for reactivity with purified brain blood vessels, effects on transendothelial electrical resistance (TEER), and expression of tight junction proteins as well as gene regulation using human brain microvascular endothelial hCMEC/D3 cells as *in vitro* blood-brain barrier model. One blood-vessel reactive antibody was infused intrathecally by pump injection in mice to study *in vivo* binding and effects on tight junction proteins such as Occludin. Target protein identification was addressed using transfected HEK293 cells.

Results: Six antibodies reacted with brain blood vessels, three were from the same patient with $GABA_AR$ encephalitis, and the other three were from different patients with NMDAR encephalitis. One antibody from an NMDAR encephalitis patient, mAb 011-138, also reacted with cerebellar Purkinje cells. In this case, treatment of hCMEC/D3 cells resulted in decreased TEER, reduced Occludin expression, and mRNA levels. Functional relevance *in vivo* was confirmed as Occludin downregulation was observed in mAb 011-138-infused animals. Unconventional Myosin-X was identified as a novel autoimmune target for this antibody.

Discussion: We conclude that autoantibodies to blood vessels occur in autoimmune encephalitis patients and might contribute to a disruption of the blood-brain barrier thereby suggesting a potential pathophysiological relevance of these antibodies.

KEYWORDS

blood-brain barrier, autoimmunity, encephalitis, occludin, Myosin-X

Introduction

Autoantibodies associated with neurological diseases have deeply changed the clinical landscape and our understanding of immunological processes in the nervous system. Especially antibodies against neuronal surface receptors turned out to be directly pathogenic, hallmarking previously unclassified disease entities. In anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis, antibodies targeting the NR1 subunit reduce surface NMDA receptor clusters and disrupt synaptic currents (Hughes et al., 2010; Kreye et al., 2016). Patients develop psychiatric symptoms typically involving behavioral changes, catatonia, hallucination as well as autonomic fluctuations and seizures in the course of the disease (Dalmau et al., 2007). The more recently discovered anti-γ-aminobutyric acid A receptor ($GABA_AR$) encephalitis is characterized by antibodies reducing the synaptic and extra-synaptic density of $GABA_A$ receptors and exerting electrophysiological changes in cultured neurons (Ohkawa et al., 2014; Petit-Pedrol et al., 2014; Pettingill et al., 2015). Patients characteristically present with catatonia, seizures, refractory status epilepticus, cognitive impairment, and MRI abnormalities (Ohkawa et al., 2014; Petit-Pedrol et al., 2014; Pettingill et al., 2015; Spatola et al., 2017).

Despite the advances in unraveling the molecular mechanisms of anti-NMDAR and anti-$GABA_AR$ antibodies, knowledge about how they gain access to central nervous system (CNS) targets is still lacking. Possible mechanisms of immune system components transmigrating the blood-brain barrier (BBB) have been investigated in other inflammatory autoimmune disorders. In neuropsychiatric systemic lupus erythematodes (NPSLE), BBB disruption has been demonstrated to be a crucial step in disease development (Kowal et al., 2004; Huerta et al., 2006; Hirohata et al., 2014). This process is fueled by endothelial antibody binding and upregulating the expression of proinflammatory cytokines and leukocyte adhesion molecules (Meroni et al., 2003; Armitage et al., 2004; Yoshio et al., 2013). Furthermore, for neuromyelitis optica (NMO) it has been shown that BBB disruption correlates with disease severity (Tomizawa et al., 2012). Findings in NMO have uncovered monoclonal antibodies targeting Glucose-regulated protein 78 (GRP78), which after repeated administration, caused extravasation of serum albumin, IgG, and fibrinogen in mouse brains (Shimizu et al., 2017). Shortly after, GRP78 antibodies were also discovered to impair the BBB in patients with paraneoplastic cerebellar degeneration with Lambert-Eaton myasthenic syndrome (PCD-LEMS), thereby potentially allowing access of pathogenic autoantibodies (Shimizu et al., 2019). Thus, antibodies targeting blood vessels can be directly pathogenic by inducing an endothelial pro-inflammatory phenotype, can cause BBB dysfunction, and possibly even promote the transition of macromolecules through the BBB.

Additionally, studies of recombinant human monoclonal antibodies (mAbs) have demonstrated that recombinant mAbs from CSF-derived antibody-secreting cells and memory B-cells especially in patients with NMDAR encephalitis do not only target the disease-defining autoantigen (Kreye et al., 2016, 2021). Rather, the majority of antibodies strongly react with further brain epitopes. Their possible involvement in the disease pathomechanism remains unclear. Hence, with our non-biased approach using recombinant production of CSF-derived mAbs (Kreye et al., 2016, 2021), we aimed to further investigate the intrathecal human monoclonal antibody repertoire. Using immunofluorescence methods on murine brain tissue we identified a subgroup of blood vessel reactive mAbs to a similar extent in patients diagnosed with NMDAR encephalitis and $GABA_AR$ encephalitis.

To our knowledge, this is the first characterization of autoantibodies to brain blood vessels in NMDAR encephalitis and $GABA_AR$ encephalitis patients. We illustrate binding to brain blood vessels *in vitro* and replicate characteristic binding *in vivo*. Furthermore, in this qualitative study, we identified Myosin-X as the target antigen for one selected mAb and showed its functional effects *in vitro* and *in vivo*. Collectively, we provide the first evidence for a putative contribution of brain blood vessel reactive mAbs to disease development.

Materials and methods

Immunohistochemistry on mouse brain sections

For tissue sections of unfixed mice brains, animals were sacrificed, brains were removed and snap-frozen in −50°C cold 2-methyl butane. Twenty micrometer sagittal sections were cut and processed as described previously (Kreye et al., 2016, 2021).

Indirect immunofluorescence assay on monkey and rat brain sections

Stainings were performed using slides with a biochip screening array of brain tissue cryosections (cerebellum of rat and *Macaca mulatta*). Each biochip mosaic was incubated with 35 µl of PBS-diluted sample at 4°C for 16 h in a humidity chamber. In the second step, Alexa488-labelled goat anti-human IgG (Jackson Research, Suffolk, United Kingdom), was applied and incubated at RT for 2 h. Results were evaluated independently by two observers using a fluorescence microscope (EUROStar II, Euroimmun AG, Lübeck, Germany).

Generation of human monoclonal antibodies

Human monoclonal antibodies were previously generated as recombinant proteins from patients with NMDA receptor encephalitis or $GABA_A$ receptor encephalitis (Kreye et al., 2016, 2021; Nikolaus et al., 2018). The diagnosis was confirmed by: (i) the presence of autoantibodies against the respective autoantigen in the patient's cerebrospinal fluid as detected in a commercial cell-based assay (EUROIMMUN, Lübeck Germany); and by (ii) typical neurological symptoms. For monoclonal antibody isolation, we used established methods (Kreye et al., 2016, 2020, 2021; Reincke et al., 2020). In brief, from patients' cerebrospinal fluid, single antibody-secreting cells and B cells were isolated using fluorescence-activated cell sorting. From single-cell cDNA variable immunoglobulin encoding genes were amplified, sequenced, and cloned into expression vectors. Pairs of functional heavy and light chain vectors were used to transfect human embryonic kidney (HEK293T) cells using Polyethylenimine (Polysciences, Inc., Warrington, USA). On day three/four and day seven after transfection cell culture supernatants were harvested, then centrifuged at 2,000× *g* for 5 min at 4°C to remove cell debris before sodium acid was added to a concentration of 0.05% to prevent bacterial growth. The human IgG concentration in cell culture supernatants was determined using a commercial ELISA kit (Mabtech, Nacka Strand, Sweden) following the provider's instructions. For functional assays, mAbs were purified from supernatants using Protein G Sepharose beads (GE Healthcare), then dialyzed against PBS and sterile-filtered using 0.2 μm filter units (GE Healthcare). Recombinant mGO53 antibody served as a control antibody in *in vitro* and *in vivo* experiments.

Commercial antibodies

A monoclonal anti-Myosin-X antibody was purchased from Santa Cruz Biotechnology (# sc-166720, St. Cruz, USA). A monoclonal anti-smooth muscle actin (SMA) antibody was from Agilent Dako (#M0851, Santa Clara, USA). A rat monoclonal anti-CD31 antibody was from BD Biosciences (#553708, Franklin Lakes, NJ, USA). A monoclonal anti-CD34 antibody was from Arigo Biolaboratories (#ARG52756, Hsinchu City, Taiwan). A polyclonal anti-Collagen IV antibody was purchased from Abcam (#ab6586, Cambridge, UK). Polyclonal anti-Occludin and anti-Claudin5 antibodies were from Thermo Fisher Scientific (#71-1500 and #34-1600; Waltham, USA). A monoclonal anti-VE-cadherin antibody was from Cell Signaling (#2500, Danvers, USA). A mouse monoclonal anti-ZO-1 antibody was from Thermo Fisher Scientific (#33-9100).

Cell culture of human cerebral endothelial cells

Immortalized human cerebral microvascular endothelial cells hCMEC/D3 (Weksler et al., 2005) obtained from CELLutions Biosystems Inc. (#CLU512, Burlington, Ontario, Canada) were grown to confluence on coverslips. Cells were then used for either immunofluorescence staining on fixed cells or live incubation. For the fixed approach, endothelial cells were incubated with 4% PFA for 10 min and subsequently washed twice with PBS. Primary human antibodies (5 μg/ml) remained on the coverslips overnight at 4°C. For live incubation, a patient antibody was added to the medium at 5 μg/ml overnight at 36°C and 5% CO_2. After fixation with 4% PFA for 20 min cells were incubated with a secondary antibody for 2 h at RT in the dark.

Transfection

Human embryonic kidney (HEK) 293 cells were cultured in 24-well multiplates to 70% confluency and transfected with 1 μg plasmid cDNA coding for eGFPC1-hMyoX (Addgene, #47608) per well for 24 h using Polyethylenimine as transfection reagent. Transfected cells were fixed with 4% paraformaldehyde for 20 min at 4°C. Cells were subsequently permeabilized with 0.1% Triton X-100. Thereafter, cells were incubated with commercial antibodies at indicated concentrations or human monoclonal antibodies at 5 μg/ml.

Purification of mouse brain vessels

Purification of mouse brain vessels was performed following a previously described protocol (Boulay et al., 2015). In brief, myelin was removed from adult mice brain homogenate using an 18% dextran solution and density gradient centrifugation. From the resulting suspension brain blood vessels between 30 and 100 μm and >100 μm were collected by a sequence of filtering steps and directly used for immunohistochemistry or lysed for further analysis.

Measurement of TEER (transendothelial electrical resistance)

Human hCMEC/D3 cells were seeded onto Matrigel-coated trans-well inserts (0.4 μm pore size, Corning) at a density of 40×10^3 in ECM Medium (PELOBiotech, Martinsried, Germany) supplemented with 5% FCS. After 5 days ECM was depleted of growth factors and FCS was reduced to 0.5% for differentiation. Subsequently, endothelial cells were treated with patient antibodies at the indicated concentrations or left untreated for an additional 24 or 48 h. TEER measurements across each trans-well were conducted using chopstick electrodes (STX-PLUS, WPI, Sarasota, Fl, USA) and an Epithelial-Volt/Ohm-Meter (EVOM3, WPI). The TEER values of blank filters were subtracted from the measured values before calculations. Values were measured in triplicates.

Real-time PCR

RNA was isolated using the NucleoSpin® RNA Isolation Kit (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The following TaqMan probes (Thermo Fisher Scientific) were used: Hs00901465_m1 (CDH5, VE-cadherin), Hs01558409_m1 (CANX, Calnexin), Hs00533949_s1 (CLDN5,

Claudin5), Hs00202485_m1 (MYO10, Myosin-X), Hs00170162_m1 (OCLN, Occludin), Hs01551861_m1 (TJP1, ZO-1) with the TaqMan® Fast Advanced Master Mix in the QuantStudio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific). CANX was used as an endogenous control. The relative expression was calculated using the comparative Ct method with QuantStudio™ Real-Time PCR Software v1.7.1.

Membrane preparations

hCMEC/D3 cells were grown to confluency, seeded in 6-well plates, and cultured for 48 h with the respective antibodies as indicated. Following the removal of medium, the cells were washed with PBS, harvested, homogenized, and lysed in a glass/teflon homogenizer under hypoosmotic conditions. Homogenates were centrifuged at 1,043× *g* for 10 min to obtain a postnuclear supernatant. The resulting supernatant was centrifuged at 267,008× *g* for 30 min to obtain highly enriched cellular membranes. Membrane fractions were subjected to immunoblot analysis.

Immunoprecipitation

Triton-X100 (1%) lysate from monkey (*Macaca mulatta)* cerebella was centrifuged at 21,000× *g* at 4°C for 15 min and clear supernatants were incubated with the patient's serum (diluted 1:33) at 4°C for 3 h. The samples were then incubated with Protein G Dynabeads (ThermoFisher Scientific, Dreieich, Germany) at 4°C overnight to capture immunocomplexes. The beads were washed 3 times with PBS and eluted with NuPage LDS sample buffer (ThermoFisher Scientific) containing 25 mmol/L dithiothreitol at 70°C for 10 min. Carbamidomethylation with 59 mM iodoacetamide (Bio-Rad, Hamburg, Germany) was performed prior to SDS-PAGE (NuPAGE, ThermoFisher Scientific). Separated proteins were visualized with Coomassie Brillant Blue (G-250; Merck), and identified by mass spectrometric analysis or were applied for Western Blot.

Intrathecal antibody infusion

Experimental animals were randomized for the different treatment groups by an independent investigator. 10–12 weeks old C57BL6/J mice received either mAb 011-138 or control mAb mGO53 (100 μg over 7 days, 200 μg over 14 days, 1 μg/h). Antibody cerebroventricular infusion was performed using osmotic pumps (model 1002, Alzet, Cupertino, CA). Pump characteristics included: volume 100 μl and flow rate 0.25 μl/h. For pump implantation, mice were placed in a stereotaxic frame and a cannula was inserted into the right ventricle (coordinates: 0.2 mm posterior and ± 1.00 mm lateral from bregma, depth 2.2 mm). The cannula was connected to a pump, which was subcutaneously implanted in the interscapular space of the animals. After pump implantation, the animals were daily monitored to assess clinical symptoms and weight variations. Mice were sacrificed either on day 7 or day 14, brains were removed and snap-frozen in 2-methylbutan for immunohistochemistry. In addition, blood samples were collected for serum preparation (2,000× *g* for 15 min, RT). Unfixed sections from mouse brains were either incubated with FITC-coupled anti-human IgG secondary antibody alone or serum from treated mice prior to the application of secondary antibody.

Fluorescence intensity measurements

To analyze the intensity of IgG binding to the brain blood vessel images were taken at 40× magnification using a Leica DMLB epifluorescence microscope. Image areas attributed to vessel walls were cropped and average gray scale brightness values were calculated by the histogram function of Adobe Photoshop CS6 software.

Results

Autoimmunity to brain blood vessels and Purkinje cells

We have previously reported that the CSF antibody repertoire from patients with NMDAR encephalitis and $GABA_AR$ encephalitis does not only include mAbs that are autoreactive to the disease-defining antigen. Besides neuronal and glial binding, several CSF antibodies exhibit autoreactivity to brain blood vessels with yet unknown functional relevance. Here, we systematically screened 149 CSF-derived mAbs from six autoimmune encephalitis patients, including 67 from $GABA_AR$ encephalitis, 61 from NMDAR encephalitis, and 21 from non-$GABA_AR$/non-NMDAR encephalitis patients for blood vessel autoreactivity in murine brain tissue. We identified six mAbs with prominent brain blood vessel reactivity, of which three (113-109, 113-111, 113-126) had been isolated from a $GABA_AR$ encephalitis patient (Nikolaus et al., 2018; Kreye et al., 2021) and three (080-221, 003-151, 011-138) from NMDAR encephalitis patients (Kreye et al., 2016). Patient details can be obtained from these previous publications.

Among the three blood vessel reactive antibodies obtained from one young patient with $GABA_AR$ encephalitis, mAb 113–109 provided remarkably strong staining of vessels of all diameters throughout the entire brain (**Figure 1A**). The homogeneous staining of mAb 113–109 throughout brain blood vessels of all sizes suggested an epitope structure present up to the capillary level. Blood vessel staining was further confirmed using co-stainings against the basal membrane constituent Collagen IV and the blood vessel endothelial cell marker CD31 and is shown exemplarily (**Figure 1B**). In contrast, mAbs 113-111 and 113-126 obtained from the same patient stained primarily large-sized vessels (**Figures 1C,D**, see also **Supplementary Figure 1** for co-stainings with CD31). Additionally, in blood vessels isolated from adult mouse brains, all three antibodies showed clear staining on the 30–100 μm fraction of brain blood vessels (**Figure 1E**).

An additional three blood vessel reactive mAbs were detected among the recombinant mAbs derived from the CSF of anti-NMDAR encephalitis patients. These include mAb 080-221 which showed prominent staining of vessels of all sizes including the capillaries (**Figure 2A**, for capillary staining, see inset a, see also **Supplementary Figure 1**). In contrast, the other two mAbs predominantly stained mid to large-size blood vessels. Vessels stained by mAb 003–151 showed a less homogeneous and more speckled pattern (**Figure 2B** and **Supplementary Figure 1**). In addition to the strong reactivity

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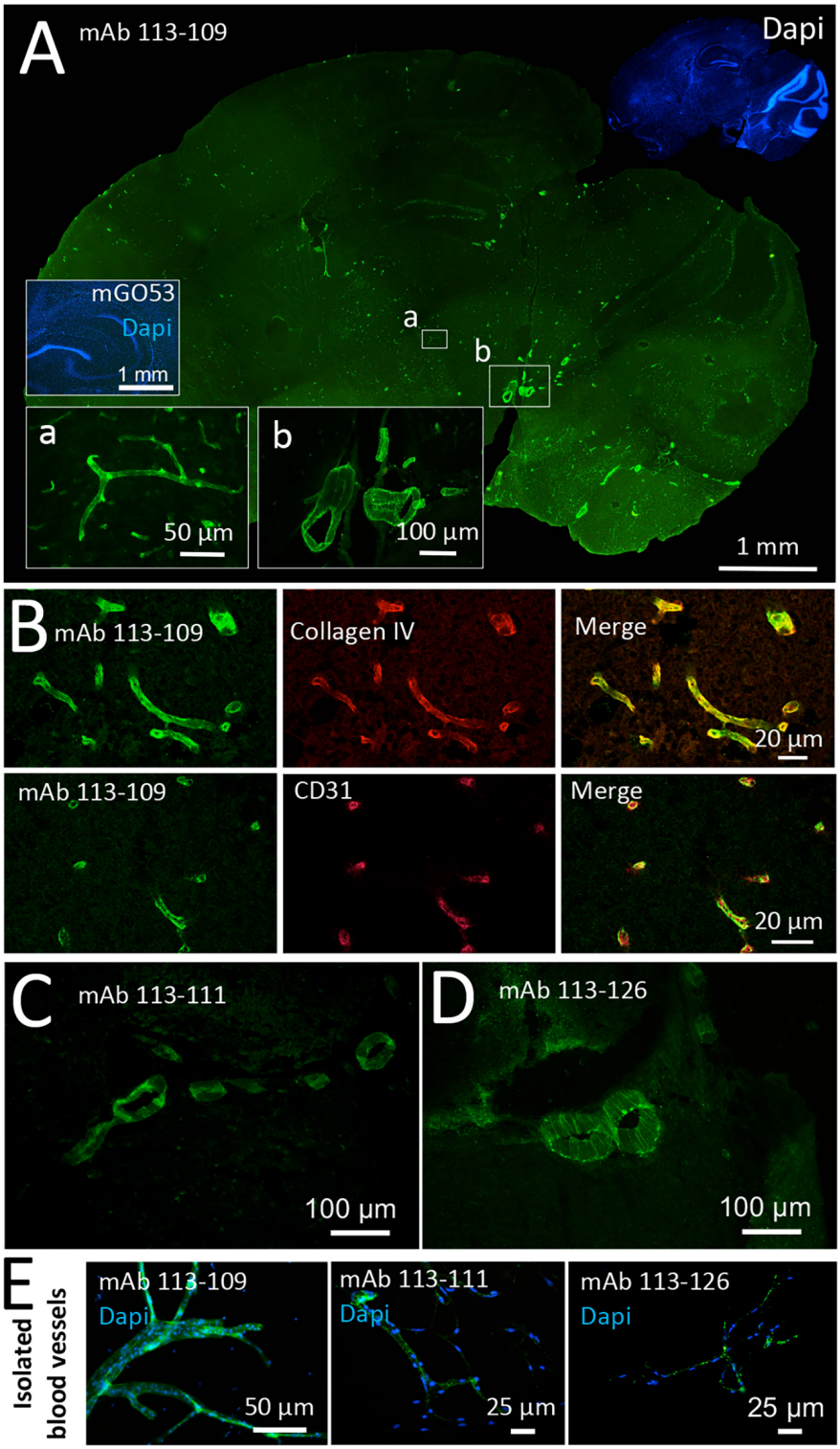

FIGURE 1
Recombinant monoclonal antibodies from a $GABA_AR$ encephalitis patient bind to brain blood vessels. Sections from unfixed and unpermeabilized adult mouse brains were incubated with 5 µg/ml of the respective monoclonal antibodies obtained from a patient diagnosed with $GABA_AR$ encephalitis. Visualization of tissue binding was performed using a FITC-coupled anti-human IgG secondary antibody. **(A)** Sagittal brain section incubated with human monoclonal antibody (mAb) 113-109. Prominent staining of blood vessels of all diameters in all brain regions was obtained (see insets a and b). Incubation with control mAb mGO53 yielded no tissue staining (inset). **(B)** Double stainings against Collagen IV (upper panel) and CD31 (lower panel) confirm immunoreactivity of mAb 113-109 to blood vessels. **(C)** Sagittal brain section incubated with mAb 113-111. Prominent staining of large blood vessels was obtained in all brain regions (the area between the cerebellum and midbrain is shown). **(D)** Sagittal brain section incubated with mAb 113-126. Again, prominent staining of large blood vessels in all brain regions was obtained (the area between the cerebellum and midbrain is shown). **(E)** Stainings of isolated brain blood vessels. Blood vessels were obtained from homogenized mouse brains by combinatory centrifugation and filtering steps. A 30-100 µm filter size fraction incubation with all three antibodies resulted in clear staining of the vessels within this fraction. Note that the diameter of mounted blood vessels is subject to shrinking artifacts during the staining procedure.

to brain blood vessels (Figure 2C), mAb 011-138 reacted with cerebellar Purkinje cells, leading to pronounced somatic staining (Figure 2C, inset d). To further characterize mAb 011-138 reactivity, we double-stained with smooth muscle actin (SMA) on murine brain slices (Figure 2D). The mAb 011-138 signal exhibited a high degree of overlap with SMA-positive smooth muscle cells of the vessels supporting our previous observation that the patient mAb predominantly recognizes large to mid-size vessels. Again, Collagen IV and CD31 stainings were applied to mark blood vessels (Figure 2E). Additionally, reactivity to brain blood vessels was confirmed using purified murine brain vessels sized 30–100 μm (Figure 2F).

In this cohort of mAbs, antibody 011-138 stood out due to its combinatory reactivity to brain blood vessels and a defined group of neuronal cells—cerebellar Purkinje cells, making mAb 011-138 a particularly interesting candidate for further investigation in terms of target identification as well as possible mechanistic effects.

Monoclonal antibody 011-138 reduces TEER in an *in vitro* BBB model and decreases the expression of cell junction protein occludin

To test for putative pathophysiological effects on blood vessels we applied an *in vitro* model for the analysis of BBB disruption. Human cerebral microvascular endothelial cells (hCMEC/D3; Weksler et al., 2005) represent an established model to mimic the *in vivo* phenotype of the BBB and are commonly used to investigate pathomechanisms and transport processes (Helms et al., 2016). Functional evaluation of endothelial monolayer integrity in response to antibody treatment was quantified with transendothelial electrical resistance (TEER) measurements. For our purposes, hCMEC/D3 cells were grown to confluency as exemplarily shown by fluorescent staining of adherens junction protein VE-Cadherin and endothelial cell marker CD34 (Figures 3A,B). We assessed TEER changes in response to 24 h and 48 h of treatment with patient mAbs in comparison to non-reactive control mAb mGO53 (Figures 3C–E). When hCMEC/D3 cells were exposed to mAbs obtained from the patient with $GABA_AR$ encephalitis as well as mAbs 080-221 and 003-151 from patients with NMDAR encephalitis, TEER values did not significantly decrease, and barrier breakdown could not be observed. For 24 h treatment with mAb 080-221 an increase in TEER was seen (Figure 3C). Conversely, the results showed that treatment with mAb 011-138 led to a significant reduction of TEER after 48 h but not after 24 h (Figure 3E). A significant reduction of TEER after 48 h was achieved using 5 μg/ml and was slightly stronger with a concentration of 10 μg/ml.

In the following, we investigated the fluorescent binding pattern of mAb 011-138 in hCMEC/D3 cells. Incubation of fixed and permeabilized cells revealed a stress fiber-like filamentous staining pattern that included the plasma membrane region not observed with control mGO53 (Figures 3F,G). Incubation of live cells with 5 μg/ml mAb 011-138 resulted in a plasma membrane-like staining of the cell periphery (Figure 3H). The reactiveness of antibody 011-138 to hCMEC/D3 cells was also tested using Western Blot analysis. Blotted cell lysates were incubated with 5 μg/ml of either mAb 011-138 or mGO53 for control (Figure 3I). Incubation with antibody 011-138 yielded a major immunoreactive band slightly above 200 kDa and a few lower bands around the 130 kDa marker. Incubation with control mAb mGO53 yielded no bands.

To investigate the underlying mechanisms of BBB disruption, the expression of barrier-constituting junctional proteins was evaluated after treatment with mAb 011-138 for 48 h in comparison to the control antibody (Figure 3J). Resulting Western blots yielded a significant decrease of Occludin expression in hCMEC/D3 cells. The decreased expression of Occludin was confirmed on the mRNA level using qPCR (Figure 3L). Downregulation of Occludin by mAb 011-138 treatment was not only observed in whole cell homogenates but was also evident for plasma membrane-enriched cell fractions (Figure 3K). VE-Cadherin, Claudin5, and Zonula occludens protein-1 (ZO-1) expression was not significantly altered. Concordantly, qPCR experiments did not show significant genetic changes (Figure 3L).

As an integral component of tight junctions, Occludin may therefore contribute to the decreased TEER.

In vivo reactivity of mAb 011-138 to brain blood vessels and decreased expression of occludin

To first confirm the detectable presence of recombinant mAb 011-138 in patients CSF, we stained CSF-011 on unfixed murine brain sections. This showed simultaneous reactivity in vessels and Purkinje cells as well in addition to the typical NMDAR distribution (Figure 4A). Prompted by these findings and to investigate the effects of mAb 011-138 *in vivo*, mice were intrathecally infused with mAb 011-138 or isotype control using an osmotic pump system for continuous delivery into the CNS (Figure 4B). Detection of human IgG after 7 and 14 days of infusion (100 μg for 7 days and 200 μg for 14 days were administered) showed mid to large-size blood vessels staining throughout different brain regions in mAb 011-138 infused animals (exemplary vessel staining of 14-day infusion: Figure 4C). In contrast, sections of animals that received the control antibody mGO53 for infusion did not show any staining (Figures 4D,F), whilst the presence of vessels containing the target vessel wall structures was ensured by SMA-staining. From the same animals, serum was collected to check for the access of antibodies to the bloodstream to provide a source of blood vessel reactive antibodies. Staining of unfixed wild-type murine brain slices with sera from mAb 011-138 treated animals showed the same staining pattern of large to mid-size blood vessels as the previously described secondary antibody treated brain sections from animals that received mAb 011-138 intrathecally (Figures 4E,G). These findings demonstrate the capacity of antibody 011-138 to bind to its target structure *in vivo* and suggest access to the blood system from the CSF compartment in our experimental paradigm. This could represent an antibody effect as well as a lesion-induced phenomenon or be due to a physiologically occurring FcRn-mediated process.

Additionally, we conducted Western blot experiments to assess the expression of Occludin in brain tissue of animals intrathecally infused with mAb 011-138 compared to the control antibody. We found Occludin expression to be significantly decreased, matching our *in vitro* findings using hCMEC/D3 cells (Figure 4H). As already observed *in vitro*, other tight junction proteins such as VE-Cadherin,

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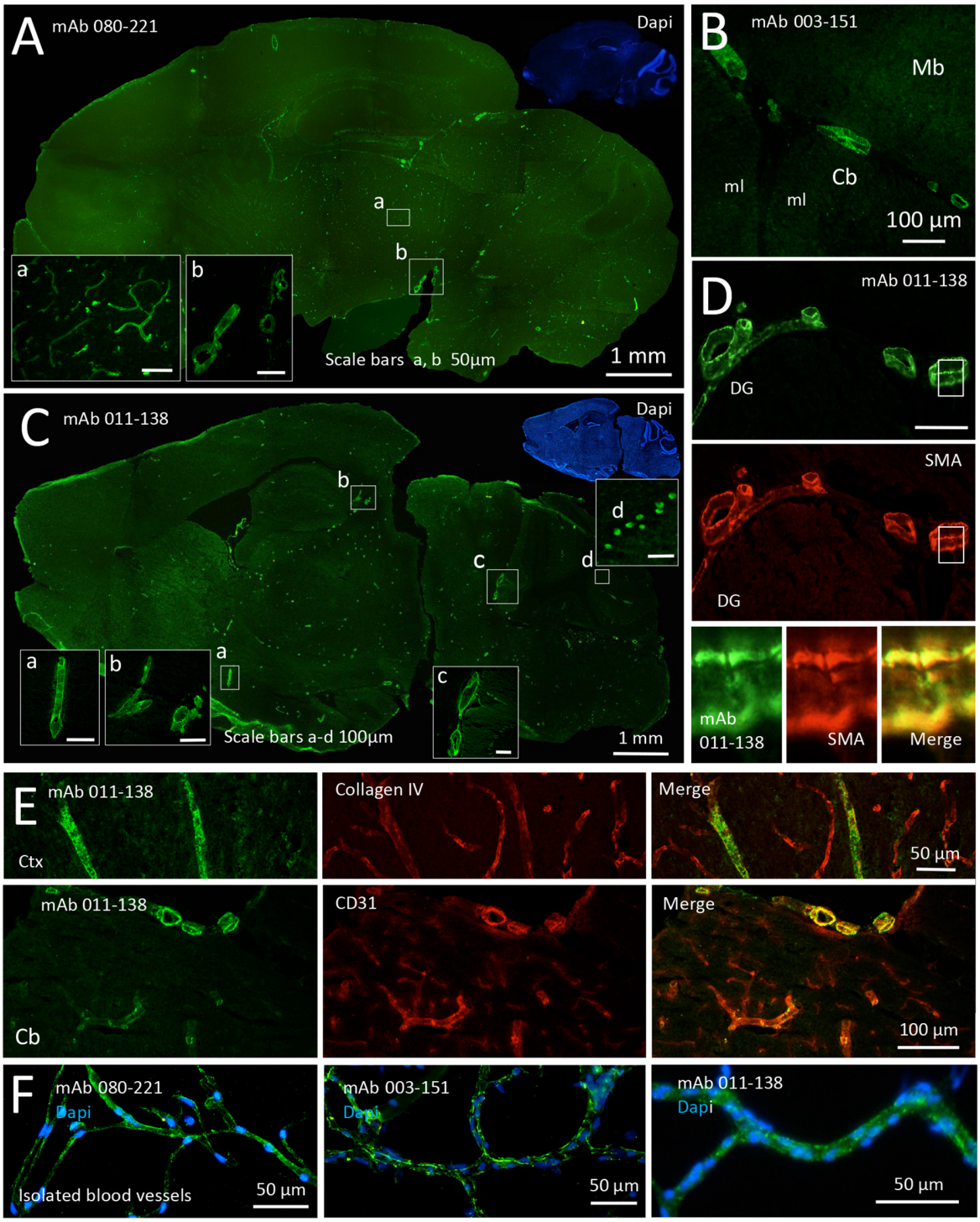

FIGURE 2
Recombinant monoclonal antibodies from NMDAR encephalitis patients bind to brain blood vessels and Purkinje cells. Unfixed and unpermeabilized sections from adult mouse brains were incubated with 5 µg/ml of the respective human monoclonal antibodies (mAbs) obtained from three different patients diagnosed with NMDAR encephalitis. Visualization of tissue binding was performed using a FITC-coupled anti-human IgG secondary antibody. **(A)** Sagittal brain section incubated with mAb 080-221. Prominent staining of large to small blood vessels including capillaries was obtained in all brain regions (see insets a and b). **(B)** Sagittal brain section incubated with mAb 003-151. Prominent staining of large to mid-size blood vessels in all brain regions was obtained (a section of cerebellum, *Cb*, and midbrain, *Mb*, is shown; *ml* molecular layer). **(C)** Sagittal brain section incubated with mAb 011-138. Prominent staining of large to mid-size blood vessels in all brain regions was obtained (see insets a, b, and c). In addition to blood vessel staining cerebellar Purkinje cells showed marked somatic staining (see inset d). **(D)** Double staining against human IgG and SMA (smooth muscle actin) in a brain section adjacent to the dentate gyrus (*DG*) of the hippocampus formation. Bound human IgG was mainly detected within the SMA-positive muscle layer of the vessels. **(E)** Double stainings against Collagen IV (upper panel) and CD31 (lower panel) confirm immunoreactivity of mAb 011-138 to blood vessels. *Ctx Cortex* **(F)** Staining of isolated brain blood vessels. Blood vessels were obtained from homogenized mouse brains by combinatory centrifugation and filtering steps. A 30–100 µm filter size fraction incubation with all three antibodies resulted in clear staining of the vessels within this fraction. Note that the diameter of mounted blood vessels is subject to shrinking artifacts during the staining procedure.

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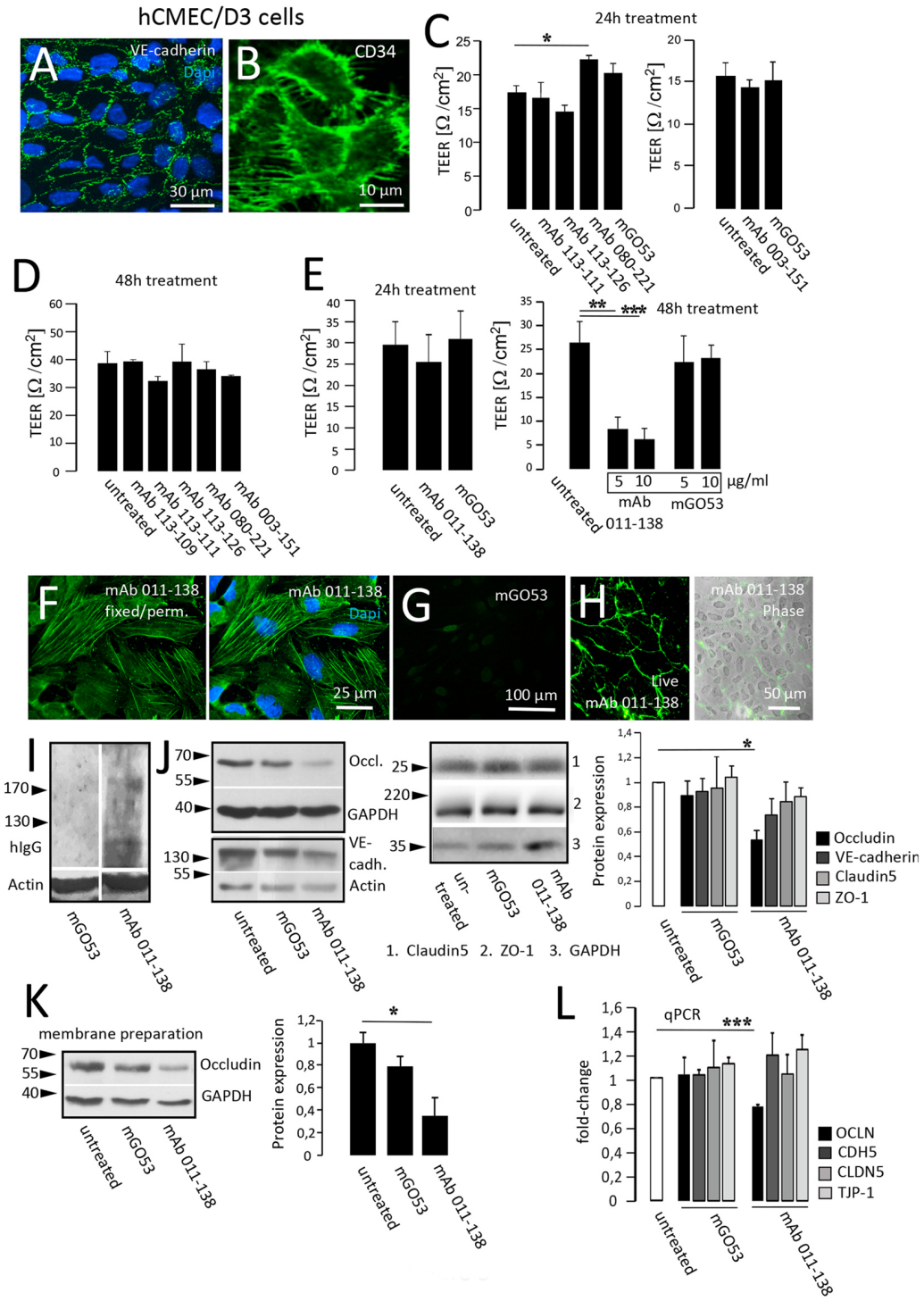

FIGURE 3
Antibody 011-138 decreases transendothelial electrical resistance and downregulates tight junctional Occludin. **(A,B)** Human cerebral microvascular endothelial cells (hCMEC/D3) were used as an *in vitro* blood-brain barrier model. Tight junction protein VE-cadherin and the endothelial cell marker CD34 were clearly expressed and depicted adjacent cell boundaries. The following functional experiments were carried out on live cells grown to confluence. **(C)** After 24 h of antibody treatment at 5 µg/ml, none of the human monoclonal antibodies (mAb) decreased TEER, only mAb 080-221 increased TEER. **(D)** No effects on TEER were observed after incubation for 48 h with 5 µg/ml with the respective antibodies. **(E)** Incubation with 5 µg/ml mAb 011-138 for 24 h had no significant effects on TEER. Conversely, incubation for 48 h resulted in a highly significant reduction of electrical resistance by mAb 011-138, but not mGO53, used as control Ab. Values analyzed in triplicates are expressed as means ± SEM from a representative experiment that was at least repeated once per condition **(C–D)** or from three independent experiments **(E)**. **(F–H)** Immunofluorescence detection of mAb 011-138 binding (5 µg/ml) to either live or fixed and permeabilized hCMEC/D3 cells. Fixed cells revealed a filamentous signal of mAb 011-138, and live cells showed a rather surface localized signal. Incubation with mGO53 resulted in no signal under either condition (**G**, shown for fixed cells). **(I)** Western blotting of hCMEC/D3 cell homogenates with either mAb 011-138 or control mGo53. Bound monoclonal 011-138 expressed higher molecular weight bands with a prominent signal at or above 200 kDa, mGO53 revealed no immune signal. **(J)** Confluent hCMEC/D3 cells were incubated for 48 h

(*Continued*)

FIGURE 3 (Continued)
with 5 μg/ml mAb 011-138 or mGO53. Western blotting for detection of Occludin, VE-cadherin, Claudin5, and ZO-1 expression. GAPDH or Actin were used as loading control. Incubation with mAb 011-138 resulted in a significant downregulation of Occludin expression exclusively. Values adjusted to loading are expressed as means ± SEM from 4–5 independent experiments. **(K)** Membrane preparations were performed from hCMEC/D3 cells following incubation with 5 μg/ml mAb 011-138 or mGO53. Western blot analysis revealed a significant removal of Occludin from the membrane compartment largely consisting of plasma membrane fractions following incubation with mAb 011-138. Western blot values adjusted to loading are expressed as normalized means ± SEM from three independent experiments. **(L)** Additionally, quantitative RT-PCR was performed to check for alterations in gene regulation of tight junction proteins. Calnexin (CANX) was used as endogenous control. Significantly decreased mRNA levels were found for Occludin (OCLN), the other genes (CDH5 VE-cadherin; CLDN5 Claudin5, and TJP-1 ZO-1) remained unchanged. Quantitative PCR values are expressed as means ± SEM from three independent experiments. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

Claudin5, and ZO-1 were not significantly altered by the injection of mAb 011-138 (**Supplementary Figure 2**), thereby confirming the specificity of the effects on Occludin.

Unconventional Myosin-X represents a target epitope of mAb 011-138

In brain biochip tissues of monkey and rat cerebellum, mAb 011-138 yielded a very similar staining pattern to the one observed in mouse brain (**Figure 5A**). To identify the target of mAb 011-138 we performed immunoprecipitation studies with rodent aorta lysates, purified mouse brain vessels, and monkey brain lysates as antigen-providing tissues and repeatedly received various conventional and unconventional myosins. Exemplarily, a Western blot is shown for an immunoprecipitation experiment using monkey brain lysate (**Figure 5B**). Incubation of the precipitated protein fraction with mAb 011-138 showed a distinct band around 240 kDa corresponding to the molecular weight of many myosin isoforms, together with a lower band around 80 kDa. Reactivity to the precipitating heavy chain around 55 kDa was shared also by other precipitating human monoclonal antibodies used for control. We were aware of the fact that myosins tend to be "sticky" and, therefore, are often pulled down unspecifically during immunoprecipitation. However, the combined occurrence of strong immunoreactivity in brain blood vessels together with the reactivity to cerebellar Purkinje cells matched the published brain distribution of unconventional Myosin-X (Sousa et al., 2006) and therefore prompted us to test for reactivity of mAb 011-138 to Myosin-X.

The binding of mAb 011-138 to Myosin-X was first investigated in a cell-based assay. Expression of eGFP-tagged Myosin-X in HEK cells was verified using a commercial anti-Myo-X antibody (**Figure 5C**). Incubation with mAb 011-138 resulted in specific binding to transfected cells with a high degree of signal overlap between eGFP-MyoX and patient antibody signals (**Figures 5D–F**). Furthermore, confirmation of CSF immunoreactivity to Myo-X was received by prominent staining of transfected cells with CSF of patient 011 (**Figure 5G**). Further supporting our finding, patient antibody 011-138 and a commercial Myosin-X antibody stained mouse brain Purkinje cells and blood vessels in a very similar fashion with a high degree of signal overlap between both antibodies (**Figures 6A,B**). Incubation of hCMEC/D3 cells with commercial anti-Myo-X antibody revealed a similar, although not fully identical staining pattern (**Figure 6C**). Double staining with mAb 011-138 revealed a partial overlap of both signals being most prominent at the cell periphery (**Figure 6C** insets a and b). Expression of Myosin-X protein in hCMEC/D3 cells was verified by Western blotting (**Figure 6D**). Furthermore, while mRNA levels remained unchanged, we detected a decrease in Myosin-X expression at the protein level in mAb 011-138 treated cells (**Figure 6D**).

Discussion

This qualitative study is the first to characterize a subgroup of brain blood vessel reactive autoantibodies in autoimmune encephalitis patients. Our data shows a range of binding patterns which point towards a bandwidth of possible target antigens. Results from our *in vivo* experiments suggest that blood vessel reactive autoantibodies are capable of binding to brain vessel epitopes when administered to the CSF. Furthermore, our results indicate –though still limited to a small number of cases- ways of potential principal contribution from blood vessel reactive antibodies to the pathomechanisms of autoimmune encephalitis as a setscrew in BBB disruption.

The growing interest in the pathomechanisms of autoimmune encephalitis has strongly fueled research efforts and thereby improved our understanding—especially of disease-defining autoantibodies targeting extracellular proteins. However, studies have shown that additional antibodies coexist in patients' CSF. In fact, non-disease-defining autoantibodies make up the majority of the antibodies generated (Kreye et al., 2016). Furthermore, antibody-associated neurological diseases typically present with a wide range of clinical symptoms (Titulaer et al., 2013), with the result that variable clinical pictures can be associated with the same antibody. Since antibody titers only partially correlate with the clinical course (Gresa-Arribas et al., 2014), other contributing factors are suspected. Such as differential epitope specificity, strong differences in affinity, and contribution of low-affinity antibodies currently eluding diagnostic observation (Ly et al., 2018; Wagner et al., 2020). The contribution of coexisting non-disease-defining antibodies remains unresolved. Utilizing recombinant human monoclonal antibodies allowed for this study to vastly eliminate unspecific effects of serological components and attribute observed binding patterns and functional effects to single mAbs. This unbiased approach of screening mAbs on murine unfixed brain sections has proven useful in previous exploratory antibody studies (Kreye et al., 2016, 2021).

In this study, we focused on CSF-derived mAbs with reactivity against blood vessels from patients with autoimmune encephalitis. $GABA_AR$ and NMDAR encephalitis patient-derived mAbs exhibited diverse binding to brain blood vessels, suggesting that mAbs likely target several different antigens on the brain vasculature. Interestingly, even within the same $GABA_A$ receptor encephalitis patient (113) blood vessel reactive antibodies showed differing binding patterns. This is in line with the observed variability of non-$GABA_A$ receptor reactive neuronal antibodies, shown previously on murine brain tissue (Kreye et al., 2021). However, overall blood vessel reactive mAbs can be roughly divided into two "pattern groups". One comprised of mAbs with reactivity to mid- to large size vessels,

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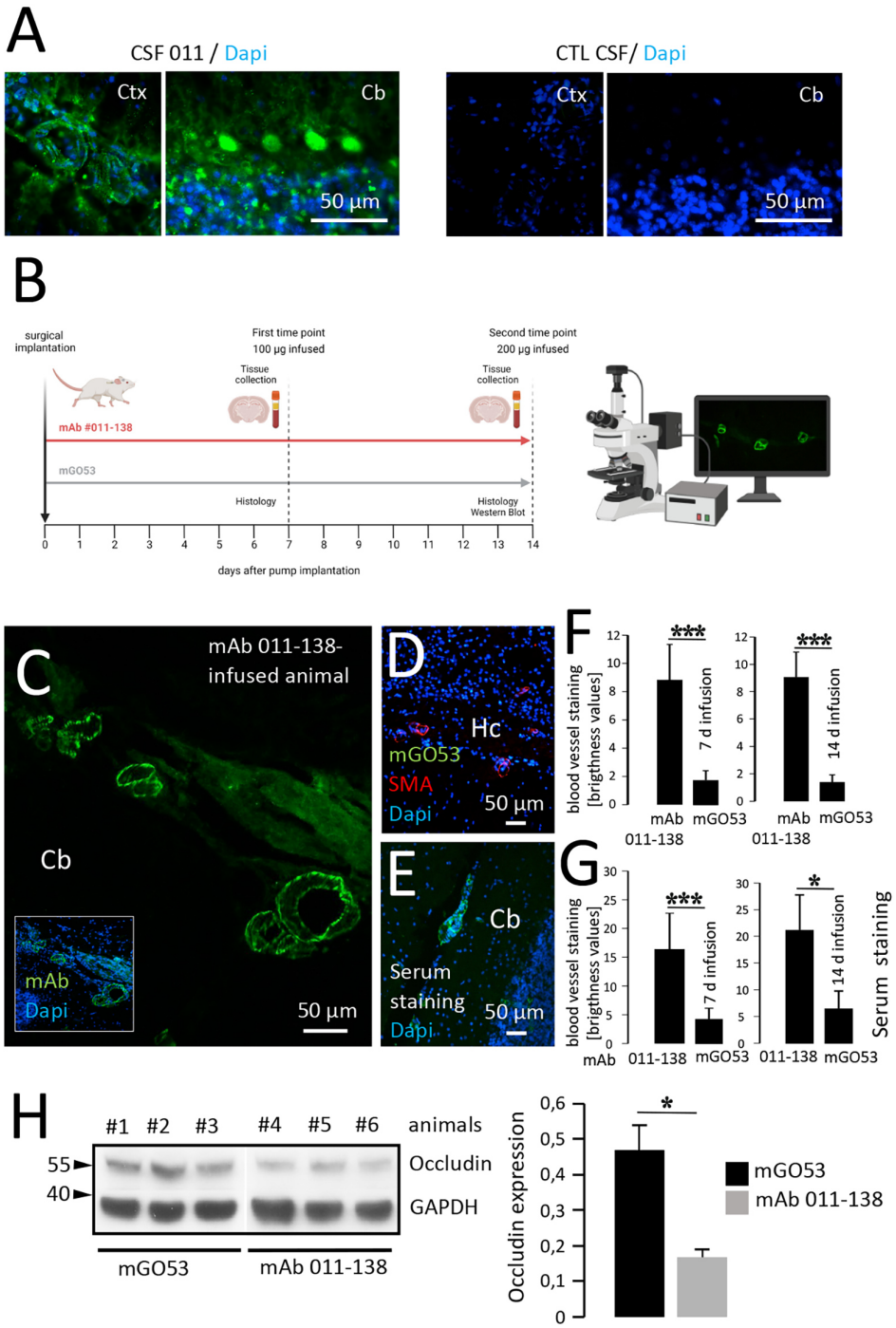

FIGURE 4
Intrathecal application of antibody 011-138 leads to *in vivo* blood vessel binding and Occludin downregulation. **(A)** Sections from unfixed adult mouse brains were incubated with CSF of patient 011 and an age-matched control patient at a dilution of 1:2. Incubation with 011 CSF resulted in IgG staining of blood vessels and cerebellar Purkinje cells. Incubation with control CSF yielded no staining. **(B)** Adult mice were either administered a dose of 100 µg of human monoclonal antibody (mAb) 011-138 into the right lateral ventricle for 7 days or 200 µg for 14 days. Animals were sacrificed, brains were removed, and immediately frozen for immunohistochemistry. In addition, blood was collected to obtain serum. **(C)** Representative sagittal brain section from an animal treated for 14 days with mAb 011-138 was incubated with FITC-coupled anti-human IgG. Clear staining of large to mid-size blood vessels in all brain regions was visible (shown for the cerebellum, *Cb*). **(D)** Sagittal brain section from one animal treated with control antibody mGO53 for 14 days. Incubation with a secondary antibody revealed no staining *Hc = hippocampus*. **(E)** Sagittal unfixed brain section from an untreated adult mouse was incubated with serum (dilution 1:200) from an animal that had received mAb 011-138 for 14 days. Visualization with secondary antibody revealed staining of larger to mid-size blood vessels by the mouse serum. **(F)** Quantification of blood vessel IgG immunoreactivity. Data are given as means ± SEM from three animals per condition. Per condition, between 38 and 43 blood vessel sections were analyzed. ***$p \leq 0.001$. **(G)** Quantification of serum blood vessel IgG immunoreactivity on naive brain sections. Data are given as means ± SEM from three animals per condition. Per condition, between 22 and 45 blood vessel sections were analyzed. *$p \leq 0.05$, ***$p \leq 0.001$. Brightness levels of mGO53 staining were within the background range. **(H)** Downregulation of Occludin by mAb 011-138. In brains of mice treated with mAb 011-138 for 14 days protein expression was strongly reduced by 65% compared to animals that had received mGO53. Data are given as means ± SEM adjusted to loading from seven animals per condition from two independent experiments. *$p \leq 0.05$. Three animals per condition from one experiment are exemplarily shown.

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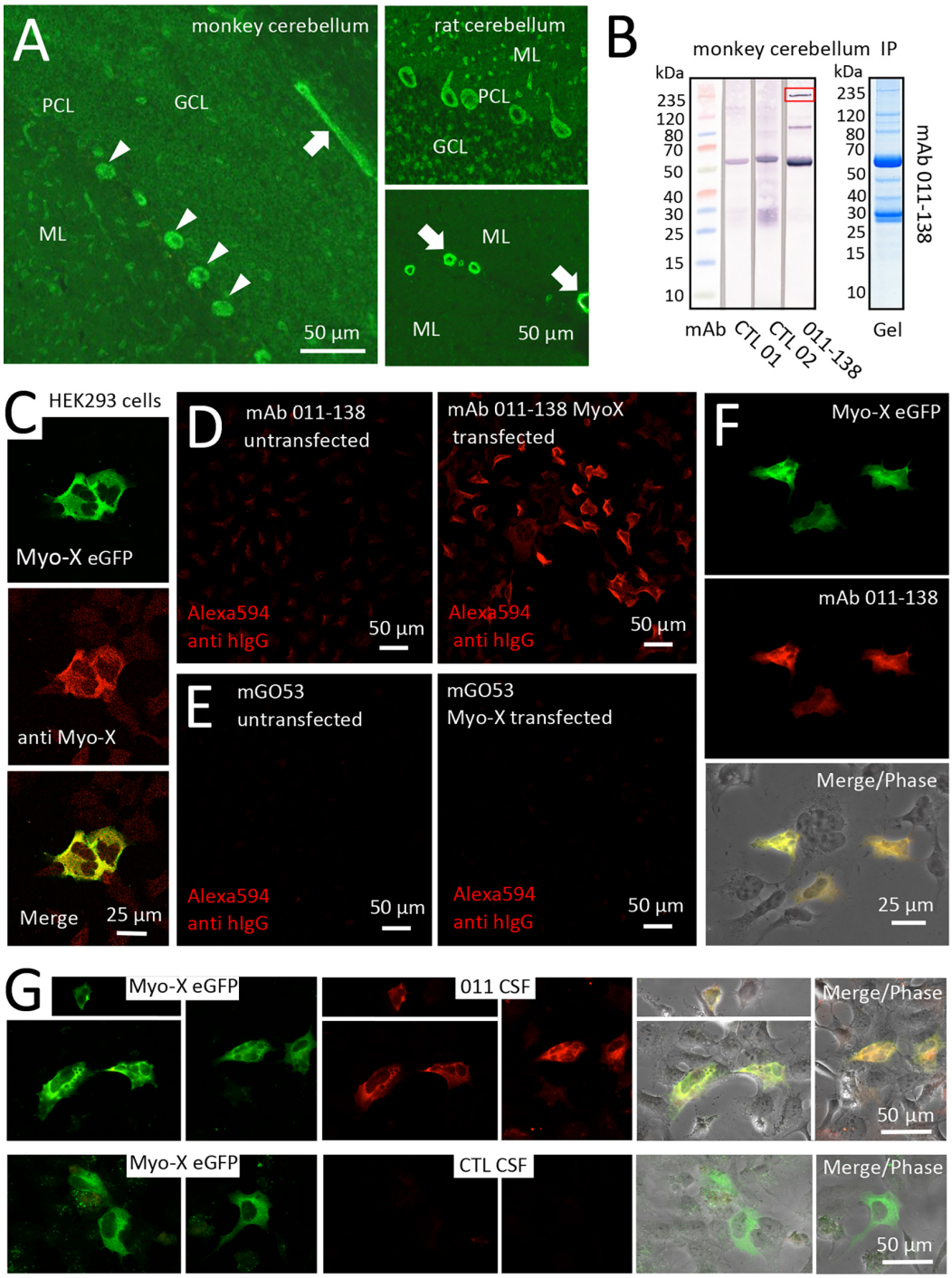

FIGURE 5
Antibody 011-138 targets Myosin-X in transfected HEK cells. **(A)** Monkey and rat brain biochip cryosections (EUROIMMUN AG) were incubated with human monoclonal antibody (mAb) 011-138 (1:100) and exhibited pronounced staining of blood vessels (left panel and lower right panel arrows) and Purkinje cells (left panel arrowheads and upper right panel). **(B)** Immunoprecipitation analysis using monkey brain lysates. Lysates were incubated with either mAb 011-138 or two other non-vessel reactive human monoclonal antibodies for control. Dynabeads were used to precipitate antibodies together with bound antigens. Elute fractions were again incubated with the precipitating antibody followed by Western blotting. The marked band at 55 kDa in all three lanes presumably resulted from detection of the precipitating heavy chain by the detecting secondary anti-human IgG conjugate. In addition, mAb 011-138 showed two distinct bands at 80 and 240 kDa (boxed), respectively. The corresponding Coomassie gel is shown for mAb 011-138. **(C)** HEK 293 cells were transfected with an eGFP construct of human Myosin-X (Myo-X) plasmid DNA for 24 h. Expression of Myo-X was verified in fixed cells using a commercial monoclonal anti-Myo-X antibody showing a very high degree of signal overlap (confocal imaging). **(D)** Incubation of Myo-X-transfected cells with 5 µg/ml of 011-138 antibody showed binding to fixed transfected cells that were absent in untransfected cells. A secondary anti-human IgG antibody coupled to Alexa594 was used for detection. **(E)** For negative control untransfected and Myo-X-transfected cells were incubated with 5 µg/ml of mGo53 antibody. No staining was observed under either condition. **(F)** In transfected cells incubated with 011-138 antibody the human IgG signal showed a very high degree of overlap with eGFP-Myo-X signal. **(G)** HEK 293 cells were transfected with an eGFP construct of human Myo-X plasmid DNA for 24 h. Incubation of Myo-X-transfected cells with patient-CSF 011 showed binding that was absent after incubation with control-CSF. The right panel shows a high degree of overlap of patient-CSF 011 signal with eGFP-Myo-X signal.

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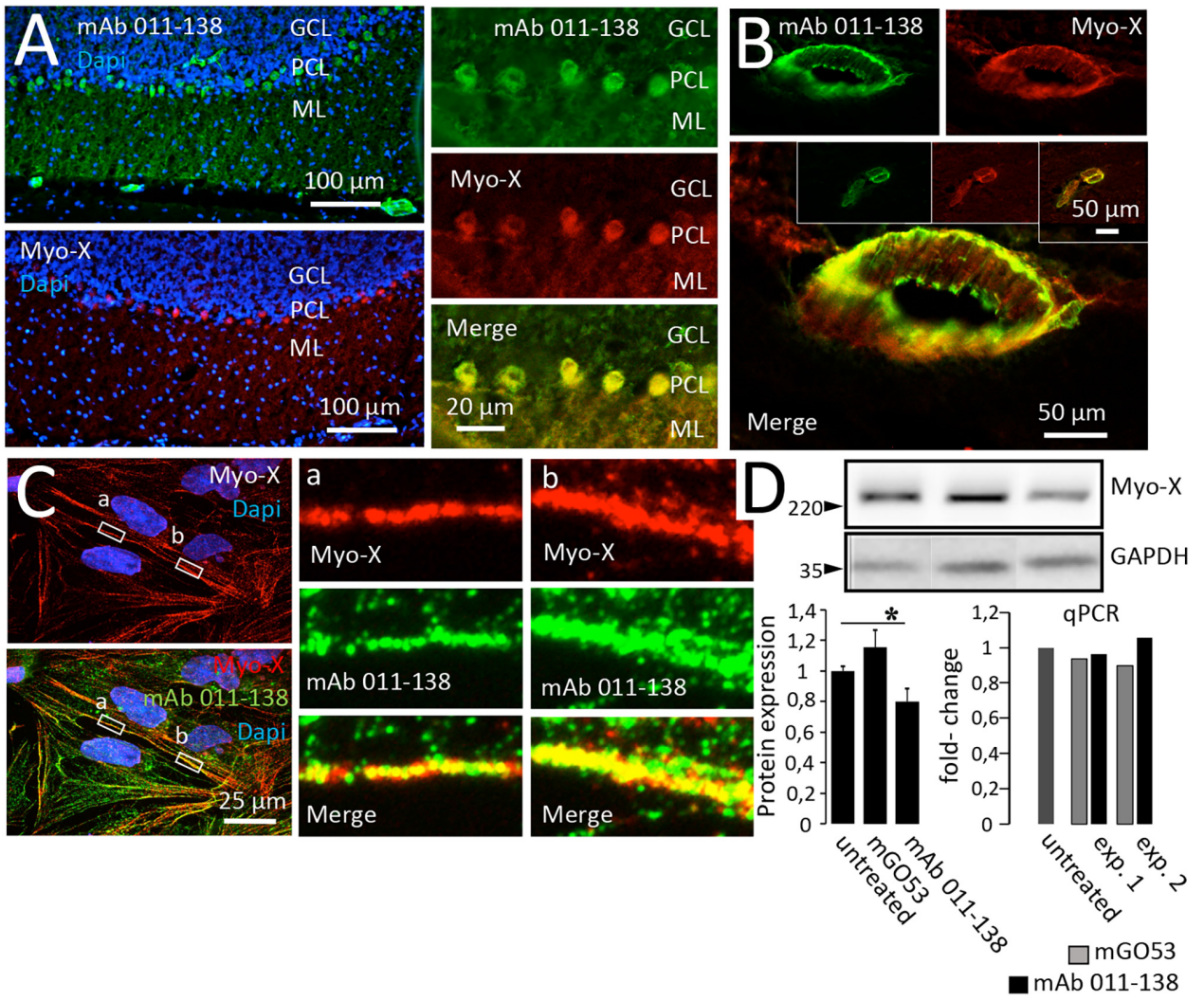

FIGURE 6
Antibody 011-138 colocalizes with commercial anti-Myosin-X antibodies in brain sections and hCMEC/D3 cells and downregulates Myosin-X. **(A)** Unfixed and unpermeabilized sections from adult mouse brains were incubated with 5 µg/ml of human monoclonal antibody (mAb) 011-138 and a commercial monoclonal mouse antibody directed against unconventional Myosin-X (Myo-X). Shown is the 3-layer cerebellar cortex consisting of the granule cell layer (GCL), Purkinje cell layer (PCL), and outermost molecular layer (ML). Incubation with mAb 011-138 resulted in staining of the Purkinje cell somata and larger blood vessels (upper panel). Staining with commercial anti-Myo-X antibody showed a comparable pattern (lower panel, no larger vessels present). Double staining revealed a high degree of signal overlap between patient 011-138 and commercial MyoX antibodies in Purkinje cells. **(B)** Likewise, double staining revealed a high degree of signal overlap between patient 011-138 and commercial Myo-X antibodies in mid-size (insets) to larger blood vessels. **(C)** Double incubation of fixed hCMEC/D3 cells with antibody 011-138 and commercial anti-Myosin-X IgG. Both stainings yielded a similar staining pattern with partially overlapping signals (see insets). **(D)** Incubation of hCMEC/D3 cells with mAb 011-38 results in the downregulation of Myo-X. Following incubation of hCMEC/D3 cells with 5 µg/ml of mAb 011-138 or mGO53 as control for 48 h cells were homogenized and subjected to Western blotting and quantitative PCR to check for protein expression and gene regulation. Incubation with mAb 011-138 decreased protein expression of Myo-X by 20% (left chart), gene regulation was unaltered (right chart). Data are given as normalized means ± SEM adjusted to loading from two individual experiments analyzed in duplicates (Western blot) and as two individual experiments (qPCR). *$p \leq 0.05$.

while the second group mAbs reacted to vessels of all sizes including capillaries. The binding pattern in the second group points towards a potential target that is present throughout the entire vascular tree. Since this study had an exploratory approach and investigated only a small number of patients, the frequency of blood vessel reactive antibodies in autoimmune encephalitis remains to be ascertained in future studies targeting exactly this subgroup of antibodies in larger patient cohorts.

The BBB represents a critical gatekeeper between blood circulation and brain tissue. Its function is maintained mainly by an endothelial cell layer tightly sealed by Claudins, Occludin, and junction adhesion proteins like VE-cadherin (Rubin et al., 1991; Corada et al., 1999; Vorbrodt and Dobrogowska, 2003). Human hCMEC/D3 cells are among the most commonly used and best characterized *in vitro* BBB models (Helms et al., 2016). The integrity of the endothelial cell layer was measured through transendothelial electrical resistance (TEER), which is a widely used and accepted method (Srinivasan et al., 2015; Burek et al., 2019). We found mAb 011-138 to significantly decrease TEER values in the hCMEC/D3 BBB model after treatment for 48 h in comparison to the control antibody, indicating a disruption in the BBB integrity, which was not observed when other antibodies were applied. Mab 011-138 treated cells also showed a significant decrease in Occludin expression. No significant changes were observed for other junctional proteins, amongst them

VE-cadherin, which represents the main player at adherens junctions (Corada et al., 1999). Occludin is a tight junction specific protein with regulatory functions at the BBB. Among other mechanisms, altered expression of VE-cadherin and Occludin has been found to influence TEER and to associate with increased permeability of brain endothelial cells (Wang et al., 2001; Xu et al., 2012; Hebda et al., 2013; Mishra and Singh, 2013).

Our investigations *in vivo* show that intrathecally applied mAb 011-138 can reach its target in brain blood vessels. We replicated the mAb 011-138 characteristic binding pattern using patient CSF on unfixed murine brain sections. This ensures that the patient's CSF indeed contains mAb 011-138. Our *in vivo* experiments thus replicated our *in vitro* findings and support the concept of antibody-antigen binding of mAb 011-138 to brain blood vessel targets when present in patient CSF. Moreover, in line with previous *in vitro* findings, we detected decreased Occludin expression in brain lysates of mAb 011-138 treated animals. This undermines the involvement of Occludin in the effects of antibody treatment to endothelial layer integrity. Considering this ability of mAbs to decrease electrical resistance and change the expression of junction components it could be further speculated that they might also enhance the permeability of larger molecules, although this needs to be assessed in future studies. Specifically utilizing monoclonal antibodies in the future will allow for the attribution of effects to certain mAbs.

We provide conclusive evidence to propose Myosin-X as a target of blood vessel reactive patient mAb 011-138. Myosin-X is currently the first and only known representative of unconventional myosin class X (Berg et al., 2000). It is expressed in most tissues, although at low levels, including brain cerebellar Purkinje cells (Berg et al., 2000; Sousa et al., 2006). MAb 011-138 predominantly stained mid- to large sized blood vessels in unfixed unpermeablized tissue, which initially showed binding to vascular smooth muscle cell layer rather than to endothelial cells. However, previous studies have shown Myosin-X expression and function in endothelial cells (Almagro et al., 2010). This was also reflected in our detection of Myosin-X in hCMEC/D3 cells using Western blot and qPCR analysis. Since Myosin-X is expressed at low levels, it may have evaded indirect immunofluorescence staining by patient mAb 011-138 in unpermeabilized endothelial cells. In permeabilized hCMEC/D3 cells we were able to detect a distinct staining pattern with commercially available Myosin-X antibody, resembling filamentous structures. There remains the possibility that mAb 011-138 binds to multiple types of myosin. We are aware of the fact that intracellular targets such as Myosin-X intuitively appear to be shielded from antibody binding at first glance. Nevertheless, some naturally occurring antibodies such as anti-DNA antibodies in SLE possess the ability to penetrate living cells (Noble et al., 2016). Among the diverse mechanisms by which cell-penetrating antibodies enter the cell, antibodies targeting intracellularly located synapsin in patients presenting with limbic encephalitis (Piepgras et al., 2015) were identified that utilize Fcy II/III receptor mediated endocytosis to reach their cytosolic target (Rocchi et al., 2019). Moreover, further intracellular autoimmune targets have been identified such as glutamate decarboxylase 65 (GAD65) and Amphiphysin in patients with the brainstem, extrapyramidal and spinal cord dysfunction, and in stiff-person syndrome respectively (Pittock et al., 2006; Geis et al., 2010).

Consistent with other classes of myosins, Myosin-X acts as an actin-based molecular motor. Nevertheless, it is presumed to have further functions including actin-membrane interaction due to its unique domain composition (Berg et al., 2000). Myosin-X is present at regions of highly dynamic actin such as the tips of filopodia (Berg et al., 2000; Berg and Cheney, 2002) and has been proposed as a candidate for trafficking VE-cadherin (Almagro et al., 2010). Knockdown of Myosin-X in developing kidney epithelial cells leads to delayed recruitment of junction proteins E-cadherin and ZO-1 reflected by a delayed peak transepithelial electrical resistance (Liu et al., 2012). The same study found that even after maturing, the epithelial monolayers showed a higher paracellular permeability. These findings together with our observation of reduced TEER and downregulation of Occludin and Myosin-X by antibody treatment support the concept of Myosin-X playing an important role in the dynamics and kinetics in polarized cells such as endothelial cells. As a player in cytoskeleton trafficking and membrane interactions, Myosin-X represents an exciting target structure for further investigations in the context of antibody-mediated diseases. In addition to blood vessel binding, cerebellar Purkinje cells were also targeted by mAb 011-138. This cell type exhibits a marked Myosin-X expression throughout development (Sousa et al., 2006). Functional implications, however, were not addressed in this study and are subject to further investigations.

The involvement of BBB disruption in autoimmune encephalitis has been proposed previously (Dalmau et al., 2007; Kreye et al., 2021). In other antibody-mediated neurological diseases such as NPSLE breakdown of the BBB has been verified as a key feature (Kowal et al., 2004; Huerta et al., 2006; Hirohata et al., 2014). Additionally, recent findings of anti-GRP78 antibodies in NMO and PCD-LEMS have revealed that endothelial targeting antibodies enhance the transmigration of pathogenic IgG (Shimizu et al., 2017, 2019). A proceeding endothelial activation as part of an inflammatory response as shown in these studies is a conceivable mechanism in our patient cohort, although this needs to be further investigated. Studying vessel targeting antibodies is therefore particularly interesting, as they potentially unfold new perspectives on the development, progression, and variability of autoimmune encephalitis.

In conclusion, we have identified vessel-targeting antibodies in autoimmune encephalitis patients, identified a corresponding antigenic structure and demonstrated the potential cause of the observed endothelial disruption. Therefore, our findings provide additional qualitative evidence for the concept of antibody-mediated BBB disruption as a further identified mechanism in neuroinflammatory diseases.

Data availability statement

The original contributions presented in the study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving human participants were reviewed and approved by Institutional review board Charité-Universitätsmedizin Berlin (EA1/096/12). The patients/participants provided their written informed consent to participate in this study. The animal study was

reviewed and approved by the Landesamt für Gesundheit und Soziales in Berlin, Germany (approval number 0078/19) and performed in compliance with relevant national and international guidelines for care and humane use of animals.

Author contributions

LL, JK, MB, CC-G, SR, HP, and MH: contributed to the conception and design of the study. LL, JK, MB, CC-G, PB, ES-S, H-CK, DS, MS, PM, HP, and MH: contributed to the acquisition and analysis of data. LL, JK, MB, CC-G, PB, HP, and MH: contributed to drafting the text and preparing the figures. All authors contributed to the article and approved the submitted version.

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Conflict of interest

MS was employed by the company EUROIMMUN AG, Lübeck, Germany.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2023.1077204/full#supplementary-material.

SUPPLEMENTARY FIGURE 1
Co-stainings of monoclonal patient antibodies and CD31. Double stainings of mAbs 113-111, 113-126, 080-221, and 003-151 (5 μg/ml) with CD31 on unfixed murine brain sections confirm immunoreactivity of all four monoclonal antibodies to cerebral blood vessels.

SUPPLEMENTARY FIGURE 2
Tight junction proteins VE-cadherin, Claudin5, and ZO-1 are not downregulated *in vivo* by treatment with mAb 011-138. In brains from mice treated with mAb 011-138 or mGO53 (5 μg/ml) for control for 14 days, protein expression of VE-cadherin, Claudin5, and ZO-1 were unaltered. Data are given as means ± SEM adjusted to loading from four animals per condition. Immune signals from one animal per condition are exemplarily shown.

References

Almagro, S., Durmort, C., Chervin-Petinot, A., Heyraud, S., Dubois, M., Lambert, O., et al. (2010). The motor protein myosin-X transports VE-cadherin along filopodia to allow the formation of early endothelial cell-cell contacts. *Mol. Cell. Biol.* 30, 1703–1717. doi: 10.1128/MCB.01226-09

Armitage, J. D., Homer-Vanniasinkam, S., and Lindsey, N. J. (2004). The role of endothelial cell reactive antibodies in peripheral vascular disease. *Autoimmun. Rev.* 3, 39–44. doi: 10.1016/S1568-9972(03)00087-9

Berg, J. S., and Cheney, R. E. (2002). Myosin-X is an unconventional myosin that undergoes intrafilopodial motility. *Nat. Cell Biol.* 4, 246–250. doi: 10.1038/ncb762

Berg, J. S., Derfler, B. H., Pennisi, C. M., Corey, D. P., and Cheney, R. E. (2000). Myosin-X, a novel myosin with pleckstrin homology domains, associates with regions of dynamic actin. *J. Cell Sci.* 113, 3439–3451. doi: 10.1242/jcs.113.19.3439

Boulay, A. C., Saubamea, B., Decleves, X., and Cohen-Salmon, M. (2015). Purification of mouse brain vessels. *J. Vis. Exp.* 113:e53208. doi: 10.3791/53208

Burek, M., König, A., Lang, M., Fiedler, J., Oerter, S., Roewer, N., et al. (2019). Hypoxia-induced microRNA-212/132 alter blood-brain barrier integrity through inhibition of tight junction-associated proteins in human and mouse brain microvascular endothelial cells. *Transl. Stroke Res.* 10, 672–683. doi: 10.1007/s12975-018-0683-2

Corada, M., Mariotti, M., Thurston, G., Smith, K., Kunkel, R., Brockhaus, M., et al. (1999). Vascular endothelial-cadherin is an important determinant of microvascular integrity in vivo. *Proc. Natl. Acad. Sci. U S A* 96, 9815–9820. doi: 10.1073/pnas.96.17.9815

Dalmau, J., Tüzün, E., Wu, H., Masjuan, J., Rossi, J. E., Voloschin, A., et al. (2007). Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. *Ann. Neurol.* 61, 25–36. doi: 10.1002/ana.21050

Geis, C., Weishaupt, A., Hallermann, S., Grünewald, B., Wessig, C., Wultsch, T., et al. (2010). Stiff person syndrome-associated autoantibodies to amphiphysin mediate reduced GABAergic inhibition. *Brain* 133, 3166–3180. doi: 10.1093/brain/awq253

Gresa-Arribas, N., Titulaer, M. J., Torrents, A., Aguilar, E., McCracken, L., Leypoldt, F., et al. (2014). Antibody titres at diagnosis and during follow-up of anti-NMDA receptor encephalitis: a retrospective study. *Lancet Neurol.* 13, 167–177. doi: 10.1016/S1474-4422(13)70282-5

Hebda, J. K., Leclair, H. M., Azzi, S., Roussel, C., Scott, M. G., Bidère, N., et al. (2013). The C-terminus region of β-arrestin1 modulates VE-cadherin expression and endothelial cell permeability. *Cell Commun. Signal.* 11:37. doi: 10.1186/1478-811X-11-37

Helms, H. C., Abbott, N. J., Burek, M., Cecchelli, R., Couraud, P. O., Deli, M. A., et al. (2016). in vitro models of the blood-brain barrier: an overview of commonly used brain endothelial cell culture models and guidelines for their use. *J. Cereb. Blood Flow Metab.* 36, 862–890. doi: 10.1177/0271678X16630991

Hirohata, S., Arinuma, Y., Yanagida, T., and Yoshio, T. (2014). Blood-brain barrier damages and intrathecal synthesis of anti-N-methyl-D-aspartate receptor NR2 antibodies in diffuse psychiatric/neuropsychological syndromes in systemic lupus erythematosus. *Arthritis Res. Ther.* 16:R77. doi: 10.1186/ar4518

Huerta, P. T., Kowal, C., DeGiorgio, L. A., Volpe, B. T., and Diamond, B. (2006). Immunity and behavior: antibodies alter emotion. *Proc. Natl. Acad. Sci. U S A* 103, 678–683. doi: 10.1073/pnas.0510055103

Hughes, E. G., Peng, X., Gleichman, A. J., Lai, M., Zhou, L., Tsou, R., et al. (2010). Cellular and synaptic mechanisms of anti-NMDA receptor encephalitis. *J. Neurosci.* 30, 5866–5875. doi: 10.1523/JNEUROSCI.0167-10.2010

Kowal, C., DeGiorgio, L. A., Nakaoka, T., Hetherington, H., Huerta, P. T., Diamond, B., et al. (2004). Cognition and immunity; antibody impairs memory. *Immunity* 21, 179–188. doi: 10.1016/j.immuni.2004.07.011

Kreye, J., Reincke, S. M., Kornau, H.-C., Sánchez-Sendin, E., Corman, V. M., Liu, H., et al. (2020). A therapeutic non-self-reactive SARS-CoV-2 antibody protects from lung pathology in a COVID-19 hamster model. *Cell* 183, 1058–1069.e19. doi: 10.1016/j.cell.2020.09.049

Kreye, J., Wenke, N. K., Chayka, M., Leubner, J., Murugan, R., Maier, N., et al. (2016). Human cerebrospinal fluid monoclonal N-methyl-D-aspartate receptor autoantibodies are sufficient for encephalitis pathogenesis. *Brain* 139, 2641–2652. doi: 10.1093/brain/aww208

Kreye, J., Wright, S. K., van Casteren, A., Stöffler, L., Machule, M.-L., Reincke, S. M., et al. (2021). Encephalitis patient-derived monoclonal GABAA receptor antibodies cause epileptic seizures. *J. Exp. Med.* 218:e20210012. doi: 10.1084/jem.20210012

Liu, K. C., Jacobs, D. T., Dunn, B. D., Fanning, A. S., and Cheney, R. E. (2012). Myosin-X functions in polarized epithelial cells. *Mol. Biol. Cell* 23, 1675–1687. doi: 10.1091/mbc.E11-04-0358

Ly, L.-T., Kreye, J., Jurek, B., Leubner, J., Scheibe, F., Lemcke, J., et al. (2018). Affinities of human NMDA receptor autoantibodies: implications for disease mechanisms and clinical diagnostics. *J. Neurol.* 265, 2625–2632. doi: 10.1007/s00415-018-9042-1

Meroni, P. L., Tincani, A., Sepp, N., Raschi, E., Testoni, C., Corsini, E., et al. (2003). Endothelium and the brain in CNS lupus. *Lupus* 12, 919–928. doi: 10.1191/0961203303lu503oa

Mishra, R., and Singh, S. K. (2013). HIV-1 Tat C modulates expression of miRNA-101 to suppress VE-cadherin in human brain microvascular endothelial cells. *J. Neurosci.* 33, 5992–6000. doi: 10.1523/JNEUROSCI.4796-12.2013

Nikolaus, M., Knierim, E., Meisel, C., Kreye, J., Prüss, H., Schnabel, D., et al. (2018). Severe GABAA receptor encephalitis without seizures: a paediatric case successfully treated with early immunomodulation. *Eur. J. Paediatr. Neurol.* 22, 558–562. doi: 10.1016/j.ejpn.2018.01.002

Noble, P. W., Bernatsky, S., Clarke, A. E., Isenberg, D. A., Ramsey-Goldman, R., and Hansen, J. E. (2016). DNA-damaging autoantibodies and cancer: the lupus butterfly theory. *Nat. Rev. Rheumatol.* 12, 429–434. doi: 10.1038/nrrheum.2016.23

Ohkawa, T., Satake, S., Yokoi, N., Miyazaki, Y., Ohshita, T., Sobue, G., et al. (2014). Identification and characterization of $GABA_A$ receptor autoantibodies in autoimmune encephalitis. *J. Neurosci.* 34, 8151–8163. doi: 10.1523/JNEUROSCI.4415-13.2014

Petit-Pedrol, M., Armangue, T., Peng, X., Bataller, L., Cellucci, T., Davis, R., et al. (2014). Encephalitis with refractory seizures, status epilepticus and antibodies to the GABAA receptor: a case series, characterisation of the antigen and analysis of the effects of antibodies. *Lancet Neurol.* 13, 276–286. doi: 10.1016/S1474-4422(13)70299-0

Pettingill, P., Kramer, H. B., Coebergh, J. A., Pettingill, R., Maxwell, S., Nibber, A., et al. (2015). Antibodies to GABAA receptor α1 and γ2 subunits: clinical and serologic characterization. *Neurology* 84, 1233–1241. doi: 10.1212/WNL.0000000000001326

Piepgras, J., Holtje, M., Otto, C., Harms, H., Satapathy, A., Cesca, F., et al. (2015). Intrathecal immunoglobulin A and G antibodies to synapsin in a patient with limbic encephalitis. *Neurol. Neuroimmunol. Neuroinflamm.* 2:e169. doi: 10.1212/NXI.0000000000000169

Pittock, S. J., Yoshikawa, H., Ahlskog, J. E., Tisch, S. H., Benarroch, E. E., Kryzer, T. J., et al. (2006). Glutamic acid decarboxylase autoimmunity with brainstem, extrapyramidal and spinal cord dysfunction. *Mayo Clin. Proc.* 81, 1207–1214. doi: 10.4065/81.9.1207

Reincke, S. M., Prüss, H., and Kreye, J. (2020). Brain antibody sequence evaluation (BASE): an easy-to-use software for complete data analysis in single cell immunoglobulin cloning. *BMC Bioinform.* 21:446. doi: 10.1186/s12859-020-03741-w

Rocchi, A., Sacchetti, S., Fusco, A. de., Giovedi, S., Parisi, B., Cesca, F., et al. (2019). Autoantibodies to synapsin I sequestrate synapsin I and alter synaptic function. *Cell Death Dis.* 10:864. doi: 10.1038/s41419-019-2106-z

Rubin, L. L., Hall, D. E., Porter, S., Barbu, K., Cannon, C., Horner, H. C., et al. (1991). A cell culture model of the blood-brain barrier. *J. Cell Biol.* 115, 1725–1735. doi: 10.1083/jcb.115.6.1725

Shimizu, F., Schaller, K. L., Owens, G. P., Cotleur, A. C., Kellner, D., Takeshita, Y., et al. (2017). Glucose-regulated protein 78 autoantibody associates with blood-brain barrier disruption in neuromyelitis optica. *Sci. Transl. Med.* 9:eaai9111. doi: 10.1126/scitranslmed.aai9111

Shimizu, F., Takeshita, Y., Sano, Y., Hamamoto, Y., Shiraishi, H., Sato, T., et al. (2019). GRP78 antibodies damage the blood-brain barrier and relate to cerebellar degeneration in Lambert-Eaton myasthenic syndrome. *Brain* 142, 2253–2264. doi: 10.1093/brain/awz168

Sousa, A. D., Berg, J. S., Robertson, B. W., Meeker, R. B., and Cheney, R. E. (2006). Myo10 in brain: developmental regulation, identification of a headless isoform and dynamics in neurons. *J. Cell Sci.* 119, 184–194. doi: 10.1242/jcs.02726

Spatola, M., Petit-Pedrol, M., Simabukuro, M. M., Armangue, T., Castro, F. J., Barcelo Artigues, M. I., et al. (2017). Investigations in GABAA receptor antibody-associated encephalitis. *Neurology* 88, 1012–1020. doi: 10.1212/WNL.0000000000003713

Srinivasan, B., Kolli, A. R., Esch, M. B., Abaci, H. E., Shuler, M. L., and Hickman, J. J. (2015). TEER measurement techniques for in vitro barrier model systems. *J. Lab. Automation* 20, 107–126. doi: 10.1177/2211068214561025

Titulaer, M. J., McCracken, L., Gabilondo, I., Armangué, T., Glaser, C., Iizuka, T., et al. (2013). Treatment and prognostic factors for long-term outcome in patients with anti-N-Methyl-D-Aspartate (NMDA) receptor encephalitis: a cohort study. *Lancet Neurol.* 12, 157–165. doi: 10.1016/S1474-4422(12)70310-1

Tomizawa, Y., Yokoyama, K., Saiki, S., Takahashi, T., Matsuoka, J., and Hattori, N. (2012). Blood-brain barrier disruption is more severe in neuromyelitis optica than in multiple sclerosis and correlates with clinical disability. *J. Int. Med. Res.* 40, 1483–1491. doi: 10.1177/147323001204000427

Vorbrodt, A. W., and Dobrogowska, D. H. (2003). Molecular anatomy of intercellular junctions in brain endothelial and epithelial barriers: electron microscopist's view. *Brain Res. Rev.* 42, 221–242. doi: 10.1016/s0165-0173(03)00177-2

Wagner, F., Goertzen, A., Kiraly, O., Laube, G., Kreye, J., Witte, O. W., et al. (2020). Detailed morphological analysis of rat hippocampi treated with CSF autoantibodies from patients with anti-NMDAR encephalitis discloses two distinct types of immunostaining patterns. *Brain Res.* 1747:147033. doi: 10.1016/j.brainres.2020.147033

Wang, W., Dentler, W. L., and Borchardt, R. T. (2001). VEGF increases BMEC monolayer permeability by affecting occludin expression and tight junction assembly. *Am. J. Physiol. Heart Circ. Physiol.* 280, H434–H440. doi: 10.1152/ajpheart.2001.280.1.H434

Weksler, B. B., Subileau, E. A., Perriere, N., Charneau, P., Holloway, K., Leveque, M., et al. (2005). Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J.* 19, 1872–1874. doi: 10.1096/fj.04-3458fje

Xu, R., Feng, X., Xie, X., Zhang, J., Wu, D., and Xu, L. (2012). HIV-1 Tat protein increases the permeability of brain endothelial cells by both inhibiting occludin expression and cleaving occludin via matrix metalloproteinase-9. *Brain Res.* 1436, 13–19. doi: 10.1016/j.brainres.2011.11.052

Yoshio, T., Okamoto, H., Hirohata, S., and Minota, S. (2013). IgG anti-NR2 glutamate receptor autoantibodies from patients with systemic lupus erythematosus activate endothelial cells. *Arthritis Rheum.* 65, 457–463. doi: 10.1002/art.37745

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of this dissertation for reasons of data protection.

Publication List

Reincke, S. M., Wardenburg, N. von, Homeyer, M. A., Kornau, H.-C., Spagni, G., **Li, L. Y.**, Kreye, J., Sánchez-Sendín, E., Blumenau, S., Stappert, D., Radbruch, H., Hauser, A. E., Künkele, A., Edes, I., Schmitz, D. and Prüss, H. (2023)

'**Chimeric autoantibody receptor T cells deplete NMDA receptor-specific B cells**',

Cell, vol. 186, no. 23, 5084-5097.e18.

IF: 64.5 (2022)

Barthel, P. C., Staabs, F., **Li, L. Y**., Buthut, M., Otto, C., Ruprecht, K., Prüss, H. and Höltje, M. (2023)

'Immunoreactivity to astrocytes in different forms of dementia: High prevalence of auto-antibodies to GFAP',

Brain, behavior, & immunity - health, vol. 29, p. 100609.

IF: N/A

Li, L. Y., Kreye, J., Burek, M., Cordero-Gomez, C., Barthel, P. C., Sánchez-Sendín, E., Kornau, H.-C., Schmitz, D., Scharf, M., Meybohm, P., Reincke, S. M., Prüss, H. and Höltje, M. (2023)

'**Brain blood vessel autoantibodies in patients with NMDA and GABAA receptor encephalitis: identification of unconventional Myosin-X as target antigen**',

Frontiers in cellular neuroscience, vol. 17, p. 1077204.

IF: 6.15

Kreye, J., Reincke, S. M., Kornau, H.-C., Sánchez-Sendin, E., Corman, V. M., Liu, H., Yuan, M., Wu, N. C., Zhu, X., Lee, C.-C. D., Trimpert, J., Höltje, M., Dietert, K., Stöffler, L., Wardenburg, N. von, van Hoof, S., Homeyer, M. A., Hoffmann, J., Abdelgawad, A., Gruber, A. D., Bertzbach, L. D., Vladimirova, D., **Li, L. Y**., Barthel, P. C., Skriner, K., Hocke, A. C., Hippenstiel, S., Witzenrath, M., Suttorp, N., Kurth, F., Franke, C., Endres, M., Schmitz, D., Jeworowski, L. M., Richter, A., Schmidt, M. L., Schwarz, T., Müller, M. A., Drosten, C., Wendisch, D., Sander, L. E., Osterrieder, N., Wilson, I. A. and Prüss, H. (2020)

‘**A Therapeutic Non-self-reactive SARS-CoV-2 Antibody Protects from Lung Pathology in a COVID-19 Hamster Model’**,

Cell, vol. 183, no. 4, 1058-1069.e19.

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Li, L. Y., Burek, M., Kreye, J., Reincke, S. M., Cordero-Gomez, C., Sánchez-Sendín, E., Meybohm, P., Barthel, P. C., Prüss, H. and Höltje, M.

‘**Autoimmunity to brain blood vessels in patients with encephalitis: antibody binding pattern and functional implications**‘

Abstracts from the Virtual Symposium “Signal Transduction at the Blood–Brain Barriers”. **Fluids Barriers CNS 18** (Suppl 1), 58 (2021)

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