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„Characterization of primary human mixed glial cells with the aim to establish a diagnostic cell-based assay for autoimmune disorders"

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Declaration

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List of Abbreviations

AID	Autoimmune disease
BBB	Blood-brain barrier
MS	Multiple sclerosis
CNS	Central nervous system
PNS	Peripheral nervous system
GBS	Guillain-Barré-Syndrome
CIDP	Chronic inflammatory demyelinating disease
OPCs	Oligodendrocyte Precursor cells
MBP	Myelin basic protein
PLP	Proteolipid protein
GalC	Galactosylceramide
ADEM	Acute disseminated encephalomyelitis
AIE	Autoimmune encephalitis
MRI	Magnetic Resonance Imaging
NMDAR	**N-M**ethyl-**D-A**spartat-Receptor
MDEM	Multiphasic disseminated encephalomyelitis
ADEM-ON	Acute disseminated encephalomyelitis with optic neuritis
NMOSD	Neuromyelitis optica spectrum disorders
MOG	Myelin oligodendrocyte glycoprotein
IgG	Immunoglobulin G
IVIg	Intravenous Immunoglobulin Therapy
IQ	Intelligence quotient
HIV	human immunodeficiency virus
MMN	Multifocal motor neuropathy
MGUS-P	Monoclonal gammopathy of uncertain singnificance
CINs	Chronic inflammatory neuropathies

PE	Plasma exchange
TNF-α	Tumour necrosis factor
NF	Neurofascin
CNTN	Contactin1
Caspr1	Contactin-associated protein 1
NMO	Neuromyelitis optica
CSF	Cerebrospinal fluid
EAE	Experimental autoimmune encephalitis
CBA	Cell-based assay
AB	Antibody
TBA	Tissue-based assay
FCD	Focal cortical dysplasia
DMEM	Dulbecco's modified eagle medium
ECM	Extracellular matrix
PBS	Phosphate buffered saline
PFA	paraformaldehyde
PLL	Poly-L-Lysine
HEK	Human embryonic kidney
DNA	deoxyribonucleic acid

1. Introduction

1.1 Autoimmunity

Autoimmune diseases (AIDs) are caused by an immune response against the host's own cell- or tissue components. Over 80 disorders have been classified but their etiology still remains unclear. Despite the broad spectrum of AIDs, the involvement of common genes and defects in the autoimmune system are shared factors among all patients [6].

Healthy individuals are tolerant to their self antigens. Lymphocytes which are able to recognize the host's own antigens are usually either killed or inactivated. A failure in self-tolerance results in an immune response against self antigens and is called *autoimmunity* [7].

Both, humoral and cellular immune responses are controlled by effector mechanisms, which protect the integrity of local tissue compartments. Therefore the self-tolerance needs to be maintained. In autoimmune diseases self-tolerance fails and an immune attack is activated, causing organ-specific tissue damage [8].

Under physiological conditions the brain is protected by the blood-brain barrier (BBB) and neuronal or glial proteins are not able to cross this border. Different mechanisms may interfere with this immune-privileged status, 1) tissue damage or inflammation may lead to a collapse of the BBB and nerve and glial cells can be recognised as foreign antigens [9], 2) necrosis caused by tissue infection may lead to tissue injury and altered display of self-antigens to the immune system that may trigger autoreactive lymphocytes [10], and 3) a failure in the deletion or inactivation process of self-reactive lymphocytes during maturation may cause a loss of self-tolerance [11]. Autoimmune diseases are very often chronic, self-perpetuating and progressive. The triggering self antigens for these reactions are persistent and an ongoing autoimmune response is perpetuated by various activated amplification mechanisms. Once the tissue is attacked by an autoimmune response against a self antigen, more of the same antigen can be released and other tissue antigens can be altered. In addition, lymphocytes specific for these altered antigens are released and lead to an exacerbation of the disease. This mechanism is called *epitope spreading* and may be the reason for the self-perpetuating character of an autoimmune disease [12].

Depending on the distribution of the recognized autoantigens, autoimmune diseases can be systemic or organ specific. Systemic autoimmune diseases (e.g. systemic lupus erythematosus) may for example be caused by circulating immune complexes containing self nucleoproteins. An immune response against self antigens with restricted tissue distribution on the other hand leads to organ specific diseases like myasthenia gravis or multiple sclerosis (MS) [13], [14]. Other possible affected organs are the thyroid gland (e.g. M. Basedow, Hashimoto thyreoditis), the parotis (e.g. Sjörgren syndrome), the lung (e.g. goodpasture syndrome), intestines (e.g. M. Crohn, Colitis ulcerosa), the skin (e.g. pemphigus vulgaris), the peripheral and central nervous system and many other organ-systems [15].

The responsible mechanisms causing the pathological effects differ between the different diseases and usually include a combination of humoral and cellular autoimmunity [5]. Antibodies play various roles in autoimmune diseases, in some as triggering elements, in others only as epiphenomenon. Autoreactive T-cells are also playing a role in some autoimmune diseases. Furthermore a disease might involve different pathomechanisms during its initial phase or the nadir of disease [16].

Both, the central nervous system (CNS) as well as the peripheral nervous system (PNS) can be affected by autoimmune diseases and may activate cellular and/or humoral effector mechanisms. Multiple Sclerosis (MS) is a demyelinating disease of the CNS and the initial inflammation is probably driven by autoreactive T-cells, but humoral mechanisms including autoantibodies are needed to explain the full range of symptoms. Guillain-Barré-Syndrome (GBS) and chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) are demyelinating diseases of the PNS and probably involve cellular and humoral factors [5].

Similar disease patterns can be found in the paraneoplastic neurological syndrome (PNS), where systemic tumours express proteins (onconeural proteins), which are normally restricted to immune-privileged tissues. For instance, limbic encephalitis associated with anti-Hu antibodies may be generated by a small cell lung cancer that shares the Hu antigen with neurons. Other mechanisms include molecular mimicry, in which antibodies or T cells are generated in response to an infection and cross-react with self-antigens, or epitope spreading, where autoimmunity against a certain antigen can expand to other antigens [17], [8],[18].

1.2 Oligodendrocytes and myelination

Myelination of axons in the central nervous system (CNS) and peripheral nervous system (PNS) makes fast signal transport possible. In the CNS the myelin is produced by oligodendrocytes and in the PNS by Schwann cells. The myelin wraps around the axons of neurons and in between nodes of Ranvier can be formed and enable saltatory impulse propagation [19]. Oligodendrocytes undergo a very distinct and complex development, which makes them very vulnerable [20]. Oligodendrocytes and Schwann cells differ in their regions of origin. While Schwann cells derive from the neural crest, oligodendrocytes derive from the neural tube [21]. The majority of information that we already have about oligodendrocytic differentiation and myelination is known from rodents since it's one of the best model organisms for human health research [20]. Embryonic oligodendrocyte precursors cells (OPCs) arise in three waves and are found mainly in the telencephalon, cerebral cortex and postnatal cortex [22]. Differentiation as well as migration of OPCs are guided processes and regulated by distinct signals. Once the OPCs reach their target destination, they either develop into adult OPCs or turn into myelin-producing oligodendrocytes [20]. While the oligodendrocytes choose which axons to wrap (diameters over 0,2µm) [23], the starting point of myelination in the CNS is settled by the maturation state of the neurons [24] and additionally supported by the electrical activity of neurons [25]. The chances for myelination are limited to a very short window of 12-18h [26]. The enormous amount of synthesis, sorting and moving of proteins requires a complex system. Myelin-associated proteins like myelin basic protein (MBP), proteolipid protein (PLP) and galactosylceramide (GalC) are transported to the myelin sheaths under the control of neurons [20], [27], [28].
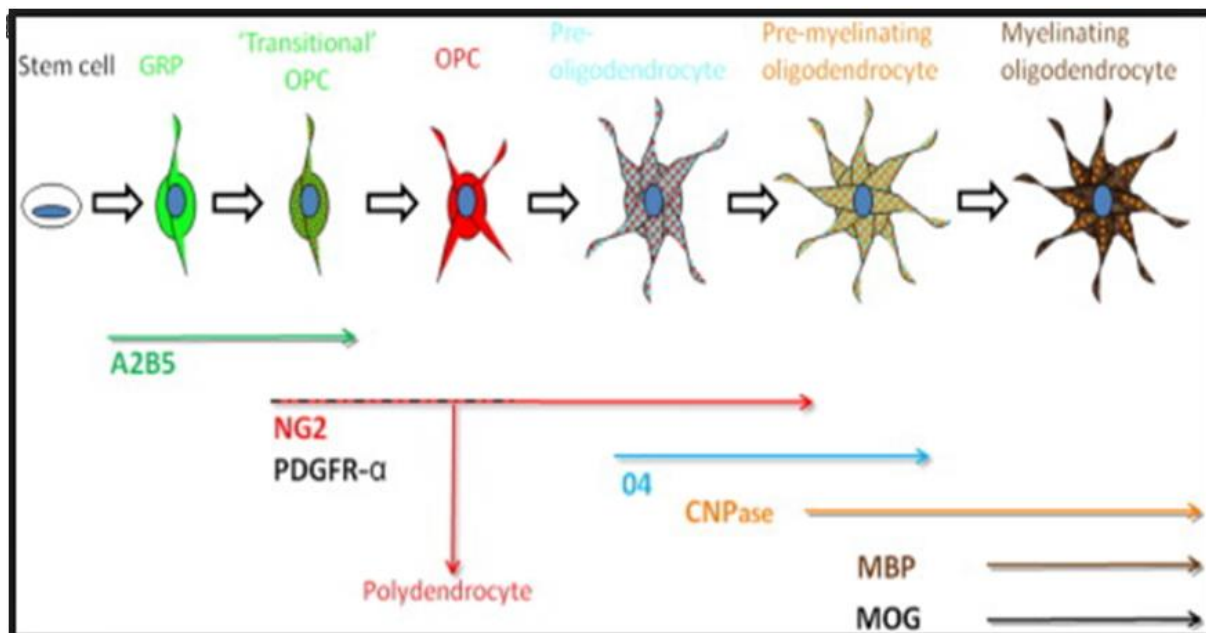
Figure 1 Development from stem cells to myelinating oligodendrocytes and present antigens [1].

After neurons have been ensheathed and the axons are insulated from electricity, a clustering of sodium channels can be observed at the node of Ranvier [29], [30].

The CNS is capable of remyelination of demyelintated axons. However, remyelination is not achieved by mature oligodendrocytes [31] but depends on adult quiescent OPCs, which bring new mature oligodendrocytes forth [32], [20].

The white matter in the CNS is mainly formed by myelinated axons, astrocytes, microglia and precursor cells. If the myelin sheaths of the white matter are attacked by autoantibodies it can result in an acute disseminated encephalomyelitis (ADEM) with neurological symptoms like epileptic seizures, confusion, headaches, hemiparesis and paraparesis [33].
Since patients with antibody-associated demyelination such as ADEM or neuromyelitis optica require a different treatment compared to multiple sclerosis (MS), it is important to test the patients' cerebrospinal fluid (CSF) or serum for antibodies [1].

1.3 Disorders associated with neuronal and glial antibodies

1.3 .1 Paraneoplastic syndromes

Paraneoplastic neurological syndromes describe immune-mediated disorders of the central or peripheral nervous system that are triggered by an underlying malignant tumour remote from the nervous tissue [34]. Paraneoplastic neurological syndromes can affect various targets such as distinct parts of the nervous system (e.g. limbic encephalitis), a special cell type (e.g. Purkinje cells) or it can have a diffuse pattern where multiple levels of the CNS are affected [35]. The underlying tumours originate usually in immunomodulatory tissues such as the thymoma, organs with neuroendocrine proteins (small-cell lung cancer) or are able to produce nervous tissue components on their own such as teratomas [36]. Previous studies have shown that paraneoplastic syndromes are mediated by a misdirected immune response. Tumour proteins similar or identical to neuronal antigens (onconeuronal antigens) trigger an immune response that on the one hand attacks the tumor cells but at the same time also affects brain regions that express the onconeuronal antigen. Antibodies that arise during the immune response can be categorized into those that target nuclear and cytoplasmic antigens and those that recognize surface antigens. Antibodies against intracellular antigens (onconeuronal antibodies) are not considered to be pathogenic but cytotoxic T-cells mediate an immune attack, which leads to irreversible neuronal damage However, the onconeuronal antibodies can be used as a diagnostic tool and might lead to a search for an underlying tumour [37]. Although there is a broad spectrum of pathological characteristics, individual antibodies can be connected with certain types of cancer and clinical syndromes (e.g. limbic encephalitis) [36]. In some paraneoplastic syndromes antibodies against surface antigens can be found. These antibodies are supposed to be pathogenic due to direct interaction with their antigen. Patients with surface autoimmunity often show a good response to immunotherapy [38].

The diagnosis of paraneoplastic neurological syndromes is based on clinical criteria [39],[40] and the antibodies in cerebrospinal fluid and sera of the patients may serve as sensitive and specific biomarkers [41],[42].

1.3.2 Autoimmune Encephalitis (AIE)

Antibody-related brain disorders were initially described in paraneoplastic neurological syndromes and non-infectious encephalitis in association with antibodies targeting intracellular epitopes [35]. Despite the assumption that these antibodies are not actually pathogenic but activate a T-cell mediated immune response [43], the detection of involved antibodies is crucial to enable an early diagnosis of a possible underlying malignancy. Neuronal dysfunction can also be caused by binding of autoantibodies to intracellular synaptic proteins. The binding leads to a disruption of synaptic transmission and the loss or neurons [44]. Over the last decade a novel group of anti-neuronal antibodies associated with autoimmune encephalitis syndromes was described and characterized. Extracellular neuronal structures such as surface receptors and synaptic proteins are the target of these antibodies [45]. The binding of the easy accessible epitopes with the antibodies causes a change in neuronal function and can lead to a limitation of the target antigen. The detection of autoantibodies at an early stage can help to find the appropriate treatment and diagnosis [46].

It is sometimes difficult to come up with the correct diagnosis and other diseases such as viral infections or dementia with similar clinical presentation like impaired memory and cognition have to be excluded [47]. Since psychiatric symptoms often occur at the onset of the disease as well as over the course, patients are initially often admitted to psychiatric institutions, which may delay the diagnosis [48].

Although the pathological effects can be very severe, the removal of the tumour and antibodies by immunotherapy causes a recovery of the neuronal dysfunction [2].

Acute encephalitis is accompanied by rapid progressive encephalopathy, which develops over a time of six weeks. Further it can be characterized by an inflammatory response in the brain and people of all ages can be affected. In high-income countries an estimated incidence rate of 5-10 people per 100 000 inhabitants occurs [49],[50]. Although the majority of the encephalitis cases are caused by infections, over the last decade a rapid increase of autoimmune mediated encephalitis (AIE) could be observed due to the discovery of antibody-mediated surface autoimmunity. The main targets of these autoantibodies are neuronal proteins such as surface antigens and synaptic proteins. As already mentioned the direct interaction of antibodies with the surface proteins causes an alteration of neuronal functions. These alterations can lead to symptoms similar to infectious encephalitis but with further

clinical features like changes in mood, behaviour and memory. Neurological symptoms such as seizures and impaired consciousness can be observed. Specific syndromes or distinct clinical features can be linked to already identified antibodies, which facilitates a diagnosis and therefore a suitable therapy. In contrast to classical paraneoplastic syndromes that are characterized by immune attacks against intracellular proteins, the AIE is associated with good response to immunotherapy and therefore a reversibility of all symptoms, since no neuronal cell loss occurred [51],[2],[52].

Although the majority of AIE are idiopathic, some surface antibodies can be triggered by systemic tumours, which express antigens similar to neuronal antigens and result in a misdirected immune response against the brain. Other triggering factors include virus infections such as the herpes simplex virus, which can lead to an anti-NMDAR encephalitis. The patients recover after immunotherapy [2]. Other factors that can trigger the development of AIE are hormones, genetic predispositions (HLA association) and already existing demyelinating CNS disorders [53], [54],[55].

1.3.3 Acute disseminated encephalomyelitis (ADEM)

ADEM is a demyelinating disorder of the CNS which often occurs after infections or vaccinations mainly in children at the age of 5-8 at the onset with male predominance [56], [57]. It is generally characterized by a monophasic disease course and symptoms like polyfocal clinical CNS events, encephalopathy and brain MRI abnormalities showing demyelination within the first 3 months but no additional changes later on. Neurologic symptoms include ataxia, optic neuritis, seizures, impairment of speech and fever [58],[57],[59]. Demyelination of both central and peripheral nervous system has been observed [60]. Worldwide the incidence of ADEM is 0,3-0,6 per 100 000 per year [61], [62]. Additionally to the monophasic ADEM, a subset of multiphasic disseminated encephalomyelitis (MDEM) has been described. One type is defined by 2 episodes with a gap of 3 months in between. A second type (ADEM-like event) is characterized as a chronic relapsing demyelinating disorder and can be accompanied by additional disorders such as optic neuritis (ADEM-ON), neuromyelitis optica spectrum disorders (NMOSD) or multiple sclerosis (MS) [63],[64],[65]. About 75% of NMOSD patients harbour anti-aquaporin-4 (AQP4) IgG antibodies in serum, up to 8% of the AQP4-negative cases have anti-MOG antibodies [66],[65]. Antibody positivity also plays a role in ADEM-ON. If a relapsing phenotype of ADEM can be observed, the patient should be tested

for anti-MOG antibodies [63]. Since the diagnosis between ADEM, NMOSD and MS is difficult, the testing of anti-MOG and anti-AQP4 antibodies can be helpful, since a seropositivity of MOG-IgG or AQP4-IgG argues against MS [67],[68],[69]. Generally, the diagnosis is done with the support of MRI studies and remains a diagnosis of exclusions [58]. In a next step CSF and blood samples are screened for antibodies, infectious agents and CSF cell count as well as a complete blood count are analysed. Another difference between MS and ADEM patients is the early onset in children and the occurrence of fever, headache and vomiting in ADEM [58].

The pathology of ADEM is defined by perivenular sleeves of demyelination caused by infiltrates of myelin-laden macrophages due to inflammation, T and B lymphocytes and granulocytes. The axons are relatively better preserved [70].

Treatment is in most cases accomplished with high-dose corticosteroids as first-line therapy [71]. Additionally IV immunoglobulin treatment has been used in some cases or for patients who did not respond to corticosteroids as a second-line treatment [72],[73]. Other possibilities are plasma exchange or rituximab [74]. Neurologic improvement and full recovery can be observed in the majority of children [58],[75]. Nevertheless a death-rate of 1-3% [76] and long-term deficits in attention, verbal abilities, behaviour and IQ (especially in patients with an onset before the age of 5) have been reported [77],[78].

1.3.4 Chronic Inflammatory Demyelinating Polyneuropathy (CIDP)

CIDP is considered as an autoimmune demyelinating disease of the peripheral nervous system [79], [80]. It shows several subtypes which can be categorized due to various and complex pathogenesis. If genetic, toxic, or metabolic sources can be excluded and the electroneurography shows demyelinating features, the polyneuropathy is most likely to have an inflammatory origin [81], [82].

CIDP is considered as the chronic form of the Guillain-Barré-syndrome (GBS) with a disease evolvement longer than 8 weeks [80]. That's why cases with an acute-onset are hard to differentiate from GBS, especially if the GBS shows treatment-related clinical fluctuations [82]. Once a GBS diagnosis deteriorates after 8 weeks, CIDP should be considered as an alternative diagnosis [83]. It is the most frequent inflammatory and therefore also treatable peripheral

neuropathy [82],[84]. CIDP is known to affect spinal nerve roots, spinal nerves, major plexuses, and proximal nerve trunks but sometimes inflammation of more peripheral nerves like intramuscular nerves and terminal autonomic nerves can occur [8]. Since the disease pattern as well as the progression of CIDP is very heterogeneous, typical and atypical symptoms are taken together in the CIDP spectrum [85].

10-30% of CIDP cases are also linked to infections, vaccination and diseases like HIV, chronic hepatitis, systemic lupus erythematosus, Hodgkin-Lymphoma, melanoma within 6 months before onset [8], [17], [80]. As already mentioned CIDP is a heterogeneous disorder and it is not yet clear whether distinct clinical phenotypes represent subgroups of one disease or rather should be regarded as a different disease entity. Patients may differ in their neurological symptoms as well as response to therapy, disease progression (relapsing, progressive), concomitant diseases (diabetes mellitus) and paraclinical features (antibodies) [85],[86],[87]. Some authors distinguish a pure sensory CIDP, which differs from neuropathies with monoclonal gammopathy as they also present predominantly sensory symptoms. In contrast to the gammopathy, sensory CIDP typically responds well to immune modulating treatment [80].

The mean age of onset is 57 years and the prevalence is increasing with age reaching a maximum between the age of 70 and 79 [82],[88]. CIDP occurs slightly more often in men [80]. CIDP, multifocal motor neuropathy (MMN), and monoclonal gammopathy of uncertain significance (MGUS-P) are the most common chronic inflammatory neuropathies (CINs) [4],[89],[90]. All of them are characterized by an immune-mediated attack of the peripheral nerves [79].

The symptoms in CIDP develop over months or years in a mostly relapsing and remitting but sometimes also a progressive form [5],[80]. In contrast to GBS the peak is reached after eight weeks or later [80]. The symptoms of CIDP include progressive symmetrical motor weakness of distal limbs, hypo- or areflexia and glove- or sock-shaped paraesthesia. Sensory involvement mainly affects large myelinated fibres. Paraesthesia is more distinct in CIDP than in GBS whereas involvement of cranial nerves, especially bulbar involvement, is less common than in GBS. Respiratory insufficiency occurs very rarely [80],[82].

Demyelinating processes also cause prolongation of distal motor latencies, absence or prolongation of F-waves (an indicator for nerve conduction velocity in proximal regions)

[80],[82]. F-wave latency is the most promising indicator to differentiate between CIDP and other neuropathies (diabetic neuropathy, MGUS-P) [91]. Abnormalities in the CNS are rarely found in MRI studies [92].

Best outcomes of treatments have been found in three treatments: corticosteroids, plasma exchange (PE), and intravenous immunoglobulins (IVIg). About 25% of patients don't response properly to the available treatments. Significant placebo studies in this field with alternative immunosuppressants are still missing [93]. Nevertheless 40% of treated patients stay dependent to medication and multifocal deficit is an additional risk factor. Immunoglobulins have been showing a more suitable response for treatment-dependent patients compared to a corticosteroid therapy [94].

Corticosteroids on the other hand showed a more promising long-term effect compared to IVIg, concerning the delay of a relapse [95]. PE appears to be the most effective therapy although relapse can't be fully excluded. Nevertheless IVIg and corticosteroids should be considered first since PE is an invasive and special center-depending treatment [82]. Since a long-term treatment of CIDP has a poor outlook and patients suffer from relapses very often, immunsuppressive and immunomodulatory drugs are being prescribed. Best results have been showed by methotrexate, rituximab and alemtuzumab [82]. For a classic CIDP, rituximab has the most promising results in treatment [96],[80].

Some studies suggest that the type of chronic polyneuropathy influences the outcome of immunotherapy and that various types of antibodies also response differently. In fact, only 17-38% of CIDP patients show a response to immunomodulator and immunosuppressive treatments with no significant difference of the used drug. Additionally there was no evidence found that axonal damage, age of onset, or the duration of the disease had an impact on the responsiveness of the medication [97].

In CIDP both cellular as well as humoral immune responses are active in the pathomechanisms [98],[82]. The presence of macrophages and T-cells in perivascular inflammations and infiltrates may be an indicator for their leading role in demyelination. T- cells produce cytokines which together with tumour necrosis factor (TNF)- α have been observed to have a higher concentration in CIDP patients, as well as chemokines and soluble adhesion molecules. These findings indicate that an active migration of T-cells across the blood-nerve barrier might be a possible mechanism [8]. Recently antibodies against neuronal and glial proteins that are expressed in the node of Ranvier in the PNS and CNS have been detected in CIDP patients [15].

Recent studies suggest that these patients may benefit from treatment with steroids or rituximab (135) [82]. A systematic search for novel antibodies and target antigens will be necessary to identify different disease subtypes within the heterogeneous group of immuno-neuropathies and may be helpful to improve the diagnosis and disease monitoring.

1.4 Autoantibodies

The humoral immunity is an important factor in demyelinating neuropathies [8]. This assumption has been supported by a study which showed that passive transfer of patients' IgG to rat sciatic nerves can lead to demyelination [99]. Some autoantibodies such as anti-gangliosid antibodies have already been described in association with peripheral demyelinating diseases. More recently, antibodies against the node of Ranvier have been discovered in patients with CIDP and GBS, which are useful biomarkers with diagnostic, prognostic, and therapeutic implications. In general, the role of autoantibodies in autoimmune diseases is not always clear and a pathological relevance or even protective function has been discussed [15].

1.4.1 Neurofascin antibodies

Neurofascin (NF) 186 and 155 are both proteins located at the nodes of Ranvier [100]. They are two splicing-variants, differing in their fibronectin-type and their mucin-like domain that is missing in NF155 [101]. NF186 is a neuronal protein located at the axonal paranodes and nodes and is responsible for sodium channel clustering [5],[102],[103]. It contributes to the unique shape of the node of Ranvier and its special property, the saltatory conduction [104]. NF155 is a Schwann cell and oligodendroglial protein, which is located at paranodes, and is crucial for paranodal junction formation and has a connection to voltage-dependent sodium channels which play a role in transmission of saltatory conduction [103],[105]. NF155 is found in both CNS and PNS in the terminal loops of myelin and binds to the CNTN1/Caspr1 complex, which is responsible for axonal cell adhesion. These molecules ensure the stability of septate-like junctions which are separating sodium- and potassium-channels. These structures are crucial for the transmission of saltatory conduction [106],[5]. Antibodies against the NF155 were first described in patients with MS but later also found in serum of patients with CIDP

and GBS, Other paranodal antibodies such as anti-CNTN1 and anti-CASPR1 were only found in CIDP and GBS [107]. As shown in animal models, an immune response against NF186 seems to cause a temporary disruption of conduction, which leads to axonal damage [104],[108]. Two animal studies showed that antibodies against NF155 cause electrophysiological blocking [100],[109]. CIDP patients with NF155 antibodies often show an aggressive motor neuropathy, predominantly distal with a slow, disabling action tremor (42%). 74% of the patients suffer from sensory ataxia, 13% of cerebellar ataxia with nystagmus. The majority (80%) of these patients show poor response to treatment with IVIg but good response to rituximab [110],[111]. Additionally, up to 8% of patients with NF155 antibodies may show a combination of central and peripheral demyelination [112].
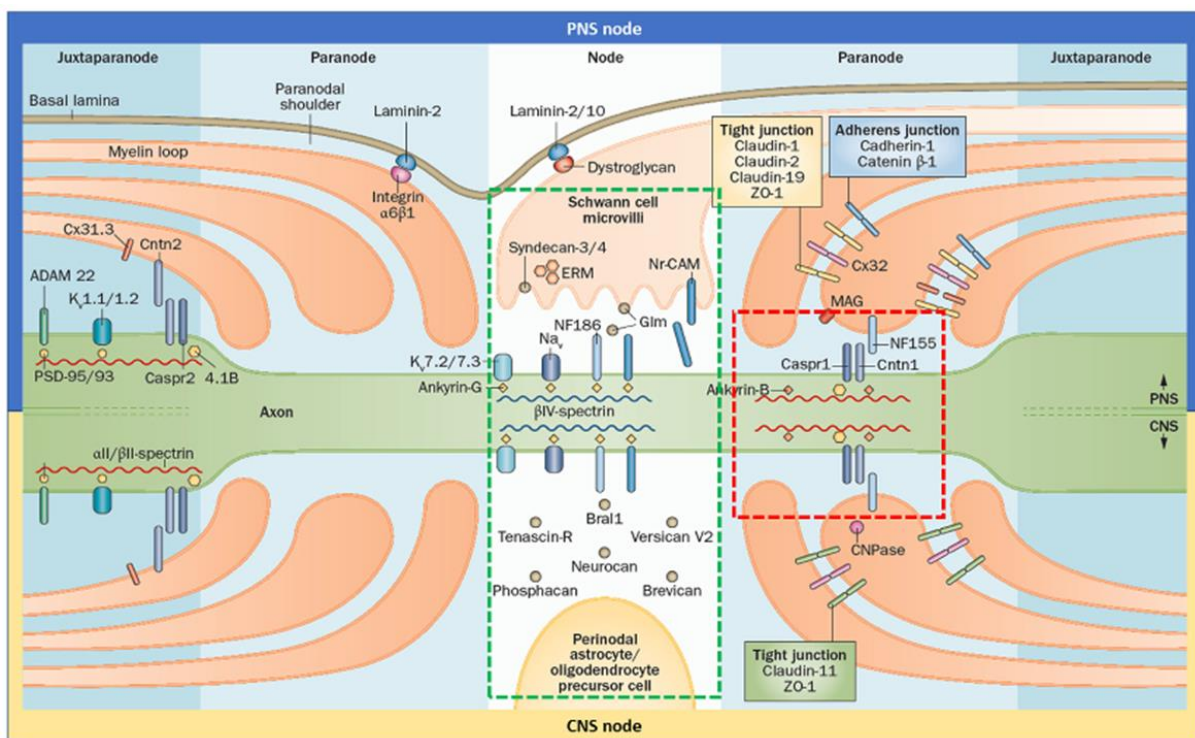

Figure 2 Node of Ranvier in CNS and PNS: In CIDP IgG4 autoantibodies cause a disruption of the septate-like junction at the paranode by binding to the contactin-1 (CNTN1) - NF155 complex which enables axoglial attachment [3], [4], [5].

1.4.2 Myelin Oligodendrocyte Glycoprotein (MOG) antibodies

Myelin oligodendrocyte glycoprotein (MOG) is a target of demyelinating autoantibodies and may play an important role in inflammatory demyelinating diseases of the central nervous system [113]. Disorders like multiple sclerosis (MS) and other less frequent inflammatory demyelinating diseases like neuromyelitis optica (NMO), acute disseminated

encephalomyelitis (ADEM), Marburg disease, Balo concentric sclerosis and Schilder disease are considered as acquired CNS inflammatory demyelinating diseases [114], [115]. Differences between these pathologies can be found in their age of onset, the progression, in the characteristics of their pathology and cerebrospinal fluid (CSF) and most importantly treatment. Nevertheless the clinical characteristics are to a large extent very similar which makes a clear diagnosis, especially at the onset, quite difficult. Similarities include features of the MRI and serum/CSF pathology [113]. Although diagnostic and prognostic tools for antibody-specific diseases are established, the role of antibodies for a differentiated diagnosis in demyelinating inflammatory CNS diseases remains developable [113],[116]. In autoimmune attacks of the CNS, various myelin as well as non-myelin antigens are affected [116],[117],[118],[119]. Within these, MOG is one of the most well examined antigens and might play an important role to understand the pathogenesis of MS and its property to be used as a diagnostic and prognostic biomarker [113]. Since MOG is a highly conserved protein between species, animal models have been a possible research strategy [120]. MOG is only found in the outer myelin sheath and the plasma membrane of oligodendrocytes in the CNS and functions can be used as a maturation marker since it's expression is limited to the phase of myelination [121], [122]. Within an organism MOG might be responsible for regulation and stabilization of microtubules, cell adhesion and a cross-link of the immune system and myelin [122]. Evidence for the importance of MOG in demyelinating diseases was firstly found in experimental autoimmune encephalomyelitis (EAE), where MOG was identified as a main target protein whose attack can lead to demyelination [123],[124],[125]. Experiments in a transgenic mouse model have shown an activation of MOG-specific CNS inflammation caused by gut bacteria. It is supposed that the inflammation, recruitment and activation of MOG-specific B cells takes place in the periphery, subsequently passes the BBB and initiates a demyelinating process. In humans a similar mechanism can be observed. On the one hand a primary CNS infection can lead to a leakage of CNS antigens to the periphery which then can cause an immune response against MOG. On the other hand, comparable to the mouse model, T cells from the CNS can be activated in the periphery by molecular mimicry which can also lead to epitope spreading [126],[113]. For the appropriate diagnosis of anti-MOG antibodies, it is important to use specific detection methods that preserve the three-dimensional structure of the protein. Cell-based assays (CBAs) with human as well as murine cells expressing the full-length human MOG protein have shown that humoral immune

responses against MOG more likely occured in patients with various demyelinating diseases than in the control cohort [127], [128]. In another study antibodies against MOG have been detected in ADEM patients, but only in a small number of adult patients with MS [129]. Additionally a young (5-19 years) MS cohort with ADEM-like symptoms and an adult MS group have been compared to juvenile controls (2-18 years). Using CBAs, anti-MOG antibodies were mainly found in paediatric MS and ADEM patients, mostly with additional conditions such as multiple sclerosis spectrum disorders or NMO [130],[131]. These findings lead to the assumption that the screening for MOG antibodies especially in paediatric patients can be helpful. Since ADEM is a monophasic disease, the detection of MOG antibodies at an early onset can indicate a possible development of recurrent optic neuritis (long-lasting chronic disease) and can therefore help to find the best medication [113],[131],[63]. Despite the promising results of previous studies, a standardized method for antibody detection hasn't been established so far and is needed to generate comparable data [113].

1.5 Current detection methods of auto-antibodies

A subgroup of autoimmune-mediated neurological disorders is associated with autoantibodies targeting intracellular or cell surface epitopes of neuronal cells. These autoantibodies either play a direct pathogenic role in disease development (surface antibodies) or merely occur as epiphenomenon (intracellular antibodies). The identification of
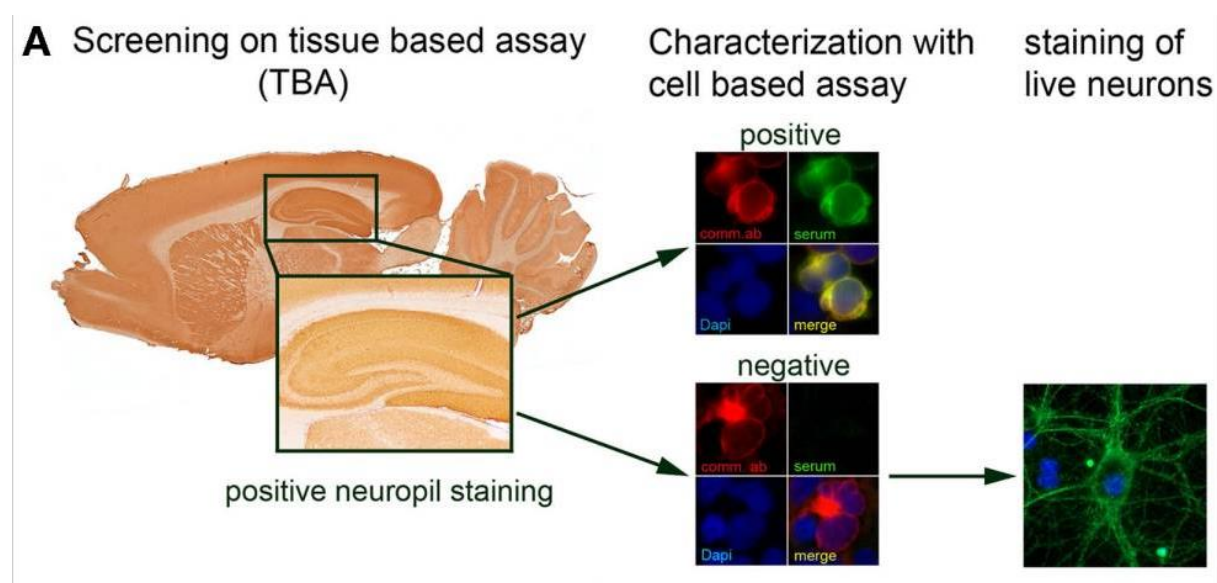

Figure 3 Current detection methods for antibodies including tissue- based assay on rat brain slides, cell based assay with transfected cells and live labelling of neuronal rat cell culture [2].

these autoantibodies is important to initiate the appropriate treatment approach. Routine detection methods include antibody screening on rat brain sections with immune histochemical techniques (IHC) and identification of the antibody type with immunofluorescence (IF) labelling of transfected cells expressing the target antigen (cell-based assay, CBA) [2].

The testing of autoantibodies is very important for the proper diagnosis of an AIE but the complexities of the tests need to be considered and the results should always be interpreted in context with the clinical presentation [47].

Since some autoantibodies such as anti-MOG were shown to recognise a human-specific epitope, the establishment of a mixed glial cell culture of primary human cells may help to identify novel autoantibodies that play a role in demyelinating diseases [132]. A co-culture with neurons mimics the physiological surroundings of glial cells and therefor can enhance myelination and expression of myelin antigens.

1.6 Aim

The aim of this study was to expand the rat brain tissue- based screening assay (TBA) to a mixed glial cell culture cell-based assay (glial-CBA), to identify novel antibodies to oligodendrocyte, astrocyte or oligodendrocyte progenitor cell antigens.

Two cell cultures were obtained from human brain surgery specimens from paediatric patients who underwent epilepsy surgery. White matter pieces were removed from both patients who were diagnosed with focal cortical dysplasia (FCD) type IIB (N1617-16 cells) and mild malformations of cortical development (N931-16 cells). Additionally MO3.13 cells, an immortalized cell line with oligodendroglial features and an OPC cell line were cultivated. We wanted to characterize all four cell cultures with commercial antibodies. In a second step the cell cultures were added to three-week-old hippocampal rat neurons and cultured for another three weeks to gain myelinating co-cultures. The co-cultures then have been characterized with commercial antibodies and were tested with sera from 3 patients, patient 1 with anti-NF155/186 antibodies, patient 2 with a yet uncharacterised antibody against the node of

Ranvier (as demonstrated with teased nerve fibers, data not shown), and patient 3 with high titers of anti-MOG antibodies. All cell cultures have been treated with an oligodendroglial differentiation medium for three weeks to enhance maturation of the cells.

2. Material and Methods

2.1 Material

All experiments were performed in the Institute of Neurology, Medical University of Vienna. Used equipment and reagents are listed in table 1-4. Sera of 3 patients, one with anti-NF155/186, one with a yet uncharacterised antibody against the node of Ranvier, and one with anti-MOG antibodies were obtained from the biobank of the Institute of Neurology, Medical University of Vienna (EK 1123/2015). All samples were sent for routine-diagnostic screening for anti-neuronal anti anti-glial antibodies.

Table 1: Laboratory equipment

Laboratory equipment	Company
Water jet pump	AKH equipment
Light microscope	Nikon Y-IDP with Axio Cam, Zeiss
Incubator	Thermo Scientific Heracell 150i
Fluorescence microscope	Zeiss Axio Imager.Z1
Freezer combination (+4°C and -20°C)	Liebherr
freezer (-80°C)	Sanyo, Vip series -86°C
Lamina	HERAsafe
Inverse microscope	Olympus
Forceps	KLS martin, germany, 23-941-15-07
15 ml tubes	Corning Science
Pipettes (10 µl, 100 µl, 1000 µl)	Eppendorf Research
Pipette tips	Thermo Scientific
Staining chamber	Semadeni Plastics Market
Microcentrifuge tubes	Costar, Sigma Aldrich
Serological pipettes	Nerbe Plus
Culture dishes (Ø 60mm, Ø 100 mm)	Corning Science
Glass cover slides CBA	Thermo Scientific, Menzel Gläser, Ø 12mm
Neubauer counting chamber	Optik Labor, Neubauer improved
Parafilm	Bemis, PM-996
Microscope slides for CBA	Assistent, Micro slides 76x26

Pasteur Pipettes	VWR
T25, T75 flasks	Thermo Fischer Scientific
24-well plates, 6-well plates	Costar

Table 2: Reagents used in the experiments

Reagents	Company
Ethanol 70%	provided by AKH pharmacy
10x phosphate buffered saline (PBS)	stock 10x PBS, Morphisto pH 7.2
Poly L-Lysine	SIGMA-Aldrich, 15µg/ml
HEK 293T cells	kindly provided by the institute
Dulbecco's Modified Eagle Medium (DMEM) high glucose	SIGMA, D6429 1L
Fetal bovine serum (FBS)	GIBCO, 10270
Penicillin Streptomycin (PenStrep)	SIGMA, P4333, 100 ml
L-Glutamin	GIBCO, 200mM (100x)
Aqua bidest	BRAUN, 500ml
Trypsin EDTA	SIGMA T4049
5 % Trypan blue	kindly provided by the institute
Plasmids	kindly provided by the institute
OPTIMEM	GIBCO, reduced serum medium (1x)
Lipofectamin 2000	INVITROGEN, 1 mg/ml
4% paraformaldehyd (PFA)	Affymetrix, 1 L
Bovine serum albumin (BSA)	GIBCO
Papain	SIGMA, P3125
Triton X 100	Merck, 1L
Aquatex mounting medium	Merck, 25 ml
Aqua dest	Produced at clinical institute of neurology
Mounting medium	Thermo Scientific, Shandon Consul-mount, 500 ml
Human OPC Expansion Complete Media	Millipore

Matrigel	Sigma-Aldrich, E1270
Oligodendrocyte differentiation medium	Millipore
Insulin	Provided by AKH pharmacy
Antibody-Diluent	Dako, S0809
Aqua-Poly/Mount	Polysciences Inc., 18606-20
DAPI	Invitrogen, D1306

Table 3: Used primary antibodies

Primary Antibody	Host	Dilution	Company	Code number
Anti-NG2	Rabbit	1:300	EMD Millipore	AB5320
Anti-CNPase	Mouse	1:100	Millipore	MAB326
Anti-MAP2	Mouse	1:1000	Millipore/Chemicon	MAB3418
Anti-GFAP	Mouse	1:1000	Millipore/Chemicon	MAB3402
Anti-O4	Mouse	1:50	R&D Systems	MAB1326
Anti-α Tubulin	Rabbit	1:200	Thermo Scientific	MJ1475262
Anti-A2B5	Mouse	1:100	Abcam	GR137594-7
Anti-PDGFR-α	Rabbit	1:500	Abcam	GR262424-2
Anti-MOG	Mouse	1:5000	Sigma LifeScience	02940
Anti-Pan-Neurofascin	Mouse	1:50	Millipore	MABN621
SMI 32	Mouse	1:000	ANOPOLI	SMI-32P

Table 4: Used secondary antibodies in immunofluorescence

Secondary Antibody	Dilution	Company	Code number
goat anti-mouse AF488	1:800	Jackson Immuno Research	115-545-166
goat anti-rabbit AF488	1:800	Jackson Immuno Research	111-545-144
Goat anti-rabbit Cy3	1:800	Jackson Immuno Research	111-165-144
Goat anti-mouse Cy3	1:800	Jackson Immuno Research	115-165-166
Goat anti-human AF488	1:800	Molecular Probes	1668688
Goat anti-human AF 594	1:800	Thermo Fisher Scientific	A-11014

2.2 Methods

2.2.1 Primary human cell cultures

Primary mixed-glial cell cultures were obtained from human brain surgery specimen. The material was collected from paediatric patients with FCD type IIB (N1617-16) and mild malformations of cortical development (N931-16) who underwent epilepsy surgery at the Department for Neurosurgery of the Medical University of Vienna. 5mm white matter pieces were cut off fresh brain pieces and each was transferred to a conical tube containing of Dulbecco's modified Eagle's medium (DMEM). The cell isolation was done according to the murine protocol as preciously published [133]. The conical tubes were put in a 37°C water bath for 3minutes. Afterwards the brain pieces were put in a sterile petri dish under the culture hood and chopped into smaller pieces with a sterile scalpel. When the brain material was able to pass a P1000 pipette tip without disrupting a smooth flow, OPC papain solution (DNase1, L-Cysteine, Papain, H2O) was added. The tubes were again incubated in a 37°C water bath for 20 minutes and inverted continuously to prevent tissue aggregation. Afterwards, mixed glial culture media was added to each tissue suspension to inactivate the papain solution. The tubes were centrifuged at 1200rpm for 5 minutes. The supernatant was discarded and the cell pellets were carefully resuspended with mixed glial cell culture media (containing Dulbecco's modified Eagle's medium (DMEM SIGMA, D6429, GlutaMAX), supplemented with 50 units/ml penicillin, 50 µg/ml streptomycin (SIGMA, P4333), and 10 % fetal bovine serum (GIBCO, 10270). The cell suspensions were transferred to a T25 flasks and incubated at 37°C until a layer of human primary cells was visible. The cell culture reached confluence after 2-3 weeks.

2.2.2 MO3.13 cell culture

The MO3.13 (CLU301, Cellutions Biosystems Inc., Cedarlane) are an immortalized human cell line which expresses various phenotypic characteristics of primary oligodendrocytes. The cells were thawed in a 37°C water bath and mixed with cell culture medium (containing Dulbecco's modified Eagle's medium (DMEM SIGMA, D6429, GlutaMAX), supplemented with 50 units/ml penicillin, 50 µg/ml streptomycin (SIGMA, P433), and 10 % fetal bovine serum (GIBCO, 10270)). The cell suspension was centrifuged at 1000rpm for 3 minutes, resuspended with cell

culture medium and transferred to a T25 flask. The cells were incubated at 37°C in a 5% CO_2 cell culture incubator. The culture reached confluence after 4-5 days.

2.2.3 OPC cell culture

Oligodendroglial cultures were established from frozen cell suspensions. EMD Millipore's Human Oligodendrocyte Progenitor Cells (OPCs, Cat. No. CS204496) are human ESC-derived OPCs. The tube, containing about >500 000 viable cells, was thawed in a 37°C water bath, the cells were transferred to a 15ml conical tube and 9ml of pre-warmed Human OPC Expansion Complete Media (freshly made with the Human OPC Expansion Media Kit; Millipore, Cat. No. SCM107) was added dropwise in a laminar flow hood. The mixed cell suspension was centrifuged at 1000rpm for 3 minutes. After the supernatant was removed, the cell pellet was resuspended in a total volume of 5ml of Human OPC Expansion Complete Media (pre-warmed to 37°C) and put on a Matrigel-coated T25 flask. Therefore Matrigel (Sigma-Aldrich Cat. No. E1270) was pipetted with a pre-cooled pipette tip to prevent solidifying of the extracellular matrix (ECM) gel, which is only liquid at 4°C, and mixed with 49ml of cold DMEM in a 50ml conical tube. The Matrigel-mix was put in the T25 flask and coated for 3 hours at room temperature. The remaining solution was discarded and after 3 hours the coating solution in the flask was removed. The flask was rinsed with 1xPBS before adding the cells. The cells were incubated at 37°C in a 5% CO2 humidified incubator. The media was exchanged every 2 to 3 days and reached confluence after 6 weeks.

2.2.4 Surface coating

After all cell types reached confluence, they were transferred onto poly-l-lysine (PLL; 15 µg/ml, Sigma-Aldrich) precoated glass cover slides (Ø 12 mm in 24-well plates, Costar, Cambridge, MA, USA) to prevent cell detachment during the staining process and to enhance cell growth.

2.2.5 Seeding of cells

The primary cell culture and the MO3.13 were cultivated in T75 flasks until they reached 80-100% confluency. The media was removed and the cells were washed two times with PBS. Cells were detached with trypsin, inactivation occurred with cell culture media followed by a

centrifugation step (at 1000rpm for 3 minutes). Afterwards, the cell number in the suspension was count with a haemocytometer. The primary cell cultures were seeded with 10 000 cells and the MO3.13 with 100 cells per well on a 6-well plate. Each cell line was cultivated for 21 days at 37°C in a 5% CO2 humidified incubator. To create a wet chamber, only the inner 10 of the 24 wells were filled with the PLL coated cover slides and cells, the outer wells were filled with PBS.

2.2.6 Co-Culture

In a second step, the 3 cell cultures were cultivated in T25 flasks and seeded to a primary neuronal rat cell culture. The neuronal cells (40 000 cells/well) were also seeded onto PLL-coated glass slides in a 24-well plate. All 24 wells were used for cell cultivation and after 3 weeks the primary cell cultures (10 000 cells/well) and the MO3.13 (100 cells/well) were seeded onto the rat neurons. An oligodendrocyte differentiation medium (Millipore, Human OPC Spontaneous Differentiation Media Kit, SCM106) was used and exchanged every 3-4 days. In a second step the same co-cultures have been incubated with additional Insulin (1mg/ml) to enhance myelination.

2.2.7 Immunofluorescence

2.2.7.1 Primary antibodies

The 4 cell cultures were incubated with commercial antibodies to characterize the antigen epitopes on the cell surfaces. All incubation steps were performed in a wet chamber and at room temperature. The media was removed with a water jet pump and the cells have been fixed with 4% PFA for 15 minutes. After a washing step with 1x Phosphate-Buffered Saline (PBS) (Morphisto pH 7) the cells were permeabilized with 0,1% Triton X-100 (Merck) for 5 minutes and blocked with 10% Antibody-Diluent (Dako, S0809) for 30 minutes. Primary antibodies were diluted in 10% PBS and the cells were incubated for 45 minutes.

2.2.7.2 Secondary antibodies

Secondary antibodies were diluted in 10% Antibody-Diluent. After a washing step the cells were incubated for 45 minutes in the dark. Afterwards the glass slips were washed with PBS

again and nucleus staining was done with DAPI (Invitrogen, D1306).

2.2.8 Mounting

After the DAPI staining, the glass coverslips were washed 3 times with PBS and mounted onto a microscope slide with Aqua-Poly/Mount (Polysciences Inc., 18606-20).

2.2.9 Seeding of HEK 293T cells on Poly-D-Lysine coated cover plates

The HEK 293T cells were cultured in a $\emptyset$ 100 mm cell culture dish until they reached 80% confluency. For cell detachment, the medium was withdrawn and cells were incubated with 2 ml Trypsin-EDTA for 3 minutes at 37° C. The enzymatic reaction was stopped with fresh cell culture medium. The cell suspension was mixed thoroughly and two aliquots were taken for cell counting. The aliquots were each resuspended in Trypanblue solution to distinguish between dead and live cells. Cell counting was performed with a Neubauer counting chamber. To achieve optimal cell density for the assay, 1.2 Mio cells were seeded in $\emptyset$ 60mm cell culture dish containing pre-coated glass cover slides. 5 ml of cell culture medium was added and cells were incubated overnight at 37 °C and 5 % $CO2$ until transfection was performed.

2.2.9.1 Transfection of HEK 293T cells with NF 155, NF186 and MOG

Neurofascin 155 (Myc-tagged human NF155; NM_001160331.1; kindly provided by Luis Querol), Neurofascin 186 (full-length human NF186 C-terminally fused with super green fluorescent protein; kindly provided by Edgar Meinl) and MOG plasmids (full-length MOG C-terminally fused with EGFP; kindly provided by Markus Reindl) were obtained in an aqueous solution. To achieve a sufficient transfection rate, HEK 293T cells had to be transfected with 8 µg Plasmid DNA per $\emptyset$ 60mm cell culture dish.

Transfection of HEK 293T cells was performed with Lipofectamin® 2000 transfection reagent. The plasmid-Lipofectamin complexes were made in Opti-MEM® reduced serum medium. 20 µl of Lipofectamin reagent and the appropriate volume of Plasmid DNA were each mixed with 500 µl Opti-MEM and incubated separately for 5 minutes at room temperature until solutions were combined. During an incubation period of 20 min at room temperature plasmid-

Lipofectamin complexes were formed. The DNA-lipid complex was transferred to the Ø 60mm cell culture dish and HEK 293T cells were transfected overnight in the incubator until the CBA was performed.

2.2.9.2 Fixation of transfected HEK 293T cells

Before staining cells with patients' samples, cells had to be fixed with PFA. Cell culture dishes, containing cover slides with transfected HEK 293T cells, were brought to room temperature and medium was sucked up with a water jet pump. The cells were washed once with 1x PBS before covering the glass plates with 4 % cold PFA for exactly 10 min at room temperature. After fixation, PFA was withdrawn and cells were washed three times with 1x PBS. To permeabilize the cell membrane, cover slides were covered with 0.3 % Triton X-100 for 5 min at room temperature. Followed by a washing step (3x with 1xPBS), cells were incubated with 1 % BSA for 1 h 45 min at room temperature to reduce unspecific binding during the staining procedure.

3. Results

3.1 Characterisation of primary human cells and MO3.13

In a first step the primary human cells (N1617-16 and N931-16) and the MO3.13 cells were cultivated in T25 flasks until they reached confluence. To characterize the maturation of the cells we used antibodies which are known to be present in maturing oligodendrocytes and their progenitor cells (Fig.1), in addition anti α-Tubulin was used to show the cell morphology. The following antibody panel was used for characterisation (Fig.2):

αTubulin binds to microtubules in eukaryotic cells which are important for the vital processes like mitosis, meiosis, intracellular transport and maintenance of the cell shape. All three cell cultures showed a constant and reliable staining. The staining with αTubulin showed a difference in the size of the human cells and the MO3.13. Another difference is the very small cytoplasm area of the MO3.13 compared to the primary human cells.

NG2 (Anti-NG2 chondroitin sulfate proteoglycan) is a surface antigen found on premature and developing glial cells. The staining showed a clustering of fluorescent signal in the outer areas of the cell bodies in the N1617-16 and the N931-16 cells and spike-like outgrowths are visible.

O4 is an early oligodendrocyte marker and is expressed in the surface of semi-mature oligodendrocytes.

CNPase is an oligodendrocyte and Schwann cell marker.

A2B5 and PDGF-α are antigens found in immature oligodendrocytes and were also tested in all three cell cultures but no positive staining occurred.

MBP (myelin basic protein) was also tested but no positive staining was found, indicating that the cell cultures didn't start myelinating processes yet.

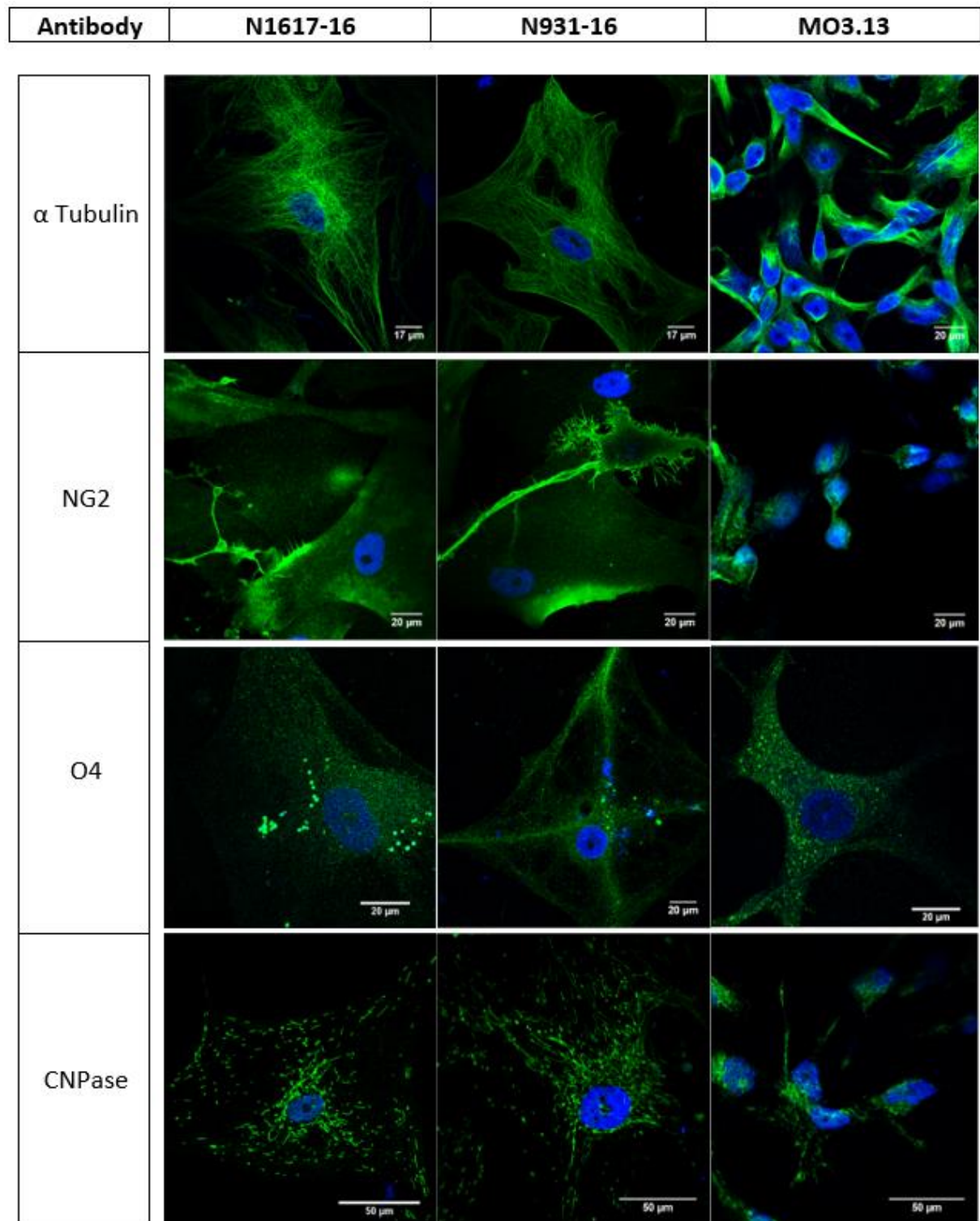| Antibody	N1617-16	N931-16	MO3.13
α Tubulin			
NG2			
O4			
CNPase			

Figure 4: Characterization and positive staining of the primary human cells N1617 and N931 and the immortalized human cell line MO3.13 with oligodendroglial antibodies and α-Tubulin. Nuclear staining was done with DAPI.

3.2 OPC cell culture

Despite several repetitions the OPC cell culture didn't grow fast enough under used conditions to establish a staining protocol and was excluded from further testing methods.

3.3 Characterization of co-cultures with hippocampal rat neurons

In a second step the 3 cell cultures were added to a 3 week-old rat-neuronal-culture and cultivated for another 21 days. The cells were incubated with an oligodendrocyte differenciation media. Additionally to the already established oligodendrolial antibodies, we tested for **anti-MBP** (myelin basic protein), a myelin associated protein. SMI32 and MAP2 have been used as neuronal markers. The human cells showed the expected positivities for the oligodendroglial proteins and also for MBP (see Fig. 3). MAP2, which is usually used as a neuronal marker, has been found in human oligodendroglial progenitor cells and mature oligodendrocytes. SMI32 was used as a substitute for MAP2 but didn't show satisfying results.
In the stainings with NG2, in all three cell cultures differentiated oligodendrocytes have been detected. Generally a broad spectrum of different maturation stages and cell types have been observed and led to the result, that a co-culture with mixed hippocampal rat cells in combination with the differentiation media has a positive outcome for our primary human cells. According to these findings and the positive staining of MBP, we tested sera from patients with suspected antibodies against myelin structure proteins such as MOG and NF.
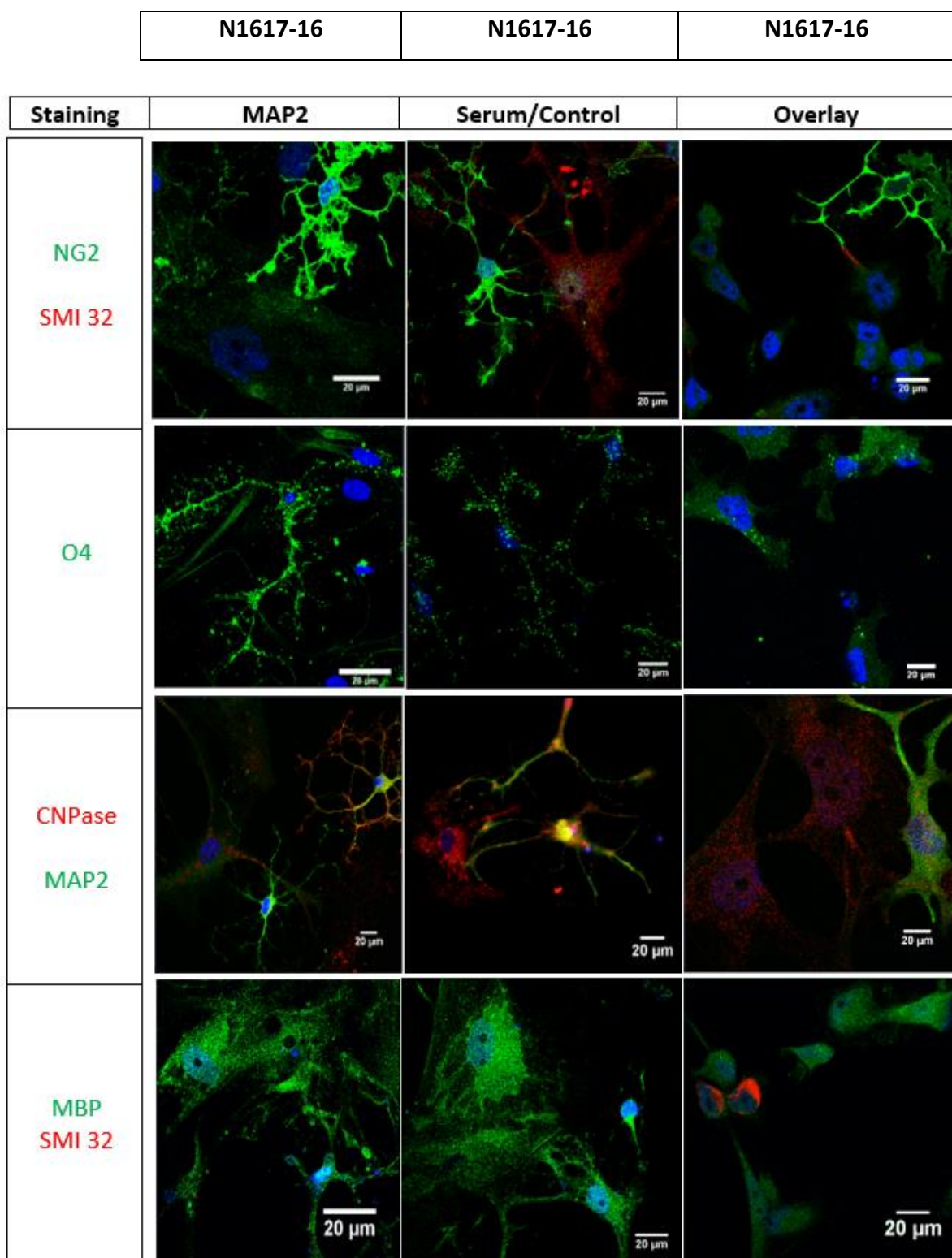
Staining	MAP2	Serum/Control	Overlay
N1617-16	N1617-16	N1617-16	
NG2 / SMI 32			
O4			
CNPase / MAP2			
MBP / SMI 32			

Figure 5 Positive staining of already established oligodendroglial antibodies in a co-culture with rat neurons. Neurons were marked with SMI32 or MAP2. Nuclear staining was done with DAPI.

3.4 Transfection of HEK cells

HEK 293T cells were successfully transfected with plasmids of MOG, NF155, and NF186. The successful transfection for MOG was demonstrated with the green fluorescent staining of the GFP-tagged MOG plasmid (figure 6). The successful transfection for NF155 and NF186 was demonstrated with a commercial anti-Myc antibody labelled with Alexa 488 (NF155) (figure 7) and the green fluorescent staining of the GFP tagged NF186 (figure 8). In addition, the commercial antibody pan-neurofascin was used to demonstrate the surface expression of NF155 and NF186. The respective cell-based assays showed a positive staining with the patient's sera with MOG (figure 6) and NF155 antibodies (figure 7). An additional serum of a patient with a yet uncharacterized antibody against the node of Ranvier was tested for NF155 and NF186 but remained negative (figure 8).
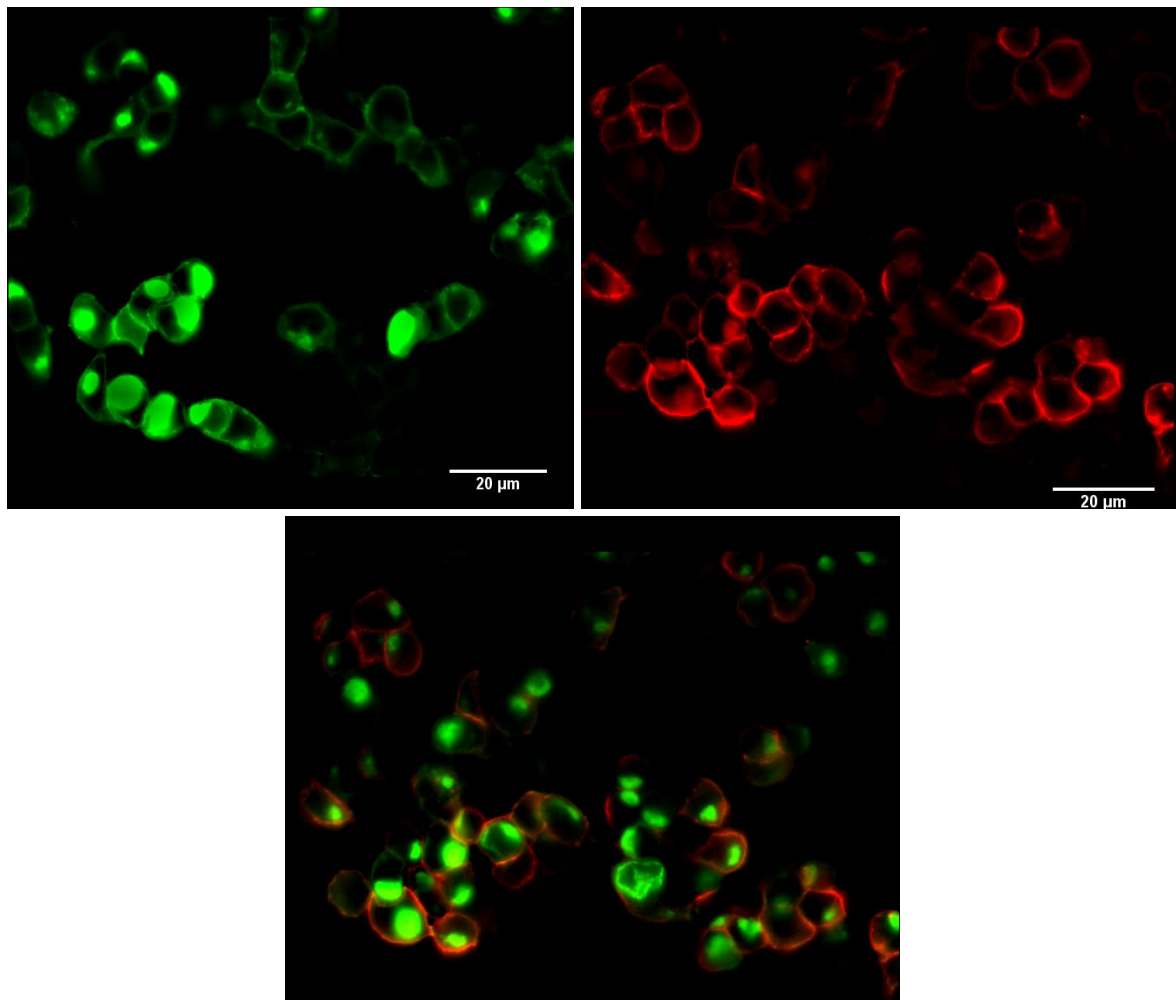
Figure 6 Positive staining of transfected HEK cells with a MOG plasmid (green) and patient's serum with anti-MOG antibodies (red).
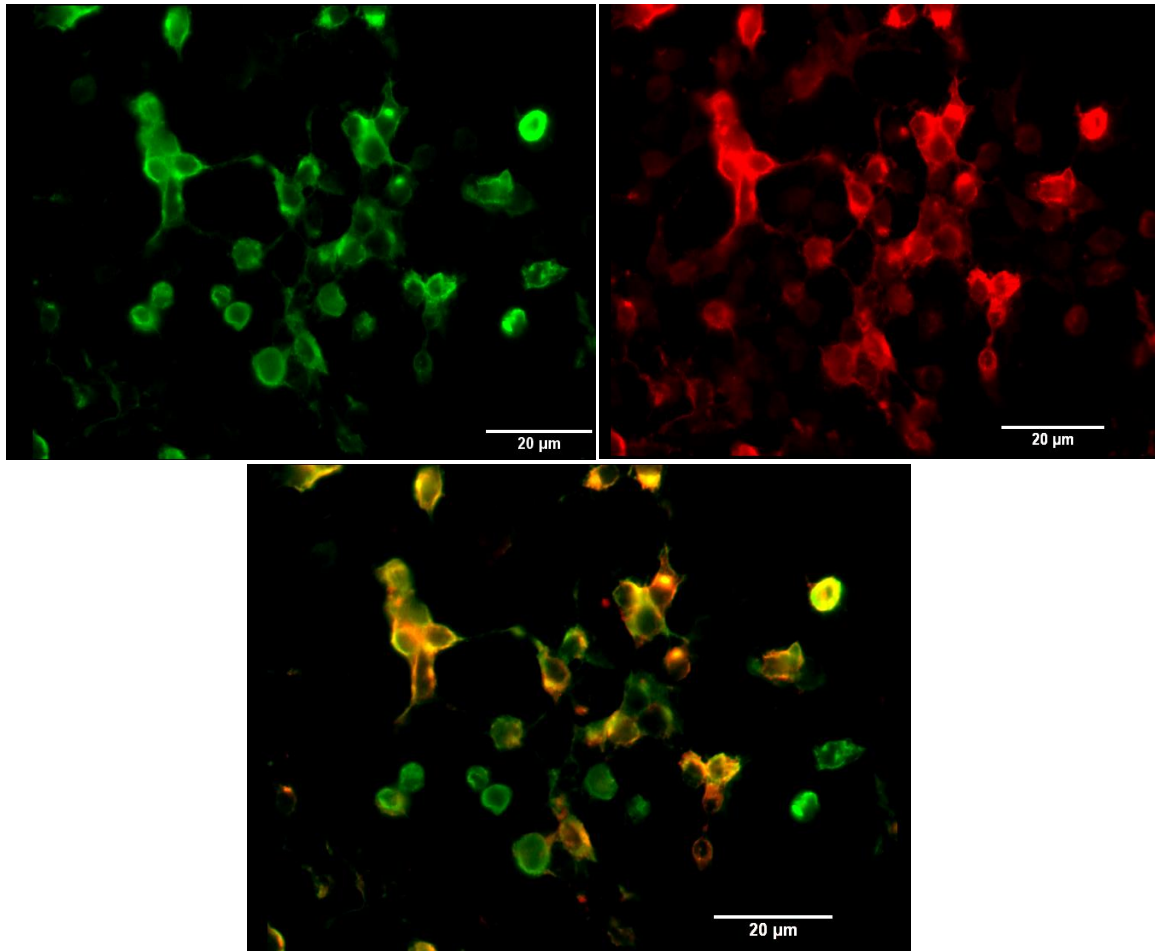
29

Figure 7 Positive staining of transfected HEK cells with a NF155 plasmid (green) stained with NF155 positive patient's serum (red).
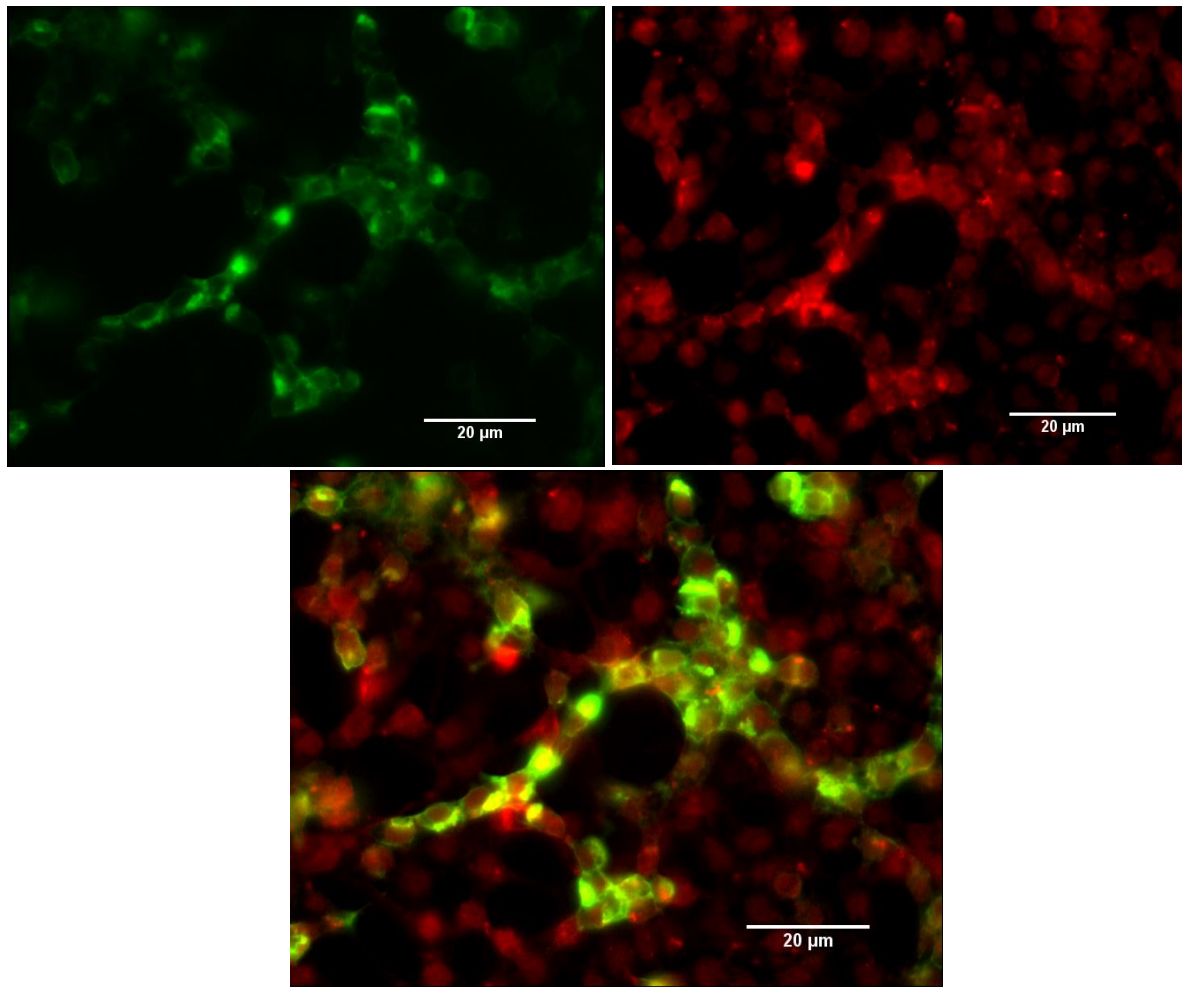
Figure 8 Negative staining of transfected HEK cells with a NF186 plasmid (green) and stained with patient's serum (red).

3.4 Screening of patients' sera on co-cultures

Since the results of the N1617-16 cells have been most promising and the cell growth correlated very well with the cultivation of the rat cells, we decided to focus on these cells for the screening of patients' sera. We used serum of a patient with anti-NF155/186 antibodies (patient 1), serum of a patient with a yet uncharacterised antibody against the node of Ranvier (patient 2), and serum of a patient with anti-MOG antibodies (patient 3).

Both, patient 1 and patient 2 were diagnosed with CIDP with progressive disease course and cerebellar ataxia, compatible with CIDP associated with paranodal antibodies. Patient 3 was diagnosed with recurrent episodes of optic neuritis since 30 years and subsequent development of confluent demyelinating brain lesions with pronounced perivenous accentuation.

Serum from patient 1 was tested positive for anti-neurofascin155 antibodies in the CBA (figure 7). This sample also showed a positive staining of oligodendrocytes in the mixed glioneuronal co-cultures (figure 9).

The serum of patient 2 with a yet uncharacterized antibody against the node of Ranvier did not show a positive staining of the N1617-16 cells. Figure 9 shows a negative staining of an oligodendrocyte of the co-culture.

The expression of NF155 in the oligodendrocytes of our co-culture was confirmed with a commercial antibody against pan-neurofascin.

The serum of patient 3 with anti-MOG antibodies showed a positive staining of myelin sheaths of oligodendrocytes in the mixed glio-neuronal co-culture of N1617-16 (figure 10).
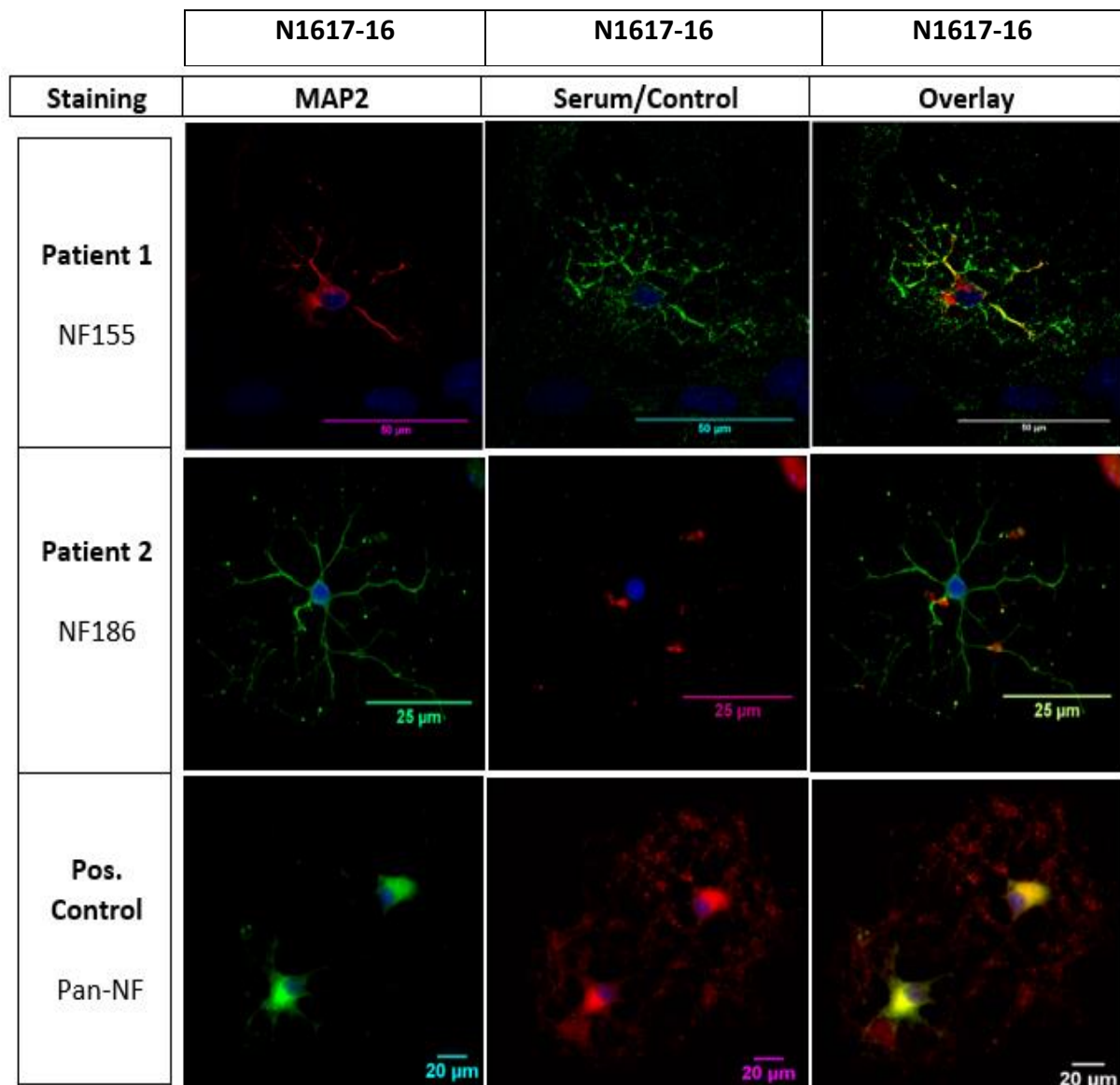
Staining	N1617-16 MAP2	N1617-16 Serum/Control	N1617-16 Overlay
Patient 1 NF155			
Patient 2 NF186			
Pos. Control Pan-NF			

Figure 9 Positive staining of an oligodendrocyte with a commercial anti-MAP2-antibody and serum of patient 1 with NF155 antibodies. Negative staining of a MAP2-positive oligodendrocyte with a serum with a yet uncharacterised antibody to the node of Ranvier. Positive staining of oligodendrocytes with a commercial antibody against pan-NF
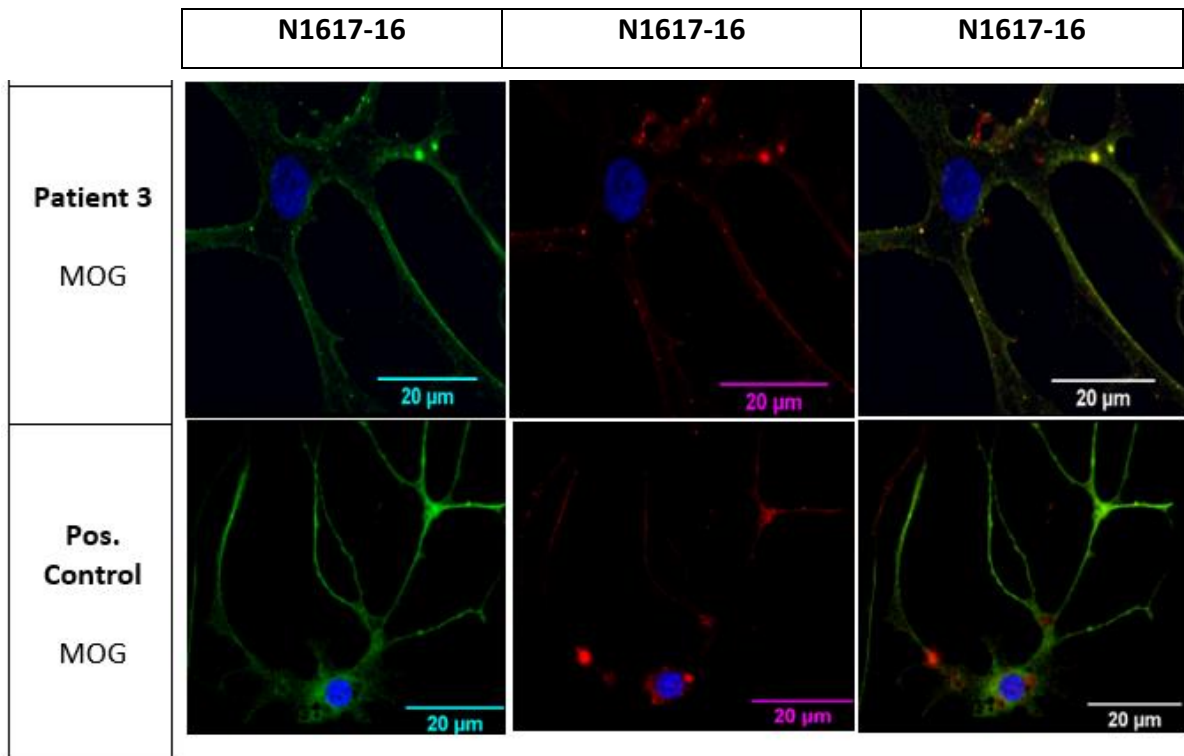
Figure 10 Positive staining of oligodendrocytes from the mixed glioneuronal co-culture with N1617-16 cells with MOG antibody-positive patient´s serum (patient 3) and a commercial MOG antibody (pos. control), as well as MAP2 (red). Nuclear staining was done with DAPI.

4. Discussion

This study was performed to establish a novel cell-based assay to detect human-specific surface antibodies against glial cells in sera or CSF from patients with demyelinating diseases. Therefore we first characterised the present antigens on the surface of two primary cell cultures (N1617-16 and N931-16) and on an immortalized human cell line, which expresses various phenotypic characteristics of primary oligodendrocytes (MO3.13). We chose antibodies which are known to be present in oligodendrocyte progenitor cells and mature oligodendrocytes and α-Tubulin to show the general cell morphology.

The N1617-16 cells as well as the N931-16 cells were obtained from human brain surgery specimen from epilepsy patients. The N1617-16 cells derive from a patient with focal cortical dysplasia type IIB (FCD IIB), which is an isolated lesion characterized by balloon cells and dysmorphic neurons [134].

The N931-16 cell line derives from a patient with a mild malformation of the cortical development (MMCD), which is characterized by subtle abnormalities e.g. heterotopic neurons in layer I of the neocortex or neurons located within the subcortical white matter. Myelination is not altered but some patients show also an oligodendroglial hyperplasia [135].

The MO3.13 cells were hard to coordinate under co-culture conditions with the primary rat neurons, because of the high proliferation rate. The fast growth rate resulted in clustered cell piles with the side effect that the rat neurons weren't detectable anymore under the MO3.13 cell layer. On the other hand, the commercial OPCs needed six weeks until they were confluent enough, but still weren't at the expected stadium after another 21 days of co-culture. The N931-16 cells were promising after the first antibody characterisation, but also grew too slow to create a balanced co-culture of primary human cells and rat neurons. Since we wanted to establish a diagnostical tool for regular serum and CSF screening, the practical usage and cell growth were important factors for the possibility of regular staining procedures. Since we used a media with oligodendrocyte-growth factors, variant stages of oligodendrocyte development can be observed within each batch. As A2B5 and PDGF- α wasn't expressed in our cells it is likely that they are already more differentiated. NG2, O4 and CNPase was observed in all cell cultures.

In the double stainings of the mixed cell cultures with the neuronal rat cells MAP2 and SMI32 have been used as neuronal markers. Although microtubule-associated proteins (MAPs) are

neuron-specific proteins, we observed the localisation of MAP2 in oligodendroglial progenitor cells as well as in a GFAP-stained cell, which is likely to be an astrocyte progenitor cells. The presence of MAP2 in immature and mature myelin-forming cells has a functional significance of the cytoskeleton during oligodendrocyte differentiation, process outgrowth, and myelin formation [136]. Additionally the treatment with oligodendroglial growth factors like PDGF-AA and NT3 in the medium might have dedifferentiated the rat neurons and made neuronal stainings insufficient.

The various intensities of MAP2 in the progenitor cells might also be linked to the maturation of the cells. Little expression of MAP2 might be more mature cells compared to a high expression of MAP2 in immature progenitor cells.

In a next step the cell cultures were incubated with sera from patients with diagnosed CIDP (patient 1 and 2) and atypical multiple sclerosis (patient 3) with antibodies against oligodendroglia (NF155 and MOG). Stainings with a commercial antibody against both isoforms of neurofascin (NF155 and NF186) were positive in oligodendrocytes in the co-cultures with N1617-16 cells, which confirms the oligodendroglial-specific expression of NF155. Staining of the cell culture with the serum of patient 1 with NF155 antibodies successfully labelled the oligodendrocytes, whereas serum of patient 2 with a yet uncharacterised paranodal antibody remained negative. This result suggests that the antibody of patient 2 recognises an antigen that is not expressed at the glial but the axonal side of the node of Ranvier [137]. Interestingly, also the neuronal cells remained negative, which may be due to the treatment of the cell culture with the oligodendroglial differentiation medium. Further tests with different cell-based assays such as CASPR1 will be necessary to characterise the targeted antigen.

Stainings with a commercial antibody against MOG was positive in oligodendrocytes in the co-cultures with N1617-16 cells, which confirms the expression of MOG on the surface of myelin sheaths in our culture. Staining of the cell culture with the serum of patient 3 with anti-MOG antibodies successfully labelled the oligodendrocytes. Since it has been shown that patient´s anti-MOG antibodies recognise a human specific epitope, this result indicates that our co-culture is useful for the screening of human specific antibodies [132]. Further sera of patients with demyelinating diseases have to be screened to detect novel antibodies that may play a pathogenic role in disease evolution.

4.1 Conclusion

We successfully characterised three different human glial cell cultures and established a co-culture with rat hippocampal neurons to induce myelination. Our experiments showed that the presence of neurons had positive effects on the myelination and that the co-cultures create a suitable test system for MOG and NF155-antibodies and might be applicable for other myelin-associated proteins, which we did not test so far. The co-culture may not be useful for the screening of anti-neuronal antibodies, since the oligodendrocyte differentiation medium may cause a dedifferentiation of co-cultured neurons with loss of neuron-specific proteins.

4.2 Outlook

In this study we established a primary human glial cell culture for the screening of anti-glial surface antibodies. This cell culture may provide a promising screening tool for the identification of novel human-specific antibodies that play a role in demyelinating CNS and PNS diseases. Further studies with a large number of patients´ sera are necessary to identify more samples with a positive staining pattern. Subsequent immunoprecipitation and mass spectrometry experiments may enable the characterisation of novel target antigens.

5. References

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Abstract

Recently antibodies against proteins of the node of Ranvier were detected in patients with chronic inflammatory demyelinating polyneuropathy (CIDP) and myelin oligodendrocyte glycoprotein (MOG) antibodies in patients with various central demyelinating diseases. These autoantibodies are supposed to play a direct pathogenic role in disease development. The identification of these autoantibodies is important to initiate the appropriate treatment approach. Routine detection methods include antibody screening on rat brain sections with an immune histochemical technique (IHC) and identification of the antibody type with immunofluoresce (IF) labelling of transfected cells expressing the target antigen (cell-based assay, CBA). Antibodies that recognise a human specific epitope such as anti-MOG-antibodies are negative on rat brain sections and can only be tested in specific CBAs that are transfected with the human full-length protein. The aim of this study was to expand the rat brain tissue-based screening assay (TBA) to a human mixed glial cell culture (glial-CBA), to identify novel human-specific antibodies to oligodendrocyte, astrocyte or oligodendrocyte progenitor cell antigens. We isolated glial cells from epilepsy surgery specimens (focal cortical dysplasia-FCD, mild malformation of the cortical development-MMCD) and used immunofluorescence to characterise the cultures with various commercial antibodies. Afterwards, we established a co-culture system with primary rat neurons to enhance the myelination of the primary human cells. The myelinating co-cultures were then used to screen patients´ sera with already known antibodies against oligodendroglial proteins, like Neurofascin155 (NF155) and MOG. The characterisation of the primary human cells and MO3.13 revealed a strong expression of alpha-Tubulin, NG2, O4 and CNPase, whereas A2B5, PDGF-alpha and MBP were negative. Co-cultures with hippocampal neurons showed a strong expression of MBP and MOG, indicating a maturation of myelin sheaths. The oligodendrocytes additionally showed a strong labelling with anti-MOG and anti-NF155 positive patients´ sera. In conclusion, we established a primary human glial cell culture for the screening of anti-glial surface antibodies. This cell culture may provide a promising screening tool for the identification of novel human-specific antibodies

Zusammenfassung

Kürzlich wurden Antikörper gegen Proteine des Ranvier'schen Schnürrings bei Patienten mit chronischer inflammatorischer demyelinisierender Polyneuropathie (CIDP)- und Myelin-Oligodendrozyten-Glykoprotein (MOG)-Antikörper bei Patienten mit verschiedenen zentralen demyelinisierenden Erkrankungen nachgewiesen. Diese Autoantikörper sollen eine direkte pathogene Rolle bei der Krankheitsentwicklung spielen. Die Identifizierung dieser Autoantikörper ist wichtig, um den geeigneten Behandlungsansatz zu initiieren. Routinedetektionsverfahren umfassen das Antikörperscreening an Rattenhirnschnitten mit immunhistochemischen Techniken (IHC) und die Identifizierung des Antikörpertyps mit Immunofluoreszenz (IF) -Markierung von transfizierten Zellen, die das Zielantigen exprimieren (zellbasierter Assay, CBA). Antikörper, die ein menschliches spezifisches Epitop wie Anti-MOG-Antikörper erkennen, sind negativ auf Rattenhirnschnitten und können nur in spezifischen CBAs getestet werden, die mit dem humanen Volllängenprotein transfiziert sind. Das Ziel dieser Studie war es, den Rattenhirngewebe-basierten Screening-Assay (TBA) auf eine humane gemischte Gliazellkultur (Glia-CBA) zu erweitern, um neue humanspezifische Antikörper gegen Oligodendrozyten-, Astrozyten- oder Oligodendrozyten-Vorläuferzellen-Antigene zu identifizieren. Wir isolierten Gliazellen aus epilepsiechirurgischen Proben (fokale corticale Dysplasie -FCD, milde Malformation der corticalen Entwicklung- MMCD) und verwendeten Immunfluoreszenz, um die Kulturen mit verschiedenen kommerziellen Antikörpern zu charakterisieren. Anschließend haben wir ein Co-Kultursystem mit primären Rattenneuronen etabliert, um die Myelinisierung der primären menschlichen Zellen zu verbessern. Die myelinisierenden Co-Kulturen wurden dann verwendet, um Patientenseren mit bereits bekannten Antikörpern gegen oligodendrogliale Proteine wie Neurofascin 155 (NF155) und MOG zu screenen. Die Charakterisierung der primären humanen Zellen und MO3.13 ergab eine starke Expression von alpha-Tubulin, NG2, O4 und CNPase, während A2B5, PDGF-alpha und MBP negativ waren. Co-Kulturen mit Hippocampus-Neuronen zeigten eine starke Expression von MBP und MOG, was auf eine Reifung von Myelinscheiden hinweist. Die Oligodendrozyten zeigten zusätzlich eine starke Markierung mit anti-MOG- und anti-NF155-positiven Patientenseren. Zusammenfassend haben wir eine primäre humane Gliazellkultur für das Screening von anti-glialen Oberflächenantikörpern etabliert. Diese Zellkultur könnte ein vielversprechendes Screening-Werkzeug für die Identifizierung neuer human-spezifischer Antikörper sein.