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CLAUDINS IN INFLAMMATORY CNS DISEASE

Muhammad Aslam

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Vorsitzender:	Univ-Prof. Dr. J. Winkelmann
Prüfer der Dissertation:	
1.	Univ-Prof. Dr. B. Hemmer
2.	Univ-Prof. Dr. Chr. Stadelmann-Nessler, Universitätsmedizin Göttingen

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Declaration

I declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any university or other institution of higher education. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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Summary

The immune privilege of central nervous system (CNS) is severely undermined in inflammatory CNS disease such as multiple sclerosis (MS) when a coordinated immune attack across the blood brain barrier (BBB) results in demyelination, gliosis and neuronal injury. Claudins (Cldns) are a family of tetrapasnin tight junction (TJ) proteins comprising of 23 members. Among claudin family members expressed in the CNS, oligodendrocyte specific protein (OSP or claudin-11) stabilizes compact myelin structure and claudin-3, -5 and -12 are expressed at inter endothelial tight junctions guarding the paracellular permeability across BBB. Experimental work presented in this thesis comprises two separate studies addressing the involvement of claudins in different aspects of inflammatory CNS disease.

Study 1

Oligodendrocyte specific protein (OSP/Cldn11) is a candidate autoantigen in MS and CSF reactivity towards OSP has been reported in MS patients. The binding specificity (to linear or conformation dependent epitopes) of anti-OSP antibody response in MS was determined using sensitive cell based assays. Further this study compares binding charactiristics and clinical relevance of the anti-OSP antibodies and antibodies targeting myelin oligodendrocyte glycoprotein (MOG) another candidate autoantigen in MS. Whereas, a unique and highly specific antibody response to native MOG (nMOG, presenting conformational MOG epitopes) was detected in children affected by the first demyelinating event (CIS or ADEM) using cell based flow cytometry assay, the reactivity against OSP/Cldn11 was only detected in ELISA based on denatured antigen indicating that autoantibodies to OSP recognized linear epitopes or denatured OSP protein. Clinical (predictive) potential of anti-nMOG and anti-OSP antibody response was also addressed in this study. Taken together the results presented here demonstrate that anti-OSP antibodies represent an epiphenomenon in MS as compared to anti-MOG antibodies that recognise

native glial MOG expression and therefore may have pathologic relevance in inflammatory CNS demyelinating diseases.

Study 2

Cldn3, -5 and -12 stabelize interendothelial tight junctions (TJs) that play a crucial role in the regulation of BBB permeability under physiological, as well as pathological conditions. Loss of BBB integrity due to molecular alteration of TJs is cardinal feature of inflammatory CNS diseases however the molecular mechanism is less characterized. Previous studies indicated the role of inflammatory cytokines. We investigated the effect of cytokines relevant to inflammatory CNS disease on the cerebral endothelial expression of claudins whereby Cldn5 was identified as a direct target of TNFα induced signaling. A strong reduction in Cldn5 mRNA and protein expression was observed in cerebral microvascular endothelial cells upon TNFα treatment whereas the expression of Cldn3 and -12 was unaffected. An ultra-conserved TNFα responsive 129 bp sequence element (-892/-763) harbouring NFκB-p65 (RelA) subunit binding sites was identified in mouse Cldn5 promoter. Furthermore, the TNFα dependent reduction in Cldn5 promoter activity could be mimicked in murine cerebral endothelial cells by the cotrasfection of NFκB-p65 (RelA) expression plasmid. In conclusion, these studies provide an insight into the molecular mechanism that may underlie loss of BBB-TJ integrity in the inflammatory CNS diseases.

Zusammenfassung

Bei entzündlichen Erkrankungen des Zentralnervensystems (ZNS) wie bei der Multiplen Sklerose (MS) kommt es zu einer Störung der Bluthirnschranke. Dadurch wird das physiologische Immunprivileg des ZNS aufgehoben und durch das Zusammenspiel von Entzündungszellen kommt es zu Demyelinisierung, Gliose und axonalem Schaden. Claudine (Cldns) gehören zur Familie der sogenannten Tetraspanin-Proteine, von denen es insgesamt 23 Mitglieder gibt. Unter den Claudin-Proteinen stabilisiert beispielsweise Claudin-11, auch Oligodendrozyten-spezifisches-Protein (OSP) genannt, die Kompaktheit des Myelins. Die Claudine-3,-5 und -12 werden an den endothelialen „tight junctions“ (TJ) exprimiert, wodurch die Zell-Zellkontakte stabilisiert werden und dadurch die Bluthirnschranke gesichert wird. Die beiden hier vorgestellten experimentellen Arbeiten über die Claudine beschäftigen sich mit zwei unterschiedlichen Aspekten in der Pathogenese entzündlicher ZNS-Erkrankungen.

Projekt 1

Das Oligodendrozyten-spezifische-Protein (OSP/Cldn11) ist ein mögliches Autoantigen bei der MS, da über eine Reaktivität gegen dieses Protein im Liquor betroffener Patienten berichtet wurde. Im Rahmen dieses Projekts haben wir die Bindungsspezifität spezifischer Antikörper gegen OSP mithilfe zellbasierter Assays gegen lineare und sogenannte konformationale Epitope bei der MS untersucht. Zudem haben wir die Bindungsspezifität und klinische Relevanz dieser Autoantikörper mit solchen Anitkörpern gegen das Myelin-Oligodendrozyten-Glykoprotein (MOG) bei der MS verglichen. Dabei fanden wir eine hochspezifische Antikörperantwort gegen natives MOG (nMOG) in Kindern, die an einem ersten MS-Schub (~klinische isoliertes Syndrom=CIS) oder an einer akuten disseminierten Encephalomyelitis (ADEM) erkrankt waren. Allerdings konnten wir keine spezifische Antikörper-Reaktivität gegen natives OSP feststellen. Im Vergleich zur Antikörperreaktion gegen MOG, wo eine Reaktivität gegen das konformationale Epitop bestand, lag im Falle der OSP-Antikörper eine Reaktivität gegen lineare Proteinepitope bzw. denaturierte OSP-Proteine vor. Die prädiktive klinische Relevanz der Antikörperreaktion gegen nMOG

wurde zudem im Rahmen des Projekts untersucht. Unsere Ergebnisse zeigen, dass anti-OSP-Antikörper somit mit keinem nativen OSP (nOSP) reagieren und daher am ehesten einem Epiphänomen bei der MS darstellt. Verglichen damit erkennen die anti-MOG-Antikörper ein natives auf glialen Zellen exprimiertes Protein und offenbaren daher eine klinische und pathologische Relevanz im Rahmen entzündlich-demyelinisierender ZNS-Erkrankungen.

Projekt 2

"Tight junctions" (TJ) als Teil der endothelialen Zell-Zellkontakte spielen eine entscheidende Rolle bei der Regulation und Aufrechterhaltung der Bluthirnschranke unter physiologischen und pathologischen Bedingungen. Eine Störung der Bluthirnschranke durch molekulare Veränderungen im Bereich des TJs spielt zudem eine wichtige Rolle in der Pathogenese entzündlicher ZNS-Erkrankungen. Die TJs der Bluthirnschranke werden dabei zum großen Teil durch Proteine aus der Claudin-Familie (Claudin-3,-5,-12) gebildet. Im Rahmen des zweiten Teilprojekts haben die Effekte proinflammatorischer Zytokine auf die Expression endothelialer TJ-Proteine untersucht. Dabei fiel auf, dass das Zytokin TNFα direkt die intrazelluläre Signalkaskade von Claudin-5 reguliert. Zudem zeigte sich eine verminderte Expression von Claudin-5 durch Abnahme der mRNA durch Behandlung von sogenannten murinen zerebralen mikrovaskulären endothelialen Zellen (MVEC) durch TNFα. Im Gegensatz dazu war die Expression anderer Claudine (Claudin-3 und -12) unbeeinflusst. Wir konnten ferner eine sogenannte evolutionär konservierte Promotor-Region (ECR) mit einer Bindungsstelle für NFκB (subunit p65) im Mauspromoter von Claudin-5 identifizieren, die durch TNFα reguliert wird. Basierend auf diesen Untersuchungen über das Expressionsmuster von Claudin-5 in Endothelzellen und zusätzlich durchgeführten immunhistochemischen Färbungen in experimentellen spinalen entzündlichen Läsionen folgern wir, dass Claudin-5 eine wichtige Rolle im Rahmen entzündlicher ZNS-Erkrankungen im Hinblick auf die Regulation der TJs in der Bluthirnschranke zukommt.

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List of Abbreviations

ADCC	Antibody-dependent cell-mediated cytotoxicity
ADEM	Acute disseminated encephalomyelitis
AQP4	Aquaporin 4
BBB	Blood brain barrier
bEND.3	Brain endothelioma 3 cell line
BM	Basement membrane
CIS	Clinically isolated syndrome
CLDN	Claudin
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTR	Control
Dex	Dexamethasone
DTT	Dithiothreitol
EAE	Experimental autoimmune encephalomyelitis
ECGS	Endothelial cell growth supplement
ECR	Evolutionary conserved region
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
FCS	Fetal calf serum
HC	Healthy control
HRP	horseradish peroxidase
IFNγ	Interferon gamma
IL-1	Interleukin 1
KDa	Kilodalton
LETM	Longitudinally extensive myelitis
MAG	Myelin-associated glycoprotein

MBP	Myelin basic protein
MFI	Median fluorescence intensity
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
MVEC	Microvascular endothelial cells
NIND	Non-inflammatory neurological disease
NMO	Neuromyelitis optica
nMOG	Native myelin oligodendrocyte glycoprotein
nOSP	Native oligodendrocyte specific protein
OCB	Oligoclonal band
OD	Optical density
ON	Optic neuritis
OND	Other neurological diseases
OSP	Oliodendrocyte specific protein
PDTC	Pyrrolidinedithiocarbamate
PPMS	Primary progressive form of multiple sclerosis
RION	Relapsing isolated optic neuritis
RRMS	Relapsing remitting form of multiple sclerosis
SPMS	Secondary progressive form of multiple sclerosis
TFBS	Transcription factor biding site
TJ	Tight junction
TM	Transverse myelitis
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor

Chapter 1. INTRODUCTION

1.1 Antibody responses in inflammatory CNS disease

1.1.1 Spectrum of inflammatory CNS disease

Inflammatory CNS diseases comprise a broad spectrum of heterogeneous disorders each with a characteristic clinical course, severity and chronicity (1). Multiple sclerosis (MS) is most common of inflammatory CNS disease and has been further classified based on clinical course into clinically isolated syndrome (CIS), relapsing remitting MS (RRMS), secondary progressive MS (SPMS) and primary progressive MS (PPMS) (2). Other common inflammatory CNS disease sometimes referred as MS variants include transverse myelitis (TM), longitudinally extensive myelitis (LETM), relapsing isolated optic neuritis (RION), neuromyelitis optica (NMO) and acute disseminated encephalomyelitis (ADEM). To date no specific aetiology has been determined for these clinical entities however there are certain common features attributed to each of them. Most important of these common features involve a complex inflammatory process leading to demyelination primarily involving white matter (3). Differential diagnosis and prognosis of these disorders is currently based on combining careful clinical observation with neuroimaging. Recently biomarker criterion has been introduced to split these clinical entities based on unique immune effecter mechanism such as presence of a specific autoantibody (4).

1.1.2 Multiple sclerosis

Multiple sclerosis (MS) is an autoimmune inflammatory demyelinating disease of CNS characterized by loss of myelin, gliosis, axonal loss, oligodendrocyte pathology, and progressive neurologic dysfunction.

1.1.2.1 Epidemiology

Multiple sclerosis affects young adults and the age of its onset peaks between 20 to 40 however 3-5 % of all individuals affected with MS experience the onset of disease prior to age 16 (5). There is a very specific geographic distribution of this disease around the world.

A significantly higher incidence of the disease is found in the northernmost latitudes of the northern and the southern hemispheres compared to southernmost latitudes. This observation is based on the incidence of the disease in Scandinavia, Germany, northern United States and Canada, as well as Australia and New Zealand (6). The underlying cause for this geographical distribution is currently unknown, but both genetic and environmental factors are thought to be important.

1.1.2.2 Etiology

The precise etiology of MS is not yet known however several studies have investigated the involvement of genetic factors. The incidence of MS in first degree relatives is 3-5 times higher than in general population, suggesting the influence of genetic factors on the disease. Monozygotic twin studies show the concordance rate of 30-40%. Dizygotic twins show concordance rate of less than 5% (7). These results combine with epidemiological data suggest that both the genetic factors and environmental exposure are important in disease expression.

1.1.2.3 Pathogenesis

MS is a heterogeneous disorder with variable clinical and pathologic features ranging from acute inflammation induced demyelination to more chronic symptoms caused by axon degeneration and neuronal dysfunction (8). Most widely accepted immunopathogenesis theory of MS defines it as an inflammatory autoimmune disorder mediated by autoreactive lymphocytes. MS features supporting immunopathogenesis theory are summarized as under:

a) Active inflammation, blood brain barrier disruption and demyelinating white matter lesions
b) Accomulation of inflammatory T cells, B cells, and macrophages observed in histopathologic examination of MS lesions (9)
c) Increased oligoclonal IgM and IgG levels in the cerebrospinal fluid (CSF) of MS patients (10)
d) Oligoclonal T cells and B cells in MS plaques and in the CSF of MS patients (11)
e) Recruitment of T-helper 17cells (12)

f) Antibody deposition and complement activation in MS lesions (13)
g) Reduction in MS disease activity as demonstrated with immunomodulatory drugs (i.e., interferon beta and natalizumab, 14)

1.1.3 Role of B cells in inflammatory CNS pathology

During the past few decades the focus of research investigating inflammatory CNS pathogenesis has been on the role of T cells. This choice has been based on long standing observation of the dominance of activated T cells within the inflammatory lesions of multiple sclerosis (MS). Additionally, in animal model of MS the disease is T cell mediated and myelin specific T cells can transfer the disease (15). The involvement of B cells was however mostly considered as the consequence of the breakdown of T cell tolerance. However, immunological findings such as the role of B cells in antigen presentation and coordination with T cells combined with clinical observations such as deposition of antibodies and complement products in MS lesions support the role of B cells in MS pathology. Moreover the efficacy of B cell depletion and plasma exchanges in the treatment of MS has led to a renewed interest in the role of B cellhumoral immunity in inflammatory CNS pathology (16). A recent study described formation of ectopic B cell follicles in the meninges of MS patients, suggestive of intrathecal differentiation of B cells (17, 18). Furthermore, B cells can contribute to inflammatory CNS pathology from the periphery, by releasing antibodies directed against myelin or neuronal antigens into the circulation. The trafficking of these circulating antibodies into the brain then occurs as a result of blood brain barrier hyperpermeability in inflammatory CNS disease (19).

Increased oligoclonal IgG synthesis as detected by electrophoretic methods (oligoclonal bands; OCBs) is present in the patients of several inflammatory CNS diseases including MS (20). The OCBs are found in > 95 % of cerebrospinal fluid (CSF) from MS patients; however the corresponding antigens are unknown. In addition to OCBs, a multitude of antibodies reacting to self and non-self antigens that do not correspond to the OCBs have been identified in the serum and CSF of a variety of inflammatory CNS disease (21).

1.1.4 Classification of Antibody responses

Based on antigen reactivity the antibody responses in inflammatory CNS disease can be broadly classified into following classes.

1.1.4.1 Anti-myelin antibodies

Myelin is composed of oligodendrocyte membranes tightly wrapped around axons, forming a layer of electrical insulation. Myelin sheaths, together with the nodes of Ranvier (short sections of unmyelinated axon that separate different segments of the myelin sheath) form a prerequisite for rapid conduction of nerve impulses over relatively long distances (salutatory conduction). In addition, myelin supports axonal growth and axonal survival.

Antibodies produced by activated B cells in the CNS or periphery may target numerous self-antigens including protein components of the CNS myelin such as myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), myelin oligodendrocyte glycoprotein (MOG) and oligodendrocyte specific protein (OSP) (22, 23). Although molecular function of many of myelin proteins remains obscure but some of these proteins are relevant to myelin structure and compaction. Therefore the antibodies targeting these proteins could in principle affect compaction of myelin sheaths resulting in demyelination, a major characteristic of all inflammatory CNS disease. A schematic depiction of myelin sheath with protein targets of antibody responses in MS is given in Figure 1.1.1.

In addition antibodies directed against lipid components of myelin such as Galactocerebroside and Phosphatidylcholine have been described in MS (24, 25).

1.1.4.2 Anti-neuronal antibodies

Antigens of myelinated axons are normally hidden from the immune system, and only become exposed after demyelination when they become antigenic and induce the production of neuron-specific antibodies. The neuronal antibody targets include cell surface proteins (neurofascin), surface gylcolipids (gangliosides), cytoskeletal components (neurofilaments), various intracellular proteins (β-arrestin, glutamate decarboxylase, heat shock proteins) and nuclear antigens (26).

1.1.4.3 Anti-glial antibodies

The Astrocyte end-feet protein Aquaporin 4 (AQP4) has been identified as antibody target in NMO spectrum disease (27). Antibodies to surface antigen of oligodendrocyte progenitor cells (AN2) were described in MS (28).

1.1.5 Clinical relevance of antibody responses

Based on their clinical relevance the antibody responses in inflammatory CNS disease can be categorised into certain biomarker classes.

1.1.5.1 Diagnostic biomarker

Circulating autoantibodies represent a pertinent source of early diagnostic and presymptomatic biomarkers for a number of pathologies including certain cancers and autoimmune diseases. In case of inflammatory CNS disease sera from patients have been used in indirect immunofluorescence assays involving brain sections to identify diagnostic biomarkers specific for different disease entities. This strategy has been successfully applied to identify anti-AQP4 serum reactivity in NMO patients (NMO-IgG). The clinical relevance of NMO-IgG has been shown as diagnostic in that it differentiates NMO from classical forms of MS (29).

1.1.5.2 Predictive or prognostic biomarker

In the absence of specific predictive biomarkers, the prognosis of inflammatory CNS disease relies heavily on careful monitoring of clinical history and application of expensive imaging techniques that are still evolving. Identification of serum antibody (predictive) biomarker would therefore provide rapid, inexpensive, and precise prognostic tool to monitor disease course and to predict therapeutic response applicable in diverse clinical settings because serum is easier to obtain and use of serum facilitates serial sampling. Recently NMO-IgG has been shown as a predictive marker in recurrent ON (30).

1.1.5.3 Pathological antibody responses

Current hypothesis on the pathogenesis of inflammatory CNS disease suggest that a T cell mediated immune response is responsible for the inflammatory component. For the

development of the demyelinating plaques and axonal damage however, additional immune mechanisms are required, which may involve pathogenic antibodies. This concept is strongly supported by complement activation and IgG deposition in active MS lesions. Additional evidence on the pathogenic role of antibodies has been provided by disease exacerbation studies in animal models elucidating the role of anti-MOG antibody in demyelination and axonal damage and anti-AQP4 antibody in myelinolysis and perivascular astrocyte depletion (31, 32).

Antibody mediated effector functions that can contribute to the pathogenesis of inflammatory CNS disease may include antibody-dependent cell-mediated cytotoxicity (ADCC), antibody mediated phagocytosis or opsonization and activation of complement cascade pathway.

Recent studies have implicated all these effector functions in autoantibody mediated demyelination in inflammatory CNS disease (33-35).

1.1.5.4 Protective antibody responses

In contrast to pathogenic antibody responses in inflammatory CNS disease some autoantibodies can be tissue protective. Studies involving experimental autoimmune encephalomyelitis (EAE) model have shown that monoclonal IgM antibodies to CNS glial cells promote remyelination (36), and polyspecific autoreactive IgM antibodies elevated in MS patient sera are needed for an efficient phagocytosis and removal of myelin debris by macrophages in demyelinating lesions (37).

1.1.5.5 Epitope spreading and epiphenomenon

After an initial exposure of pathogenic epitope and associated tissue injury, inter and intra-molecular epitope spreading occurs. This results in the accumulation of antibody specificities that may or may not contribute in exacerbation of pathogenesis and disease progression (23). Structural studies on anti-MOG antibodies in MS have revealed that not all antibodies produced in the CNS inflammation are related to disease pathogenesis or progression (38). The anti-MOG antibodies with binding specificity to conformational MOG epitopes are pathogenically relevant (39). The antibody responses to linear or

partially folded epitopes are therefore described as epiphenomenon because they fail to bind native forms of their tagret antigens expressed in the in-vivo conditions.

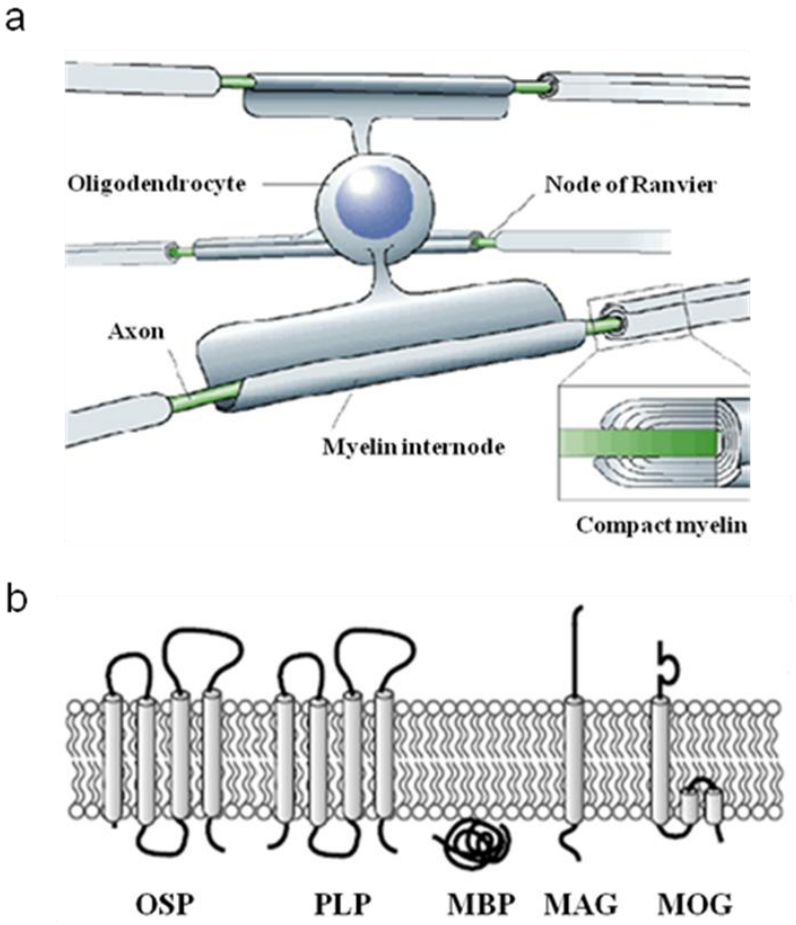


Figure 1.1.1 Myelin and myelin proteins in CNS

(a) An ultrastructural drawing of a CNS *oligodendrocyte* contributing to *myelin internodes* of several axons. A myelin sheath is interrupted by *node of Ranvier*, where myelin is absent and the *axon* is exposed. Myelin internodes are formed by wrappings of oligodendrocyte plasma membrane. The box shows a longitudinal section through a myelinated axon, illustrating *compact myelin* (adapted from Popko B., 2003). **(b)** Schematic representation of CNS myelin protein antigens in MS (adapted from Bunge M. et al., 1968).

1.1.6 Oligodendrocyte specific protein or claudin-11 (OSP / Cldn11)

1.1.6.1 Discovery and structure

A cDNA subtractive screen of mouse spinal cord designed to identify genes involved in myelinogenesis led to the discovery of a novel cDNA clone whose expression was specifically restricted to myelinating oligodendrocytes in CNS (40). Therefore the protein encoded by this novel cDNA clone was named as oligodendrocyte specific protein (OSP). A protein profile search of OSP in GenBank suggested a relationship with peripheral myelin protein 22 (PMP-22) which implies that OSP is an important molecule in CNS myelination. Structural analysis of both PMP-22 and OSP proteins indicated common features including four hydrophobic transmembrane domains embedded in membrane and two extracellular loops with both N- and C-terminal in cytoplasm (41, 42). A new family of proteins was later identified that can form tight junctions (TJs) its members were termed claudins (43). Based on sequence searches OSP was found to share considerable homology with these claudins and to have the ability to form TJs and therefore was added to the claudin family and renamed as (OSP / Cldn11) (44).

1.1.6.2 Expression of OSP / Cldn11

In CNS during myelinogenesis OSP / Cldn11 is targeted to oligodendrocyte processes shortly after they contact axons and persist in mature myelin sheaths as relatively abundant protein comprising 7 % of total protein in rodents (45). However its expression is not restricted to CNS and it is broadly expressed during development as well as in several adult tissues.

1.1.6.3 Role of OSP / Cldn11 in myelin structure and function

In myelinated axons OSP / Cldn11 based tight junctions form spiral channels at each end of myelin sheaths in paranodal regions. A loose spiral channel runs along the sheath between the two paranodes and short tight spirals originate from this channel at regular intervals and

course through the Schmidt-Lantermann incisures (Schematic depiction of this organization of OSP / Cldn11 based tight junctions in mature myelin is given in Figure 1.1.2).

The intramembraneous continuous spiral organization of OSP / Cldn11 tight junctions around the perimeter of the myelin sheath is ideally placed to prevent passive diffusion of solutes and ions that might otherwise enter at the outer or inner edges of the myelin along the internodes or at the paranodes (Figure 1.1.2). In effect OSP / Cldn11 tight junctions not only seal and stabilize the multilammelar structure of compact myelin but also generate an impermeable lipid insulator with the high resistance and low capacitance electrophysiological properties favorable for salutatory conduction. This relevance of OSP / Cldn11 to myelin structure and function is further supported by the perturbed conduction velocities along the CNS myelinated fibers in *claudin-11* null mice (46).

1.1.6.4 OSP / Cldn11 as an autoantigen in MS

The generally accepted classical criteria for self proteins to qualify as candidate autoantigens in an autoimmune disease (47) include:

a) Evidence for humoral or cellular autoreactivity in patient clinical samples
b) Capacity for inducing an analogous autoimmune disease in laboratory animals.

In case of MS, OSP / Cldn11 qualifies these criteria.

Antibodies to recombinant OSP/Cldn11 were detected in the CSF samples of RRMS patients using immunoblot assays (48). To identify the dominant B cell epitopes within OSP/Cldn11 to which these antibodies were directed, pools of CSF from RRMS patients were tested in peptide ELISA assays against a panel of overlapping synthetic peptides representing the amino acid sequence of OSP/Cldn11. Two overlapping peptides delineated a focus of strong antibody binding in the amino acid 114-120 region of OSP/Cldn11 protein. Other weaker antibody interactions were dispersed along the length of the OSP/Cldn11 molecule.

To establish an OSP / Cldn11 induced experimental autoimmune encephalomyelitis (EAE) a model for MS, a panel of 20-mer synthetic peptides with 10-amino acid overlaps was injected together with appropriate adjuvants into SJL/J (H-2s) mice (49). Six different peptides elicited clinical features characteristic of EAE. A dominant OSP / Cldn11 directed

T cell epitope was later identified in a similar EAE model (50). Recently, encephalogenicity of OSP / Cldn11 was also shown in rhesus macaques (51).

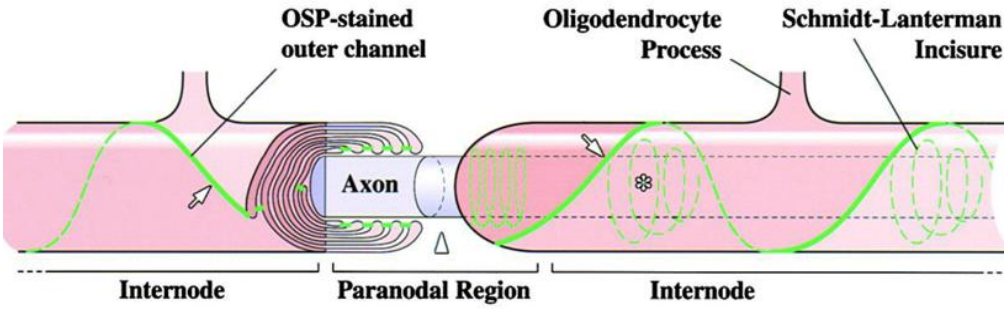


Figure 1.1.2 Organization of OSP / Cldn11 tight junctions in CNS myelin sheaths

(a) An ultrastructural drawing of a CNS *oligodendrocyte* contributing *myelin internodes* to several axons. A myelin sheath is interrupted by *node of Ranvier*, where myelin is absent and the *axon* is exposed. Myelin internodes are formed by wrappings of oligodendrocyte plasma membrane. The box shows a longitudinal section through a myelinated axon, illustrating *compact myelin* (adapted from Popko B., 2003). **(b)** Schematic representation of CNS myelin protein antigens in MS (adapted from Bunge M. et al., 1968)

1.1.7 Myelin oligodendrocyte glycoprotein (MOG)

1.1.7.1 Discovery and structure

Myelin oligodendrocyte glycoprotein (MOG) was identified initially as a target for autoantibody mediated demyelination in EAE induced by CNS tissue homogenates (52). Cloning of human *MOG* gene allowed identification of 12 mRNA variants synthesized from complex alternate splicing of single primary transcript encoding the major transmembrane MOG isoform (53). Structural and topological studies of MOG indicated a highly glycosylated N-terminal extracellular domain, a transmembrane hydrophobic region and a second hydrophobic region buried in lipid bilayer to generate two short cytoplasmic domains, a short hydrophilic loop and a cytoplasmic C-terminal tail (54)

1.1.7.2 Expression and molecular function

MOG is localized on the surface of myelin and oligodendrocyte processes. Developmental expression of MOG increases with increase in myelinogenesis. Unlike other myelin proteins MOG is distributed evenly on the external lamellae in mature myelin sheaths (55). To date no molecular function has been assign to MOG. Mice deficient in MOG develop normally and show no discernable neurological disability (56). It has been proposed on the basis of the presence of extracellular IgG like domain and a highly conserved C-terminal cytoplasmic tail that MOG might be a surface receptor capable of carrying out signal transduction although no interacting partners to these MOG regions have been determined so far.

1.1.7.3 MOG as an autoantigen in MS

In the EAE model, MOG is the only CNS antigen to initiate both demyelinating antibody response and an encephalitogenic T cell response. The clinical and immunopathological similarities between MOG induced EAE and MS suggest that MOG may also be target autoantigen in human disease. It was suggested that MOG induced immune responses are enhanced in some patients and may directly participate in the formation of inflammatory

lesions (57). Immunopurified MOG was then used to provide the first indication that MOG-specific T cell response together with MOG-specific B cell response preferentially sequestered in the CNS compartment in MS (58).

Elevated autoantibody responses to linear MOG epitopes were detected with enzyme linked immunosorbent assays (ELISA) and western blotting assays in sera and CSF of adult MS patients (59). Due to the lack of clinically relevant biomarkers in MS identification of this antibody response raised interest among researchers and many studies were carried out to explore the pathogenic or predictive potential of anti-MOG antibody response in MS. It was shown that CIS patients with and without anti-MOG and anti-MBP antibodies have different clinical course towards developing MS (60). However this finding was not confirmed by others (61). The conflicting results regarding the clinical relevance of anti-MOG antibodies in MS were attributed atleast in part to the detection methods used in these studies. The conventional assay systems used to detect anti-MOG antibodies, such as ELISA and western blot could not differentiate between antibody responses that recognize linear MOG epitopes from those responses that recognize conformational epitopes presented by native MOG (62). Therefore the clinical relevance of anti-MOG antibodies in MS remained controversial until and new assays were developed to distinguish conformational epitope specific anti-MOG antibody responses from those responses that target linear epitopes (31, 63).

1.2 Role of claudins in blood brain barrier integrity

1.2.1 Blood Brain Barrier (BBB)

Paul Ehrlich and Edwin E. Goldman discovered in 1880s that upon the injection of protein bound dyes into the vascular system, the dyes were taken up by all organs with the notable exception of brain and spinal cord (64), whereas the same dyes when injected into the cerebrospinal fluid readily stained CNS tissue but were excluded from all other organs (65). It was therefore suggested that a vascular endothelial barrier between blood and brain, the blood-brain barrier (BBB) exists. Later studies identified that the function of BBB is to maintain homeostasis of CNS microenvironment.

Permeability of BBB endothelium is extremely low as compared to peripheral endothelial barriers. This is because of a low pinocytotic activity at BBB endothelial cells that inhibits transcellular passage and an elaborate network of complex interendothelial tight junctions (TJs) that restrict paracellular diffusion of molecules (66). Contrary to peripheral endothelial barriers, BBB endothelium expresses specific transport systems for the transport of metabolites and toxic metabolic products. These BBB properties combined with the lack of lymphatic vessels (67) determine the immuno-privilege of CNS.

1.2.2 Cellular and acellular organization of BBB

Functionally, BBB operates at the level of capillaries and post-capillary venules formed by highly specialized endothelial monolayer and associated cellular and acellular components. The cellular and acellular layers of BBB are shown schematically in Figure 1.2.1. At the level of post-capillary venules, BBB consists of an inner endothelial cell layer with basement membrane (BM), bordered by the meningeal epithelium and its BM, and an outer astroglial BM and astrocyte endfeet. Meningeal and astroglial BMs are collectively termed the parenchymal BM as they delineate the border to the brain parenchyma (68). The endothelial and parenchymal BMs define the inner and outer limits of the perivascular (Virchow-Robin) space. However at the level of capillaries these BMs are indistinguishable (69) and fuse to form one composite BM. Other cellular components include pericytes that

are embedded in endothelial BM of post-capillary venules. The pericytes play important role in the induction and regulation of BBB (70).

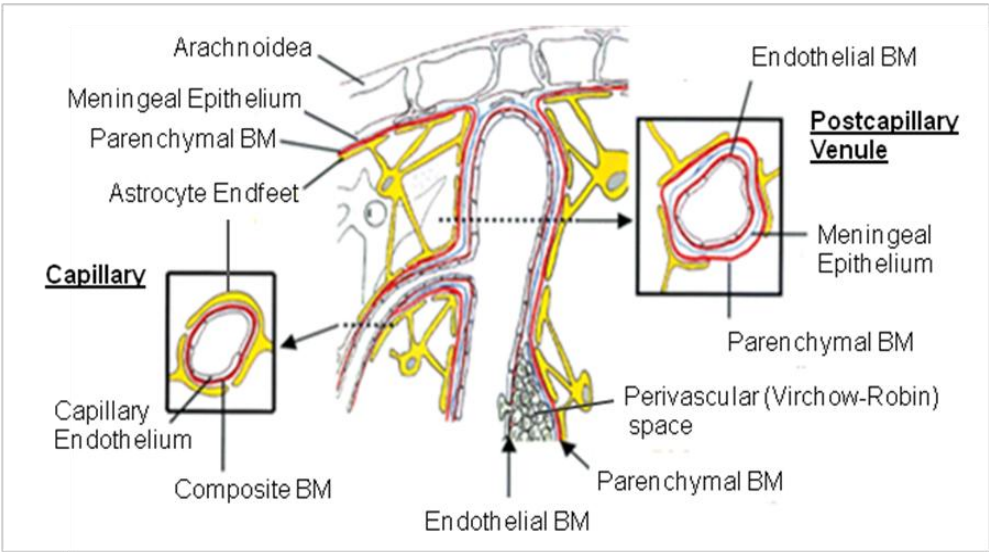


Figure 1.2.1 Cellular and acellular organization of BBB

Illustration of cellular layers and basement membranes (BM) of CNS postcapillary venule and capillary. Postcapillary venule consists of an inner endothelial cell layer with endothelial BM, bordered by the meningeal epithelium and its BM and an outer astroglial BM and astrocyte endfeet. The meningeal and astroglial BMs are collectively termed the parenchymal BM. Endothelial and parenchymal BMs are distinguishable during CNS inflammation and define the inner and outer limits of the perivascular (Virchow-Robin) space. The CNS capillaries (microvessels) where no epithelial meningeal contribution occurs appear to have a composite BM (adapted from Sixt M. et al., 2001).

1.2.3 Interendothelial junctions of BBB

The interendothelial space of BBB is characterized by the presence junctional complexes that include tight junctions (TJs) adherens junctions (AJs) and gap junctions. Electron microscopic studies involving the injection of electron-dense tracers into CNS show that tracer dyes are distributed into the interendothelial clefts upto TJs indicating that BBB TJs are primarily responsible for paracellular impermeability and barrier properties of BBB (71). The BBB AJs are thought to mediate the adhesion of endothelial cells to each other and mediate contact inhibition during vascular growth, remodeling and angiogenesis. The role of AJs in paracellular permeability to circulating leukocytes has also been suggested (72). The gap junctions mediate intercellular communication and allow the passage of small molecular weight solutes between neighboring endothelial cells.

Freeze-fracture electron microscopic studies indicate that BBB TJs are more complex than the interendothelial TJs elsewhere in the body. The BBB TJs form parallel and highly interconnected strands (73) confined to the apical part of the lateral membranes of adjacent endothelial cells. Another distinguishing feature of BBB TJs is their protoplasmic (P-face) association as compared to the exocytoplasmic membrane leaflet (E-face) associated with peripheral interendothelial TJs (74, 75). This unique cytoplasmic (P-face) anchoring of BBB TJs is thought to be important for its barrier function (76).

1.2.4 Molecular phenotype of BBB TJs: Role of Claudins

The BBB TJs consist of three main groups of proteins. They are transmembrane proteins (Tetraspanins occludin and claudins, and junctional adhesion molecule, JAM-1), accessory proteins (ZO-1 /2 /3 etc) and cytoskeletal proteins (actin etc). All three groups interact to maintain functional TJs (Figure 1.2.2).

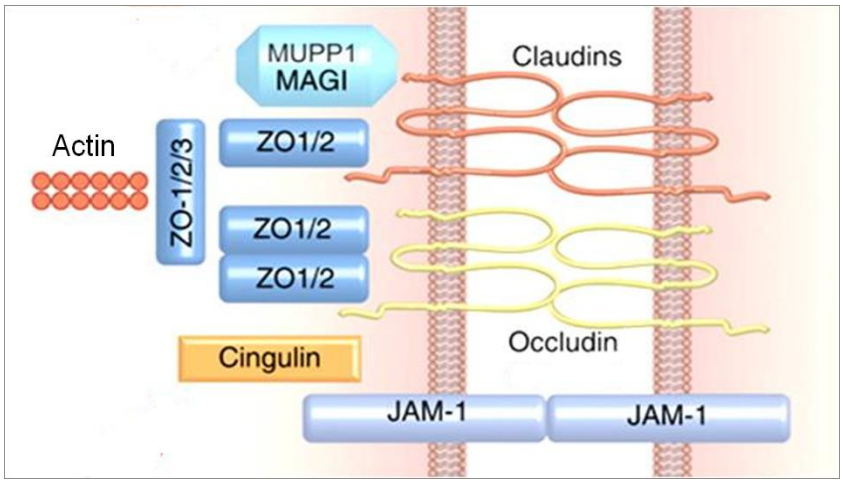


Figure 1.2.2 Molecular architecture of BBB tight junctions

The transmembrane proteins of BBB TJ include tetraspanins (occludin and claudins) and Ig-like adhesion molecules (JAM-1). The transmembrane proteins are linked to the cytoskeleton (actin) via a complex network of adaptor proteins (adapted from Niessen C., 2007).

Occludin was the first transmembrane tetraspanin protein to be localized within TJs (77). It was however found that occludin knockout mice are viable and have morphologically normal BBB TJs indicating that occludin may not be essential for proper TJ formation (78). Adhesion molecules such as JAM-1 and the newly discovered endothelial cell-selective adhesion molecule (ESAM) are localized at BBB TJs as well (79). These molecules are involved in organizing the tight junctional structure and in leukocyte extravasation across BBB (80).

Presence of morphologically and functionally normal TJs in the absence of occludin in occludin deficient mice led to the discovery of tetraspanin claudins (Cldns). Claudins share tetraspanin structure with occludin, but do not contain any sequence homology to occludin. To date 23 members of claudin family have been described (43). Claudins seem to fulfill the task of establishing barrier properties of endothelia as well as epithelia. When transfected with claudins the cells that normally do not form TJs such as L-fibroblasts exhibit very long TJ strands morphologically resembling in vivo TJ strands of endothelial and epithelial barriers (81). Similar transfection experiments indicated that combination and stoechiometry of claudin species directly determine the barrier function and morphological outcome of TJs (82). Claudins are not randomly distributed and show a tissue specific expression pattern. The BBB TJs express Cldn3, 5 and 12 and this composition directly determines the paracellular barrier properties and low transendothelial resistance of BBB (83-85).

1.2.5 BBB TJ hyperpermeability in inflammatory CNS disease

In the inflammatory CNS disease such as MS, the loss of BBB integrity leads to dysregulation of CNS interstitial fluid exchange and vasogenic edema causing a flow of antibodies, plasma and complement proteins in CNS parenchyma. Breakdown of BBB is considered as marker of active lesions in MS as well as development of new lesions (86). The changes in BBB integrity during CNS inflammation have also been modelled in EAE where increased transport of tracers across BBB is observed in inflamed areas of brain (87).

The BBB leakiness during CNS inflammation may be either due to TJ opening or enhanced pinocytotic activity of endothelial cells. Whereas the latter has not been reported as yet, there is considerable evidence of structurally defective hyperpermeable BBB TJs in CNS inflammation. Immunostaining of TJ proteins (occludin and ZO-1) in CNS vasculature associated with MS lesions manifest TJ abnormalities as beading, interruption and separation of junctional contacts between neighboring endothelial cells (88). Dual immunostaining with plasma components indicated that these abnormal TJs are indeed associated with BBB hyperpermeability in MS (89).

1.2.6 Inflammatory cytokines and angiogenic mediators cause BBB TJ hyperpermeability

Proinflammatory cytokines such as tumor necrosis factor (TNFα), interferon (IFNγ) and interleukin (IL-1) have been detected in CNS tissue of MS patients and EAE animals (90). Based on in vitro studies in which these cytokines have been shown to induce increased paracellular permeability of tracers across monolayer of endothelial cells isolated from CNS it has been suggested that they might also disrupt BBB in vivo (91). Administration of TNFα to an in vitro model of BBB resulting in increased paracellular permeability accompanied with reorganization or reduced synthesis of TJ protein components has been reported in many studies (92-95). In addition, the role of TNFα in BBB disruption via TJ hyperpermeability has also been implicated in context of inflammatory CNS diseases (96). Inflammatory cytokines also activate BBB endothelium for the recruitment and extravasation of inflammatory cells.

Recently, the role of vascular endothelial growth factor (VEGF) has been defined in BBB disruption during MS and EAE (97). The VEGF is a major inducer of angiogenesis and was originally described as propermeability factor in stroke and head injury (98). In acute and chronic MS and EAE lesions, the expression of VEGF was found to be upregulated. In vitro studies show that VEGF disrupt BBB by downregulation or altering the assembly of TJ proteins occludin and Cldn5 (99).

Chapter 2. SPECIFIC OBJECTIVE

The experimental work performed in this thesis consists of two studies exploring different aspects for inflammatory CNS disease.

2.1 Study I: Antibody responses in inflammatory CNS disease

Antibody responses against OSP/Cldn11 and MOG have been reported in MS however the binding specificity (linear or conformational epitopes) and clinical relevance of these responses has been less characterized. Therefore, the specific objectives of this study include:

a) To design assays to characterize binding specificity of anti-OSP/Cldn11 antibody response in MS.
b) To compare specificity and clinical relevance of anti-OSP/Cldn11 antibody response with anti-MOG antibody response in MS.

2.2 Study II: Inflammatory cytokine regulation of claudins in cerebral endothelial cells

The claudin family proteins Cldn3, 5 and 12 stabilize BBB tight junctions (BBB-TJs), the paracellular permeability route across cerebral endothelium. Exposure of cerebral endothelium to inflammatory cytokines leads to BBB-TJ hyperpermeability however the molecular mechanism underlying this effect is not well characterized. Objective of this study is to investigate the effect of cytokines relevant in inflammatory CNS diseases (IL-1β, IFNγ and TNFα) on the regulation of cerebral endothelial expression of claudin family members (Cldn3, 5 and 12).

Chapter 3. RESULTS

3.1 Antibody responses in inflammatory CNS disease

3.1.1 Validation of native glial expression of OSP/Cldn11 and MOG

The LN18 (human glioblastoma) cell line was transduced with full length human OSP/Cldn11 or MOG cDNA containing lentiviral constructs and an empty lentiviral vector to obtain LN18-OSP, LN-18-MOG and LN18-CTR cell lines as described in methods (see section 6.2). All cell lines were kept under the same conditions and solely differed from each other by the expression of native glial proteins (nOSP or nMOG). Surface expression of nOSP and nMOG was confirmed by immunofluorescence and flow cytometry with corresponding monoclonal antibodies (Fig. 3.1.1).

3.1.2 Efficacy of serum and CSF antibody screening assays

Titration curves were generated to assess the efficacy of screening assays. A highly reproducible surface staining was observed for LN18-MOG and LN18-OSP even at very low concentrations of corresponding monoclonal antibody (Fig. 3.1.2 a, b) demonstrating that these cell lines can be used to quantify serum and CSF for antibody responses to native MOG and OSP in a flow cytometry based assay.

The efficacy of ELISA assays to detect antibody responses against conformational and linear epitopes was also determined in titration experiments. For ELISA assay based on native OSP presenting conformational epitopes, monoclonal antibody (similar to flow cytometry assay) was used to obtain the titration curve (Fig. 3.1.2 c). For ELISA assay based on denatured or linear epitopes of OSP the LN18-OSP (and LN18-CTR) cell lysates were processed to obtain membrane fraction enriched cell lysates. The cell lysates were heat denatured and additional chemical denaturants were added. The denatured membrane proteins were used as antigen source in ELISA to characterize binding specificity of serum and CSF anti-OSP antibody responses against linear, partially denatured epitopes. A

titration curve with polyclonal anti-OSP antibody represents validity of this assay to determine antibody responses to denatured OSP (Fig. 3.1.2 d).

The titration curves were generated for all the assay systems before each round of screening to make sure the stability of assay systems.

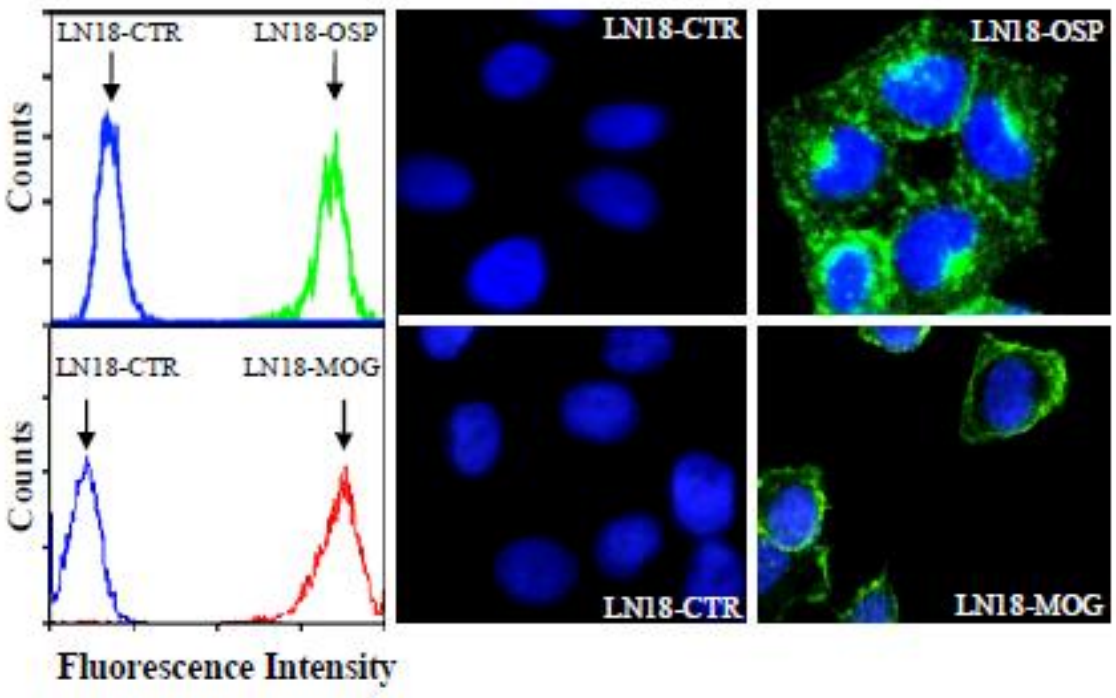


Figure 3.1.1 Validation of native glial expression of OSP/Cldn11 and MOG

Flow cytometry histograms showing anti-human OSP/Cldn11 monoclonal antibody staining with the LN18-CTR (Blue line) and LN18-OSP (Green line) cells (upper left panel) and similar staining of LN18-CTR (Blue line) and LN18-MOG (Red line) with monoclonal antibody (mAb) 8-18C5 (lower left panel). Immunofluorescence staining of LN18-CTR [Middle panels (×600)] with anti-human OSP/Cldn11 monoclonal antibody (Upper) and monoclonal antibody (mAb) 8-18C5 (Lower). Immunofluorescence labelling of LN18-OSP cells [Upper right (×600)] and LN18-MOG cells [Lower right (×600)] with corresponding monoclonal antibodies is shown.

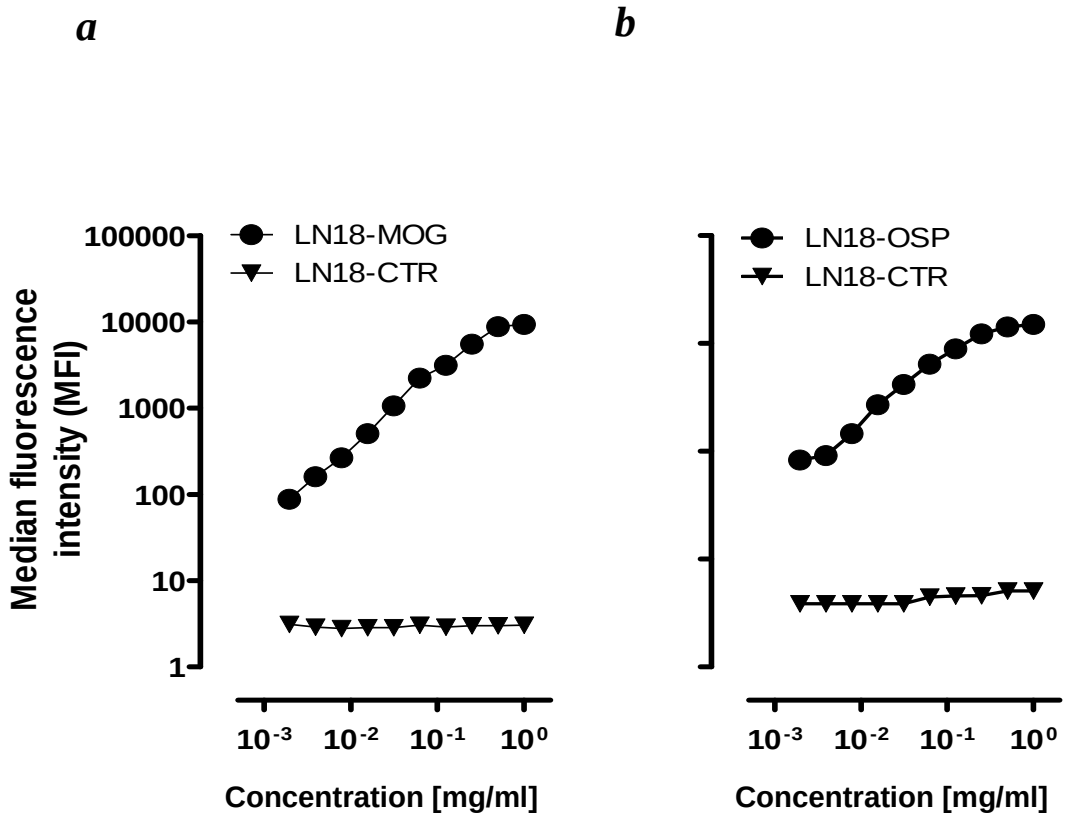
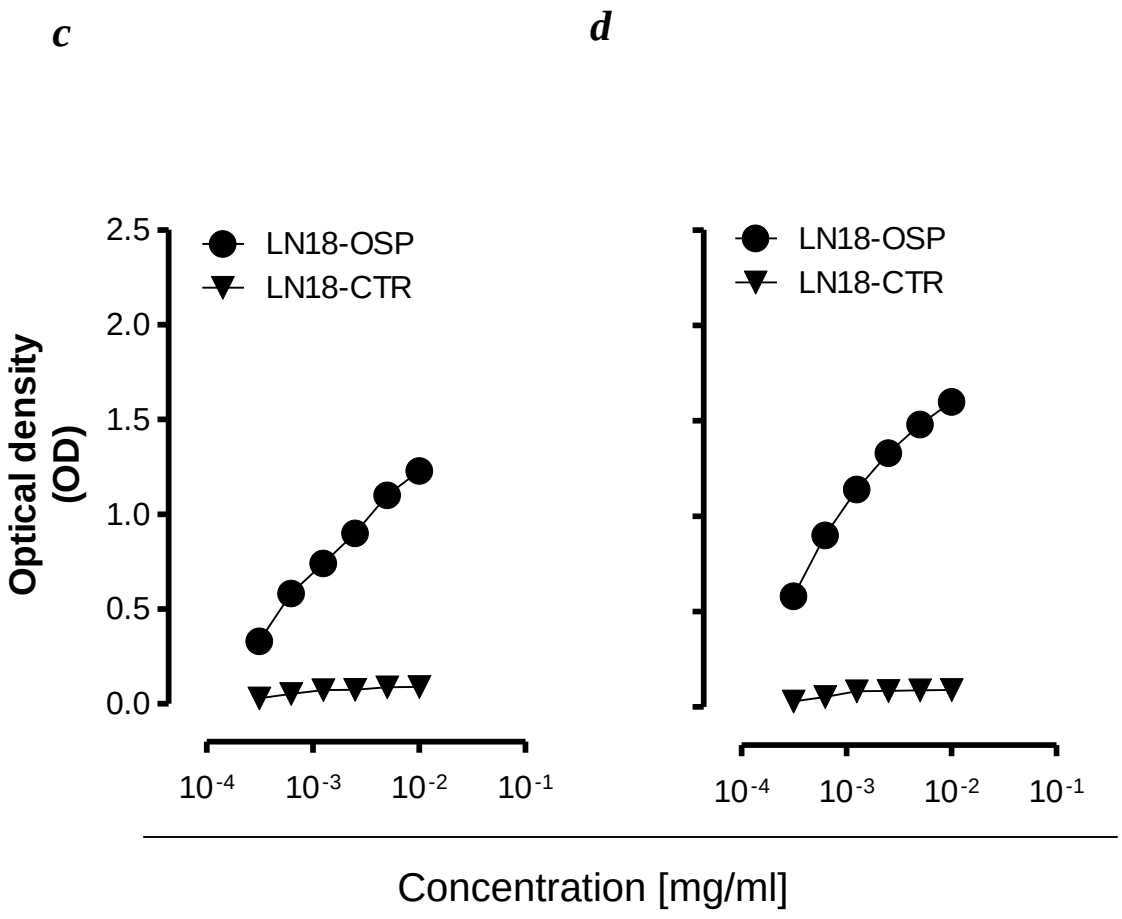


Figure 3.1.2 Efficacy of serum and CSF screening assays

Titration curve showing median fluorescence intensity (MFI) as obtained by LN18-CTR and LN18-MOG cells stained with various concentrations of monoclonal antibody (mAb) 8-18C5 (*a*) and LN18-CTR and LN18-OSP cells stained with various concentrations anti-human OSP/claudin-11 monoclonal antibody (*b*). Titration curve showing OD values as obtained by LN18-CTR and LN18-OSP cells with various concentrations of anti-human OSP/Cldn11 monoclonal antibody in native OSP based ELISA.(*c*). Titration curve for denatured OSP based ELISA is also shown indicating reactivity of denatured LN18-CTR and LN18-OSP cell lysates with different concentrations of anti-human OSP/Cldn11 polyclonal antibody (*d*).

Figure 3.1.2; continued



3.1.3 Elevated MS serum and CSF reactivity to denatured but not native OSP/Cldn11 protein

MS serum (n=50) and CSF (n=40) were tested against control serum (n=28) and CSF samples (n=18) for anti-OSP antibody reactivity. All serum and CSF samples were adjusted to a total IgG concentration of 20 mg/L. Serum and CSF reactivity to OSP/Cldn11 was quatified by ΔOD in denatured cell lysate and native OSP based ELISA, OD in OSP $_{114\text{-}120}$ peptide based ELISA and ΔMFI in cell-based flow cytometry assay (see methods section 6.4 and 6.5). In ELISA assays based on denatured cell lysate presenting linear OSP epitopes, a higher anti-OSP reactivity in sera of MS patients (mean ΔOD 0.45±0.16) was observed as compared to controls (mean ΔOD 0.32±0.08), ($P<0.05$, Fig. 3.1.3a). A similar trend was also observed in the CSF analysis ($P<0.05$, Fig. 3.1.3b). Furthermore, elevated anti-OSP $_{114–120}$ peptide reactivity in serum and CSF from MS patients as compared to controls was seen. A subgroup analysis was carried out to determine clinical relevance of anti-OSP antibodies. Statistically significant difference in anti-OSP 114–120 reactivity was achieved in sera and CSF from RRMS patients compared to controls (Fig. 3.1.3, c and d).

To test MS serum or CSF reactivity towards native OSP (nOSP) presenting conformation specific epitopes two different assay systems were applied. In cell based ELISA assay mean ΔOD values of MS serum (0.29±0.10) and CSF (0.18±0.06) did not differ significantly to that of control serum (0.31±0.12) and CSF (0.20±0.07) (Fig. 3.1.3, e and f). Absence of MS serum and CSF reactivity towards nOSP was confirmed in a cell based flow cytometry assay. The mean ΔMFI values from MS serum (7.8±3.2) and CSF (3.6±2.1) did not differ significantly from control serum (7.4±3.8) and CSF (3.8±1.9) respectively (Fig. 3.1.3, g and h).

a

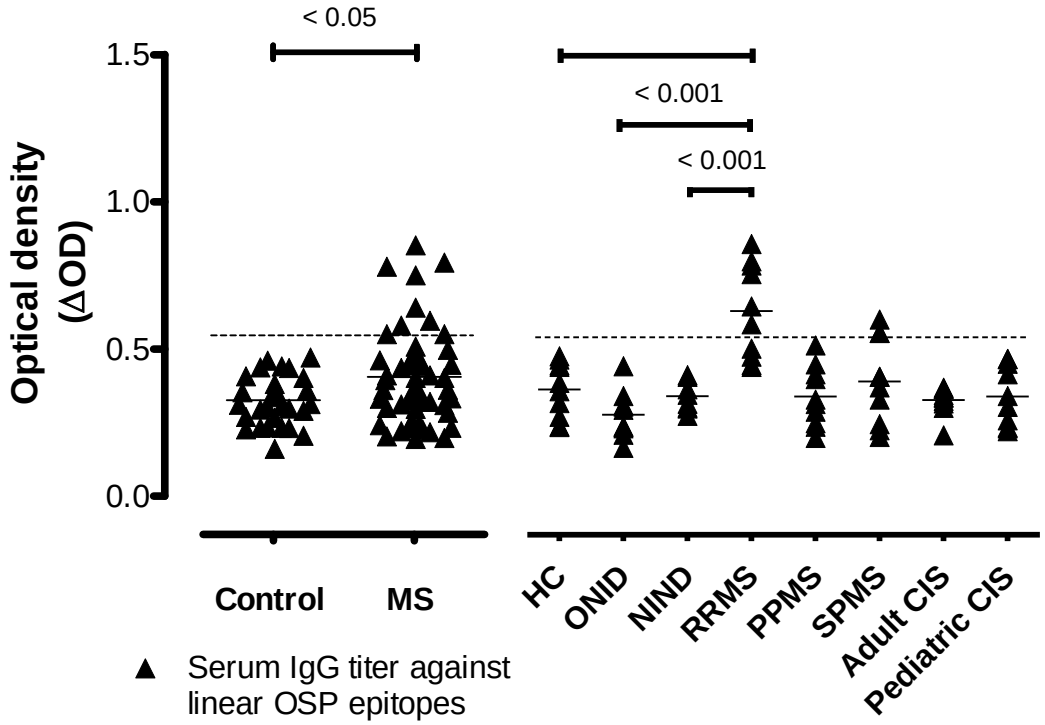
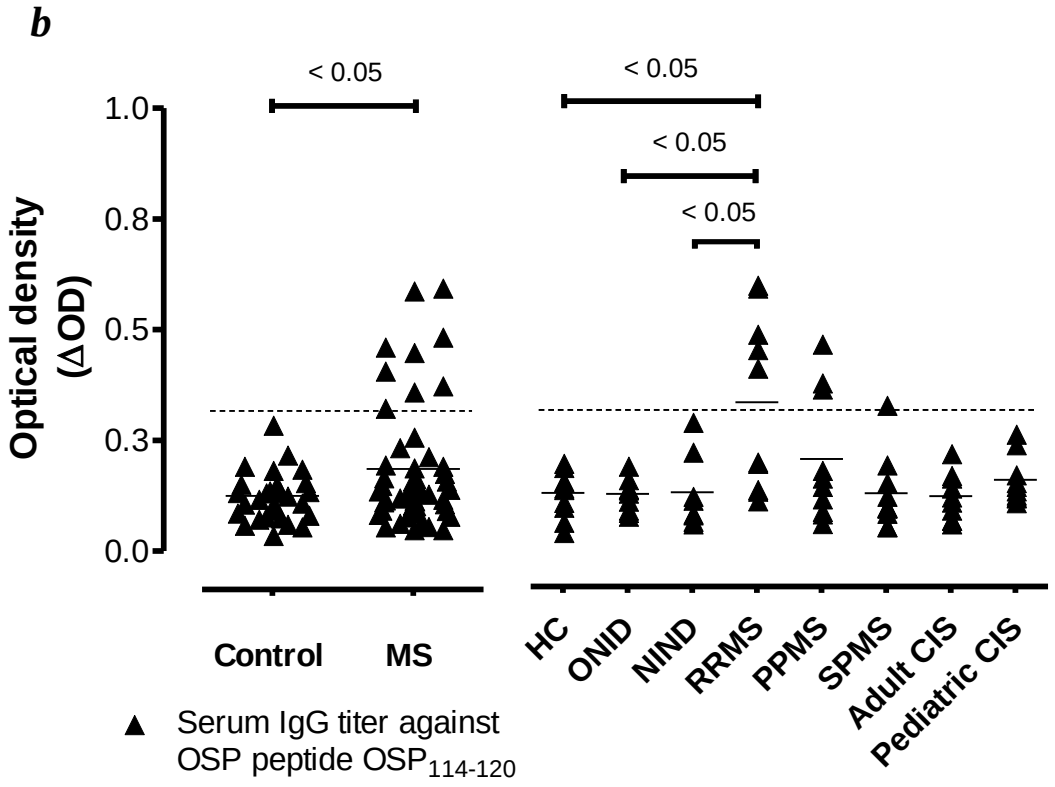


Figure 3.1.3 Serum and CSF antibodies to denatured OSP/Cldn11 and OSP peptide (OSP $_{114\text{-}120}$)

Serum and CSF reactivity towards denatured OSP (*a, b*; left panels) and OSP $_{114–120}$ peptide (*c, d*; left panels) was compared between MS patients and controls using an ELISA assay based on denatured OSP or OSP 114–120 respectively. Horizontal bars indicate median. Mann–Whitney U test is used to compare groups. P values are indicated. Comparison of antibody reactivity against denatured OSP (a, b; right panels) and OSP $_{114–120}$ peptide (*c; d*; right panels) in serum and CSF between controls and MS patients stratified for disease course and age of onset. P values are only shown for significant difference between patients and controls in the subgroup analysis. Horiontal lines indicate threshold for anti-OSP reactivity in serum and CSF samples (se methods section 6.13). Serum and CSF reactivity towards native OSP was compared between MS patients and control individuals in ELISA assay based on native OSP (*a, b*) and cell based flow cytometry assay (*c, d*). Horizontal bar indicates median. Mann–Whitney U test was used to compare groups. Stratified analysis of different subgroups is also shown (right panels). Threshold for native OSP serum/CSF reactivity is indicated by horizontal line (Threshold = mean delta OD/delta MFI of control group + 3 standard deviations).

Figure 3.1.3; continued



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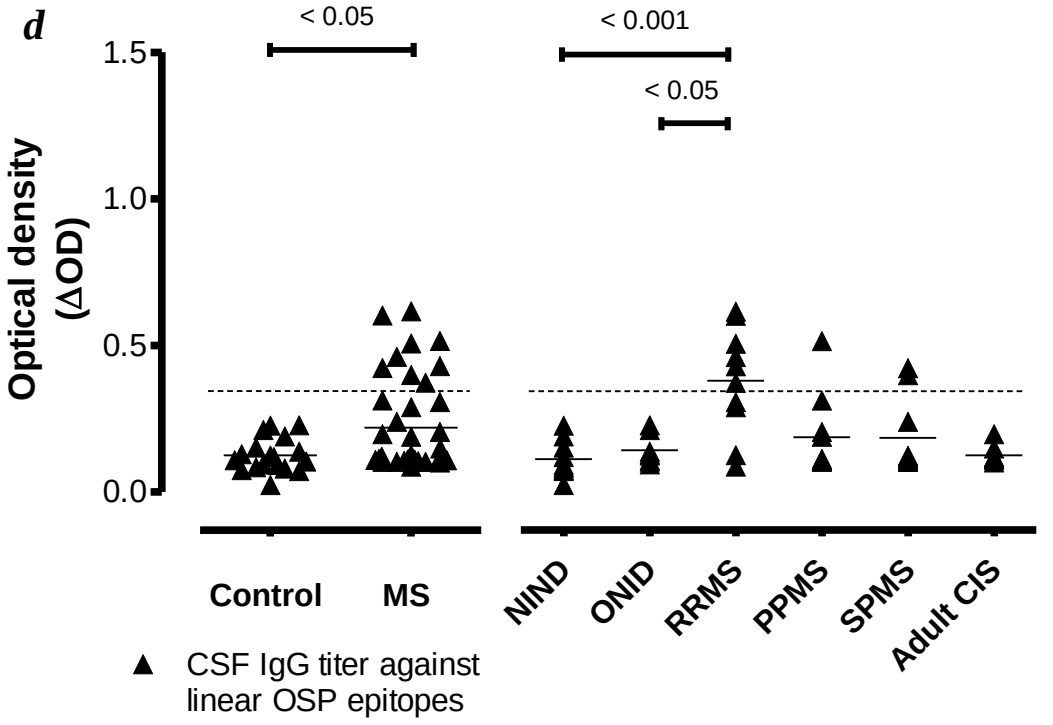

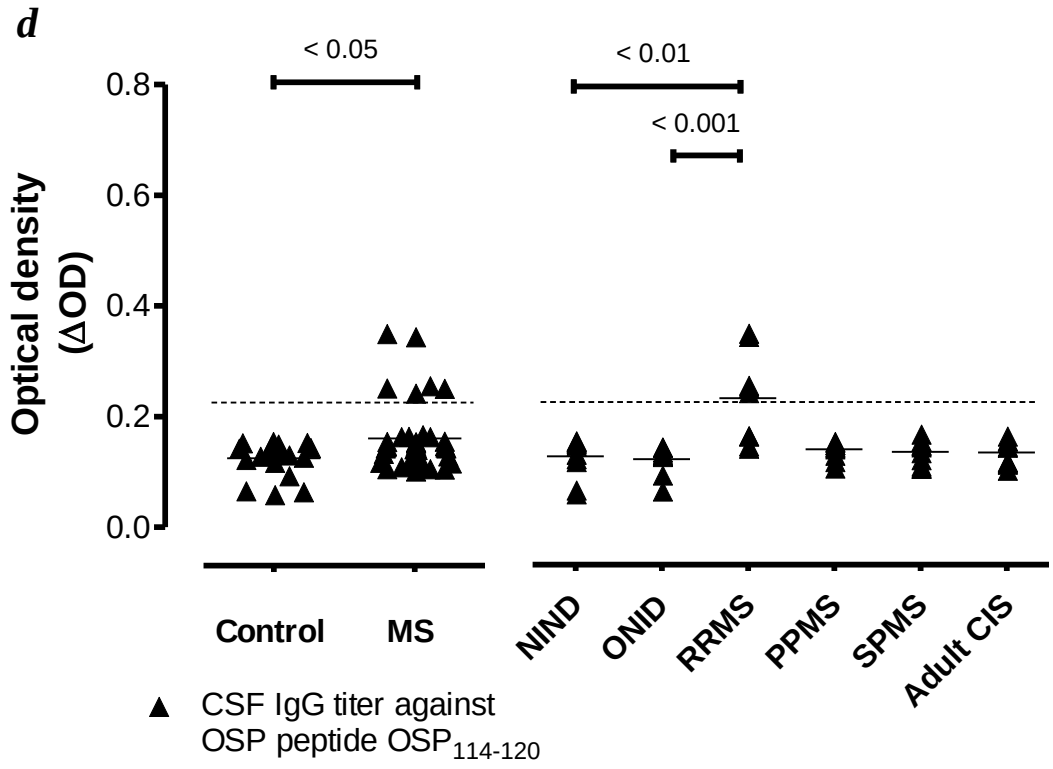

Figure 3.1.3; continued

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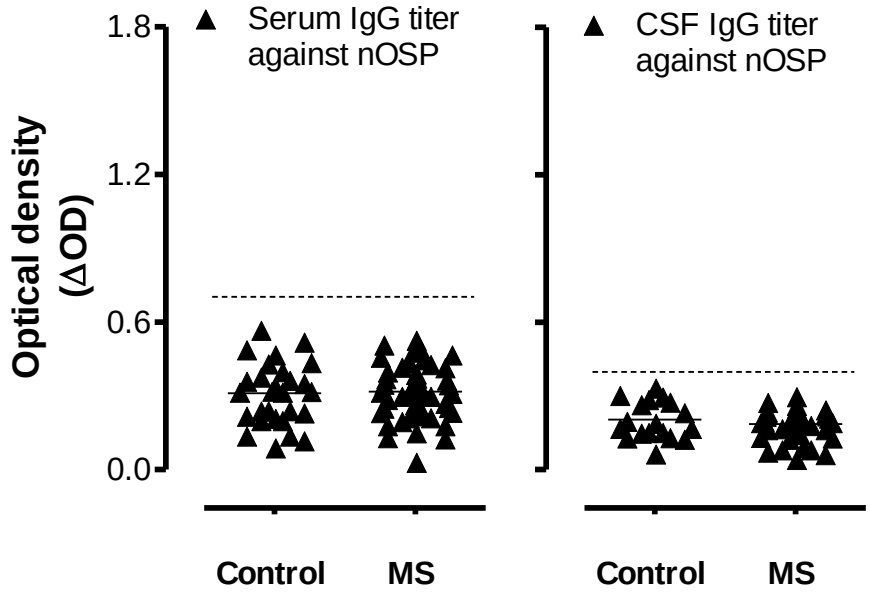

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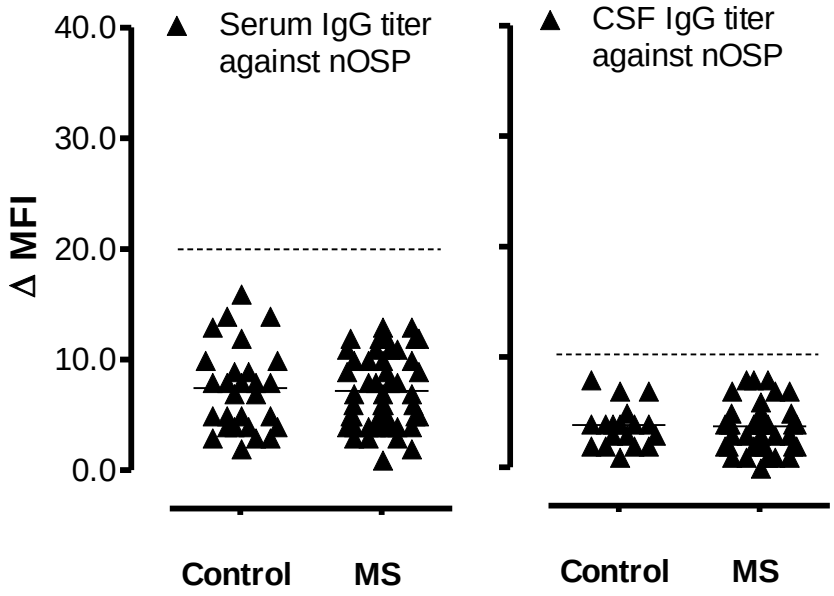


3.1.4 Elevated antibodies to native MOG (nMOG) in childern with first inflammatory demyelinating event (CIS/ADEM)

To study the presence of autoantibodies to native MOG in inflammatory CNS disease the study group included serum samples from adult and pediatric patients exhibiting first inflammatory demyelinating event (CIS and ADEM) adult MS patients and controls. A cell based flow cytometry assay was used for the detection of antibody responses against native MOG based conformational epitopes. Serum reactivity to nMOG was quantified as ΔMFI (Fig. 3.1.4 a). A comparative analysis of the the serum IgG antibody reactivity to nMOG indicated that children with a first inflammatory demyelinating event have higher anti-MOG titres as compared to either children with other neurological diseases (OND), healthy controls (HC) or adult CIS and MS patients (Fig. 3.1.4 b). The IgG titers in pediatric patients with first inflammatory demyelinating event (CIS/ADEM), pediatric HC, pediatric OND and adult CIS/MS patients were [median ΔMFI, 24.5; range, 1.0 –1896.3], [median ΔMFI, 2.2; range, 0–10.7], [median ΔMFI, 2.1; range, 2.1–21.5] and [median ΔMFI, 3.4; range, 0.3–107.1] respectively. Based on the titer observed in pediatric controls, a threshold was determined for nMOG IgG seropositivity at ΔMFI = 38.1 [median + 95% percentile of pediatric HC and ONDs], and a "high" titer threshold was defined as ΔMFI = 109.8 [median + 99% percentile, indicated by broken line in Fig. 3.1.5 b].

The IgG antibodies to nMOG were detected in 46.8% of CIS/ADEM children (22 of 47) and 6.9% (2 of 29) of OND children. No pediatric HC or adult MS patient was found to be positive for IgG antibody according even at the lower threshold (ΔMFI = 38.1). High titers were found in 40.4% of CIS/ADEM children (18 of 47 patients). A stratified analysis of pediatric CIS or ADEM patients indicated similar IgG antibody titers to nMOG (Fig. 3.1.4 d). The anti-nMOG IgG antibodies in some pediatric CIS/ADEM patients were detectable at a serum dilution as low as 1:10,000 (Fig. 3.1.4 c). In contrast titers with MFI > 100 were found in only < 3%, and titers > 500 in only < 1% of adult CIS and MS patients.

a

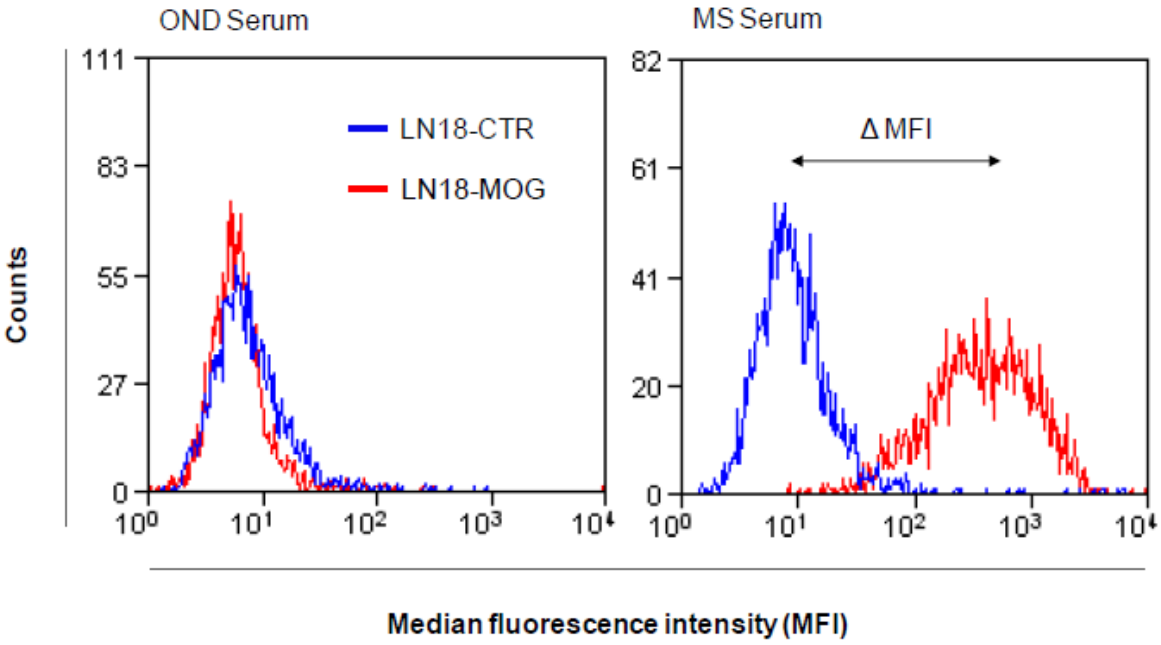


Figure 3.1.4 Serum antibody titer to nMOG in children and adults with a first inflammatory demyelinating event (CIS or ADEM)

Antibody reactivity to nMOG was determined in sera from all patients and controls by incubating LN18-MOG and LN18-CTR cells with serum at a dilution of 1:100. Secondary staining was performed with Alexa Fluor 488-conjugated anti-human IgG antibody. Representative flow cytometry histograms for negative (*a*: left histogram) and positive (*a*: right histogram) are shown. The Δ MFI is difference between median fluorescence intensity obtained with LN18-MOG and the MFI obtained with LN18-CTR cells. Immunoglobulin (Ig) G antibody titers to nMOG were determined in sera of healthy children (HC), children with other noninflammatory neurological diseases (ONDs), children with a first inflammatory demyelinating event (CIS/ADEM), and adults with MS (*b*). A stratified analysis of patients into pediatric CIS or ADEM shows similar IgG antibody titers to nMOG (*c*). Representive titration curves with anti-nMOG positive serum from pediatric and adult CIS patients (*d*). Antibody titers were compared among groups by the Kruskal-Wallis. Plain bars display median titer from the whole group. The p-Values are given.

Figure 3.1.4; continued

b

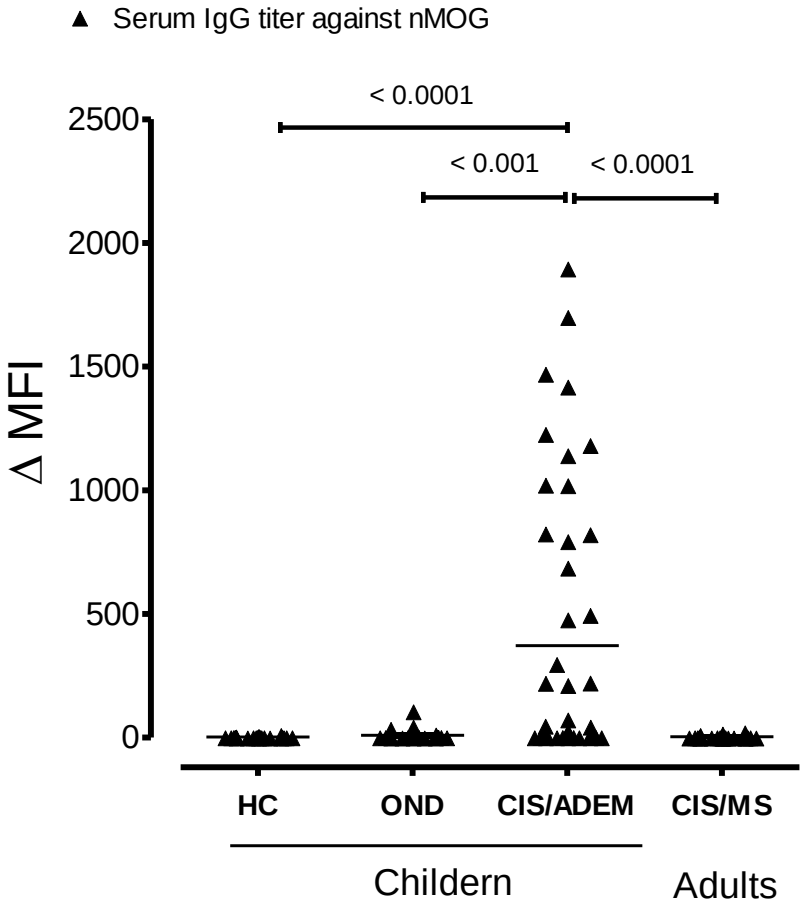
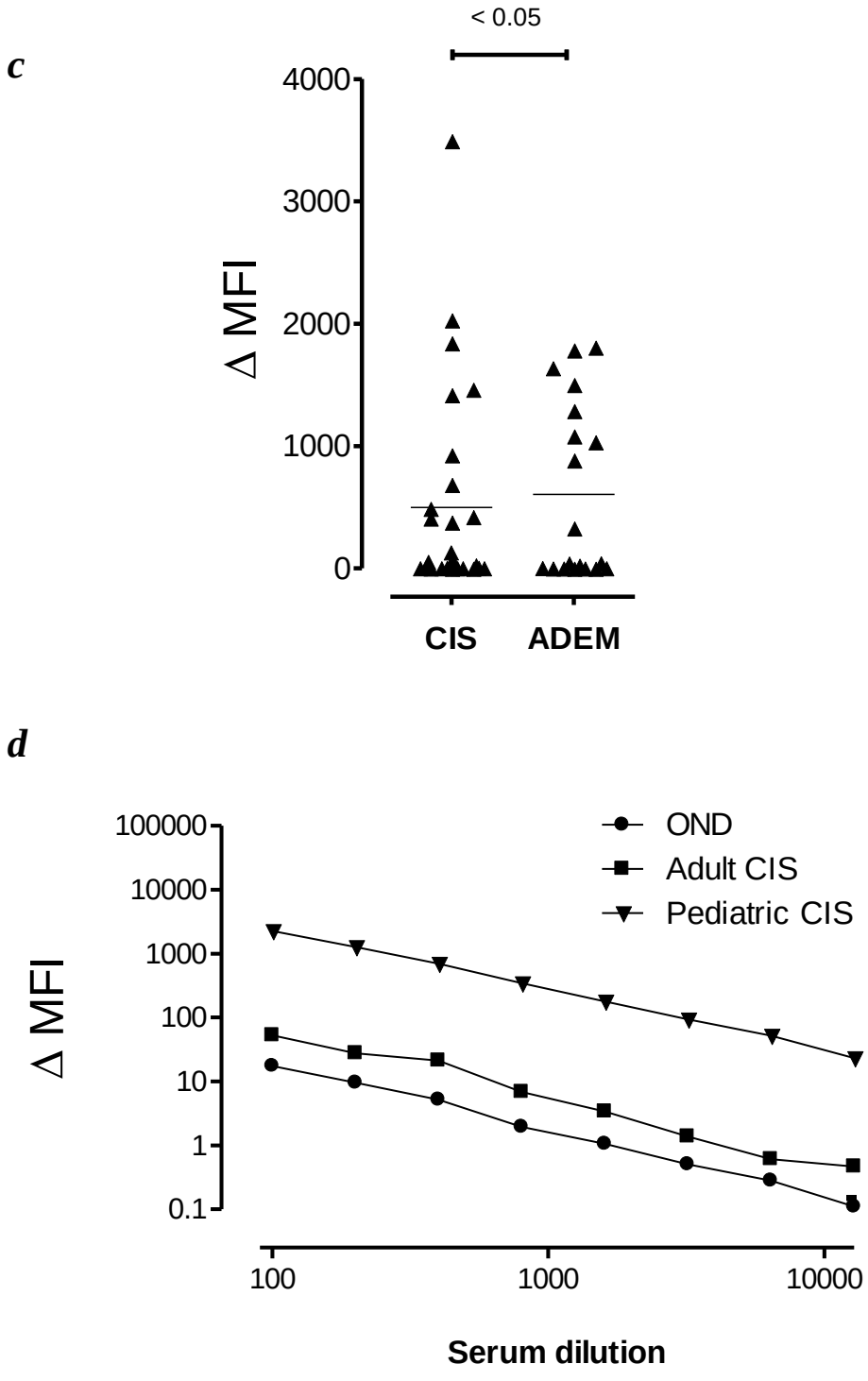


Figure 3.1.4; continued



3.1.5 Higher Antibodies to nMOG show negative correlatation with the age of onset in pediatric patients with first inflammatory demyelininating event

Further analysis indicated that the age of the patients with high anti-nMOG IgG (ΔMFI > 109.8, high titer threshold) was significantly less than that of patients with low or negative IgG antibody titer to nMOG. Both pediatric ADEM and CIS patients indicated this relationship (Fig. 3.1.5). The negative correlation (r = 0.46, p = 0.023) was observed between serum titer and age in children with anti-nMOG antibodies (ΔMFI > 38.1, anti-nMOG seropositivity threshold)

3.1.6 Antibodies to nMOG in pediatric and adult patients with first inflammatory demyelinating event do not predict progression to MS

Patients with CIS exhibit an increased risk of developing clinically definite MS. Conversion from CIS to MS is often unpredictable and is either characterized by a second clinical relapse or newly formed lesions disseminated in time and space (100). Pediatric ADEM patients may also exhibit multiple non ADEM relapses and are reclassified as MS (101). In the current study, pediatric patients with a first inflammatory CNS demyelinating event (CIS/ADEM, n = 47) were followed up for a mean duration of 2 years (range, 0.5-4.6 years). Conversion to MS was observed in 14/47 (~ 30 %) of these patients. Serum samples were obtained and tested for anti-MOG titers in pediatric CIS/ADEM patients that progressed to MS and those that did not progress to MS whereby no difference was observed (Figure 3.1.6 a).

Clinical relevance of anti-nMOG antibodies was also assessed in adult CIS patients. On average adult patients presented lower anti-MOG titers than pediatric patients. Anti-nMOG titers were meaured in adult CIS patients (n = 410) and the titers were classified into three quantiles, negative (ΔMFI < 10, n = 232 patients), intermediate (ΔMFI < 10-17.3, n = 75 patients) and positive (ΔMFI > 17.3, n = 103 patients). All adult CIS patients (n = 410) were followed up for a mean duration of three years and reclassified as clinically definite

MS after they met clinical criteria. The cox propotional hazard regression model was used to assess the predictive value of anti-nMOG antibodies for CIS to MS conversion. Anti-nMOG negative patients (ΔMFI < 10) did not differ significantly in survival distribution function estimates for remaing free of clinically definite MS (CDMS) from patients with positive (ΔMFI > 17.3) and intermediate (ΔMFI < 10-17.3) anti-nMOG titers (Log-rank test p values 0.96 and 0.82 respectively), in three year follow up period (Fig. 3.1.6 b).

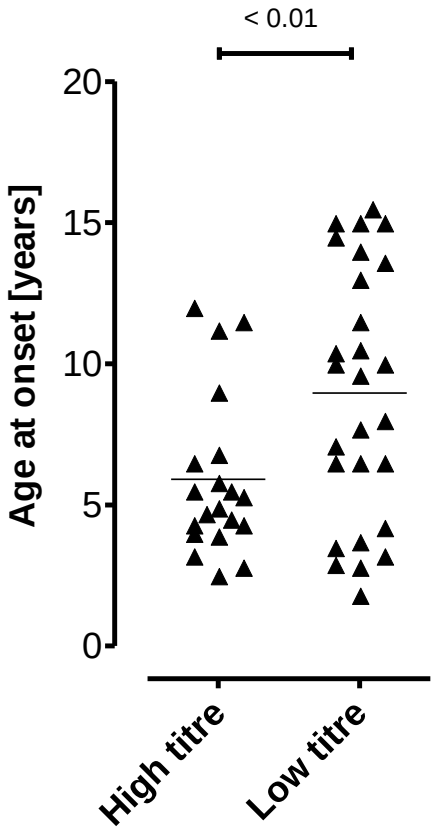


Figure 3.1.5 Negative correlation between age of onset and anti- nMOG antibody titre in children with a first demyelinating event (CIS/ADEM)

Children with a first inflammatory demyelinating event (CIS or ADEM) were stratified into 2 groups, with either high anti-MOG titer in serum (Δ MFI > 108.9) or negative/low titer, and were compared according to age of disease onset.

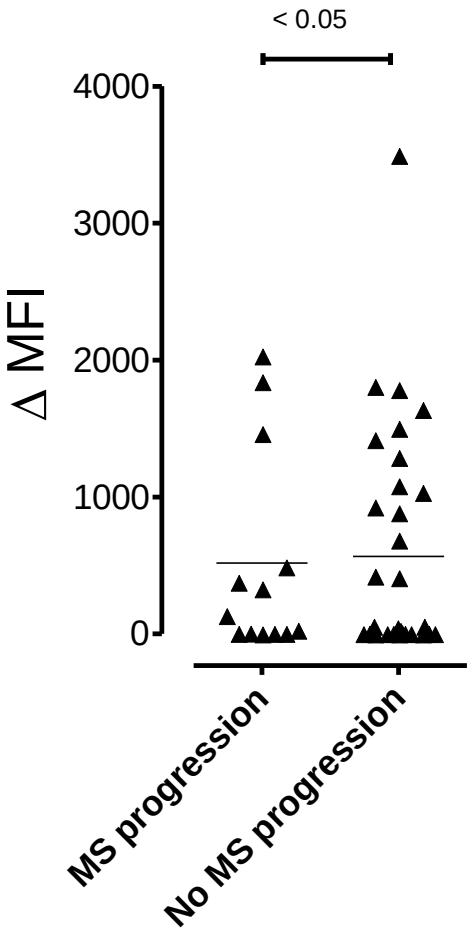
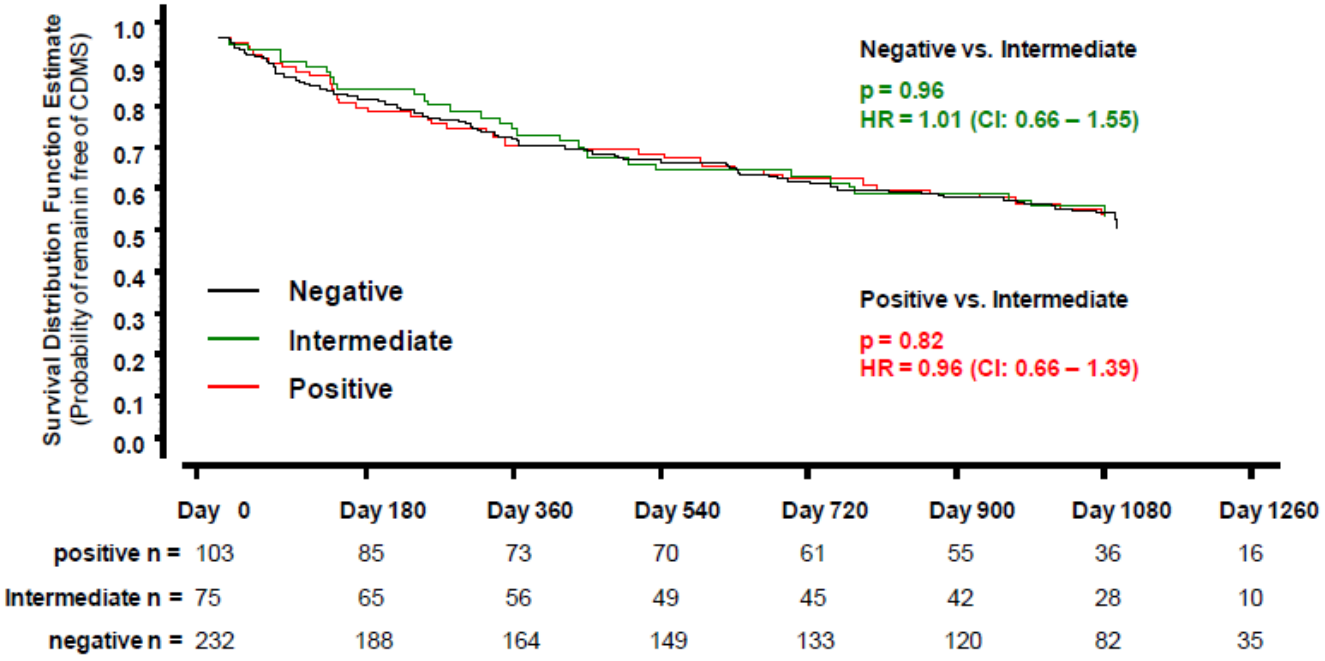


Figure 3.1.6 No association between anti-nMOG titer and disease progression (CIS to clinically definite MS) in pediatric and adult patients

Comparison of anti-nMOG IgG titre was carried out between children with first demyelinating episode (CIS or ADEM) who did and who did not progress to clinically definite multiple sclerosis (MS) (*a*). Antibody titers were compared between groups using the Mann-Whitney U test. Plane bars indicate median values of each group. The p-value is given. Cox propotional hazard regression analysis with adjustment of disease related time dependent covariates for adult CIS patients are shown (*b*). The p values indicate comparison between anti-nMOG intermediate and positive titer patient groups with anti-nMOG negative patients (obtained with log-rank test). Hazard ratio (HR) with 95 % confidence interval (CI) is given. Number of patients remaining free of clinically definite MS (CDMS) at each follow up time is shown for all three groups.

Figure 3.1.6; continued

b



3.2 Inflammatory cytokine regulation of claudins in cerebral endothelial cells

3.2.1 TNFα downregulates Cldn5 expression in MVEC

Murine microvascular endothelial cells (MVEC) isolated from C57/BL6 mice were grown to confluence and incubated in medium containing 10 ng/ml of cytokines relevant to neuroinflammatory disease including IL-1β, IFNγ and TNFα (or vehicle control) for 12-48 hours. Western blot analysis (Fig. 3.2.1 a) demonstrated that Cldn5 protein (detected as 22 kDa band) is strongly reduced upon treatment with TNFα. Expression of Cldn3 and 12 was not affected by cytokine induction. No change in the expression of Cldn3, 5 and 12 was observed upon treatment with IL-1β and IFNγ. Parallel MVEC cultures were maintained and treated with TNFα (or vehicle control) for 12 hr and realtime PCR was performed for absolute quantification of Cldn5 mRNA. A 50-60 % reduction in Cldn5 mRNA transcripts was observed in time dependent manner (Fig. 3.2.1 b). Downregulation of Cldn5 by TNFα could be observed through surface staining. Double immunofluorescence staining using antibodies against Cldn5 and endothelial marker pecam-1 indicated partial loss of Cldn5 staining whereas surface staining for endothelial marker pecam-1 (cd31) was relatively stable upon TNFα treatment (Fig. 3.2.1 c). Collectively, these results identify Cldn5 as a target of TNFα induced down regulation in murine cerebral endothelial cells.

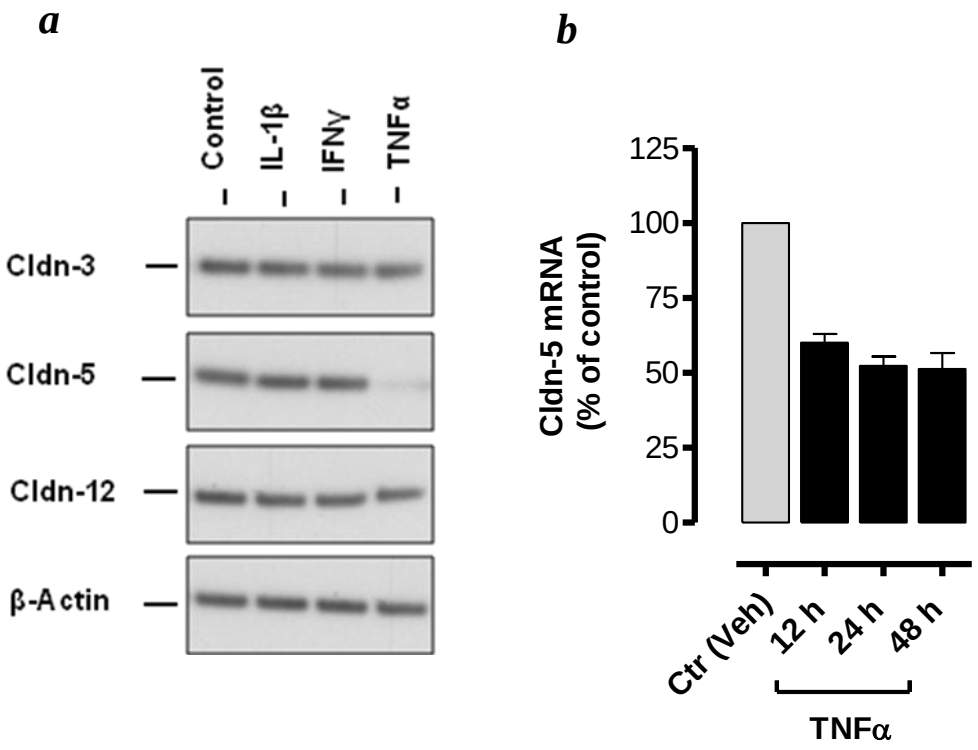
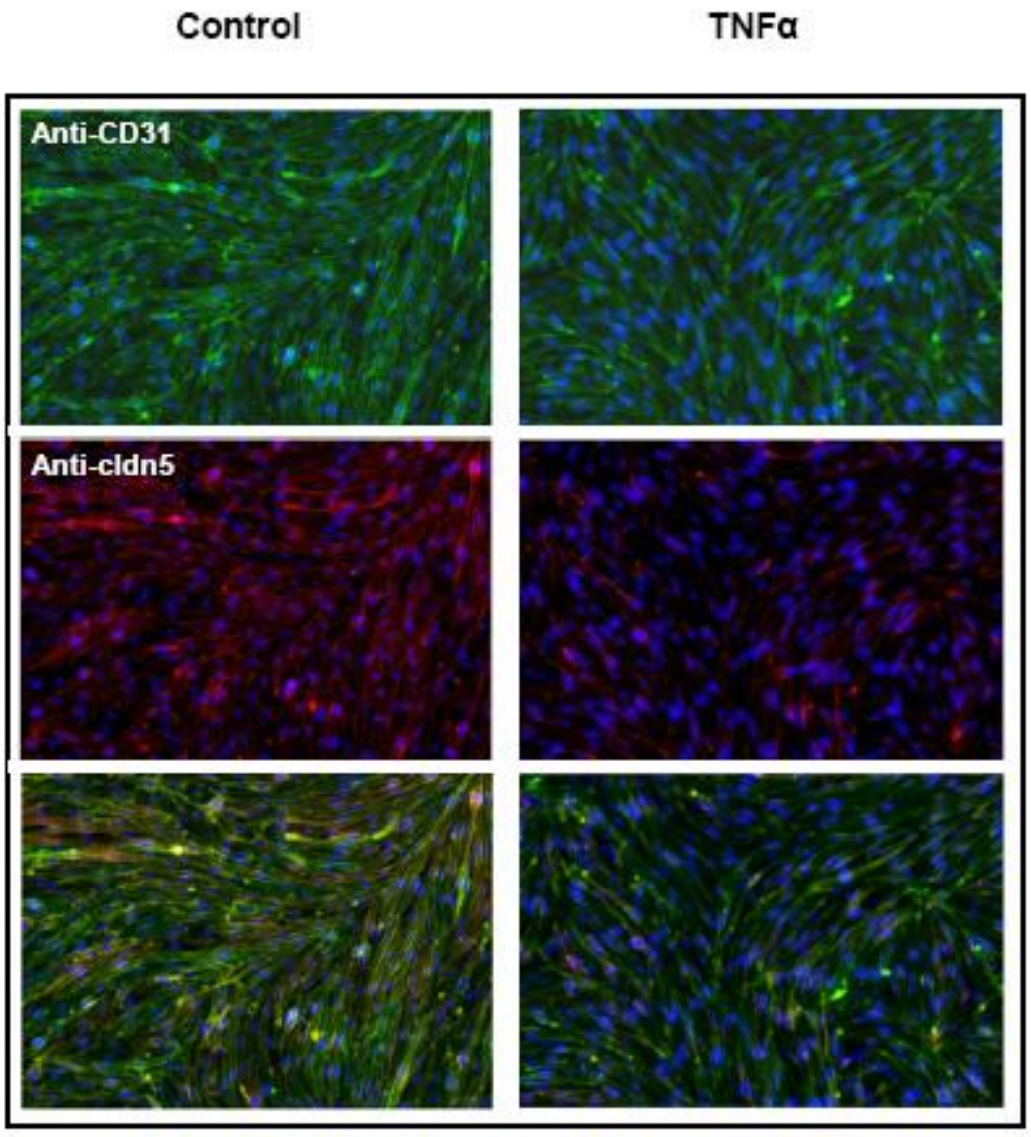


Figure 3.2.1 TNFα induces Cldn5 down regulation in murine MVECs

Western blots of protein extracts from murine MVECs treated for 24 h with 10 ng/ml cytokines (IL-1β, IFNγ and TNFα) or vehicle control are shown (*a*). Note that MVEC expression of Cldn5 protein is strongly reduced with TNFα treatment. Immunofluorescence imaging of murine MVEC treated as described earlier and immunostained for Cldn5 and endothelial marker pecam-1 (*b*). In control treatment, both proteins localize to the plasma membrane. Note that Cldn5 is downregulated by TNFα. Data is representative of at least 3 separate experiments of distinct MVEC cultures. Absolute quantification of Cldn5 mRNA (see methods) in MVECs treated with 10 ng/ml TNFα for indicated time is shown (*c*). Data is presented as percent reduction in number of copies of Cldn5 mRNA per μg of RNA extracted from TNFα treated MVECs as compared to control (set as 100 %). As control, MVECs were treated with vehicle alone (average number of copies per condition are also shown).

Figure 3.2.1; continued

c



3.2.2 Sequential deletions and bioinformatic analysis identify TNFα responsive evolutionary conserved region (ECR) in murine Cldn5 promoter

MatInspector prediction indicated putative binding sites for several cytokine-inducible transcription factors in Cldn5 promoter (Fig 3.2.2 a). To map the TFBS necessary for TNFα responsiveness we cloned successive deletion fragments of -1000/+144 sequence covering 1kb 5´-upstream and UTR sequence in front of luciferase reporter in pGL3-basic vector. The ability of each fragment to express luciferase reporter was measured in transient transfection assays in bEND.3 cells with TNFα or vehicle (0.1 % BSA in PBS) treatment. In the absence of TNFα treatment (i.e vehicle treatment) all fragments tested could promote luciferase activity above baseline control (luciferase activity in empty pGL3-basic transfection). TNFα treatment significantly reduced the luciferase activity in two of the cloned fragments both containing distal 150 bp sequence extending from -900 to -750 relative to TSS. Deletion of this region eliminated TNFα responsiveness. Further deletions (down to -300) did not reveal any additional regulatory sequence necessary for TNFα response (Fig 3.2.3 b). We therefore selected -900/-750 promoter region for further analysis. Sequence conservation analysis within this region (-900/-750) indicated evidence of ultraconservation extending from -892 to -763 relative to TSS (chr16: 18776048-18776176). A TFBS search returned with three putative nuclear factor κB sites shared by multiple species (Figure 3.2.2 b). No other potential TNFα response element was found in Cldn5 promoter.

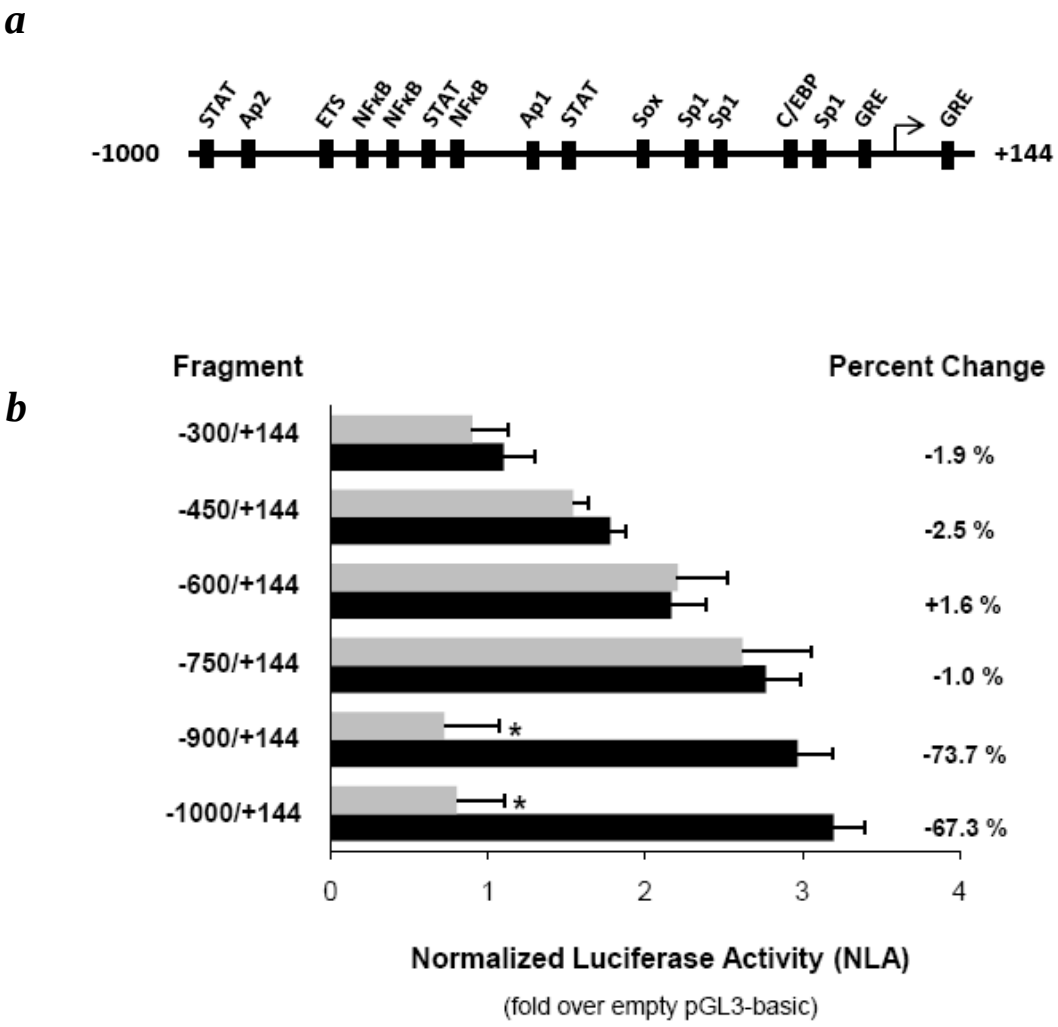
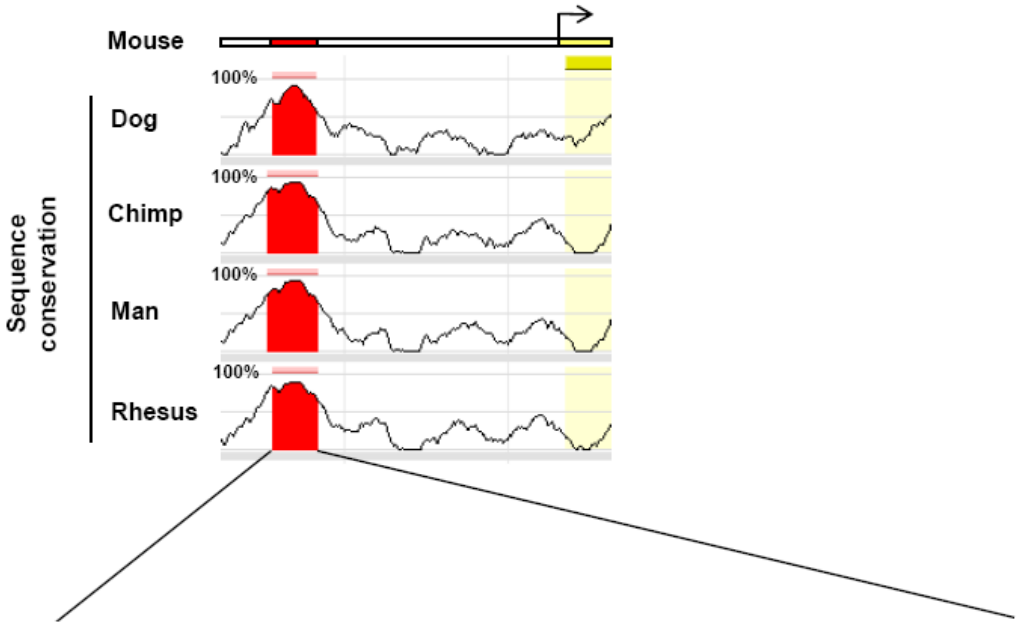


Figure 3.2.2 Sequential deletion and bioinformatic analysis of mouse Cldn5 promoter

Depiction of the −1000/+144 bp mouse Cldn5 promoter region. Cldn5 promoter represents a Typical TATA-less promoter enriched in Sp-1 binding sites. Predicted binding sites for ubiquitous transcription factors (CAAT-box, Sp-1), cytokine-inducible factors (STAT, NFκB, Ap-1) and endothelial differentiation factors (ETS, SOX18) are shown (a). Luciferase activity reflecting transcriptional activity of the deletion fragments spanning 1kb 5' flanking and UTR of mouse Cldn5 gene. Luciferase activity was measured after vehicle (grey bars) or TNFα treatment (black bars) in bEND.3 cells. Data (means ± S.D.) are expressed as fold above control (luciferase activity obtained with empty pGL3-basic), that was set at the baseline. Asterisk (*p*-value below 0.05) indicates statistically significant difference of luciferase activity between TNFα and vehicle treatments. Luciferase activity was normalized to protein content and expression of cotransfected renilla luciferase (b). The ultraconserved sequence (> 95 % identity) in Cldn5 distal promoter region is shown. Alignment of the ultraconserved 129 bp element (-892 to -763 relative to TSS) of mouse Cldn5 promoter with corresponding region in human Cldn5 promoter is given (lower panel) with predicted κB sites underlined (plus or minus sign indicate strand orientation). Asterisks indicate nucleotides conserved in evolutionary distant mammalian species (c).

Figure 3.2.2; continued

c

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                                                    -859 NFκB(-)
Mouse    AGAAATGGCTGGGCCATTGTGCAGAAGAATGCCCGGAAATCCCGCGGCTCCCTCCTCCACCGAGG
Human    AGAAATGGCTGGGCCATTGTGCAGAAGAATGCCCGGAAATCCCGCGCCTCCCTCCTCCAGCAAGG
         ******************************************** * ************ * ***

            -824 NFκB(+)          -804 NFκB(-)
Mouse    ATGGGGGCTCTTCCTCCTGGCCAGGAAACTCCAAGTTGGCTTCCGGAGGGTGGCCTGGGGGCTG
Human    ATGGGGGCTCTTCCTCCTGGCCAGGAAACTCCAAGTTGGCTTCCGGAGGGTGGCCTGGGGGCTG
         ************************************************************ ***
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3.2.3 TNFα induced Cldn5 promoter repression involves NFκB activation

Molecular effects of TNFα at the nuclear level in the endothelial cells are mediated by the members of NFκB (predominantly homo and heterodimers of p50 and p65 subunit) and AP-1 (Fig. 3.2.3 a). In order to determine molecular signaling mechanism involved in TNFα induced Cldn5 promoter repression two sets of experiments were performed involving chemical or molecular inhibition of NFκB pathway.

For chemical inhibition of NFκB activation anti-oxidant pyrrolidinedithiocarbamate (PDTC) and anti-inflammatory glucocorticoid dexamethasone (Dex) were used (102, 103). Transient transfection experiments involving TNFα responsive mouse Cldn5 promoter construct were performed in combination with PDTC or dexamethasone treatment to determine the effect of the chemical inhibition of NFκB activation on TNFα induced Cldn5 promoter repression. As shown in Fig. 3.2.3 b, PDTC mediated inhibition of NFκB activation resulted in loss of Cldn5 promoter TNFα response. Dexamethasone however seems to induce Cldn5 promoter activity above basal levels (broken line) independent of TNFα stimulation. This effect was further confirmed by western blot experiment showing a time dependent increase in Cldn5 protein in MVECs in response to dexamethasone treatment Fig. 3.2.3 c. In the second set of experiments molecular abrogation of NFκB activation in bEND.3 cells was achieved by over expression of degradation resistant ΔN-IκBα. The IκBα degradation kinetics in response to TNFα was monitored in bEND.3 cells transfected with pRC/CMV plasmid expressing ΔN-IκBα by western blot analysis. A time dependent reduction of the endogenous IκBα (~ 42 KDa) but not the transfected ΔN-IκBα protein (~ 37 KDa) was observed (Fig. 3.2.3 d) indicative of NFκB pathway inhibition**.** As a further proof of principle NFκB inducible 3xκB-Luc (see methods section 5.10) and ΔN-IκBα expressing plasmid co-transfection was performed. Induction of 3xκB-Luc by TNFα was lost upon ΔN-IκBα expressing plasmid co-transfection indicating that this assay functionally blocks the NFκB signaling (Fig. 3.2.3 e, open bars). Similar co-transfection experiment was performed involving TNFα responsive mouse Cldn5 promoter construct

and ΔN-IκBα expressing pRC/CMV. The Cldn5 promoter derived luciferase activity was comparable to the basal activity in case of ΔN-IκBα expressing plasmid co-transfection (Fig. 3.2.3 e, closed bars) indicating that molecular inhibition NFκB activation results in loss of TNFα induced repression of mouse Cldn5 promoter. Overall these results implicate inflammatory cytokine induced NFκB signaling in Cldn5 down regulation in bEND.3 cells.

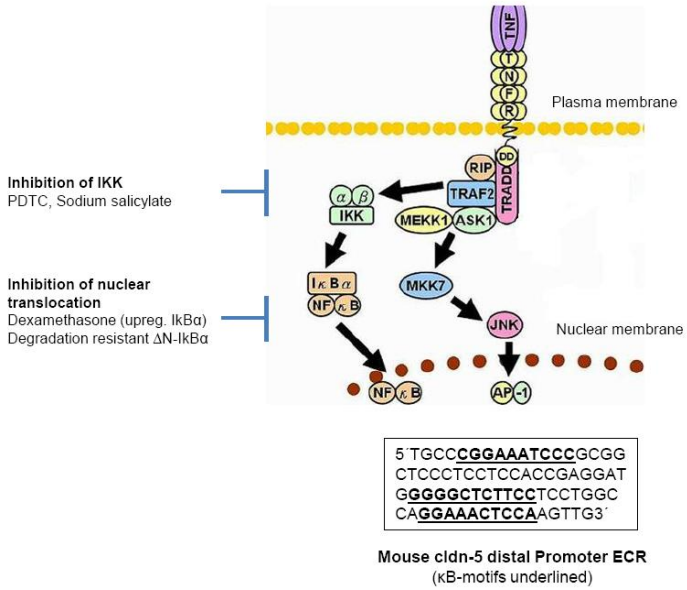
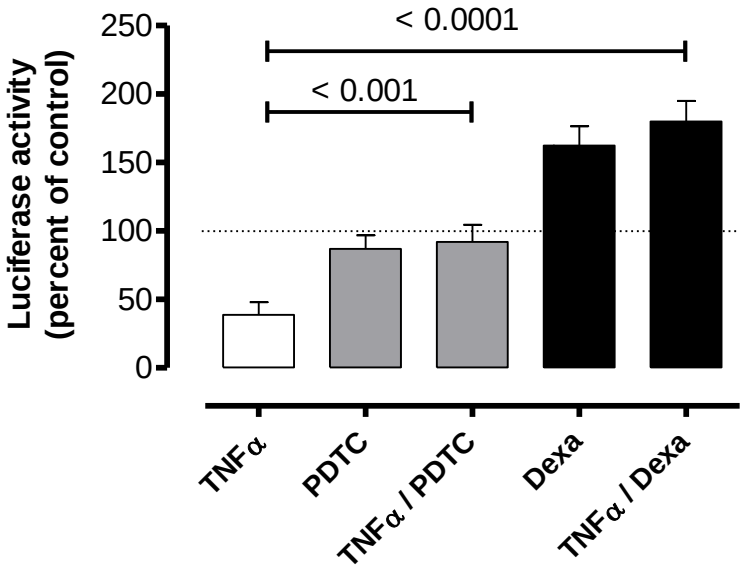


Figure 3.2.3 Chemical inhibition of NFκB pathway in bEND.3 cells

(*a*) Depiction of TNFα induced candidate transcriptional regulatory pathways in endothelial cells for Cldn5 promoter down regulation. The involvement of NFκB pathway activation in Cldn5 promoter repression was addressed by using two different abrogation/inhibition strategies (indicated in the left).
(*b*) Data from experiment involving chemical NFκB inhibitor (PDTC and dexamethasone) in combination with TNFα treatment in bEND.3 cells transfected with TNFα responsive Cldn5 promoter construct (-1000/+144) is presented . The luciferase activity is presented as percent vs control (basal activity of Cldn5 promoter in b.END3 cells, set at 100 % as indicated by broken line). The difference of luciferase activity between TNFα treated and TNFα + NFκB inhibitor treated bEND.3 cell lysates was compared by unpaired T test statistics. The p-values are indicated.
(*c*) Results of quantitative western blotting experiment showing time dependent increase in Cldn5 protein upon 100 nM dexamethasone treatment is shown. Data presented is representative of three independent experiments.
(*d*) Immunoblot showing differential degradation kinetics of overexpressed ΔN-IκBα and endogenous wild type IκBα (wt-IκBα) in bEND.3 cells after TNFα treatment.
(*e*) Data from experiment involving molecular aborgation of NFκB activation (ΔN-IκBα expressing) in combination with TNFα treatment in bEND.3 cells transfected with TNFα responsive 3xκB-luc plasmid (grey bars) or Cldn5 promoter construct (-1000/+144, black bars) is presented White bars indicate TNFα treatment response of 3xκB-luc and Cldn5 promoter. The luciferase activity is presented as percent of control. Basal promoter activity was set at 100% (shown as broken line). Unpaired T test statistics was used to compare luciferase activities. The p-values are indicated. Immunoblot showing differential degradation kinetics of overexpressed ΔN-IκBα and endogenous IκBα in bEND.3 cells after TNFα treatment.

Figure 3.2.3; continued

b



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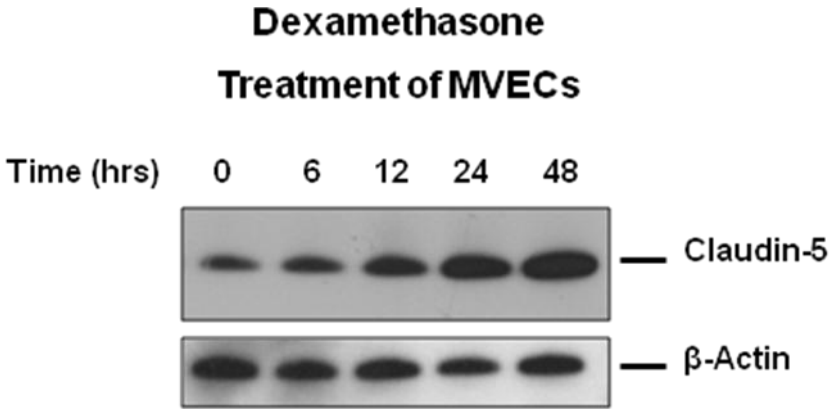

Figure 3.2.3; continued

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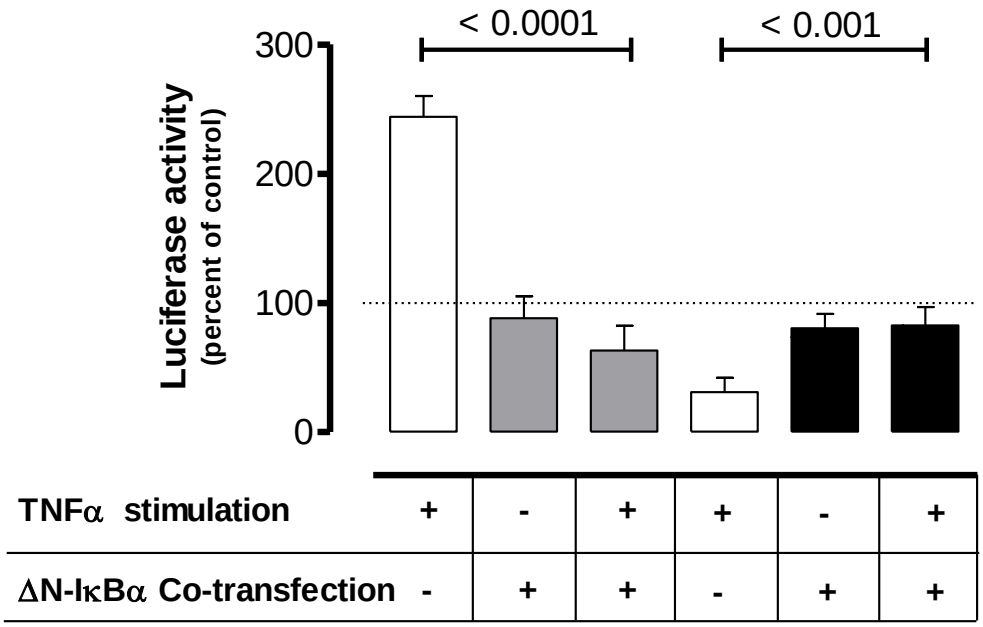

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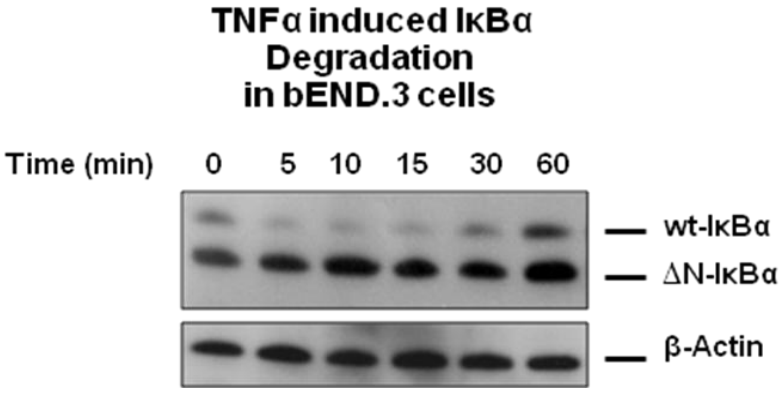


3.2.4 Direct interaction between NFκB subunit p65 and Cldn5 promoter mediates transcriptional repression

Trans-repression assays involving co-transfection of expression plasmid containing cDNA of murine p65 and p50 were carried out. Co-transfection with empty plasmid served as control. Fig. 3.2.4 a shows that trans-repression assay involving p65 expression mimics TNFα induced Cldn5 promoter repression. Because of extremely active transcriptional activation domain of p65, expression of p65 may repress Cldn5 promoter indirectly by induction of other transcriptional repressors. To explore the possible mechanism of p65 mediated repression electrophoretic mobility shift assays (EMSA) were performed with murine p65 over expressing bEND.3 nuclear lysate and mouse Cldn5 promoter derived oligos [C1, C2 and C3, for sequence see section 7.1.1]. Two of the oligos [C1 and C3] containing ultra-conserved κB motifs (CGGAAATCCC and GGAAACTCCA respectively) presented strong DNA-protein complexes (Fig. 3.2.4 b, Lane 1 and 3) that could be efficiently competed with oligoneucleotide containing κB consensus sequence (Fig. 3.2.4 b, lane 4 and 6). As control EMSA was performed with oligos containing mutated κB motifs of murine Cldn5 promoter [M1, M2 and M3, for sequence see section 7.1.1] or the bEND.3 nuclear lysate transfected with empty pcDNA3.1 vector (control nuclear lysate). We did not observe DNA-protein complexes in EMSA involving mutated oligos (Fig. 3.2.5 b, lane 7-9) or EMSA involving control nuclear lysate (Fig. 3.2.4 b, lane 10-12). Taken together these observations indicate a direct interaction between p65 and Cldn5 promoter κB motifs.

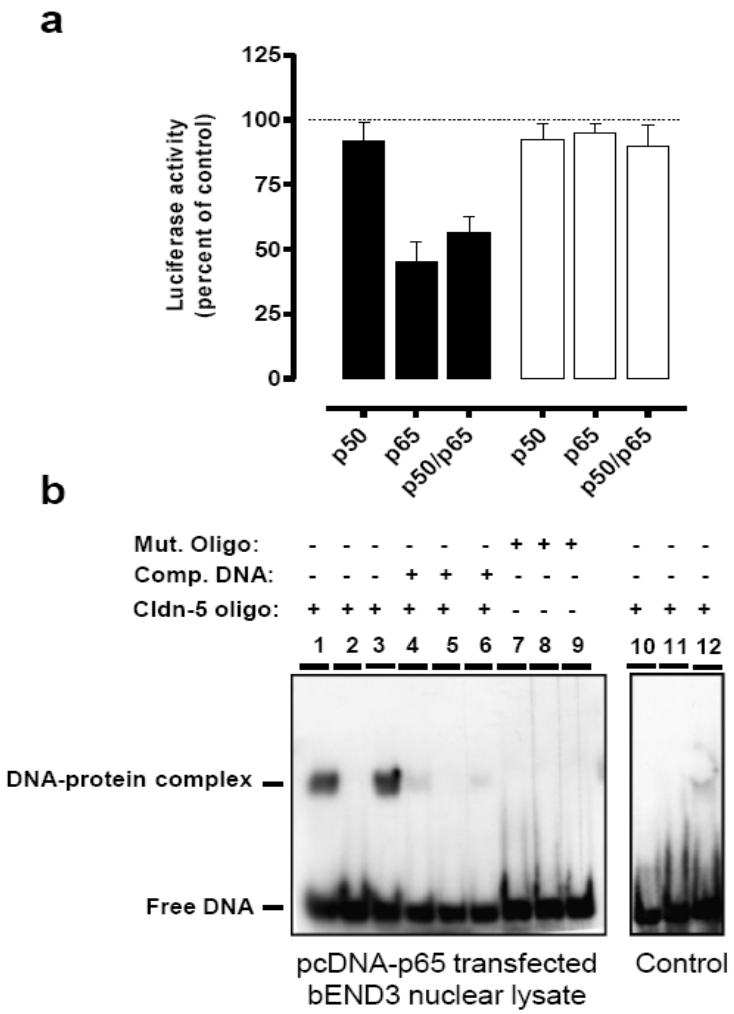


Figure 3.2.4 Trans-repression assay and EMSA show p65 involvement in TNFα induced Cldn5 promoter repression

(*a*) Result from trans-repression of TNFα responsive (-1000/+650, black bars) and unresponsive Cldn5 promoter construct (-1000/+650, white bars) with p50, p65 or p50/p65 expressing pcDNA plasmid co-transfection is shown As control empty pcDNA plasmid was co-transfected (broken line).
(*b*) Result of EMSA experiment involving Cldn5 promoter wild-type and mutant oligos with p65 over expressing bEND.3 nuclear lysate (lane 1-9). EMSA involving Cldn5 promoter wild-type oligos control nuclear lysate (lane 10-12). DNA-protein complexes observed in lane 1 and 3 were competed out in presence of competitor oligo consensus p65 binding motif (lane 4 and 6).

Chapter 4. DISCUSSION

4.1 Antibody responses in inflammatory CNS disease

The structure and function of CNS myelin is dependent on the expression of several myelin-specific proteins. In the inflammatory CNS diseases such as MS, deposits of antibodies can be detected in white matter lesions where myelin is undergoing degradation. It has been suggested that antibodies against myelin proteins can produce demyelination by mechanisms, such as antibody-dependent cell-mediated cytotoxicity (ADCC), release of inflammatory mediators through stimulation of Fc receptor on natural killer cells, macrophages, or mast cells or opsonization of myelin, which promotes phagocytosis by macrophages (104). Anitobody responses against several different myelin proteins have been described in MS. However the epitope specificity (linear or conformational), the clinical and pathological relevance of these antibody responses in MS have not yet been characterized.

The binding specificities of antibody responses to myelin proteins in MS may consist of linear or continuous epitopes, or of conformational epitopes consisting of discontinuous stretches of amino acid chain formed by localized protein folding, and the disperse epitopes formed from the different regions of the protein by complex folding. Most classical detection techniques such as western blotting, enzyme-linked immunosorbent assays (ELISA), or radioimmunoassay can readily identify linear epitope specific antibody responses; however these detection techniques fail to identify conformational and disperse epitope specific antibodies (105). This is partly because these classical methods use denatured antigen and therefore favor linear epitopes. Western blotting is even less sensitive in detecting discontinuous or conformational epitope specific antibodies due to the multiple denaturing steps involved prior to immunoreaction. In the in vivo conditions only conformational epitope specific antibodies are expected to bind native expression of target

protein and damage the expressing cells (31). Therefore the clinical and pathological relevance of antibody responses in inflammatory disease depend exclusively on their conformational epitope specificity. The conflicting reports about the clinical and pathologic relevance of myelin proteins specific antibodies may therefore be attributed to the assays system used to detect and quantify myelin protein specific antibody responses in inflammatory CNS disease.

Oligodendrocyte specific protein (OSP/claudin-11) is a transmembrane protein expressed on oligodendrocytes, testes and the inner ear (45). Due to its significance in myelin compaction and functioning, it has been considered as a strong autoantigenic candidate protein in MS. Previously autoantibodies targeting OSP peptides have been reported to be present in the CSF of 80% of relapsing–remitting MS (RRMS) patients (48). The antibody responses against OSP in MS have not been tested for their ability to bind to the native OSP protein (nOSP) expressed on the surface of glial cells presenting conformational epitopes that might occur in the in vivo conditions. Therefore their relevance to inflammatory CNS diseases remains to be elucidated and was addressed in this study.

Second objective of this study was to determine the occurrence, titers, and predictive clinical value of anti-MOG antibodies in pediatric and adult patients with inflammatory CNS disease. A cell based assay system to detect anti-MOG antibody response that targets conformational epitopes of native MOG (nMOG) protein has been described (31). This assay system was used to measure anti-nMOG antibodies in sera from pediatric and adult patients with inflammatory CNS disease.

4.1.1 The antibody responses to oligodendrocyte specific protein (OSP/Claudin-11) in multiple sclerosis

OSP/claudin-11 is an interesting candidate for autoantibody response in MS due to its expression and relevance for myelin structure and function. Because of its similarities with PMP-22, it is considered to be involved in oligodendrocyte proliferation, differentiation and migration (106). Immunization with OSP/claudin-11 peptide =OSP 103–123) induces optic neuritis in Rhesus monkeys (107). The animals also develop an antibody response to

claudin-11 peptide (OSP 103–123). In another study in SJL/J mice; immunization with soluble recombinant OSP/claudin-11 (lacking hydrophobic transmembrane domains of the native molecule) induced experimental autoimmune encephalomyelitis and optic neuritis. The IgG antibodies in these mice were directed against OSP 22–46 (108). Bronstein et al. used western blot assays to identify CSF reactivity of MS patients to recombinant human OSP expressed in E. coli. They also reported binding of pooled CSF from six RRMS patients to OSP 114–120 peptide in ELISA (48).

Sera and CSF samples of patients and controls were investigated by ELISA based on peptide (OSP 114–120), full length denatured and native OSP. A cell based flow cytometry assay was also used that allowed to detect antibodies that would bind to OSP expressed on the surface of glial cells mimicking in vivo OSP expression. We found higher antibody titers in MS patients to denatured OSP protein and the OSP peptide (Figure 3.1.3). This higher reactivity was found particularly in RRMS patients. By contrast we did not observe significant reactivity towards native OSP in any adult MS subgroup, pediatric and adult CIS patients and healthy controls (Figure 3.1.2). Our findings support the concept that such antibodies originate as a result of ongoing pathogenesis and are not relevant to MS pathology. The inability of anti-OSP antibodies isolated from CSF of RRMS patient to influence oligodendrocyte progenitor cell (OPC) migration in in-vitro migration assay despite the fact that native anti-OSP specific antibodies can inhibit OPC migration in-vitro supports our that antibody response to linear or denatured OSP epitopes do not play active role in disease progression in MS (109, 110).

This finding contrasts the results of studies on myelin oligodendrocyte protein (MOG) another candidate autoantigen in inflammatory CNS disease. Antibodies reactive to both linear and conformational epitopes are found in the sera of MOG induced EAE models and in MS patients. Little crossreactivity has been observed in anti-MOG antibodies that bind to linear and conformation specific MOG epitopes and only anti-MOG antibodies binding native MOG (nMOG) can mediate demylination in vivo (111).

Conclusion

Based on the findings of this study it is concluded that the antibody response to OSP/Cldn11 predominantly targets non-conformational, linear epitopes of the protein. Since these antibodies do not bind the native OSP expressed on glial cells, it is likely that the antibody does not contribute to antibody mediated demyelination in vivo. Since antibody titers are predominantly higher in RRMS than CIS, SPMS and other disease groups we believe that the occurrence of the antibodies is a result and not the cause of the inflammatory demyelinating attack to the CNS. Most likely the anti-OSP antibodies represent an epiphenomenon in MS pathology, an effect that may originate after initial demyelination event, when degraded myelin proteins are released to the periphery and generate polyspecific T and B cell responses.

4.1.2 The antibody responses to myelin oligodendrocyte glycoprotein (MOG) in multiple sclerosis

Flow cytometry based assay was used to detect and quantify anti-nMOG responses in pediatric and adult patients clinically diagnosed as CIS (or ADEM) characterized by first inflammatory CNS demyelinating event. Adults and pediatric patients differ in the titer as well as occurrence of anti-nMOG antibodies as detected by our assay. Higher anti-nMOG IgG titers [median ΔMFI, 24.5; range, 1.0 –1896.3] were observed in 40 % (19/47) pediatric CIS/ADEM patients as compared to the adult patients [median ΔMFI, 3.4; range, 0.3–107.1] (Figure 3.1.4). Reason for these differences between pediatric and adult patients with respect to anti-MOG antibody titers is not clear but on the basis a recent longitudinal study it is hypothesized that anti-MOG antibody titers show temporal dynamics and may change over time (112). A negative correlation was observed between age of onset and the titre of anti-MOG antibodies in children with first demyelinating event (Figure 3.1.5). This negative correlation of anti-MOG titers and age in inflammatory demyelinating CNS disease in children was also reported recently in another study (113). Expression of MOG is strictly restricted to myelinating oligodendrocytes (114) and in humans myelination is an ongoing development process that continues throughout

childhood at a higher rate than adults (115). It is therefore possible that a higher MOG synthesis rate in younger age result in higher MOG titres in patients with lower age of CIS/ADEM onset.

A first demyelinating event may be due to acute disseminated encephalomyelitis (ADEM) that includes encephalopathy and has a lower risk of progression to MS. The alternative diagnosis for first demyelinating event is clinically isolated syndrome (CIS) that does not include encephalopathy and has a higher risk of progression to MS (100-101, 116). It is generally accepted among researchers that unraveling early immunopathgenic processes that contribute to inflammatory CNS demyelinating event is not easy. As the etiology is unknown, it is difficult to identify events that cause the onset because the first demyelinating event (CIS/ADEM) may be a result of long period of subclinical pathological activity. Moreover the early immunopathgenic events leading to demyelination, may take place locally in the CNS, a compartment that is not easily accessible. For these reasons, most hypotheses on the early immunopathogenic events underlying inflammatory CNS disease are tested on experimental models or in the in vitro assays. Pathogenic relevance of anti-nMOG antibodies in CNS demyelinating diseases has been suggested and on the basis of these studies a possibility of the participation of these antibodies in early immunopathogenic events in inflammatory CNS disease, at least in a subset of pediatric CIS/ADEM patients with high titers and earlier age of onset, cannot be ruled out (31, 117).

The progression from first CNS demyelinating event (CIS/ADEM) to clinically definite MS in individual patients is often unpredictable because of the lack of clinical, biological or radiological biomarkers and anti-myelin antibodies have been suggested as one of the candidate predictive biomarker (60). Potential clinical value of anti-nMOG antibody titers was tested as predictive factor for progression to clinically definite MS in pediatric as well as adult patients with first inflammatory CNS demyelinating event. There was no difference in anti-nMOG antibody reactivity or titer in children with first demyelinating event who did or did not develop MS later on indicating that the nMOG specific antibodies do not predict progression to MS in pediatric patients (Fig 3.1.6 a). Similar results were observed in case of adult CIS patients (Figure 3.1.6 b). Presence and the level of anti-MOG titers have been

tested previously as predictive clinical marker for CIS to MS progression in many studies. However contradicting results were reported in these studies (60, 61). The controvery surrounding the clinical (prdictive) value of anti-MOG response was partly based on the assay systems used (i.e ELSIA and western blot) in previous studies that were not able to distinguish between linear vs conformational epitope specific anti-MOG antibody responses (118). With our assay based on native glial MOG expression we addressed this issue and our results support the lack of association between CIS to MS progression. Factors other than binding specificity (linear or conformation dependent epitopes) of anti-MOG antibodies may contribute to this lack of association. For instance it is known that genetic factors predispose patients for development of anti-MOG antibody response (119). The clinical (predictive) value of anti-nMOG antibodies can therefore not be excluded on the basis of these results and genetic factors need to be considered in future predictive models while assessing the role of anti-MOG antibody response in inflammatory CNS disease.

Conclusion

In contrast to anti-OSP antibody response a unique and conformational epitope specific antibody response against MOG was detected in a set of pediatric inflammatory CNS disease patients. These patients are characterized by first demyelinating event (CIS or ADEM) at a younger age of onset (mean 7.2 years). The adult CIS/MS patients exhibited lower anti-nMOG titers. However the seroprevalence of anti-nMOG antibody response in childhood CIS and ADEM and adult CIS patients was not predictive of later conversion to MS.

4.1.3 General conclusion on study I

Antibody responses are considered as potential biomarker with prognostic or predictive potential in inflammatory CNS diseases. Some of the responses such as anti-nMOG may be more important because of their conformational epitope specificity while others such as anti-OSP/claudin-11 may represent epiphenomenon. The assay systems described in this

study could be helpful in the detection of epitope specificity and functional characterization of antibody responses in inflammatory CNS diseases.

4.2 Inflammatory cytokine regulation of claudins in cerebral endothelial cells

Blood–brain barrier (BBB) separates peripheral and the CNS circulations, establishing a highly impermeable barrier to proteins, cellular elements of peripheral circulation, solutes and majority of the circulating substances in blood. The principal cellular component of the BBB is microvascular endothelial cells (MVECs) that form monolayer with extremely low paracellular activity due to an elaborate network of interendothelial tight junctions (BBB TJs). In the interendothelial TJ complex claudins are particularly important because of their apical localization within the tight junctional complex and their role in paracellular diffusion selectivity and endothelial cell polarity (120).

Among the 23 claudin family members, Cldn5 is unique because of its ubiquitous endothelial specific expression pattern (121). Basal expression of Cldn5 has been shown to be regulated by endothelial specific transcription factor SOX-18 (122). Transfection of Cldn5 cDNA into cell lines results in the induction of barrier properties, whereas Cldn5 knockout mice show selective BBB opening and die perinatally (84). The disruption of Cldn5 expression has previously been linked to barrier breakdown in glioblastoma multiforme, a CNS neoplastic condition (123). Reduction in the expression of Cldn5 combined with increase in vascular permeability as a result of the inflammatory cytokine induced signaling has recently been shown in retinal endothelial barrier which is physiologically similar to BBB (124). Our finding of TNFα induced downregulation of Cldn5 expression in cerebral endothelial cells is therefore important in the context of inflammatory CNS diseases that are characterized by BBB TJ hyperpermeability (89). BBB permeability studies in animals and in-vitro models indicate that exposure to inflammatory cytokines (IL-1β IFNγ and TNFα) disrupt the tight junctional barrier properties (125-127). Pro-permeability properties of TNFα in the in vitro BBB models are particularly important because hyperpermeability produced by TNFα across endothelial monolayer is both time- and concentration-dependent. The lag time between TNFα exposure and TNFα-mediated effects on the permeability in in-vitro models of the BBB suggest alterations at the gene

expression level (128). However, the potential intracellular signaling pathways involved are not well understood. Our study indicates that Cldn5 promoter repression upon TNFα exposure via NFκB activation in cerebral endothelial cells might be a potential molecular mechanism in inflammatory cytokine TNFα induced disruption of BBB.

TNFα is a pleitropic cytokine that activates several signaling pathways in cerebral endothelial cells including NFκB (129). Normally NFκB dimers (homo or heterodimers composed mainly of p50 and p65 subunits in endothelial cells) are sequestered and inhibited by IκB proteins (mainly IκBα). Interaction of inflammatory cytokines such as TNFα with its receptor leads to the activation of NFκB signaling cascade. Subsequently the IκB kinases (IKKs) phosphorelate IκBα leading to its. The NFκB dimers are set free upon IκBα degradation translocate to the nucleus where they bind at κB motifs of target gene promoters thereby mediating transcriptional regulation of target genes. In addition NFκB activation may regulate target gene expression indirectly through the induction of other transcriptional regulators. Using chemical and molecular abrogation of NFκB signaling, transrepression and mobility shift assays we demostrate that the repressor effect of TNFα on the murine Cldn5 promoter is mediated by direct interaction between NFκB subunit p65 and ultra-conserved κB motifs in distal promoter. Repressive effect of p65 on Cldn5 promoter is of particular interest. Regulatory effects of NFκB dimers containing p65 subunit on target gene expression mainly include transcriptional activation due to its extremely active C-terminal activation domain (130). It is however indicated in recent studies that biochemical interaction of p65 with transcription co-repressors results in down regulation of p65 target gene expression (131). A similar ineraction at Cldn5 promoter may be possible but further studies are needed to characterize those interactions. An interesting candidate for such an interaction is vascular endothelial growth factor (VEGF) induced signaling. There is a considerable overlap between VEGF and TNFα mediated signalling pathways in cereberal endothelial cells (132). VEGF induced signaling has previously been implicated in Cldn5 downregulation at transcriptional level (99). VEGF is believed to play a role in inflammatory CNS diseases and its expression is has been detected in MS plaques (97). Considering these similarities a convergence of VEGF and TNFα mediated signalling

pathways in cerebral endothelial cells leading to Cldn5 down-regulation can not be ruled out.

In MS, loss of BBB TJ permeability properties is considered as an early event that preceeds leukocyte infilteration. Therapeutic approaches that can selectively target BBB disruption are lacking. Immunomodulatory and immunosuppressive drugs such as glucocorticoids (GCs) have been used traditionally to reduce acute CNS inflammation and MS relapses possibly via suppression of leukocyte infilteration (133-135). Other mechanisms suggested for the beneficial GC effects on BBB include strengthening of interendothelial tight junctions (136). Upregulation of Cldn5 expression was observed in cerebral endothelial cells in response to dexamethasone, an anti-inflammatory glucocorticoid. Glucocorticoid induced upregulation of other junctional proteins has also been reported in cerebral endothelial cells (137, 138). GCs are however pleiotropic drugs and have wide spread side-effects. Identification of beneficial targets of GCs therefore represents a potentially important area in therapeutic research. Considering the role of Cldn5 in BBB tight junctional integrity, its vulnaribility to inflammatory signals and upregulation by anti-inflammatory glucocorticoid, Cldn5 could be a relevant molecule for inflammatory CNS diseases.

Conclusion

Abnormal vascular permeability at the BBB is considered as a marker of the development of new inflammatory CNS lesion as well as expansion of existing ones. To design effective therapeutic approaches to control BBB dysfunction, an understanding of molecular alterations at BBB TJs is important. In present study we sought to find out inflammatory cytokine inducible alteration in cerebral endothelial expression of TJ proteins claudin-3, 5 and 12. Data presented in this thesis suggest that TNFα induced transcriptional repression and down-regulation of Cldn5 could be a key event in BBB disruption during inflammatory CNS diseases.

4.3 Future studies

The data presented in this thesis highlights several areas for further investigation.

In the **study I**, we tested binding specificity (linear or conformational) antibody responses against two myelin antigens (OSP/11 and MOG) in inflammatory CNS disease. We also addressed the clinical (predictive) potential of these responses. This study reveals following areas for future investigation.

1. High throughput proteomic technologies and peptide arrays can be applied to identify autoreactive B cell epitope spreading fingerprints in MS patients (such as anti-OSP/Cldn11 antibody response). Although not pathologically relevant such information on clinically defined set of patients may still provide important diagnostic and prognostic information in inflammatory CNS disease.

2. Genetic factors predispose patients for development of antibody response (119). In previous studies (including study I in this thesis) genetic factors were not considered in predictive models used to assess clinical potential of anti-MOG antibody responses and therefore the study populations consisted of genetically heterogeneous set of patients. Similar studies involving genetically homogeneous study population with high antibody titers against CNS antigens would define the true clinical potential of antibody responses in inflammatory CNS disease.

In the **study II** presented in this thesis we addressed inflammatory cytokine induced modulation of the expression of the claudin family members known to stabilize blood brain barrier tight junctions. Applying various promoter characterization assays we implicate TNFα induced NFκB singaling and NFκB subunit p65-promoter interaction for Cldn5 down regulation in mouse endothelioma cell line bEND.3. An altered Cldn5 staining pattern of Cldn5 was observed in inflamed spinal cord sections from EAE mice as compared to control mice sections (Appendix figure). Chromatin immunoprecipitation Studies are needed to define transcriptional co-repressor interactions at Cldn5 promoter. Furthermore relevance of TNFα induced Cldn5 downregulation in cerebral micrvoascular endothelial cells to the inflammatory CNS disease needs to be addressed in vivo.

Chapter 5. METHODS

5.1 Patients and controls

All pediatric patients and controls were recruited in the UK. Adult MS patients and controls were recruited at the neurology departments in Düsseldorf and Munich. Ethics approval for the use of pediatric samples was granted by the Children's hospital at Westmead ethics committee (No HREC 07/CHW/28 and SSA 07/CHW/58). Ethics approval for the use of adult samples was granted by the local Ethics committees in Düsseldorf and Munich. Table 5.1 shows characteristics of patients and controls.

5.2 Cloning and expression of human OSP/Cldn11 and MOG proteins

The full-length human OSP/Cldn11 and MOG cDNAs were PCR amplified from human brain total RNA (BD Biosciences). The cDNAs were cloned into the plasmid pLenti6/V5 (Invitrogen) to get pLenti6/V5-OSP or pLenti6/V5-MOG constructs using primer born restriction sites SpeI / SacII (OSP/Cldn5 cloning) and BglII (MOG cloning). For the production of viral particles containing pLenti6/V5-OSP or pLenti6/V5-MOG constructs 293-FT cells were transfected with 36 μg lipofectamine (invitrogen) and 12 μg of each plasmid construct in serum free DMEM transfection medium. After 12 hour incubation at 37 ^{o} C in humidified incubator (5 % CO_2) the transfection medium was replaced with complete culture medium. After 48 hour incubation the virus-containing supernatant was harvested and used to transduce glioblastoma cell line LN18 to get LN18-OSP and LN18-MOG cell lines. A control cell line (LN18-CTR) was obtained by transducing the LN18 cell line with viral particles containing empty pLenti6/V5 vector. All cell lines were maintained in identical culture conditions in RPMI 1640 growth medium.

5.3 Assessment of native glial expression of human OSP/Cldn11 and MOG proteins

Native glial expression of OSP/Cldn11 (nOSP) and MOG (nMOG) on transduced LN18 cells was determined by immunoflourescence staining and flow cytometry.

For Immunofluorescence staining cells were grown overnight in chamber slides (Nalge Nunc). After PBS washes and 3% paraformaldehyde-fixation cell staining was performed by using 0.3 µg/ml anti-OSP/Cldn11 monoclonal antibody (LifeSpan BioSciences) monoclonal anti-MOG antibody (mAb 8-18C5) as primary antibodies. Alexa 488-conjugated goat anti-mouse IgG (Invitrogen) was used as secondary antibody. DAPI (Invitrogen) was used for the nuclear staining. Images were captured and analyzed by an Olympus IX71 microscope system (Olympus).

For flow cytometry staining 20 µl of primary monoclonal antibody (same as immunofluorescence staining) at 1 mg / ml was added in duplicates to 96-well plates containing 30,000 LN18-OSP, LN18-MOG or LN18-CTR cells in 20 µl of RPMI 1640 growth medium. After 20 minutes incubation on ice cells were washed twice with PBS containing 1 % FCS. Alexa Fluor 488-labeled goat anti-mouse IgG (Invitrogen) was applied as secondary antibody. After final washing steps cell surface staining was analyzed on a FACS cell analyzer (CyAn ADP, Beckman Coulter) using summit software (Beckman Coulter).

5.4 Enzyme linked immunosorbent assays (ELISA)

5.4.1 Peptide ELISA

For peptide ELISA, 96- well Plates (Nalge Nunc) were coated with 100 µl (10 µg / ml) OSP/Cldn11 peptide (114-120) in bicarbonate buffer (pH 9.6). To block unreacted sites, the wells were treated with 2% BSA for 2 hours at room temperature. Washing was performed with PBS-T (0.05% Tween 20 in PBS). After washing four times, serum (100 µl / well, 20 mg / l IgG) or CSF (100 µl / well, 20 mg / l IgG) was added and incubated for one hour at room temperature. The wells were washed and horseradish peroxidase labelled rabbit anti-human IgG antibody (DAKO) was added. After the final incubation and four washes, 100 µl of HRP substrate (KPL Inc.) was added. After 20 min development the colour reaction was stopped with of 2N sulphuric acid (50 µl / well) and the plates were read using an automated ELISA plate reader (Tecan).

5.4.2 Denatured antigen ELISA

For denatured antigen ELISA, LN18-OSP and LN18-CTR cells were lysed with membrane protein extraction buffer (Thermo Fisher Scientific). Cell lysates enriched for membrane proteins were heat denatured at 72 ^{o}C for 15 minutes in presence of reducing agent containing 500 mM dithiothreitol (DTT) (Invitrogen). Cell lysates were then diluted to 30 ug / ml in 100 mM sodium carbonate buffer (pH 9.6) and 100 µl of this solution was added to 96- well Nunc immobilizer™ amino plates (Nalge Nunc Inc). Plates were again heated in water bath at 75 ^{o}C for 15 minutes and left for coating at room temperature for 2 hours. ELISA procedure was performed as described earlier. specific antibody reacivity (ΔOD) was determined by the OD obtained with the lysate of LN18-OSP cell line subtracted by the OD obtained with the lysate of LN18-CTR cell line. Sensitivity and reproducibility of the assay were evaluated by titration experiments using the anti-OSP/Cldn11 polyclonal antibody (Santa Cruz Biotechnology).

5.4.3 Native antigen ELISA

To detect serum and CSF reactivity to native human OSP/Cldn11 (nOSP) in ELISA assay LN18-CTR and LN18-OSP were grown to 90 % confluence. Medium was removed and cells were washed twice with PBS. Following washing cells were incubated in 12 ml Sulfo-NHS-SS-Biotin solution in PBS (Thermo Fisher Scientific) at 4^{o} C for 30 minutes. After quenching the biotinylation reaction cells were scrapped from flask and washed twice with TBS buffer (Thermo Fisher Scientific) and 50,000 biotinylated cells per well were added to streptavidine coated microwell plates (Thermo Fisher Scientific). The wells were then washed twice and blocked with 2 % BSA. Staining was carried out as described for Peptide ELISA. specific native OSP/Cldn11 antibody reactivity (ΔOD) was defined by OD obtained by a particular serum or CSF sample with LN18-OSP minus the OD obtained by same sample with LN18-CTR cell line. Sensitivity and reproducibility of the assay were evaluated by titration experiments using the anti-OSP/Cldn11 monoclonal antibody (LifeSpan BioSciences).

5.5 Cell-based assay for quantification of antibody reactivity in MS sera and CSF

The following bioassay was used to quantify antibody reactivity of sera and CSF to nOSP and nMOG. The IgG concentration of sera and corresponding CSF were measured by nephelometry (BN ProSpec). Serum or CSF was added to 30,000 LN18-OSP, LN18-MOG, or LN18-CTR cells in RPMI1640 at final IgG concentration of 5 mg/l. Cells were incubated on ice on an orbital shaker for 20 min, and washed twice with washing buffer (1%FCS in PBS). Cells were then stained with Alexa Fluor 488-labeled goat anti-human IgG or IgM secondary antibody (Invitrogen) for 20 min on ice and washed again twice in washing buffer (PBS with 0.1 % FCS). Analysis of cell surface staining was determined by flow cytometry using CyAn ADP (Beckman Coulter) and Summit software (Beckman Coulter). Levels of Antibody titers are expressed by ΔMFI. ΔMFI was determined by the substraction of median fluorescence intensity (MFI) obtained with LN18-CTR cells from the MFI obtained with LN18-OSP or LN18-MOG cells.

5.6 Isolation of brain microvescular endothelial cells (MVEC)

Microvascular endothelial cells (MVEC) were isolated from C57/BL6 mice and cultured as described by Wu Z. et al. (139). Briefly, brains from C57/BL6 mice (age 8-10 weeks old) were aseptically collected and rinsed in Medium 131 (Invitrogen) supplemented with 2% fetal bovine serum (Invitrogen), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma). After removing white matter, large vessels and leptomeninges, cortices were minced and homogenized using Dounce tissue grinder (0.25 mm clearance) in same medium until tissue clumps disappeared (3-5 strokes). The homogenates were suspended in 15 % dextran (ave. MW 65,000-75,000, Sigma) and centrifuged at 10 000 g for 15 min. The supernatant was again mixed with 15 % dextran and centrifuged. The pellets containing microvessels and dextran were combined and digested in 15 ml 0.1% of collagenase / dispase (Roche) supplemented with 2% FBS for 6 h at 37 ^{o}C with occasional agitation. The digested microvessels were collected with centrifugation at 1000 g for 5 min. To purify microvessels and individual endothelial cells pellet were subjected to in Percoll density gradient centrifugation (45 % percoll in PBS) at 20 000 g for 10 min at 4 ^{o}C. The microvessels and

individual endothelial cells were collected from the top layer and washed with PBS. The microvessels suspended in endothelial culture medium (Medium 131 supplemented with 30 mg/ml ECGS, 10% FBS, 15 U/ml heparin, 325 mg/ml glutathione, 1 ml/ml 2-mecaptoethanol, 100 U/ml penicillin and 100 mg/ml streptomycin, all from Sigma) were seeded on plastic ware pre-coated with attachment factor (AF, invitrogen) and grown in standard cell culture conditions. The medium was changed after every 2 days. A monolayer MVEC was obtained in 8-10 days. A PBS based enzyme free cell dissociation buffer (Invitrogen) was used to for sub-culturing. All the experiments were performed on MVEC between passages 2 to 3.

5.7 Cytokines and drug treatment

Murine IL-1β, IFNγ and TNFα were purchased from R&D Systems. Dexamethasone, and pyrrolidine dithiocarbamate (PDTC) were obtained from sigma. Cells were washed with serum free medium and treated with cytokines at concentration 10 ng/ml for indicated time. As control cells were treated with vehicle alone (0.1% BSA in PBS). Treatment with dexamethasone and PDTC was carried out at concentrations 100 nM and 50 μM respectively for 24 hours.

5.8 RNA isolation and real-time PCR

Isolation of RNA from MVECs was performed using Rneasy kit, (Quiagen Hilden, Germany) and for reverse transcription reactions High-capacity RT Kit, (Applied Biosystems, USA) was used. To obtain external standard curve for absolute quantification of Cldn5 mRNA in MVEC, partial coding sequences of murine Cldn5 were amplified and cloned in pGEM-T easy vector (Promega, Madison, WI). Primers used for cloning are shown in Table 2.2.1 (primer 1-2). Realtime PCR was perfomed with StepOne Plus PCR System (Applied Biosystems) using TaqMan Gene Expression Assay (Cldn5: Mm01169675_s1). A total of 1μg RNA purified from MVEC was reverse transcribed for each condition using high capacity cDNA reverse transcription kit (Applied Biosystems). In each experiment the identity of PCR product was confirmed by DNA sequencing and gel electrophoresis.

5.9 Western blotting, Immunofluorescence and immunohistochemistry

For western blot analysis MVECs were lysed in M-PER buffer (Thermo scientific). Total protein content in each lysate was measured by micro BCA protein assay kit (Thermo scientific). Cell lysate containing total of 5 μg protein was loaded per lane of Novex 4-12 % Bis-Tris Gel (invitrogen). After transfer to PVDF membrane the blots were blocked in 3 % milk for 1 hour and incubated in primary antibody solution overnight at 4^{o} [primary antibodies: anti-mouse Cldn3 (Acris, AP06064PU-N), rabbit anti-mouse Cldn5 (Acris, DP-157) rabbit anti-mouse Cldn12 (Lifespan, LS-B2181), mouse monoclonal to IKBα (Abcam, ab12134) and mouse monoclonal β-actin (abcam, ab8226)]. After washing and incubation in HRP-conjugated secondary antibodies for 1 hour the blots were subjected to chemiluminescent ECL detection (Novex ECL, invitrogen). All western blot experiments are repeated at least three times.

For immunofluorescence staining of MVEC, rabbit anti-mouse Cldn5 (Acris, DP-157) and rat anti-mouse pecam-1 antibodies (invitrogen, RM5200) were used as primary antibodies. Alexa 594-conjugated anti-rabbit and Alexa 488-conjugated anti-rat antibodies (both from invitrogen) were used as secondary antibodies. DAPI staining was use to visualize nuclei. Images were captured and analyzed using AxioVision software system (Zeiss) on a fluorescent microscope (Zeiss).

Induction of experimental autoimmune encephalomyelitis (EAE) in C57/BL6 mice was performed by adoptive transfer of MOG 35-51 specific T cell clones as described before (140). Spinal cords were harvested from control mice and mice exhibiting different stages of EAE, fixed overnight in paraformaldehyde, embedded in paraffin and cut in 5 μm thin transverse sections. Immunohistochemistry was performed using standard protocol on at least three sections per animal using anti mouse Cldn5 antibody to assess Cldn5 staining pattern. Sections were counter stined with H&E to visualize inflammatory lesions. Light microscopic images were catptured with AxioVision software (Zeiss).

5.10 Promoter analysis and plasmid construction

The mouse Cldn5 promoter region was identified by using PromoterInspector (Genomatix Software GmbH). ECR browser (141) was used to visualize and analyze evolutionary

conserved regions (ECRs; search parameters; minimum ECR length of 100 bp and min sequence identity of 95%). Conserved transcription factor binding sites in mouse Cldn5 promoter were identified using MatInspector (Genomatix Software GmbH) and rVISTA 2.0 with stringent search parameters (142).

PCR was performed on mouse genomic DNA to amplify sequential deletion fragments of mouse Cldn5 promoter. Restriction sites 5´ XhoI and 3´ HindIII were introduced in all the promoter fragments for cloning into luciferase reporter plasmid, pGL3-basic (Promega). The cloned PCR products were sequenced to confirm the authenticity of each promoter fragment. The cDNA encoding N-terminal deletion mutant of IκBα (ΔN-IκBα) was obtained by PCR amplification and inserted into pRC/CMV vector (invitrogen) using primer born restriction sites (HindIII/XbaI). Murine p50 and p65 were PCR amplified from PMA stimulated murine lymphocytes and cloned in pcDNA3.1 vector (invitrogen) using primer born restriction sites (BamHI/XhoI for p50 and HindIII/XbaI for p65 cloning). A 3xκB-Luc plasmid (containing minimal murine *fos* promoter element and three copies of major histocompatibility complex (MHC) class I κB element TGGGGATTCCCCA) capable of inducing luciferase activity in response to NFκB activation (143), was kindly gifted by Dr. Ute Reuning (Klinikum rech der Isar, Technische Universität München).

5.11 Transfection assays and promoter modulation studies

All promoter transient transfection assays were performed in transfection competent murine endothelial cell line bEND.3. For transient transfection of promoter constructs bEND.3 cells were seeded at 5 x 10^5 cells per well in 6-well plates (TPP) and grown for 48 hours to get ~ 90 % confluence in 2 ml culture medium. Before transfection culture medium was replaced with 1.5 ml serum free medium. Transfections were performed with Lipofectamine 2000 reagent (invitrogen) according to manufacturer's instruction. In all transient transfection experiments renilla luciferase expressing pRL-SV40 and enhanced GFP (venus) expressing pCS2-venus (144) plasmids were co-transfected as internal control and to measure transfection efficiency respectively. For mouse Cldn5 promoter modulation studies transfection medium was removed after 24 hours and incubation with modulator in serum free medium was carried out for 12-24 hours. Cells were then rinsed twice with PBS

and lysed with passive lysis buffer (promega) according to manufacturer's instructions. Total protein content in cell lysates was measured by micro BCA protein estimation kit (Thermo scientific). Renilla and firefly Luciferase activity was measured in cell lysates using microplate reader (Tecan). For all promoter assays data is shown as mean and standard deviation of three independent experiments each performed in triplicates. For comparison where ever needed an unpaired T-test was used. In all cases, $P < 0.05$ was considered significant.

5.12 Electrophoretic Mobility Shift Assay (EMSA)

A nonradiolabeled, biotin-based EMSA was used to study the binding specificity of over expressed p65 to putative κB sites in mouse Cldn5 promoter. All DNA oligos were biotin labeled at the 5′ end (Eurofins MWG operon). Three oligos derived from mouse Cldn5 promoter (C1, C2 and C3) and corresponding mutant oligos (M1, M2 and M3) are were used in this study. Complementary oligos were annealed by using 1× oligo annealing buffer [10 mM Tris (pH 7.5), 50 mM NaCl, and 1 mM EDTA]. Binding reaction was performed at room temperature for 30 min in 10 µl of total reaction volume containing 1× binding buffer (Promega), 1 µg of Poly (dI-dC) (Roche Applied Science), 5 µg murine p65 over expressing bEND.3 nuclear lysate (or control lysate), and 20 ng of annealed biotin-labeled probes. For competition unlabelled oligoneucleotide (C) with standard NFκB binding consensus motif (146) was used in 20 fold excess in binding reaction. The binding reaction mixture was allowed to run in a 6% DNA retardation gel (Invitrogen) for 45 min at 100 V and was transferred onto a nylon membrane for 90 min at 30 V. After crosslinking blot was incubated with blocking buffer (125 µM NaCl, 20 µM Na_2HPO_4, 10 µM NaH_2PO_4 and 175 µM SDS in dH_2O) for 30 minutes. After blocking the blot was incubated with HRP-conjugated streptavidin solution in blocking buffer for 30 minutes. The protein-bound and free probes were detected with Novex ECL chemiluminescent substrate reagent kit (invitrogen).

5.13 Statistical Analysis

Threshold for denatured OSP reactivity in ELISA assays was calculated as mean delta OD shown by control sera or CSF plus three standard deviations of delta OD shown by control

group. Threshold for nOSP reactivity in ELISA assays was calculated as mean delta OD shown by control sera or CSF plus three standard deviations of delta OD shown by control group. Threshold for nOSP or nMOG reactivity in cell-based flow cytometry assays were calculated as mean delta MFI shown by control sera plus three standard deviations of delta MFI shown by control group. The Mann-Whitney U test was used in all serum and CSF screening experiments to compare anti-nOSP and nMOG antibody titers between patients and controls. For correlation analysis between anti-nMOG titer and age of onset the spearman rank correlation was applied. Cox proportional-hazards analysis, adjusted for potential confounding variables (age, sex, type of onset, CSF positivity for oligoclonal bands and steroid treatment at first inflammatoty CNS event) was used to test association between anti-nMOG antibody titers and development of clinically definite multiple sclerosis in adult CIS patients. For all comparisons p-values < 0.05 were considered significant. All analyses were performed by GraphPad Prism 5.00.

Table I Characteristics of patients and controls

Serum and CSF screening for anti-OSP/Cldn11 antibody reactivity		
Adult MS	**Pediatric MS**	**Control**
(n = 40, Mean age = 41)	(n = 10, Mean age = 12)	(n = 28, Mean age = 34)
RRMS (n = 10, Mean age = 40)	RRMS (n = 3, Mean age = 13)	OIND (n = 10, Mean age = 40)
PPMS (n = 10, Mean age = 44)	ADEM (n = 3, Mean age = 11)	NIND (n = 08, Mean age = 40)
SPMS (n = 10, Mean age = 46)	ON (n = 4, Mean age = 12)	HC (n = 10, Mean age = 34)
CIS (n = 10, Mean age = 34)		
Serum screening for anti-nMOG antibody reactivity		
Adult MS	**Pediatric MS**	**Control**
RRMS (n = 33, Mean age = 40)	ADEM (n = 19, Mean age = 5.2)	OND (n = 28, Mean age = 06)
PPMS (n = 19, Mean age = 44)	CIS (n = 28, Mean age = 9.5)	HC (n = 30, Mean age = 11)
SPMS (n = 01, Mean age = 46)		
CIS (n = 410, Mean age = 34)		

Chapter 6. APPENDIX

6.1 Materials

6.1.1 PCR Primers and EMSA oligos

All primers and EMSA oligos were ordered from Eurofins MWG operon. Lyophilized primers were diluted in double-destilled water and stored as a 100 mM stock solution at -20°C.

PCR primers used for OSP/Cldn11 and MOG cloning (Restriction sites in block):

Primer	Description / Position	Sequence (5′→ 3′)
11-F	Forward NM_005602	ACTAGTcatggtggccacgtgcctg
11-R	Reverse NM_005602	CCGCGGgccctcttatacgtgggcactc
MOG-F	Forward NM_206809	attgAGATCTgagatggcaag
MOG-R	Reverse NM_206609	gAGATCTcagaagggatttcg

PCR primers used for mouse Cldn5 cDNA cloning:

Primer	Description / Position	Sequence (5′→ 3′)
5-F	Forward NM_013805	gcattcagtctttagccatgg
5-R	Reverse NM_013805 c	gcccttagacatagttcttc

PCR primers used for mouse Cldn5 promoter sequential deletion fragments (Restriction sites in block). Six forward primers (F1-F6) were used with common reverse primer (R). Position with respect to mouse claudin-5 transcription start site is given. Annealing position at mouse chromosome 16 is also given.

Primer	Mouse Chr.16 position	Sequence (5′→ 3′)
F1	18775940-18775959	CTCGAGgtgttcactggaaagaggc
F2	18776041-18776060	CTCGAGcctttcaagaaatggctgg
F3	18776190-18776211	CTCGAGccacaactatttgtaggatgg
F4	18776340-18776357	CTCGAGccagaggagagcacagg
F5	18776500-18776517	CTCGAGgcttggtgtgccaaggg

F6	(-300)	18776650-18776668	CTCGAGtcatgctgctaacagtgg
R	(+144)	18777076-18777094	AAGCTTaagactgaatgctcaccc

PCR primers used for mouse ΔN-IκBα, p50 and p65 (Restriction sites are shown in block).

Primer	Description / Position	Sequence (5′→ 3′)
ΔN-IκBα-F	Forward NM_010907	AAGCTTccatgaaggacgaggagtacg
ΔN-IκBα-R	Reverse NM_010907	TCTAGActtataatgtcagacgctggcc
p50-F	Forward NM_008689	GGATCCatggcagacgatgatccctacgg
p50-R	Reverse NM_008689	CTCGAGctaatgggtgacccctgcgttgg
p65-F	Forward NM_009045	AAGCTTcttataatgtcagacgctggcc
p65-R	Reverse NM_009045	TCTAGActgtcagcaccttaggagc

Oligonucleotides used in EMSA: In mouse Cldn5 promoter derived oligos (C1-C3) predicted κB motifs are shown in block. For each site the position with respect to mouse chromosome 16 is also given. In mutant oligos (M1-M3) the specific mutation introduced is underlined. In NFκB consensus oligo (C) κB motif is shown in block.

Primer	Description / Position	Sequence (5′→ 3′)
C1	18776075-18776097	aatgccCGGAAATCCCgcggct
C2	18776110-18776133	aggatgGGGGCTCTTCCtcctgg
C3	18776130-18776153	tggccaGGAAACTCCAAgttggc
M1	18776075-18776097	aatgccCGGAAAGCACgcggct
M2	18776110-18776133	aggatgGGGGCTATGCCtcctgg
M3	18776130-18776153	tggccaGGAAACACGAAgttggc
C	NFκB consensus oligo	agttgaggGGACTTTCCCaggc

6.1.2 Cell culture and media

To develop cell-based assays for the quantification of anti-nOSP and anti-nMOG antibody responses in MS serum and CSF (study I), 293FT and LN18 cells were used. Primary microvascular endothelial cells (MVEC) isolated from C57/BL6 mice and immortalized murine endothelioma cell, bEND.3 were used in study II. The cells were grown in

humidified incubator with 5 % CO_2 at 37 ^{o}C. Culture media and supplements are given in Table II.

Cell	Basal medium	Supplements	Freezing medium
293FT	DMEM (E15-011, PAA)	10% fetal bovine serum 0.1 mM MEM Non-Essential Amino Acids 6 mM L-glutamine 1 mM MEM Sodium Pyruvate 500 µg/ml Geneticin	90% complete medium 10% DMSO
LN18	RPMI 1640 (E15-840, PAA)	10% fetal bovine serum 100 µg/ml Pen-Strep 50 µg/ml Blasticidin (after transduction)	90% complete medium 10% DMSO
MVEC	Medium131 (M-131-500, Gibco)	30 µg/ml ECGS 10% fetal bovine serum 15 U/ml heparin 325 µg/ml glutathione, 1 µl/ml 2-Mecaptoethanol 100 µg/ml Pen-Strep	--
bEND.3	DMEM (E15-011, PAA)	1.5 g/L sodium bicarbonate 4.5 g/L glucose 10 % fetal bovine serum 100 µg/ml Pen-Strep	95% complete medium 05% DMSO

Table II Cell lines and culture conditions

6.1.3 Transfection reagents, cloning kits and vectors

Following transfection reagents and cloning kits and vectors were used:

Lipofectamine 2000 reagents	Invitrogen
ViraPower lentiviral packaging mix	Invitrogen
pLenti6/V5-DEST vector	Invitrogen
pGEM-T Easy vector system	Promega
pcDNA3.1 plasmid	Invitrogen
pRC/CMV plasmid	Invitrogen
pGL3-basic plasmid	Promega
pRL-SV40 plasmid	Promega
pCS2-Venus plasmid ,	(See ref. 144)

6.1.4 Cytokines, reagents and chemicals

Following cytokines, reagents and chemicals were used:

2-mecaptoethanol	Sigma
Blasticidin	Invitrogen
Cell dissociation buffer	Invitrogen
Chemiluminescent ECL detection kit	Invitrogen
Collagenase / Dispase	Roche
DAPI	Invitrogen
Dexamethasone	Sigma
Dextran	Sigma
EMSA binding buffer	Promega
Endothelial growth supplement (ECGS)	Sigma
Ethylenediaminetetraacetate (EDTA)	Sigma
Fetal bovine serum	Invitrogen
Geneticin	Gibco
Glutathione	Gibco

Heparin	Gibco
HRP-conjugated streptavidin	Thermo scientific
MEM Non-Essential Amino Acids	Gibco
MEM Sodium Pyruvate	Gibco
Micro BCA protein estimation kit	Thermo scientific
Na_2HPO_4	Sigma
NaCl	Sigma
NaH_2PO_4	Sigma
Passive lysis buffer	Promega
Penicillin	Gibco
Percoll	Sigma
Phosphate buffer saline	PAA
Poly (dI-dC)	Roche
Pyrrolidine dithiocarbamate	Sigma
Recombinant mouse IFNγ	Invitrogen
Recombinant mouse IL-1β	Invitrogen
Recombinant mouse TNFα	Invitrogen
SDS	Sigma
Streptomycin	Gibco
Sulfo-NHS-SS-Biotin solution	Thermo scientific
Tris	Sigma
Tween 20	Sigma

6.1.5 Equipment

Following equipment was used:

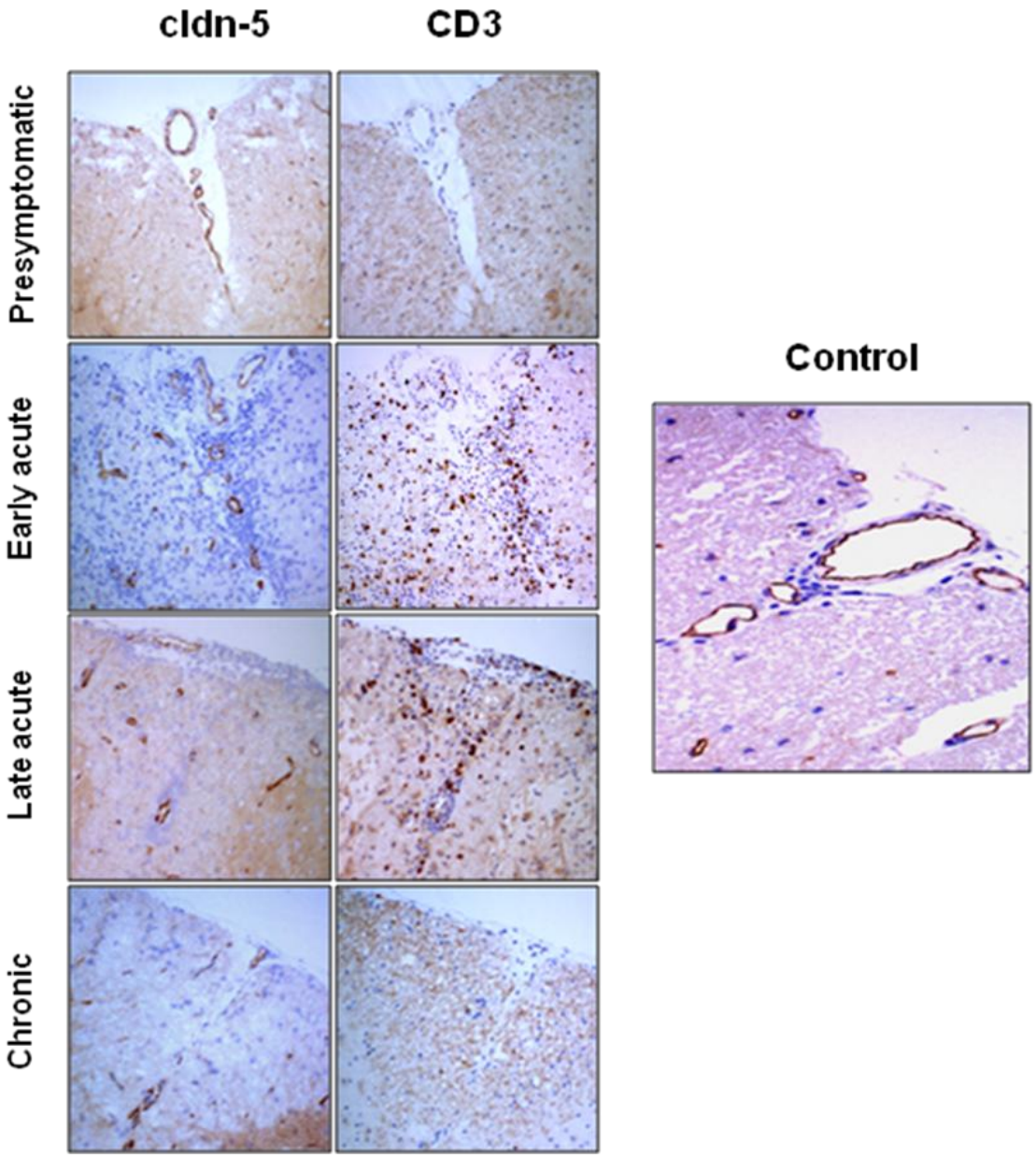
Agarose gel electrophoresis chambers	Biorad
Bacterial culture shaker	ThermoQuest
AutoMACS	Miltenyi Biotec

ELISA plate reader	Tecan
FACS cell analyzer	Beckman Coulter
Humidified cell culture incubator	Binder
iBlot™ Gel Transfer Device	Invitrogen
Microplate reader	Tecan
Olympus IX71 microscope system	Olympus
StepOne™ Real-Time PCR System	Applied biosystems
Thermocycler	Applied biosystems
UV cross-linker	Biotec-Fischer
Xcell SureLock Mini-Cell chamber	Invitrogen
NanoDrop 1000	Thermo scientific
Power supply Power Pac 300	Biorad
CAWOMAT 2000 IR	Physia

6.1.6 Softwares and analysis programs

Following softwares and programs were used:

AxioVision software	Zeiss
ECR browser	http://ecrbrowser.dcode.org
GraphPad Prism 5.0	GraphPad
MatInspector	Genomatix
PromoterInspector	Genomatix
rVISTA	http://rvista.dcode.org
StepOne	Applied biosystem
Summit software	Beckman Coulter



Appendix figure. Cldn5 staining pattern in inflammatory spinal cord lesions during EAE

Representative images of Cldn5 ands cd3 stainings performed on the spinal cord sections from from control (n = 3) and EAE (n= 3) mice (at presymptomatic, early acute, late acute and chronic stages of EAE) are shown (*a*) Patchy or discontinuous Cldn5 staining at spinal cord sections from EAE mice as compared to smooth staining in control spinal cord section. Leukocyte infiltration (cd3 staining) was observed in spinal cord sections from mice exhibitin acute EAE. For each mouse atleast 3 spinal cord sections were stained for Cldn5 and leukocyte infiltration marker (cd3).

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6.4 Curriculum Vitae

Personal details

Name	Muhammad Aslam
Date of birth	15.01.1979
Nationality	Pakistani
Marital status	Single

Education

WS 2007-present	PhD program in medical life science and technology, faculty of Medicine, Technische Universität Munich Project: Claudins in inflammatory CNS disease Advisor: Prof. Dr. Bernhard Hemmer
2002-2004	MPhil (Biochemistry/Molecular biology), Biological science department, Quaid-i-Azam university, Islamabad Project: Linkage and candidate gene studies on kindreds with autosomal recessive monogenic disorders
2000-2002	MSc (Biochemistry/Molecular biology), Biological science department, Quaid-i-Azam university, Islamabad

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