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Studies on the CNS Histopathology of EAE and Its Correlation with Clinical and Immunological Parameters

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1. Introduction

Multiple sclerosis (MS) is one of the most difficult to diagnose neurological diseases because its clinical manifestations are highly variable and the disease course also shows unpredictable individual patterns. We are far from understanding the complexities that underlie this variability, but certain patterns clearly emerge. First, it has become clear that different genetic backgrounds will lead to different manifestations of an autoimmune T cell attack on the central nervous system (CNS) (Hoppenbrouwers and Hintzen, 2010). It is also clear that differences in the CNS antigen-specificity of the T cell response can result in a differential involvement of anatomical regions of the CNS (Berger et al., 1997; Kuerten et al., 2007). Differences in lesion localization are a typical feature of MS, termed dissemination in space, and are likely to cause heterogeneity in clinical symptoms. There is evidence for the prevalence of either T cell-/macrophage- or antibody-/complement-mediated CNS demyelination versus a primary oligodendrogliaopathy in MS patients (Lucchinetti et al., 2000). While the patterns of demyelination remain the same in individual patients over time, heterogeneity is evident when comparing different patients (Lucchinetti et al., 2000). In addition to these rather defined parameters of CNS histopathology (termed “pattern I-IV” by Lucchinetti et al., 2000) there are dynamic elements of the inflammatory cascade that can result in interindividual variations of disease progression. Among these are the extent of antigen determinant spreading (Lehmann et al., 1992; McRae et al., 1995), the prevalence of antigens in different CNS regions to which the spreading occurs (Targoni et al., 2001) as well as the rate at which regulatory or compensatory reactions of the immune system surface to counterregulate the damage of the target organ (Kasper et al., 2007).

Due to the impossibility of obtaining CNS tissue samples from individual patients repeatedly over time, studies as to the pathogenesis of the human disease need to rely on suitable animal models. To study pathologic features of MS three main animal models are used: disease induction by toxic agents, viral models, and finally different types of experimental autoimmune encephalomyelitis (EAE). Toxic agents like the copper chelator

cuprizone cause demyelination in the relative absence of inflammation or axonal damage. Lesions induced in the cuprizone model typically resemble primary oligodendrocyte dystrophy in MS patients, while lacking the characteristic T cell infiltrate. The cuprizone model has no autoimmune component. Still, it is well-suited to investigate principle features of de- and remyelination in the CNS (Kipp et al., 2009). Intracerebral inoculation of Theiler's murine encephalomyelitis virus (TMEV) is used to investigate how viral infections can induce CNS autoimmunity. After an early, subtle disease period, susceptible mouse strains develop brain and spinal cord inflammation, demyelination and axonal damage. The clinical course resembles that of chronic, progressive MS (Tsunoda et al., 2010). However, EAE remains the most intensively studied animal model of MS.

2. Experimental Autoimmune Encephalitis (EAE)

EAE was introduced by Thomas Rivers and his colleagues in the early 1930s (van Epps, 2005). Since then, it has been subject to elaborate studies (reviewed in Goverman and Brabb, 1996; Steinman, 1999; Hemmer et al., 2002). Animals studied initially included guinea pigs and rats, in particular the Lewis rat, but later also involved marmoset monkeys and mice – the latter being the dominant model organisms used nowadays (Gold et al., 2006). EAE can either be induced by active immunization with CNS antigens in adjuvant (active EAE) or by the passive transfer of encephalitogenic T cells (adoptive/passive EAE). In addition, spontaneous EAE models relying on transgenic animals exist (Fig. 1).

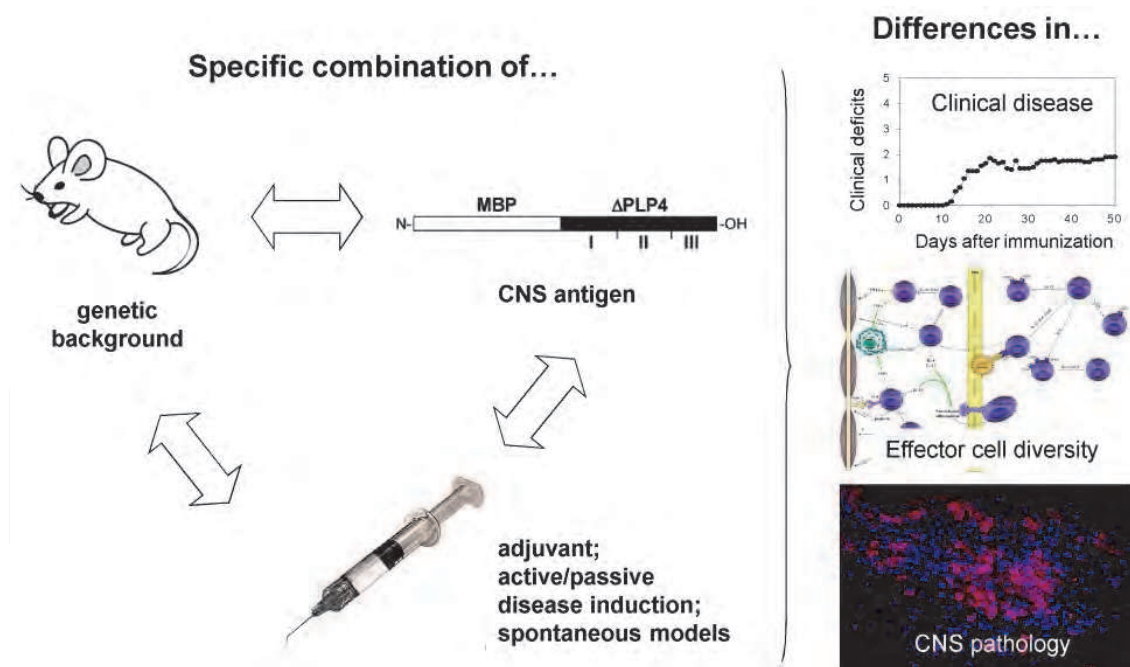


Fig. 1. The interplay between genetic background, disease triggering antigen and the mode of disease induction results in differences in EAE outcome.

Originally, whole spinal cord homogenate (SCH) (Einstein et al., 1962; Bernard & Carnegie, 1975; van Epps, 2005) was used for disease induction, before specific target antigens were defined. Early efforts to characterize the encephalitogenic antigen in SCH identified myelin basic protein (MBP) (Einstein et al. 1962; Martenson et al., 1970; Hashim et al., 1975)

comprising approximately 30 – 40% of the proteins in the myelin sheath. H-2^u mice, in particular the B10.PL and PL/J strains are highly susceptible to MBP- or MBP peptide-induced EAE (Fritz et al., 1983 and 1985), while most common mouse strains, including C57BL/6 (B6) mice are resistant to MBP-induced disease (Bernard, 1976; Fritz and Zhao, 1996; Gasser et al., 1990; Skundric et al., 1994). The in-depth characterization of the B10.PL and PL/J model revealed highly restricted T cell responses to MBP involving a single immune dominant determinant and a limited usage of T cell receptor (TCR) chains (Zamvil et al., 1988; Urban et al., 1988; Kumar and Sercarz, 1994; Radu et al., 2000). Since similar findings were made in the Lewis rat (Burns et al., 1989), hopes emerged that such features could also apply to MS, providing therapeutic possibilities. These perspectives faded as diverse T cell repertoires were found in the proteolipid protein (PLP):139-151-induced EAE of SJL mice (Kuchroo et al., 1992) and after realizing that autoimmune T cell repertoires undergo determinant spreading (Lehmann et al., 1992; McRae et al., 1995; Jansson et al., 1995; Yu et al., 1996; Tuohy et al., 1999). Recent studies of antigen-specific autoantibodies in EAE have also provided for the diversification of the autoimmune response (Stefflerl et al., 2000; Cross et al., 2001). PLP constitutes approximately 50% of the myelin proteins. As with MBP-induced EAE, only few strains were found to be susceptible to PLP-induced EAE. C57BL/6 mice were reported to be resistant (Tuohy, 1993; Mendel et al., 1995; Fritz and Zhao, 1996; Klein et al., 2000). The search for additional encephalitogenic antigens identified myelin oligodendrocyte glycoprotein (MOG) (Lebar et al., 1986; Mendel et al., 1995 and 1996; Schmidt, 1999), myelin associated glycoprotein (MAG) (Schmidt, 1999; Morris-Downes et al., 2002; Weerth et al., 1999), myelin oligodendrocyte basic protein (MOBP) (Schmidt, 1999; Holz et al., 2000; de Rosbo et al., 2004), oligodendrocyte-specific glycoprotein (OSP) (Morris-Downes, 2002), 2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase) (Schmidt, 1999; Morris-Downes et al., 2002), β -synuclein (Mor et al., 2003) as well as S100 β , which is not only expressed on astrocytes, but also in many other tissues including the eye, thymus, spleen and lymph nodes (Kojima et al., 1997; Schmidt, 1999).

Each combination of antigen with the respective susceptible strain and also considering the mode/protocol of disease induction results in a characteristic form of EAE (Goverman & Brabb, 1996; Steinman, 1999; Schmidt, 1999) (Fig. 1). The different EAE models show fundamental differences, however. For example, the MBP-induced disease in B10.PL and PL/J mice is monophasic: the mice completely recover after a single episode of short acute disease and become resistant to re-induction of EAE (Waxman et al., 1980). PLP peptide 139-151-induced EAE in SJL mice is remitting-relapsing (Hofstetter et al., 2002), while the disease elicited by MOG:35-55 in C57BL/6 mice is chronic (Eugster et al., 1999). In addition, the different EAE models involve differences in CNS histopathology and the role of antigen-specific antibodies, which will be described in detail below.

There have been extensive discussions regarding which antigen/strain combination provides the “best” EAE model for MS. The prevalent view is that none of them individually, but all of them jointly are best (Schmidt, 1999; van Epps, 2005; Hafler et al., 2005). MS does not seem to be a single disease entity, but rather involves a profound heterogeneity. As Vijay Kuchroo (Harvard University) once pointed out “each EAE model recapitulates a small piece of the human disease”, thus facilitating the analysis of each single step disrupting immune competence leading finally to a severe autoimmune disease (van Epps, 2005; Steinman and Zamvil, 2006). EAE is an appropriate model for studies of basic mechanisms that underlie autoimmune pathology because, unlike in spontaneous

autoimmune diseases, the autoantigen, the time point and the site of the ensuing autoimmune response is known, and the type of cytokine differentiation of the induced T cells can be directed (Forsthuber et al., 1996; Yip et al., 1999). Being able to control the above parameters as well as the ability to monitor the autoantigen-specific T cells in the course of the disease renders EAE suitable for studies aiming at defining the mechanisms of therapeutic interventions. Genetically-manipulated mice have been and will continue to gain increasing importance for such studies.

Traditionally, mechanistic studies have relied on the use of antibodies and on complex manipulations of mice. However, such treatments that can be applied to essentially any EAE model, do not necessarily permit unambiguous conclusions. For example, when a cell surface marker-specific antibody is injected to study the role of that molecule in EAE, the antibody might have a clinical effect on the disease, but it could be due to a multitude of mechanisms. The antibody could deplete the marker positive cells via the activation of complement, antibody-dependent cell-mediated cytotoxicity (ADCC) or apoptosis (Cebeaucuer et al., 2005), which in turn may be associated with a varying degree of inflammation causing unaccounted effects. Alternatively (or in addition) antibodies can inactivate or activate the marker positive cells, with variable bystander cell involvement. When antibodies are injected to study the role of a cytokine, the clinical effect seen can result from the neutralization of the cytokine, or on the contrary, from the prolongation of the half-life of that cytokine. For such reasons, the use of antibodies for mechanistic studies has frequently resulted in contradictory, inconclusive data (Dittmer and Hasenkrug, 1999; Silvera et al., 2001). The use of genetically-targeted mice, along with adoptive transfers of cells that express/do not express molecules of interest is increasingly becoming indispensable for mechanism-oriented studies, and the more this “tool box” expands the more powerful it will become. Most gene knock-out/knock-in mice have been generated on the 129 (*H-2^b*) background and backcrossed to *H-2* congenic C57BL/6 mice. Instead of having to move each new member of this ever expanding “toolbox” to the background of each EAE susceptible strain, it is much more effective to be able to study EAE in C57BL/6 mice. This is why MOG:35-55-induced EAE in C57BL/6 mice is increasingly becoming essential for mechanism-oriented studies – and why at the same time it is problematic to rely on this single EAE model for MS. To this end, we set out to establish and characterize additional EAE models for C57BL/6 mice. PLP protein-induced EAE has not been extensively studied; unlike the hydrophilic MBP molecule, PLP is highly hydrophobic and thus as a protein very difficult to utilize (Tuohy, 1993). PLP as an antigen for EAE induction established itself only after the encephalitogenic PLP peptide 139-151 had been identified for SJL mice (Kuchroo et al., 1992; Lehmann et al., 1992). Only recently, PLP peptide 178-191-elicited disease in C57BL/6 mice has been introduced (Tompkins et al., 2002), but this model still awaits thorough characterization. Encompassing most potential determinants of the two major myelin antigens, MBP and PLP, the MP4 fusion protein was generated as a drug candidate for MS (Elliott et al., 1996). MP4 contains the three hydrophilic loops of PLP (domains I, II and III; Fig. 2A), while the four hydrophobic transmembrane sequences have been excised. These hydrophilic domains constitute Δ PLP4 that has been linked to the 21.5 kD isoform of human MBP (Fig. 2B).

In SJL mice it has been shown that, when given under tolerogenic conditions, MP4 can prevent and revert EAE induced by MBP- and PLP-specific T cells (Elliot et al., 1996). It has also been shown that MP4 can induce EAE in SJL/J mice, and in another report (Jordan et

al., 1999) MP4 was found to be encephalitogenic in marmoset monkeys. Our studies later demonstrated that MP4 was also capable of inducing EAE in C57BL/6 mice, thus introducing a much needed alternative to the MOG:35-55 and PLP:178-191 peptide model (Kuerten et al., 2006). Overall, there are few systematic studies as to whether different EAE models can reproduce distinct features of MS histopathology. One typical problem is that – as mentioned above – the induction of EAE requires the specific combination of genetic strain and CNS antigen. Yet, it is difficult to compare results obtained in different models since it is unclear, which outcome can be ascribed to the antigen and which one depends on the genetic background (Kuerten et al., 2009). It is therefore crucial to modify only one variable at a time, that is either the antigen or the genetic background. With the introduction of the MP4 model on the C57BL/6 background, the spectrum of models on this background covered all main antigens known from MS pathology: MOG, MBP and PLP. In addition, this background offers the possibility of performing genetic modifications, facilitating mechanistic studies.

In the following the characteristic histopathological features of MOG:35-55-, MP4- and PLP:178-191-induced EAE on the C57BL/6 background will be discussed and critically evaluated in the context of MS pathology considering the three hallmarks of MS pathology inflammation, demyelination and axonal damage.

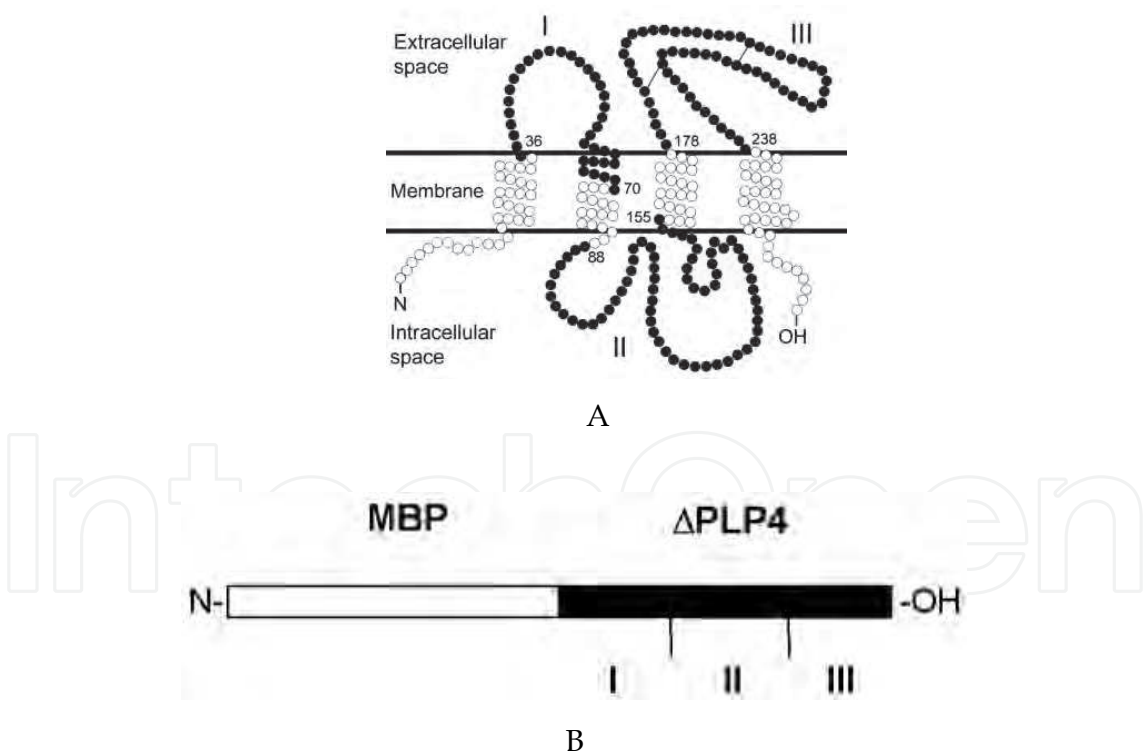


Fig. 2. The molecular structure of the MBP-PLP fusion protein MP4. (A) Structure of PLP. PLP is a transmembrane protein that consists of two extracellular (I and III) and an intracellular (II) hydrophilic domain, and four hydrophobic transmembrane sequences. (B) Structure of MP4. The three hydrophilic PLP domains have been fused to create Δ PLP4, which has been linked to the 21.5 kD isoform of human MBP.

3. Studies on the CNS histopathology of EAE and its correlation with clinical and immunological parameters

3.1 The CNS lesion topography and composition depends on the antigen used for immunization in models of C57BL/6 EAE

Inflammation is a feature of MS pathology that can essentially be reproduced in any EAE model. In principle, pathology is initiated when autoreactive T cells enter the CNS. Before, these cells need to be primed in the secondary lymphoid organs. In MOG:35-55- and PLP:178-191-induced EAE the antigen used for immunization is a single peptide and the autoreactive T cell response is directed against this peptide, while determinant spreading does not occur, which could include further determinants into the autoimmune response. The MP4 model, in contrast, is characterized by a multideterminant-specific CD4⁺ T cell response and we have shown that there is no single dominant determinant being recognized in mice immunized with MP4 (Kuerten et al., 2006). Rather, the response seems to randomly target different determinants of the MP4 protein with interindividual variation in individual mice. The advantage of multideterminant specificity in the MP4 model resides in the fact that it may be used to better mirror the heterogeneity of the T cell response present in MS patients. It has been shown that there is not a single determinant targeted by the autoimmune response in MS. Differences do not only exist between individual patients, but also develop as disease progresses, since new determinants can be engaged into the immune response through determinant spreading (Tuohy et al., 1998). In patients this is a random process, which is highly unpredictable and can at least in part account for the kinetics of disease progression.

We assume that differences in the peripheral antigen-specific response have major implications on the subsequent CNS histopathology. In all models that we analyzed, infiltration of the cerebrum with focus on the meninges close to the hippocampal region occurred already in acute disease. In addition, in MP4- and PLP peptide-induced EAE inflammation of the spinal cord meninges was evident, while in the MOG peptide model inflammation extended into the parenchyma. Cerebellar infiltration was absent in the former two models, but pronounced in the latter in the acute stage of the disease. In chronic EAE (50 days after immunization), lesion distribution shifted towards the spinal cord and cerebellar parenchyma in the MP4 model, while it decreased remarkably in the cerebrum. In MOG peptide- and PLP peptide-induced EAE the lesion topography was comparable to the acute stage. Overall, CNS inflammation was time-dependent and dynamic only in the MP4 model, shifting from the cerebrum to the spinal cord and finally involving the cerebellum, thus allows the staging of the disease (Kuerten et al., 2007). MS is characterized by lesion dissemination in time and space, for which the MP4 EAE could serve as a valuable model. In contrast, the PLP and MOG model showed rather static inflammatory patterns that remained unchanged throughout the disease.

Next to differences in lesion topography we found differences in the cellular composition of CNS lesions. In particular, these differences pertained to the numbers of CNS infiltrating B cells.

3.2 The development of tertiary lymphoid organs (TLOs) in MP4-induced EAE of C57BL/6 mice

Studying the MP4, MOG peptide and PLP peptide model systematically early and late after immunization, we found that B cell infiltration was a common feature of the MP4 model,

while in MOG peptide- and PLP peptide-induced disease B cells were scarce within the infiltrates (Kuerten et al., 2008). Moreover, it was striking that in MP4-induced disease B cells showed clustering, while in the other models they were scattered throughout the lesions (Fig. 3).

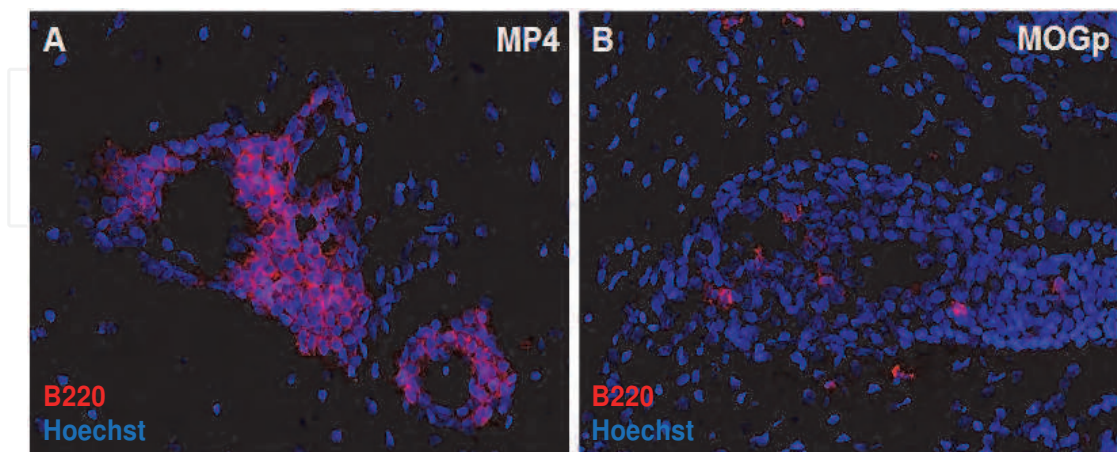


Fig. 3. Presence of CNS B cell aggregates in the MP4 model. C57BL/6 mice were immunized with 150 μ g MP4 or 100 μ g MOG peptide 35-55 (MOGp) in complete Freund's adjuvant (CFA). Pertussis toxin was given at 200 ng per mouse on the day of immunization and 48 h later. 35 days after disease onset mice were sacrificed, the CNS was removed and snap-frozen in liquid nitrogen. Seven μ m thick cryostat sections were obtained from cerebrum, cerebellum and spinal cord and stained for the presence of B cells using B220 antibody. Representative cerebellar parenchymal infiltrate from a (A) MP4-immunized and a (B) MOG peptide 35-55-immunized mouse. Images are at 400x magnification and representative for 58 mice tested in at least eight independent experiments (Kuerten et al., 2008).

The presence of B cell aggregates could be indicative of the formation of ectopic foci of lymphoid tissue – termed tertiary lymphoid organs (TLOs) – in the MP4 model. Lymphoid organization of inflamed tissue can occur in the setting of chronic inflammation and is mainly directed by the expression of lymphotoxin ($LT\alpha_1\beta_2$) by activated B and T cells that interacts with the lymphotoxin- β receptor on stromal organizer cells (Aloisi & Pujol-Borrell, 2006). The structure of TLOs has been reported to be variable (Drayton et al., 2006) and there has been controversy as to the exact definition of a TLO. Overall, TLOs are thought to resemble lymph nodes (Aloisi & Pujol-Borrell, 2006; Deteix et al., 2010). Major features of TLOs include T cell/B cell compartmentalization, the presence of high endothelial venules (HEVs) that allow naive B and T cells to enter the tissue as well as the expression of lymphoid chemokines such as CXCL13, CCL12, CCL19 and CCL21. In addition, follicular dendritic cell (FDC) networks have been associated with TLO formation and occasionally germinal centers have been found. The presence of germinal centers in TLOs points to the fact that these structures are not solely epiphenomena that emerge in chronic inflammation, but that these organs can be functional and may influence disease progression in the setting of autoimmunity, for example by the production of high-affinity autoantibodies and by the facilitation of determinant spreading (Stott et al., 1998; Armengol et al., 2001; Sims et al., 2001). Somatic hypermutation, affinity maturation, immunoglobulin class switching and B cell receptor revision are all processes that take place in secondary lymphoid organs (SLOs) and there is increasing evidence that they can also occur in TLOs, probably contributing to

the exacerbation of the chronic inflammatory state and also to the detachment from the immune response generated in SLOs. The formation of TLOs has been found under a variety of pathogenic circumstances including autoimmune diseases such as Sjögren's syndrome (Aziz et al., 1997; Salomonsson et al., 2003; Barone et al., 2005), autoimmune thyroiditis (Armengol et al., 2001) and arthritis (Takemura et al., 2001; Shi et al., 2001), infectious diseases (Murakami et al., 1999; Mazzucchelli et al., 1999), tumors (Coronella et al., 2002; Nzula et al., 2003) and transplantation (Baddoura et al., 2005). In MS B cell aggregates have been identified in the meninges of patients with secondary-progressive MS (SP-MS). Owing to the expression of CXCL13, the presence of FDCs, proliferation (indicative of germinal center formation) and plasma cells these aggregates have been described to be ectopic B cell follicles (Serafini et al., 2004; Magliozzi et al., 2007). The presence of ectopic B cell follicles has been linked to a younger age at disease onset, irreversible disability and death in addition to more pronounced demyelination, microglia activation and loss of neurites in the cerebral cortex (Magliozzi et al., 2007). In a follow-up study, Serafini et al. provided evidence for an association between B cell follicle formation in the CNS and latent Epstein-Barr virus (EBV) infection, which they suggested to contribute to B cell dysregulation (Serafini et al., 2010). These data have important implications for the disease pathogenesis since they propose a histopathological correlate for sustained disease and its chronification as well as they strongly support the viral hypothesis of MS. However, it should be noted that other researchers failed not only to detect meningeal B cell follicles and an association between meningeal inflammation and cortical demyelination, but also the presence of EBV-infected cells in the CNS of MS patients (Kooi et al., 2009; Willis et al., 2009; Perferoen et al., 2010). The debate about the actual presence and relevance of B cell follicles/ectopic lymphoid tissue in MS has not yet been resolved.

Since research involving tissue from MS patients is restricted and problems emerge when it comes to defining the exact onset and further course of the disease, studies in EAE could help clarify the controversy about B cell follicles in the disease process. In EAE, disease onset and progression are clearly defined, which facilitates the correlation of ectopic lymphoid tissue development and clinical outcome. Yet, most traditional EAE models are independent of B cells. Among these models are (as mentioned above) the traditional MOG peptide 35-55- and PLP peptide 178-191-induced EAE in C57BL/6 mice and the PLP peptide 139-151-elicited disease in the SJL/J strain. Only the human MOG (Oliver et al., 2003) and a transgenic SJL model (Pöllinger et al., 2009) have been shown to comprise a pathogenic B cell response, but have so far not proven to be helpful when it came to studying lymphoid tissue formation in the CNS. Ongoing studies in our laboratory are currently dealing with a thorough analysis of the B cell aggregates found in the CNS of MP4-immunized mice. We have obtained first evidence that these aggregates indeed take over characteristics of TLOs including B cell and T cell compartmentalization (Fig. 4).

In addition to B cell/T cell compartmentalization, CNS B cell aggregates in MP4-induced EAE showed further characteristic features of TLO formation including the presence of HEVs, FDCs and chemokine expression (Fig. 5).

Having established that MP4 immunization induces TLO formation in C57BL/6 mice, specific questions addressed in future studies will be concerned with the topography and kinetics of TLO formation in MP4-induced EAE, the correlation with the clinical disease outcome and cortical pathology. In addition, we aim at elucidating whether TLOs in MP4-

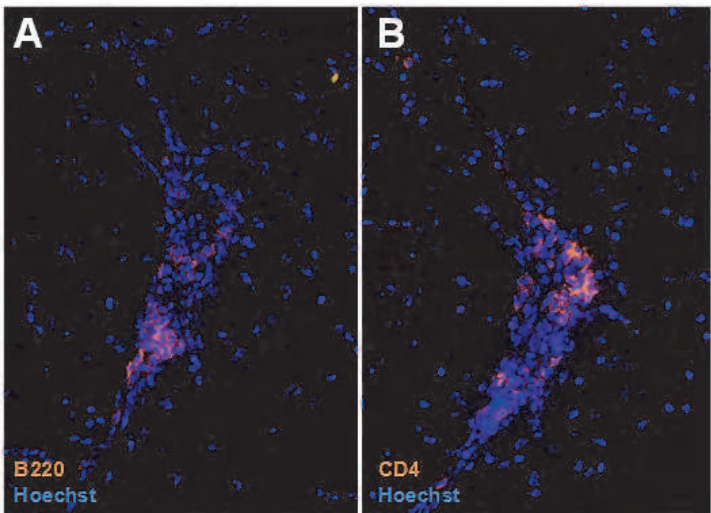


Fig. 4. Presence of B cell/T cell compartmentalization within a CNS B cell aggregate in MP4-induced EAE. C57BL/6 mice were immunized with 150 μ g MP4 in CFA. Pertussis toxin was given at 200 ng per mouse on the day of immunization and 48 h later. 52 days after disease onset mice were sacrificed, the CNS was removed and snap-frozen in liquid nitrogen. Seven μ m thick cryostat sections were obtained from the cerebrum, cerebellum and spinal cord and stained for the presence of B cells (A) and CD4⁺ T cells (B). A representative infiltrate in the cerebral meninges is shown. Images are at 400x magnification and representative for a total of 24 mice tested in six independent experiments (Kuerten et al., 2011c).

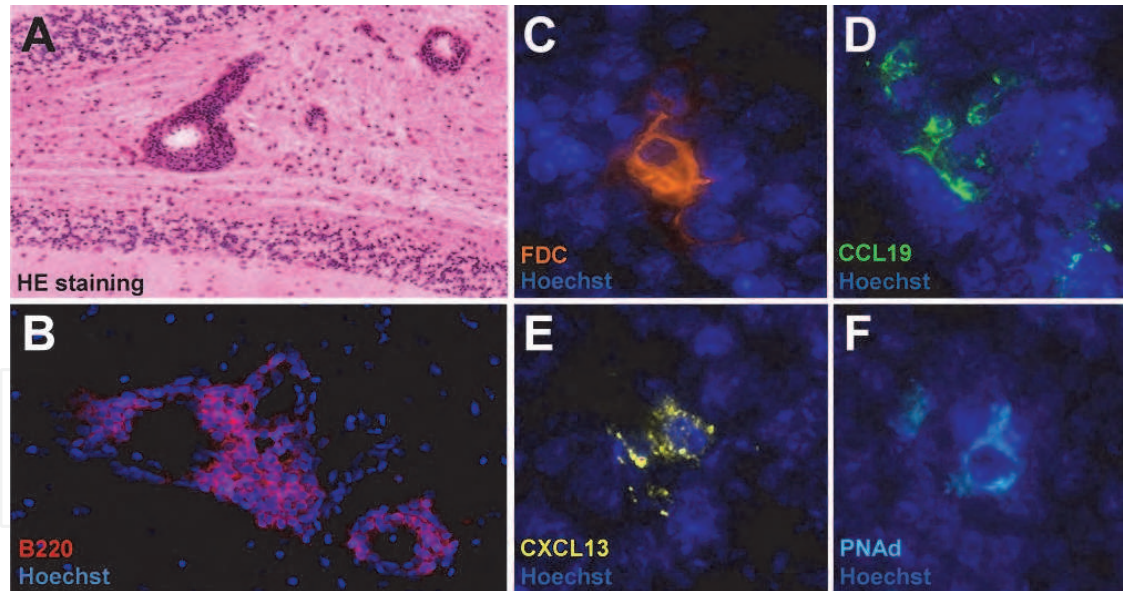


Fig. 5. Characteristics of CNS TLO formation in MP4-induced EAE. C57BL/6 mice were immunized with 150 μ g MP4 in CFA. Pertussis toxin was given at 200 ng per mouse on the day of immunization and 48 h later. 35 days after disease onset mice were sacrificed, the CNS tissue was removed and snap-frozen in liquid nitrogen. Seven μ m thick cryostat sections were obtained from cerebrum, cerebellum and spinal cord and stained for the presence of B cells (A,B), FDCs (C), CCL19 (D), CXCL13 (E) and PNAd expressed in HEVs (F). A representative cerebellar parenchymal infiltrate from a MP4-immunized is shown. (A) is at 100x, (B) at 200x and (C-F) are at 630x magnification. The images are representative for a total of 24 mice tested in six independent experiments (Kuerten et al., 2011c).

induced EAE are functional – here our focus will be laid onto the role of TLOs for determinant spreading of the T cell and B cell response. Determinant spreading can substantially contribute to the chronification and diversification of the immune response in autoimmune disease (Lehmann et al., 1992; McRae et al., 1995; Tuohy et al., 1998). Revealing TLOs as structures responsible for determinant spreading will underline the importance of studies that evaluate TLOs as therapeutic targets. The therapeutic disruption of TLO formation could be a potential means to slow down or ideally prevent disease progression in multiple sclerosis and other autoimmune disorders.

3.3 The immunopathology of MP4-induced EAE is autoantibody-dependent

We have shown that the involvement of B cells in the MP4 model is not restricted to the infiltration of these cells into the CNS and the formation of TLOs, but that it additionally includes the production of antibodies by autoreactive B cells. Antibodies have been noted in a variety of EAE models, directed against MOG, MBP and PLP (Sadler et al., 1991; Lyons et al., 2002). However, the presence of antibodies in the serum of immunized mice does not directly imply their pathogenicity. Among others, antibodies directed against MOG peptide 35-55 or rat MOG protein have been shown to be non-pathogenic (Oliver et al., 2003; Marta et al., 2005), while antibodies against human MOG protein have been associated with demyelination (Lyons et al., 2002; Oliver et al., 2003). Immunization with MP4 clearly triggered the production of MP4-specific antibodies (Kuerten et al., 2011a). Antibodies reactive to MP4 were evident as early as 15 days after immunization. The MP4-specific antibody response reached a plateau around day 50 after immunization. The MP4-specific antibodies were of the IgG1 and IgG2a isotype, with IgG1 apparently prevailing, but the difference did not reach statistical significance. In addition, these MP4-specific antibodies proved to be myelin-reactive. C57BL/6 mice were immunized with PBS in CFA or MP4 in CFA, and B cell-deficient μ MT mice were immunized with MP4. On day 40 after immunization mice were bled and serum was isolated. Consecutively, frozen longitudinal spinal cord sections obtained from naïve untreated C57BL/6 wild-type mice were incubated with the serum to evaluate myelin reactivity. Staining of the myelin sheath was only evident when incubating spinal cord sections with serum obtained from MP4-immunized wild-type C57BL/6 mice, while staining was absent when using control serum from PBS/CFA-immunized or MP4-immunized μ MT mice. In the following, we demonstrated that these myelin-reactive antibodies, however, were not able to mediate pathology on their own. When immunized with MP4, the two congenic B cell-deficient mouse strains μ MT and J_HT did not develop EAE, emphasizing the role of B cells in the MP4 model (Kuerten et al., 2011a). Transfer of MP4-reactive serum into B cell-deficient mice did not revert this resistance. The permeabilization of the blood-brain barrier (BBB) by injection of pertussis toxin in parallel to the serum transfer did not result in clinically and/or histologically evident EAE either. Merely the additional immunization of B cell-deficient mice with MP4 restored disease to the level of the wild-type mice. In this set of experiments, the serum transfer protocol as established by Lyons et al., 2002 was used. MP4-reactive serum was isolated from C57BL/6 donor mice on days 30, 50, 70 and 90 after immunization. MP4 reactivity was tested by ELISA and myelin binding capacity by incubating spinal cord tissue from naïve untreated C57BL/6 mice with each particular serum batch. Only serum that tested positive in both ELISA and immunohistochemistry was used for subsequent transfer. Serum was transferred four times, that is on days 0, 4, 8 and 12 adding up to a total of 600 μ l

of serum transferred into each recipient mouse (150 μ l of serum were transferred on each time point, diluted to a total of 500 μ l injection volume in sterile PBS).

The mechanism by which MBP/PLP-specific antibodies assert the EAE sensitizing effect in MP4-immunized mice is unclear. We have shown that MP4-induced antibodies stain myelin on tissue sections. Because the sections go through the myelin sheath, such staining, however, does not permit the distinction between intra- or extracellular binding of the antibodies. When studying CNS sections of mice undergoing EAE antibody depositions were seen in lesions only, while being absent in parts of the CNS, in which cellular infiltration and tissue damage was not evident. This finding supports the hypothesis that autoantibodies can assert local effects only in synergy with T cell-induced inflammation, which disrupts the blood-brain barrier (BBB) and also permits complement components to enter. Fig. 6 shows colocalization of demyelination, antibody and complement depositions in a spinal cord lesion induced by immunization with MP4. The data point to the involvement of the complement system in the MP4 model.

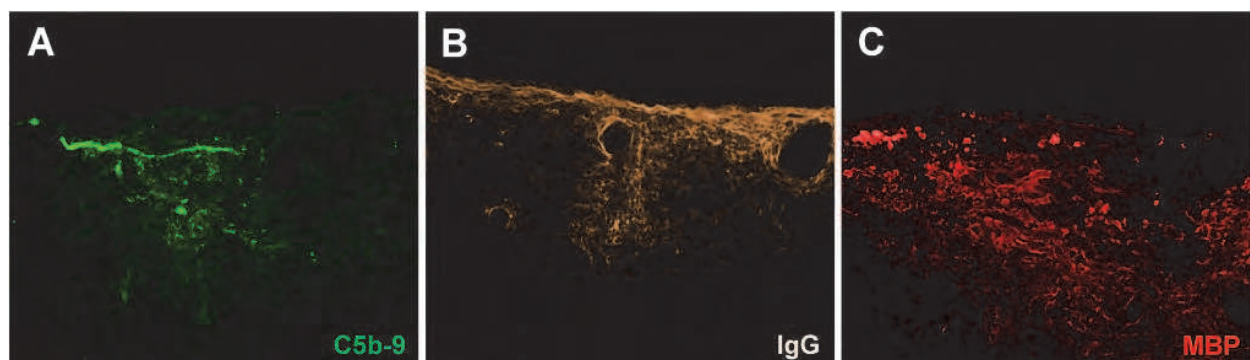


Fig. 6. Colocalization of demyelination and antibody/complement depositions in CNS lesions of MP4-immunized mice. C57BL/6 mice were immunized with 150 μ g MP4 in CFA. Pertussis toxin was given at 200 ng per mouse on the day of immunization and 48 hours later. Mice were sacrificed on day 35 after immunization, the spinal cord was removed and 7 μ m cryostat sections were obtained. The tissue was stained for the deposition of C5b-9 (the membrane attack complex MAC) (A) and IgG (B) in colocalization with demyelination (C). Results are representative for a total of $n = 6$ mice analyzed in two independent experiments. Images are at 100x magnification and refer to a mouse with a clinical score of 2.5 (Kuerten et al., 2011d).

While PLP may be the primary target of the autoimmune response due to its extracellular domains, MBP might be involved in later disease stages once myelin breakdown has occurred. It will be of interest to evaluate the relevance of various myelin antigens in different disease stages. To this end, future studies will require working with individual domains of MP4. In addition, the generation of monoclonal antibodies against individual domains of PLP and the MBP molecule will be needed to further dissect the fine-specificity of MP4-specific antibody action.

3.4 Demyelination, axonal damage and gray matter pathology in C57BL/6 EAE

Inflammation is known to be the characteristic attribute of the acute stages of the disease. However, differences have been observed when comparing different EAE models. In MOG

peptide 35-55 and PLP peptide 178-191 EAE of C57BL/6 mice three months after immunization only few disseminated infiltrating cells, in particular CD4⁺ T cells and macrophages, were present in the tissue. In the MP4 model, inflammation was more sustained: while inflammatory foci decreased over time in spinal cord and cerebrum, cerebellar infiltration was a prevalent feature of chronic EAE three months after immunization. Besides inflammation, demyelination and axonal damage are considered to be hallmark features of MS and therefore also need to be addressed in studies of the animal model. In our follow-up study we investigated the degree of demyelination, axonal damage and motor neuron pathology in MP4-, MOG peptide and PLP peptide-induced EAE of C57BL/6 mice. We demonstrated that major differences between the three models resided in (i) the region-/tract-specificity and disseminated nature of spinal cord degeneration, (ii) the involvement of motor neurons in the disease and (iii) the extent and kinetics of demyelination.

Not many studies have dealt with a systematic investigation of the differential involvement of spinal cord fiber tracts in EAE. The murine spinal cord can be subdivided into three main fiber tract systems – the anterolateral tract (that carries on pain, temperature and crude touch sensation), the dorsal tract (that transmits fine touch sensation) and the pyramidal tract (responsible for motor function). Most reports either do not provide information about which tract has actually been analyzed, or reports focus on the anterolateral tract. However, the clinical symptoms evident in the mice cannot solely be explained by anterolateral tract pathology. Mice typically present with a floppy tail initially that advances into an ascending paralysis as the disease progresses. To this end, any correlation between clinical deficits and CNS histopathology also needs to involve studies of pyramidal tract and motor neuron alterations.

Our data demonstrate that the anterolateral tract was affected in all mice in MOG peptide 35-55-, PLP peptide 178-191- and MP4-induced EAE and to a similar extent. The dorsal tract showed more gradual pathology in acute EAE, but was targeted in 100% of mice in chronic EAE. While a clinical assessment of sensory deficits in mice is hard to perform, our data show that EAE is suitable to study the pathology of this neurological quality (Kuerten et al., 2011b). Autoimmune encephalomyelitis has originally been believed to be a “white matter disease”. However, reports exist that suggest additional pathologic changes in the gray matter including loss and/or atrophy of motor neurons (Bannerman et al., 2005; Fisher et al., 2008; Derfuss et al., 2009; Rudick & Trapp, 2009). In MS patients CNS gray matter has been shown to be affected at multiple sites covering the basal ganglia, the hippocampus (Derfuss et al., 2009), spinal cord and the cortex (Kidd et al., 1999; Bo et al., 2003; Wegner et al., 2003; Kutzelnigg et al., 2005; Kutzelnigg et al., 2007). In addition, gray matter pathology has been shown to reflect functional disability better than the extent of white matter plaque formation (Wegner et al., 2003; Fisher et al., 2008). Consecutively, contactin-2 has been suggested as gray matter target antigen for the autoimmune T cell response (Derfuss et al., 2009). Motor neuron pathology as one aspect of gray matter disease has not been investigated extensively yet. Vogt and colleagues (Vogt et al., 2009) were able to demonstrate that in MS patients compound muscle action potential amplitudes and motor unit numbers were decreased compared to controls subjects, which was indicative of lower motor neuron loss. The data were confirmed by high-precision unbiased stereological quantification of spinal cord neurons in *post-mortem* MS tissue. In this material, T cells secreting TNF-related apoptosis inducing ligand TRAIL were found in close vicinity to apoptotic neurons (Vogt et al., 2009).

Studies as to whether gray matter pathology is a characteristic feature of different EAE models are scarce. Bannerman et al. (2005) have reported motor neuron atrophy in MOG peptide 35-55-induced EAE of C57BL/6 mice by staining of hypophosphorylated neurofilament H with SMI-32 antibody. In our study we extended the analysis of neurofilament H phosphorylation patterns to also include PLP peptide- and MP4-induced EAE. The data show that in PLP peptide-induced EAE the motor neuron phenotype did not show any signs of atrophy, while in the MP4 and MOG peptide model significant alterations were evident.

We agree with findings presented by Bannerman et al. (2005) in the context of MOG:35-55-induced EAE suggesting motor neuron atrophy evidenced by significantly diminished SMI-32 reactivity. In addition, this group reported that such abnormalities were much less prominent by 14 weeks post immunization. We followed up on spinal cord pathology for about 12 weeks. We share the regression of motor neuron phenotype alterations in MOG peptide-induced EAE and we delineate that in the MP4 model motor neuron pathology seems to be more persistent (similar to the chronic demyelination and axonal damage in this model) (Kuerten et al., 2011b).

Loss of spinal cord motor neurons has been reported in MBP-induced EAE of Lewis rats (Smith et al., 2000). In MOG peptide 35-55-induced EAE of C57BL/6 motor neuron loss has not been noted (Bannerman et al., 2005). On the one hand, no TdT-mediated dUTP-biotin nick end labeling (TUNEL) positive neurons were observed in any of the mice analyzed on days 14, 21 or 98 post immunization (Bannerman et al., 2005). On the other hand, there was also no difference in the densities of motor neurons counted in cross sections of L5,L6 spinal cords in MOG peptide-EAE and CFA control mice on day 98 after immunization. Finally, the analysis of L5,6-innervated skeletal muscles did not show any muscle atrophy, angulated skeletal muscle fibers no fiber type-specific grouping in MOG peptide-immunized animals on day 98 after immunization, indicating that these muscle fibers were neither denervated nor reinnervated by axonal collateral sprouting (Bannerman et al., 2005). We also performed analysis of apoptosis in motor neurons in MOG peptide 35-55-, PLP peptide 178-191- and MP4-induced EAE staining for TUNEL or caspase 3. In accordance with Bannerman et al., 2005 we did not observe motor neuron apoptosis in the three models (unpublished data). The cause of the alteration of the motor neuron perikaryal phosphorylation in the MOG peptide and MP4 model remains to be elucidated. Previous studies have shown that increased phosphorylation of neuronal neurofilament H can be induced by an increase in the extracellular concentration of glutamate (Ackerley et al., 2000). Glutamate excitotoxicity is believed to play an important pathogenic role in EAE and MS (Hardin-Pouzet et al., 1997; Matute et al., 2001; Werner et al., 2001) and could also be of importance for causing neuronal damage. Future studies clearly need to address the mechanisms underlying motor neuron alterations in EAE and possibly also MS. To further assess alterations in the motor neuron phenotype we are currently conducting ultrastructural analysis using electron microscopy. Our preliminary data show that in addition to changes in the neurofilament H phosphorylation patterns, motor neuron pathology can include nuclear membrane dissolution, vacuolization of the cytoplasm and a decrease in synaptic densities (Fig. 7). Only mild motor neuron degeneration was evident in MOG:35-55-immunized mice, characterized by an increased number of intracytoplasmic vacuoles and slight nuclear changes that encompassed an irregular undulation of the nuclear membrane. MP4-induced EAE in contrast led to more severe nuclear membrane

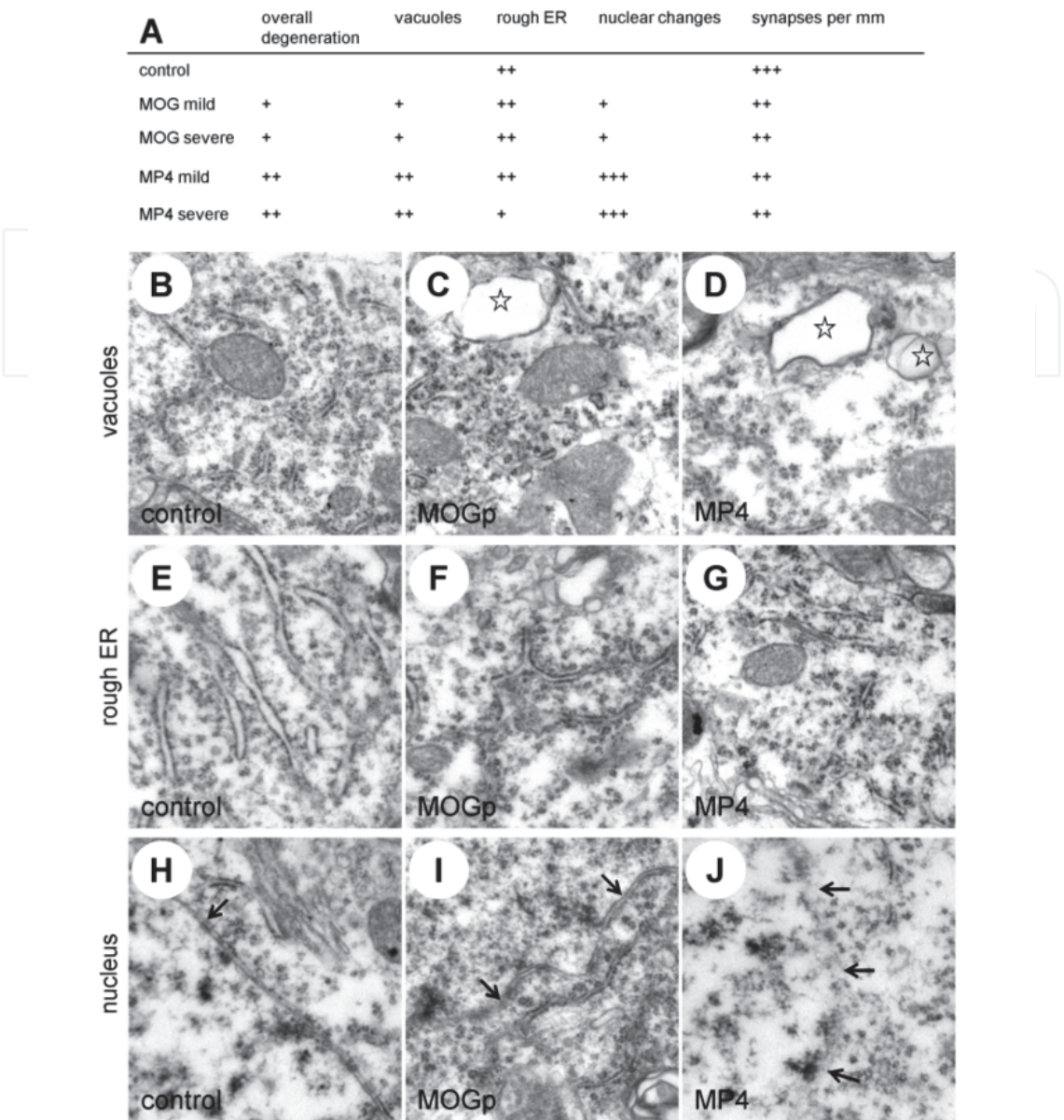


Fig. 7. MP4-induced EAE displays severe motor neuron pathology. C57BL/6 mice were immunized with 150 µg MP4 or 100 µg MOG:35-55 in CFA. Pertussis toxin was given at 200 ng per mouse on the day of immunization and 48 hours later. (A) Motor neuron pathology was assessed using a semi-quantitative scoring system, which considered the overall degree of motor neuron degeneration, the occurrence of intracytoplasmic vacuoles (B-D), rough ER (E-G) and nuclear changes (H-J) as well as the number of synapses per mm. Stars in panels (F) and (G) designate vacuoles, the arrows in panels (I) and (J) indicate the nuclear membrane. All images are at 12.000x magnification. The data refer to n = 6-8 mice tested in each group and tested in at least three independent experiments. Data obtained from EAE mice were compared to data from untreated control mice. Mild EAE encompassed the clinical scores 0.5-2, severe EAE referred to clinical scores > 2. The mean clinical score in the MOG peptide and MP4 group was similar both in mild and severe EAE, respectively (1.40 ± 0.22 versus 1.35 ± 0.03 in mild MOG peptide versus MP4-induced EAE with p = 0.593 and 2.58 ± 0.20 versus 2.66 ± 0.28 in severe MOG peptide versus MP4-induced EAE with p = 0.582).

defects up to complete nucleic resolution. The extent of rough endoplasmic reticulum (rER) alterations and the number of synapses remained largely unchanged compared to the control group. Only in severe MP4-induced EAE we noted beginning resolution of the rER. The number of synapses per mm was decreased in both MOG:35-55- and MP4-induced EAE compared to controls (Gruppe et al., 2011).

Besides motor neuron dysfunction, damage to the pyramidal tract can be responsible for the development of motor deficits. What was intriguing to see was the fact that in our light microscopic analysis of methylene blue-stained transverse spinal cord sections in the MP4 model almost exclusively motor neuron perikaryal disturbances were evident, while in the MOG and PLP peptide model the pyramidal tract equally showed degeneration (Kuerten et al., 2011b). The perikaryal disturbances were characterized by an increase in staining intensity of the Nissl substance. These microscopically visible changes could be due to a transient increase in Nissl substance due to a loss of trophic input as a consequence of EAE-induced motor neuron dendritic pathology (Zhu et al., 2003; Bannerman et al., 2005). Considering our ultrastructural data that showed a decrease of rough ER (and thus Nissl substance) over time, it is – however – more likely to favour the alternative hypothesis that the light microscopic picture is due to rough ER dissolution/fragmentation.

However, at this point it should be noted that a conclusive statement as to the pathologic changes in individual fiber tracts cannot be made without further ultrastructural analysis. Similar to our analysis of motor neuron pathology we are currently also conducting electron microscopic studies of pyramidal tract pathology. Our data indicate that pyramidal tract pathology also occurs in the MP4 model including demyelination and axonopathy, however the extent of pyramidal tract pathology seems to be less severe compared to the MOG peptide 35-55 model, which could explain the differences observed in our light microscopic analysis.

Next to the differential targeting of spinal cord fiber tracts, another differential histopathological feature applied to the extent and kinetics of demyelination in MP4-, MOG peptide 35-55- and PLP peptide 178-191-induced EAE. Chronic demyelination in the MP4 model was opposed to only transient or absent myelin pathology in MOG peptide and PLP peptide EAE. Considering these data in the context of what we have discussed above, the MP4 and MOG peptide model could help reproduce distinct demyelinative patterns. MP4-induced EAE may be a valuable tool for studying myelin pathology that relies on B cells/autoantibodies and complement activation versus demyelination primarily caused by T cells and macrophages in the MOG peptide model. In addition, the comparison of these models to the non-demyelinating disease induced by PLP peptide 178-191 may give insight into factors that actually initiate and maintain the myelin attack dependent on the antigen that is the prime target. When evaluating the activity of the lesion by staining for major histocompatibility (MHC) class II we noted the transition from active plaque regions with high expression of MHC II to chronic still demyelinated, but “MHC II low” lesions in MP4-induced EAE. The presence of highly inflammatory lesions later turning into chronic burnt-out demyelination is a hallmark of MS (Trapp et al., 1996), which still needs further investigation, for which the MP4 model may be a suitable tool.

Finally, besides all these differences in CNS histopathology, the analysis of the clinical outcome of EAE induced by MP4, MOG peptide 35-55 and PLP peptide 178-191 showed major similarities in the course and severity of the disease (Kuerten et al., 2007). While these

similarities were clearly opposed to the differential patterns of demyelination and regional spinal cord pathology that we have just discussed, we found the extent of axonal damage to be highly comparable in the three models (Fig. 8). Therefore, in accordance with data obtained in MS patients (Trapp et al., 1998), we propose axonal injury as the main structural-morphological correlate causing irreversible functional deficits, but we acknowledge that future studies on the ultrastructural level are still needed to support this notion.

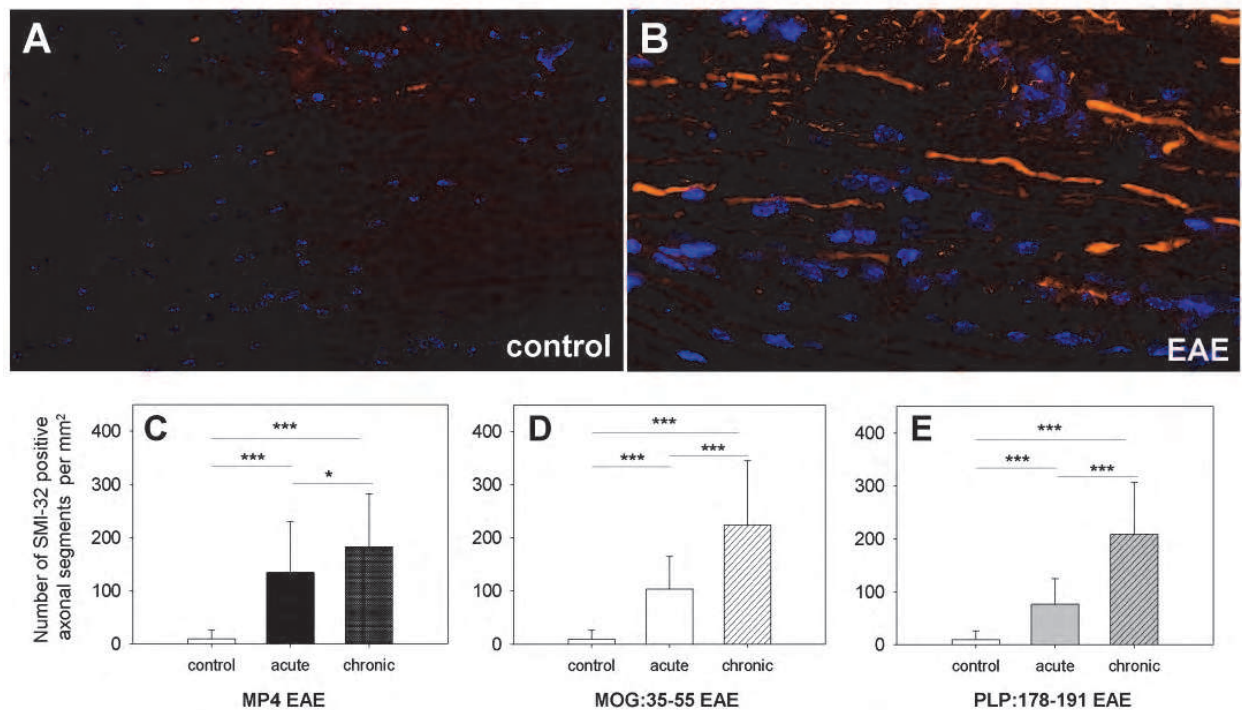


Fig. 8. Increasing axonal damage in the course of MP4-, MOG:35-55-, and PLP:178-191-induced EAE. C57BL/6 mice were immunized with 150 µg MP4, 100 µg MOG peptide 35-55 or 200 µg PLP peptide 178-191. Pertussis toxin was given at 200 ng per mouse on the day of immunization and 48 h later. Longitudinal 7 µm thick frozen spinal cord sections were stained with SMI-32 antibody. Representative images are shown for spinal cord sections from PBS/CFA control-immunized C57BL/6 mice (control) (A) and EAE tissue, here taken from a MP4-immunized mouse three months after onset of the disease with a score of 2.5 (B). Panels (C-E) display the mean number of SMI-32 positive axonal segments per mm² + SD in each model on the peak of acute EAE (approximately day 15 in the MP4 model, day 20 in the MOG peptide 35-55 model and day 30 in PLP peptide EAE) and in the chronic stage of EAE three months after immunization compared to control-immunized mice. The data refer to n = 10 mice tested in each group in three independent experiments. All images are at 400x magnification. * p = 0.05, *** p < 0.001.

In conclusion, while all three models – MP4-, MOG 35-55- and PLP:178-191-induced EAE in C57BL/6 mice – clinically display chronic disease with comparable severity and course, the CNS histopathology underlying the functional clinical deficits can follow differential patterns. Our data suggest the use of MP4-, MOG peptide 35-55- and PLP peptide 178-191-induced EAE on the C57BL/6 background as a reasonable strategy for reproducing distinct features of CNS pathology fuelling work towards a better understanding of MS diversity.

4. Future implications

Eventually, our experimental approach is meant to go beyond the investigation whether different EAE models can be reflective of different patterns of CNS pathology. A clinically highly relevant question deals with the correlation between CNS histopathology and the clinically evident outcome in each individual patient. While axonal damage is assumed to cause irreversible deficits, due to the limited access to human CNS samples and the impossibility of obtaining samples repeatedly over time there are few reports so far directly analyzing in which way or how the degree of CNS pathology defines the clinical severity. The kinetics of lesion development in patients have mostly been assessed by MRI (Inglese et al., 2011; Sicotte et al., 2011). While this approach allows to establish a correlation between overall lesion load and clinical disease severity, it does not provide information about the mechanisms underlying lesion development. Even further, it would be clinically highly relevant to investigate whether there is a correlation between CNS pathology and peripherally measurable immunological parameters.

In an initial study we set out to analyze the correlation between the magnitude of the interferon- γ (IFN- γ)/interleukin-17 (IL-17) antigen-specific response in the blood and the clinical course of the disease in two independent EAE models: the remitting-relapsing PLP peptide-induced EAE of SJL/J mice and the chronic disease of C57BL/6 mice immunized with MOG peptide 35-55. To this end, we established an experimental technique that worked with low amounts of blood, thereby permitting longitudinal and repeated testing of mice. The technique we used relied on a double-color enzyme-linked immunospot technique (ELISPOT)-based test system, for which as little as 150 μ l of murine blood sufficed. We then tested mice repeatedly over the time course of EAE to establish the kinetics of the antigen-specific blood IFN- γ and IL-17 T cell response in both the MOG peptide 35-55/C57BL/6 and the PLP peptide 139-151/SJL model. In sum, the data delineate that the dynamic course of EAE in the SJL/J model was closely reflected by dynamics in the blood T cell compartment, while chronic EAE in the C57BL/6 model was mirrored by a relatively stable antigen-specific T cell response (Kuerten et al., 2010).

As we learn more about the antigens that are actually targeted in MS, our approach may serve as a valuable approach towards more efficient prognostic and diagnostic options in patients. Despite remarkable scientific effort, MS has remained highly unpredictable and a suitable biomarker for the disease has not been found (hallmark studies are summarized in Galboiz & Miller 2002; Rinaldi & Gallo, 2005 and Reindl et al., 2006). To date, there is no possibility of determining whether a patient presenting with clinically-isolated syndrome (CIS) or radiologically-isolated syndrome (RIS) will develop definite MS. There is also no possibility of predicting the course of disease in MS patients, and in particular whether and when a patient in clinical remission will develop a relapse. In addition, it is assumed that several subpopulations of MS exist, and the contribution of CNS antigen- and in particular myelin-reactive B and T cells differs in these subpopulations. While in a majority of patients autoreactive T cells are detectable in CNS demyelinating lesions, there are also subpopulations of patients, in which a primary oligodendroglialopathy is evident. This difference in response is likely to result from differences in immune pathogenesis underlying the disease. So far, there are also no methods available that permit the prediction of treatment responsiveness in MS patients.

In an ongoing study in collaboration with the Department of Neurology, University Hospitals of Cologne, we perform measurements of CNS antigen/myelin-specific T and B cells in the blood after *in vitro* stimulation of these T cells with CNS/myelin antigen. It is our aim to determine whether such measurements can permit the prediction of: (a) whether a patient with CIS or RIS is likely to transit into definite MS, (b) whether a patient with MS is likely to show disease relapse in the near future, and/or (c) whether a patient with CIS, RIS or MS is likely to respond to/benefit from immune modulatory treatment.

While our data imply that there is indeed a correlation between blood cytokine responses and the clinical course of EAE, they leave open the question if and how the CNS cytokine response is linked to this correlation. It is tempting to speculate that the magnitude of the blood cytokine response defines the magnitude of the CNS cytokine response. On the one hand, it is conceivable that the more antigen-specific cells are present in the blood the more enter the CNS. On the other hand, it is equally possible that the more cells are present in the CNS, the less cells will be found in the blood. In addition, it needs to be defined if the magnitude of the IFN- γ /IL-17 response in the blood and/or the CNS itself is related to the degree of histopathology and if this finally defines the clinical outcome of the disease. Should we find a positive correlation between all four parameters – peripheral/CNS cytokine response, CNS pathology and clinical disease – our data would point towards the possibility of mirroring/predicting the degree of CNS pathology by simple measurements of blood responses and kinetics. It remains to be elucidated in the future whether our notion proves true.

5. Conclusion

What is to be concluded from the data presented here is that despite all the criticism about the model, EAE can be a valuable tool for studying MS. Different models can be used to selectively reflect different pathomechanisms of the disease. Most of the therapeutic options we rely on in the therapy of the disease today have been developed and/or validated in EAE and our data delineate that the model can also be used for working towards a better understanding of the interaction between the peripheral and CNS immune response and the subsequent damage to the target organ that finally defines the clinical outcome of the disease. The translation of our data into the clinical situation will show how accurate our interpretation of the disease processes presented here is.

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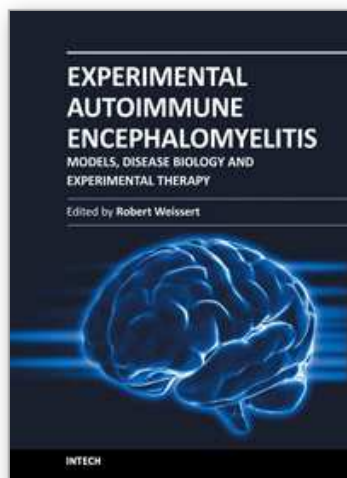
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