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Minireview

Adjuvant-free animal model for studying CNS autoimmunity

Bojan JEVTIĆ

University of Belgrade, Institute for Biological Research "Siniša Stanković" - National Institute of Republic of Serbia, Department of Immunology, Belgrade, Serbia

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Summary. Multiple sclerosis (MS) is a chronic inflammatory, demyelinating, and neurodegenerative disorder of the central nervous system. More than 2.5 million people suffer from this disease worldwide. It is assumed that the autoimmune response to myelin antigens in the CNS is the main cause of the disease. Experimental autoimmune encephalomyelitis (EAE) is a widely used animal model for studying MS. However, EAE models resemble only particular aspects of MS pathogenesis. EAE is classically induced with the CNS antigens emulsified in complete Freund's adjuvant (CFA). CFA consists of paraffin oil supplemented with Mycobacterium, and its application potentiates the innate immune response and prolongs the presence and effective transport of antigen in the lymphatic system. However, CFA has a confounding influence on the results and the translational capacity as a multiple sclerosis model. Our group has successfully excluded CFA from the immunization regime. In a recent study, we compared clinical, histological, cellular, and molecular properties between spinal cord homogenate (SCH) and SCH+CFA immunized Dark Agouti rats. We have observed a higher clinical score in rats without CFA, and a more significant number of immune cell infiltrates at the peak of EAE in the same animals. Further, a stronger myelin basic protein-specific T cell immune response is evoked in the draining lymph nodes of CFA-free compared to CFA immunized rats. In the CNS, a high abundance of CD8+ T cells is detected at the onset of the disease. Also, enrichment in CD8+ and CD4+ macrophages was observed in the CNS during EAE. Therefore, CFA-free EAE is a reliable model for studying CNS autoimmunity.

Keywords: CD8+ macrophages, CFA, experimental autoimmune encephalomyelitis, multiple sclerosis.

MULTIPLE SCLEROSIS

Multiple sclerosis (MS) is a chronic, inflammatory and neurodegenerative disease of the central nervous system (CNS). It is estimated that more than 2.5 million people are affected by this disease worldwide. The risk of developing MS is 1.5 to 2.5 times higher in the female population and affects mostly young adults. The disease manifests in various ways, including sensory, primarily visual disturbances, motor dysfunctions, fatigue, pain, and cognitive disturbances (Compston and Coles 2008). The exact cause of MS is still unknown. The disease occurs in genetically susceptible individuals under the influence of various environmental factors. The most widely accepted hypothesis about the origin of MS assumes that the damage to the CNS arises due to an autoimmune response to CNS tissue antigens, primarily myelin proteins.

Indirect evidence that confirms this hypothesis is the presence of autoreactive T lymphocytes and autoantibodies found in the lesions and peripheral blood of MS patients, as well as the fact that the most effective therapies for the disease have an immunomodulatory effect (Nakahara et al. 2010). It is assumed that the pathogenesis occurs due to the break of tolerance to myelin antigens, which can arise for various reasons

E-mail: bojanbh@gmail.com

(molecular mimicry provoked by microbes (Harkiolaki et al. 2009), activation of T lymphocytes by super-antigens (Meyer 1995), dysregulation of regulatory T cells (Danikowski et al. 2017), etc.). Following activation, T cells specific for myelin antigens pass the blood-brain barrier, reactivate in the CNS, and cause inflammation, demyelination, and neurodegeneration. MS lesions result from inflammation, primary demyelination, axonal degeneration, and neuronal loss. Although the latter can also be seen in other neurodegenerative diseases, the primary demyelination is the main feature differing MS from other disease (Fischer et al. 2013). Inflammation in the lesions is the results of the infiltration of T lymphocytes, macrophages, B lymphocytes, and autoantibodies. In MS patients, CD8+ T cell infiltrates dominate, whereas B cells and CD4+ T cells are less represented.

Furthermore, active lesions are characterized by excessive activation of microglia and astrocytes, which propagate inflammation. This leads to astrogliosis and the establishment of glial scars in the CNS (Lassmann and Bradl 2017). Despite heterogeneity and complex etiology, it is most likely that the autoimmune response that arises from breaking tolerance to CNS antigens in the periphery is the origin of MS pathogenesis.

EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

Experimental autoimmune encephalomyelitis (EAE) is the best characterized and the most commonly used animal model of MS. It was first described more than 75 years ago and can be induced actively or passively in various susceptible species and strains of rodents and primates. Active EAE is induced by immunization with CNS tissue homogenate or specific CNS antigens, such as myelin basic protein (MBP), myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), as well as peptides of these proteins that are emulsified in complete Freund's adjuvant (CFA) (Gold et al. 2006). Alternatively, the disease can be induced by the passive transfer of $CD4^+$ T cells obtained from the animals after sensitization with brain antigen into naive recipients (Ben-Nun et al. 1981). Clinical manifestations of EAE depend on the species and strain of animals but also the method of induction (Simmons et al. 2013). It is characterized by paresis or paralysis, which spreads from the tail over the hind and front limbs, leading in severe cases to the death of the experimental animal. In EAE, inflammation predominantly occurs in the spinal cord of animals, causing paralysis. Although this model provides many insights into CNS inflammation, the main drawback of EAE as a model of MS is that it does not represent the entire spectrum of inflammatory mechanisms and neurodegeneration observed in MS (Lassmann and Bradl 2017). The lack of inflammation in the brain is a fundamental difference compared to MS patients.

Furthermore, most strains, including the most used strain C57BL/6, show a monophasic disease course (Bittner et al. 2014). The most popular model of EAE is induced in C57BL/6 mice by immunization with MOG_{35-55} peptide in CFA. Except for CFA, applying pertussis toxin (PTX) is mandatory for effective EAE induction (Lassmann and Bradl 2017). EAE induced in this way results in highly reproducible acute or chronic inflammatory disease with lesions restricted to the spinal cord without or with low brain affection. Another disadvantage of this model is massive axonal degeneration with secondary demyelination, contrary to the primary demyelination seen in patients with MS (Kim et al. 2006). More reliable models are induced in rats, guinea pigs, or primates with MOG, myelin, or CNS tissue in CFA. In addition, inducing EAE in rats does not require the application of PTX. Also, primary demyelination with partial axonal loss seen in this model in many instances resemble the pathological changes in MS. In addition to the mentioned difference in the sites of CNS inflammation in MS patients and EAE-induced animals, immune cell infiltrates within the CNS are significantly different in the EAE and MS models. Thus, due to the immunization regimen, the T cell response mediated by CD4+ T lymphocytes is dominant in EAE, while CD8 + T cells are the most represented in MS (Babbe et al. 2000). Likewise, the genetic heterogeneity existing in MS patients, but not in the inbred EAE models, also appears as an issue (Mix et al. 2010). Although these and other problems limit the interpretation of the results and do not fully reflect the entire spectrum of pathological and clinical phenomena seen in MS, the EAE model is still a valuable tool for studying MS, and EAE improvement is warranted.

IMMUNOPATHOGENESIS OF MULTIPLE SCLEROSIS AND EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

An explanation of the pathogenesis of MS that supports the view that the disease originates in the periphery is in agreement with the EAE induction method, where CNS antigens emulsified in the immunization mixture when injected into the animal lead to the onset of the disease. Antigen application leads to local activation of antigen-presenting cells (dendritic cells (DCs), macrophages), antigen uptake by these cells, and presentation to naïve T lymphocytes in the draining lymph nodes (DLNs). Naïve $CD4^+$ T cells use their TCR to recognize the antigen presented as part of the Major Histocompatibility Complex II (MHC II) by antigen-presenting cells. Subsequently, guided by specific co-stimulatory signals and the cytokine milieu of the surrounding environment,

these cells are activated and differentiated into encephalitogenic helper subpopulations Th1 and Th17 (Kapadia and Sakic 2011). Th1 and Th17 cells leave the DLNs, enter the circulation and reach the CNS, where they are reactivated by local antigen-presenting cells, further leading to the recruitment of immune cells to the site of infiltration and the spreading of inflammation in the CNS (Ransohoff et al. 2003; McMahon et al. 2005). Th1 cells produce cytokines IFN-γ, which in inflammatory conditions activate macrophages and help eliminate intracellular bacteria and viruses, while Th17 cells secrete IL-17 and play a role in maintaining mucosal homeostasis and protection against extracellular bacteria and fungi (Kamali et al. 2019). However, excessive activation and malfunctioning of these cells are associated with the pathogenesis of EAE, MS, and other autoimmune diseases.

Both cell populations, Th1 and Th17, and increased cytokine levels have been identified in the CNS, serum, and cerebrospinal fluid (CSF) of MS patients (Link et al. 1992; Kebir et al. 2007). Regulatory T cells (Treg) counterbalance auto-aggressive Th1 and T17 cells. The role of these cells is to limit the immune response and maintain immune homeostasis. Different studies have shown that Treg are involved in the pathogenesis of MS and EAE due to their inability to suppress the autoimmune response efficiently (Korn et al. 2007; Frisullo et al. 2009). Microglia and macrophages are crucial cells in the effector phase of EAE, and MS. Resident microglia represent about 10% to 20% of all glial cells in the CNS. As part of innate immune responses, their role is to defend the organism from pathogens. Under physiological conditions, microglia interact with neurons, support development, and influence the maintenance of brain homeostasis (Michell-Robinson et al. 2015). However, during the autoimmune response in EAE and MS, these cells are involved in the damage of myelin and destruction of oligodendrocytes and neurons (Bogie et al. 2014). Persistent activation of microglia was confirmed in all active lesions of MS patients (Guerrero and Sicotte 2020). After infiltration into the CNS, in response to myelin antigens, Th1 and Th17 subpopulations activate macrophages and microglia.

Consequently, activated macrophages and microglia produce pro-inflammatory cytokines, express high levels of MHC I and II molecules, and propagate inflammation (Yin et al. 2017). Further, they produce a large amount of reactive oxygen (ROS) and nitrogen species (RNS) that damage lipids, proteins, and nucleic acids. The accumulation of damage caused by ROS and RNS leads to neurotoxicity and the death of oligodendrocytes and neurons. $CD8^+$ T and B lymphocytes also play an important role in the pathogenesis of MS. In the active lesions of MS patients, CD8+ lymphocytes significantly exceed the number of CD4+ T cells. The expansion of CD8+ clones specific for myelin antigens has been detected in CFS of MS patients. CD8+ T cells exhibit cytotoxicity by inducing apoptosis in the cells of the CNS (Huseby et al. 2012). B cells also contribute to the pathogenesis of MS in terms of activating T cells and producing autoantibodies and proinflammatory cytokines. A biological therapy based on neutralizing B cells appeared to be very useful in treating patients with MS. By contrast, the protective role of CD8+ and B cells has been shown in some studies (Dargahi et al. 2017). Overall, it could be concluded that the immunopathogenesis of MS is a complex process where, despite the considerable number of studies, the role of the immune system and its components has not yet been fully explored.

CFA-FREE EAE MODEL

Our research uses an active model of EAE induced by immunization of the Dark Agouti (DA) rat strain with spinal cord homogenate (SCH) emulsified in Complete Freund's adjuvant. Following immunization, animals pass through three phases of the disease: onset, peak, and recovery. After the first peak and recovery, most animals go through a second peak, which is generally weaker in symptoms than the first one, and finally enter into recovery. The described course of EAE is a very similar relapsing-remitting form of MS. Although CFA is widely used in all EAE models, there are several concerns regarding the use of CFA.

CFA is made of incomplete Freund's adjuvant (IFA) supplemented with heat-inactivated *Mycobacteria* as a massive immune stimulant. IFA itself consists of paraffin oil containing mannide mono-oleate as a surfactant (Wanstrup and Christensen 1965). Adjuvant acts in a way to prolong the presence of antigen at the injection site, stimulate phagocytosis and antigen transport in the lymphoid organs and activate innate immunity. The main immune targets of CFA are mononuclear phagocytes (MNPs) and dendritic cells. These cells express Toll-like receptors (TLRs) that bind to different pathogen-associated molecular patterns (PAMPs). Among microbial PAMPs detected in CFA, the most common are chemical constituents of the bacterial cell wall, such as muramyl dipeptide, trehalose dimycolate, and lipoarabinomannan. These compounds are responsible for the adjuvant effects of CFA as they induce an innate immune response by stimulating Toll-like receptor 2 (TLR2) on MNP. Likewise, bacterial heat-shock proteins and DNA enriched in CpG oligodeoxynucleotides potently stimulate the innate immune response, binding to TLR4 and TLR9, respectively (Billiau and Matthys 2001). Upon stimulation with PAMPs, MNPs and DCs produce IL-12, TNF and IL-6. IL-12 further stimulates the natural killer (NK) cells to produce IFN-γ (Flesch et al. 1995). Under this condition, a Th1 immune response is favoured. Besides, CFA induces a massive leucoproliferation and alters haematopoiesis.

Furthermore, mycobacterial cells embedded in oil droplets can be deposited in the liver and lungs for weeks and even months, having delayed effects. Treatment with CFA at the site of immunization causes strong and long-lasting inflammation, inducing the formation of lesions that become ulcers and cause pain. The lesions could also be formed in local and distant lymph nodes and nonlymphoid tissue together with dispersed granulomas composed of MNPs (Wanstrup and Christensen 1965; Billiau and Matthys 2001; Yamagami et al. 2001). Regarding EAE, Th1 immune response is dominant when CFA is used as an adjuvant. Additionally, CFA interferes with studying other cell populations involved in neuroinflammation. It has been shown that CFA activates glia cells by itself and provokes the production of mediators of inflammation in the CNS (Raghavendra et al. 2004).

Furthermore, CFA contributes to biochemical changes and differential gene expression in the CNS. The permeability of the blood-brain barrier is also affected by CFA (Brooks et al. 2005). Moreover, different types of *Mycobacterium* species, e.g., *M. tuberculosis* and *M. butyricum*, may impact the reproducibility of EAE results (Laman et al. 2017). So far, there have been attempts to eliminate *Mycobacteria* from the immunization protocol. EAE can be induced in certain rat strains without CFA, whereas using CFA in guinea pigs and mice is mandatory (Billiau and Matthys 2001). For example, the immunization of mice with PLP in the incomplete Freund's adjuvant induces immunological tolerance (Mills 2011). An effective autoimmune response in the CNS has been elicited in some primates by immunization with CNS antigens emulsified in IFA (Jagessar et al. 2012; Haanstra et al. 2013). When IFA alone, without antigen, applies to DA rats, it induces arthritis. In some of our experiments, we have observed arthritis-like symptoms during EAE induction in DA rats.

Due to CFA's confounding effects, our group has successfully eliminated CFA from the immunization regimen where reliable EAE is induced only with rat spinal cord homogenate (SCH-immunized) (Stosic-Grujicic et al. 2004). Our recent study compared CFA-free EAE with a classical EAE in DA rats. We have focused on the clinical course, composition, and function of the immune cells in the lymph nodes draining the site of immunization, in the blood, and within the CNS (Lazarević et al. 2021).

Compared to the model induced with SCH+CFA, the CFA-free model shows a more pronounced clinical and histological manifestation of EAE. The number of infiltrates in the whole spinal cords and the white matter was slightly but significantly higher in SCH-immunized rats. At the same time, there was no difference in the number of infiltrates in the gray matter between the groups. Results obtained from the periphery have shown that both immunization regimes led to the expansion of T cells (CD4+, CD8+), macrophages (CD11bc+, MHCII+), Treg (CD4+CD25+FoxP3), and B cells (CD45RA+) in popliteal lymph node cells (PLNCs) compared with non-immunized rats. The proportion of CD4+ and CD8+ cells expressing IL-17, IFN- γ, and IL-10 were also higher in immunized rats. However, the absolute number of PLNCs and the proportion of CD4+ cells and activated CD4+ among them was lower in SCH- vs. SCH+CFA immunized animals. Further, the total number of cytokine-producing T cells and Treg was also lower in SCH- compared to SCH+CFA. These results suggest that both immunization protocols induce strong activation of CD4+ and limited activation of CD8+ T cells in PLN following immunization. However, this effect was more pronounced in rats immunized with SCH+CFA. Despite reduced composition and activation of T cells in the PLN of CFA-free immunized rats, a higher number of infiltrates were detected in CFA-free EAE compared to SCH+CFA. This might be explained by the fact that CFA itself activates T cells and that infiltrates of SCH+CFA are „diluted" with microbial-specific T cells. Reaching the CNS, T cells specific for microbial antigens will not be reactivated in contact with the CNS antigens. Indeed, the transfer of MBP and ovalbumin-specific T cells in EAE have shown that both T cell lines can invade the CNS, but only the former could be reactivated (Kawakami et al. 2005; Bartholomäus et al. 2009).

To examine the antigen-specific response in PLN after SCH- or SCH+CFA immunization, immune cells were stimulated with MBP. In both experiments, immune cells responded with high production of IL-17 and IFN-γ cytokines after stimulation with MBP. Although the higher basal production of both cytokines was observed in immune cells obtained from SCH+CFA immunized rats, the rise in the release of the cytokines in PLNC in response to MBP was significantly higher in rats immunized only with SCH. These results suggest that CFA immunization is poorer in the expansion of MBP-specific cells, possibly due to their lower frequency in PLNCs of SCH+CFA immunized rats. A strong response to MBP in the CFA-free immunization protocol may allow us to generate an MBP-specific CD4+ T cell line. This would be particularly interesting due to the possibility of establishing passive EAE by the adoptive transfer of CD4+ T cells without CFA.

As the CNS represents an effector site of immune response in EAE, we have compared the immune cell composition in the spinal cord of SCH- and SCH+CFA immunized rats. Following immunization, spinal cord immune cells (SCIC) were isolated at the peak of disease from both groups. The results have shown no significant difference in the absolute number of isolated cells or the proportion of CD4+ cells and CD4+ T cells isolated from SCIC. The only observed

difference was in total CD8+ cells, where the ratio of these cells was higher in SCH- compared to the SCH+CFA group, while the proportion of CD8+ T cells and activated CD8+ T cells were lower. Further, the proportion of inactivated microglia (CD45-CD11bc+) was similar between groups. In summary, these results show that the composition of SCIC is quite similar in both immunized groups of animals at the peak of EAE. The main differences between SCH+CFA and SCH-immunized rats are presented in Table 1.

In an attempt to more closely determine the composition of SCIC in SCH-only immunized rats, we have compared SCIC cell composition at the onset, peak, and recovery phases of EAE. We have looked at CD8+ and CD4+ T cells, Natural killer, Natural killer T cells (NKT), B cells, granulocytes, macrophages, and microglia. Throughout the onset, peak, and recovery, there was an increase in the total number of cells isolated from the spinal cord. The proportion of CD4+ T cells was lower at the onset of the disease, followed by an increase during the peak and a slight decrease at the recovery. Contrary to CD4+ T cells, CD8+ T cells were most abundant at the onset of disease, with a decrease at the peak and recovery. Interestingly, there were also differences in the ratios of CD4+CD8+ T cells, which level was increased during EAE recovery. NK and NKT were increased at the onset and remained steady throughout the peak and recovery, whereas granulocytes were most abundant at the peak and declined sharply at the recovery. The proportion of B cells was at low levels throughout the observed periods. The proportion of inactive microglia comprised one-third of all isolated cells, but that number decreased significantly at the peak of the disease. Further, we detected CD8+CD11bc+ and CD4+CD11bc+ macrophages in SCIC. The levels of these cells were significantly increased during peak and recovery, respectively (Fig. 1).

Interestingly, these cells are also detected in the peripheral blood of animals in corresponding phases of the disease but at a substantially lower level than in the CNS. Upon entering the CNS, they significantly increase the expression of MHCII molecules on their surface. These findings show that SCIC of CFA-free rats are comprised of various immune cells where CD8+ T cells and granulocytes dominate during the onset, while CD4+ T cells are more abundant at the peak and recovery. This is of particular interest regarding the existing knowledge on the role of CD8+ in the pathogenesis of MS (Hohlfeld et al. 2016). Although CD8+ T cells are outnumbered by CD4+ T cells at peak and recovery, it would be interesting to assess how the initial abundance of CD8+ T cells at the onset determines CNS inflammation. Another significant result of this study was the observed presence of CD8+ and CD4+ macrophages in SCIC. Both cell populations have been linked to CNS inflammation. The recent findings appear to be consistent with other research, which found CD8+ macrophages in the CNS of spinal cord injury model as well as in experimental autoimmune neuritis and MOG-induced chronic EAE but not in MBP-induced acute EAE (Popovich et al. 2003; Schroeter et al. 2003; Hiraki et al. 2009). All these studies indicate that CD8+ macrophages have a pathogenic role in CNS inflammation.

On the other hand, their protective role has been reported in autoimmune glomerulonephritis in rats (Wu et al. 2014). Similar to CD8+, CD4+ macrophages/microglia have also been detected in the CNS during EAE. A correlation exists between CD4+ microglia and the ability of rats to recover from EAE (Almolda et al. 2009). The increased expression of MHCII molecule by CD8+ and CD4+ macrophages at the peak and recovery of EAE indicates their ability to acquire an antigen-presenting cell phenotype, but further research should be conducted to investigate their role in the EAE pathogenesis. Besides, CD8+ and CD4+ macrophages were identified in humans as well (Herbein et al. 1995; Gibbings et al. 2007; Gibbings and Befus 2009), but not in mice (Crocker et al. 1987; Baba et al. 2006), further supporting the use of rats as a valuable animal model for multiple sclerosis. Although B cells are rare in the CNS of the CFA-free model, their role in the pathogenesis cannot be excluded, especially because currently, the most prominent therapies in MS are based on B cell depleting (Miyazaki and Niino 2022). In addition, NK and NKT, as well as CD4+CD8+ cells, are worthy of future research because they are enriched in the CNS at the onset and recovery, respectively. It is particularly interesting because previous studies have reported the regulatory role of NK and CD4+CD8+ cells in animal models of MS (Tutaj and Szczepanik 2007; Beliën et al. 2022).

In general, the evidence from a recent study suggests that CFA possesses confounding effects and its exclusion from immunization regimens makes the EAE model more valuable for multiple sclerosis studies.

Table 1. Summary of differences between SCH+CFA and SCH-immunized rats.

SCH+CFA	SCH
↑ Immune response in the draining lymph nodes ↑ Absolute number of Th1, Th17, Treg, macrophages, B cells, CD8+ cells in the draining lymph nodes	↑ Cumulative clinical score ↑ Infiltrates in the CNS ↑ MBP-specific antigen response ↑ CD8+ cells in the CNS

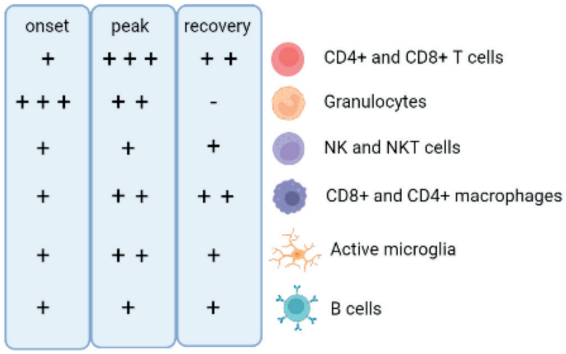

Fig. 1. Schematic presentation of SCIC cell composition at the onset, peak, and recovery of CFA-free EAE.

CONCLUSIONS

Our recent research makes several noteworthy contributions to promoting the CFA-free EAE in DA rats as a suitable animal model for the studies of multiple sclerosis (Lazarević et al. 2021). Here, we have shown that CFA is a confounding element in EAE because of its properties to skew autoimmune response. In the CFA-free model, we demonstrated a stronger MBP-specific antigen response compared to the SCH+CFA model. Also, the presence of CD8+ and CD4+ macrophages in the CNS during EAE is an advantage of the CFA-free model since these cell populations are present in humans and absent in mice. Additionally, this animal model is reliable, low cost, and highly reproducible. Moreover, by excluding CFA from immunization, animals do not develop pain or ulcers at the site of immunization. However, several limitations of the recent study need to be acknowledged. This model does not fully resemble the relapsing-remitting course of disease seen in MS patients. Nevertheless, this could be overcome with the increased number of animals used in the experiments. Although we confirmed the presence of CD8+ T cells in the CNS with or without CFA, it seems that CD8+ T cells do not participate in inflammation in a way they do in the MS. CD8+ T cell-induced brain inflammation appears to be absent in our model, but further studies should resolve this assumption. However, it has been challenging to elicit a pathogenic CD8+ T-cell autoimmune response by active immunization (Lassmann and Bradl 2017). Still, the observed abundance of CD8+ T cells in the CNS at the onset of EAE should be worthy of investigation regarding its subsequent influence on inflammation. Besides, our further research will investigate the reactivity of T cells to the CNS antigens other than the MBP, such as MOG and PLP. It would be interesting to examine both CD4+ and CD8+ T cell reactivity in our model. A greater focus on CD8+ and CD4+ macrophages could produce interesting findings that account more for their role in EAE and MS pathogenesis. As a large and growing body of literature emphasizes the vital role of the gut microbiota and gut immune cells in MS pathogenesis (Schepici et al. 2019), our ongoing studies aim to determine if CFA in DA rats influences gut microbiota and gut immune cells and how it is related to the disease. More research regarding the role of NK cells, B, and CD4+CD8+ T cells in the EAE pathogenesis would also be worthwhile. To conclude, data presented in our recent study promote the CFA-free SCH-induced EAE model in DA rats as a preferential non-primate animal model for studying multiple sclerosis.

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