

**The influence of common infections on clinical course
and neurodegeneration in an animal model of
Multiple Sclerosis**

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Dedicated to my mother...!

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ABSTRACT

The course of Multiple Sclerosis (MS), a chronic autoimmune disease, can be influenced by systemic infections. The common notion is that bacterial infections worsen disease symptoms and lead to enhanced neurodegeneration. However, the underlying biological mechanisms by which bacterial infections may lead to neurodegeneration in MS are complex and poorly understood. Here, we assessed the disease-modulating effect of an acute *Escherichia coli* and a chronic *Staphylococcus aureus* infection on the clinical course and the extent of neurodegeneration in experimental autoimmune encephalomyelitis (EAE), an animal model of MS.

In this study, we did not observe any disease modulating effect of acute *E. coli* infection on EAE when infection was induced in both the preclinical and in the clinical phase of the disease. However, we observed that local application of *S. aureus* in the preclinical phase of the disease induced a chronic systemic inflammatory response with increased T- and B-cell counts as well as systemic production of pro-inflammatory cytokines. Unexpectedly, the infection with *S. aureus* completely prevented the development of clinical EAE. Moreover, it markedly decreased the extent of inflammatory infiltrates and demyelination of the optic nerve and increased the number of surviving retinal neurons. Using a *S. aureus* strain deficient for the extracellular adherence protein (Eap) we identified this protein to be at least partly responsible for the inhibitory effect of *S. aureus* infection on autoimmune inflammation of the central nervous system. Thus our results show for the first time a beneficial effect of a chronic bacterial infection on neurodegeneration in EAE and open the avenue for the design of new neuroprotective therapy in MS.

1. INTRODUCTION

1.1: Multiple sclerosis: General background

Multiple sclerosis (MS) is an autoimmune, inflammatory, demyelinating disease of the central nervous system (CNS), which often manifests with optic neuritis (Hickman *et al.*, 2002). MS was first described in detail by Dr. Jean-Martin Charcot in 1866 and called it as “sclerose en plaques” (Compston, A. 1998). The main target of damage in MS is the myelin sheaths which are wrapped around the neurons and make efficient conduction of nerve impulse. During the disease course, there is a destruction of the myelin sheath which may lead to the impairment of impulse along the axons which further cause neurological abnormalities and typically affect motor, sensory and visual functions (Compston and Coles, 2008).

It is estimated that about 2.3 million people are affected by MS and the number of patients are increasing over the time (MS Atlas 2013). MS is present worldwide, but the prevalence of MS varies in different parts of the world. Earlier studies have indicated that the incidence of the disease increased with the distance from the equator (Kurtzke, 2005; Miller *et al.*, 1990; Simpson *et al.*, 2011). The highest prevalence of MS is seen in people from North America and Europe (>100/100 000) and it is rare amongst Asian and African (2/100 000) (MS Atlas 2013). However, the prevalence of MS also varies considerably within regions (Compston and Coles, 2008).

MS is the most common neurological disease of young adults and females are twice more affected than males (Alonso and Hernan, 2008; Rezaali *et al.*, 2013). Although with lower incidence rate of MS in male, they show slower recovery and show more aggressive nature of the disease course than females (Koutsis *et al.*, 2010; Leibowitz and Alter, 1970; Runmarker and Andersen, 1993; Weinshenker *et al.*, 1991). Due to early onset, the disease has a huge impact on the socioeconomic status of an individual. The majority of MS patients are diagnosed between the age of 20 and 50 years (National MS society). The first clinical symptoms can include fatigue, headache, limb weakness, vision abnormalities, impairment of bladder, depression and problems

with balance and coordination (Schneider, 2005). Clinical onset of disease is characterized by the neurological relapses followed by remission and this type of disease is referred as relapsing-remitting type of MS (RRMS) and found amongst 80 - 85% of MS patients (Gold *et al.*, 2010; Rovaris *et al.*, 2006). RRMS patients are clinically stable between the relapse for several years. However, there is an increasing evidence to suggest that the axonal and neuronal loss occurs in the early phase of MS (Kornek and Lassmann, 1999; Trapp *et al.*, 1998; Trapp *et al.*, 1999). The majority of RRMS patients further develop secondary progressive MS, (SPMS) where patients show axonal loss and neurological deficit without relapses (Fitzner and Simons, 2010; Rovaris *et al.*, 2006). This could lead permanent cause of neurological disability of MS patients. Approximately 10-15% of patients has no remitting phase and show progressive worsening of the disease from the start and called as primary progressive MS, (PPMS) (Andersson *et al.*, 1999; Miller and Leary, 2007). Recent advances in magnetic resonance imaging techniques improved all over the world which added the value in the diagnosis of MS patient by characterizing the lesions size and locations directly into the white matter of the brain in very early stage of disease (Patsopoulos *et al.*, 2011). Interferon-beta (IFN- β), glatiramer acetate (GA), and natalizumab, are some common medication used for the treatment of MS. Since 2010 fingolimod (FTY 720) was approved for the treatment of MS as a first oral drug. These drugs slow down the progression of disease, but they could not prevent the neuronal cell loss and in the consequences of this ongoing progression of neuronal loss patients develop the long term disability. Therefore there is a need to design of new neuro-protective therapy in MS.

1.2: Pathogenesis and pathology

MS predominantly assumed to be a T-cell mediated autoimmune disease (Wekerle, 2008) involving both CD4⁺ T helper-cell and CD8⁺ T cytotoxic-cell (McFarland and Martin, 2007). In the recent years the role of B-cells is also emphasized in MS pathogenesis (Hirotsani *et al.*, 2010). Although the sequential event taking place in the pathogenesis of MS is hardly known, but activation of auto reactive T-cells is supposed to be the initial step in the disease pathogenesis (Markovic-Plese *et al.*, 2004).

However, factors which trigger this phenomenon are not exactly known. From several clinical studies myelin reactive T-cells has been shown to be present in every individual. But these myelin-reactive T-cells, reactive to myelin basic protein (MBP), were found to be significantly higher in MS patients compared to healthy controls (Zhang *et al.*, 1994). Thus it is presumed that some environmental factors, especially the microbial antigens, which probably supposed to cross-activate T-cells specific for myelin epitopes through a mechanism called molecular mimicry in susceptible individuals (Wucherpfennig and Strominger, 1995). During the activation cascade T-cell receptor present on T-cell recognizes and binds to the myelin specific antigen present on major histocompatibility complex (MHC) of an antigen-presenting cell (APC) which delivers co-stimulatory signal leading to activation of myelin specific T-cells (Fig 1.1). These activated T-cells cross the blood-brain barrier (BBB), a physical barrier formed by the endothelial cells that form the walls of the capillaries, and enter into the CNS.

The BBB maintains highly controlled microenvironment of the CNS by regulating several functions across the CNS like passive diffusion of nutrition, maintenance ionic balance, prevention of macromolecules and neurotoxins to enter into the CNS. During several neurological disorders and in traumatic brain injury the BBB is breached, which allows the inflammatory cells to enter into the CNS and impair the neuronal function (Rosenberg, 2012). The interactions between the adhesion- and signaling-molecules present on leukocyte and endothelial cells lining the BBB leads to the recruitment of pro-inflammatory cells to the CNS (Rossi *et al.*, 2011). That includes the involvement of cytokines, chemokines and adhesion molecules expressed by both the activated endothelium and the immune cells, which especially involve the very late antigen-4 - Vascular cell adhesion molecule-1 (VLA-4 - VCAM-1) and the lymphocyte function-associated antigen-1-intercellular adhesion molecule-1 (LFA-1 - ICAM-1) adhesion systems (Xie *et al.*, 2006). Monoclonal antibody like natalizumab is in therapeutic use for MS, which targets interaction between VLA-4 - VCAM-1, and subsequently block the migration of activated T-cells across the BBB and reduces the severity of disease. Although of a majority of T-cells are crossing the BBB primarily through VLA-4 - VCAM-1 adhesion pathway, but the LFA-1 - ICAM-1 also plays an important role in downstream to this pathway.

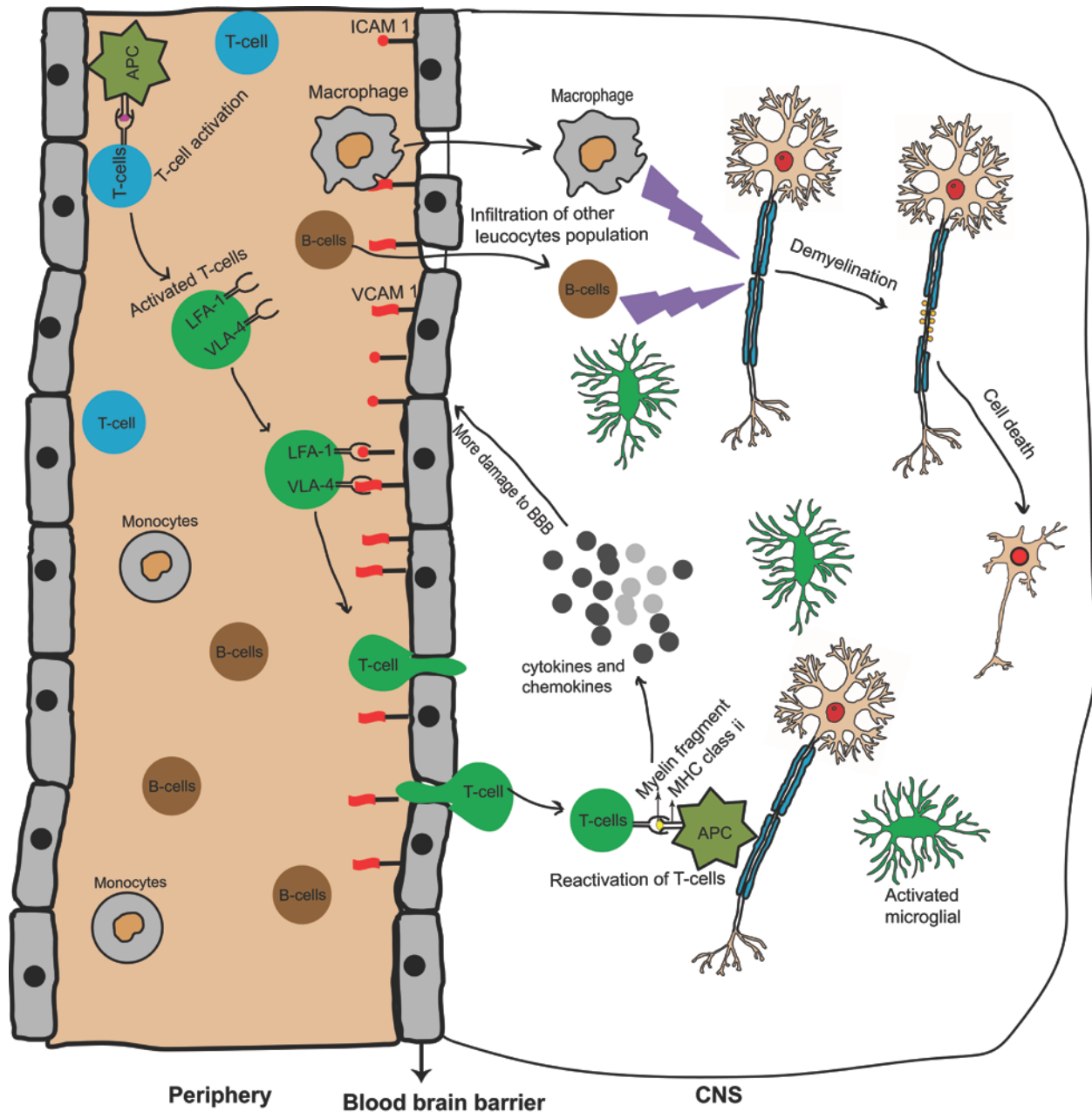


Fig 1.1: Immunopathogenesis of MS: Initial activation of T-cells takes place in the periphery by antigen presenting cells (APCs) followed by transendothelial migration of activated T-cells across the blood-brain barrier by use of different adhesion system. After crossing the BBB these cells undergo reactivation within the CNS and induce the release of several pro-inflammatory cytokines, which further cause damage to the BBB. The damaged BBB allows the other blood cells to enter into the CNS. That together with other brain cells contribute to the damage of the myelin sheath and ultimately to the death of the neuronal cell bodies.

It has been also suggested that, the integrity of the BBB could be protected by blocking the infiltration of inflammatory cell recruitment secondary to the activated T-cells (Xie *et al.*, 2006). Several studies indicated the role of ICAM in pathogenesis of MS (Bullard *et al.*, 2007; Steiner *et al.*, 2010). Eventually there is no current therapy targeting the LFA-1 - ICAM-1 adhesion pathway. However, some microbial products like extracellular adhesion protein (Eap), secreted from *staphylococcus aureus* (*S. aureus*) have been shown to have potential which specifically targeted LFA-1 - ICAM-1 but not VLA-4 - VCAM-1 mediated T-cell recruitment at the BBB in an animal model of MS (Xie *et al.*, 2006).

After crossing the BBB, T-cells get reactivated by antigen presented on resident brain antigen presenting cells (APCs), which triggers the release of pro-inflammatory cytokines (Th1 cytokine profile) like Interleukin (IL)-1, IL-2, IL-6, IL-12, IFN-gamma and TNF- α that further disrupt the BBB and resulting in a massive infiltration of other inflammatory cells from the peripheral blood circulation. The inflammatory cells further destroy the myelin sheath of the neurons and subsequently cause neurodegeneration and plaque formation in the CNS (Fig 1.1).

Since multiple sclerosis is clinically, genetically and pathologically heterogeneous disease, one can assume that more than one pathogenic mechanism leading to the disease progression. It has been shown that axonal injury and neuronal cell loss occurs in the early phase of MS lesion formation and is most pronounced in active demyelinating phase of the disease (Kuhlmann *et al.*, 2002). This slow and continuous axonal destruction may account more to the global axonal loss in MS.

A pathological hallmark of MS is multiple focal areas of myelin loss called plaques, especially in the white matter of the CNS (Ge, Y. 2006). Inflammation, demyelination, oligodendrocyte injury, astrogliosis, neurodegeneration and axonal loss are the main factors associated with the plaque formation. MS plaques, may develop anywhere in the CNS, but most frequently affected areas are the optic nerves, spinal cord, brainstem, peri-ventricular white matter regions and cerebellum (Popescu and Lucchinetti, 2012). Plaques can be in acute or in chronic form that depends on the degree of inflammation present within the plaques. Acute plaques are more common in

patient with RRMS, which shows robust inflammatory infiltration with lymphocytes (predominantly T-cell) macrophages and distributed throughout the lesions and shows parenchymal edema (Frohman *et al.*, 2006) that can be detected by gadolinium enhancement on MRI. Moreover, these plaques also contain a considerable number of oligodendrocyte precursor cells, which probably help the CNS in the restoration of myelin (Wolswijk, 1998).

Once the inflammation in the acute plaque resolves in the brain or spinal cord it forms scars and turned into a chronic form of plaque and accumulates throughout the disease course. Chronic plaques are more common in patients with progressive MS (Kutzelnigg and Lassmann, 2005). These plaques show complete demyelination, substantial loss of oligodendrocytes and axons, astrogliosis and less infiltration of lymphocytes and macrophages (Popescu and Lucchinetti, 2012).

1.3: Genetics and role of infection

The exact cause of this highly complex disease is still unknown. It has been suggested that both genetic and environmental factors are the two major determinants which contribute to the disease susceptibility. However, the precise impact of these factors is not very clear so far and that can be either harmful or protective.

Demographic studies and data from the epidemiology suggest a strong influence of genetic factors in pathogenesis of MS (Barcellos *et al.*, 2006; Chataway *et al.*, 2001; Dyment *et al.*, 1997; Sawcer *et al.*, 2011; Weinshenker, 1994). Human leukocyte antigen (HLA) locus, present on the short arm of chromosome 6, in major histocompatibility complex (MHC) region was discovered as a first potential genetic risk factor associated with the pathogenesis of MS in 1970s (Munoz-Culla *et al.*, 2013). These genes encode the cell surface glycoproteins, which are the key components of the immune system. Since 1970s a great deal of effort has been made to understand the functional and mechanistic role of the genes present in this locus (McElroy and Oksenberg, 2011). From a collaborative effort of Wellcome Trust Case Control Consortium 2 and International Multiple Sclerosis Genetics Consortium, containing large data set, it has been shown that HLA DRB1*15: 01 loci have the strongest association

with multiple sclerosis and also identified 29 novel susceptibility loci (Sawcer *et al.*, 2011). Even though with the strong genetic evidences it is also noticed that in 75% of the population only one sibling getting affected with MS which signifies the role of additional factors which play an important role in the pathogenesis of MS (Ebers *et al.*, 1986; Sadovnick, 1993; Sadovnick *et al.*, 2009).

The idea of MS pathogenesis and the role of infection came in late 1800s by Pierre Marie, and since then a number of different pathogens have been associated and then discarded due to lack of evidences. Many clinical studies have shown that common bacterial and viral infections that cause respiratory infections and urinary tract infections may alter the immune status of an individual. However, in regards to MS and its animal model, it is observed previously that infections with these bacteria and viruses significantly influence the course and severity of MS and experimental autoimmune encephalomyelitis (EAE) especially immediately after viral and bacterial infections (Tauber *et al.*, 2007).

Epstein Barr virus (EBV) infection is most promising candidates, which are gaining support for being involved in MS pathogenesis. The link between EBV and MS was come in picture from a study of Warner and Carp in 1981, who suggested that the young adults are at highest risk of acquiring MS where the chances of infectious mononucleosis are at most (Warner and Carp, 1981). EBV infection occurs in over 90% of the general population, but in an epidemiological study conducted in the past 30 years revealed that MS patients are almost universally, seropositive for EBV infection (Pender, 2011). Antibodies against EBV antigens particularly, Epstein-Barr nuclear antigen-1 was detected in the cerebrospinal fluid (CSF) of the MS patients in significantly higher amount compared to the control (13% of controls against 85% of MS patients, $p < 0.001$) (Bray *et al.*, 1992; Cepok *et al.*, 2005; Jaquier *et al.*, 2010; Rand *et al.*, 2000). There are several other viral studies which are linked to the etiology of MS like human herpesvirus-6 (Challoner *et al.*, 1995; Kim *et al.*, 2000; Opsahl and Kennedy, 2005; Panitch, 1994), adenoviruses (Andersen *et al.*, 1993), picornavirus (Kriesel *et al.*, 2004), varicella-zoster virus (Mancuso *et al.*, 2007; Ordonez *et al.*, 2004) and torque teno virus (Sospedra *et al.*, 2005).

Even though viral particle can cause the infection of the brain directly, but in most of the studies researchers could not find presence of viral particle in the brain. Moreover, several patients have been documented until now and they were sero-negative for these viruses. Hence these results suggest that these viruses are the strong risk factors for the pathogenesis of MS rather than a direct cause for acquiring this disease.

Earlier studies with bacterial infection and MS were not given importance due to development of thrust (Sibley and Foley, 1965). However, in recent time, studies with the animal model and MS patient shows that bacterial infection may lead to exacerbations in MS (Buljevac *et al.*, 2002; Herrmann *et al.*, 2006). The possible explanation for these exacerbations is that, an infection may increase the inflammatory activity in the peripheral vascular system which may lead to more sustained neurological damage over a period of time. Thus these bacterial components appear to play an important role in MS pathogenesis and were found also in the CNS of MS patients (Schrijver *et al.*, 2001). In a study by Sriram and colleagues in 1999, it was observed that 97% of CFS samples from MS patients were found positive for *Chlamydia pneumoniae* (*C. pneumoniae*) major outer membrane protein gene against 18% of control. Moreover, from the culture of the CSF they showed the presence of *C. pneumoniae* in 64% of the MS patients against 11% from control patients. In contrast, some bacterial component seems to prevent the EAE (Ben-Nun *et al.*, 1995; Ben-Nun and Yossefi, 1992).

In the last few decades with the increase in the sanitary condition in western countries showed a tremendous increase in the incidence of many immune disorders including MS (Bach, 2005). Epidemiological evidence indicates a strong correlation between improved sanitation and an increase in the number of patients with such immune disorders (Fig 1.2) (Bach, 2002; Christen and von Herrath, 2005). Hence this increase in the immune disorder against high sanitation level gives rise to new hypothesis known as hygiene hypothesis, first proposed by Strachan for allergic disorders (Strachan, 1989) and now correlates well with several other autoimmune disorders. According to the hygiene hypothesis, the children grows in the highly hygienic environments have negative effect on their immune development which may lead to an

in the incidence of autoimmune disorders like MS (Fleming and Cook, 2006; Leibowitz *et al.*, 1966). This finding also supported by migration studies which indicate that moving from a high- to a low-risk area in childhood reduces the risk of MS, while moving during late adulthood has no impact on susceptibility of MS. Migration in the opposite direction, does not seem to increase the MS risk substantially until the next generation (Ascherio and Munger, 2007; Gale and Martyn, 1995; McLeod *et al.*, 2012; Orton *et al.*, 2010).

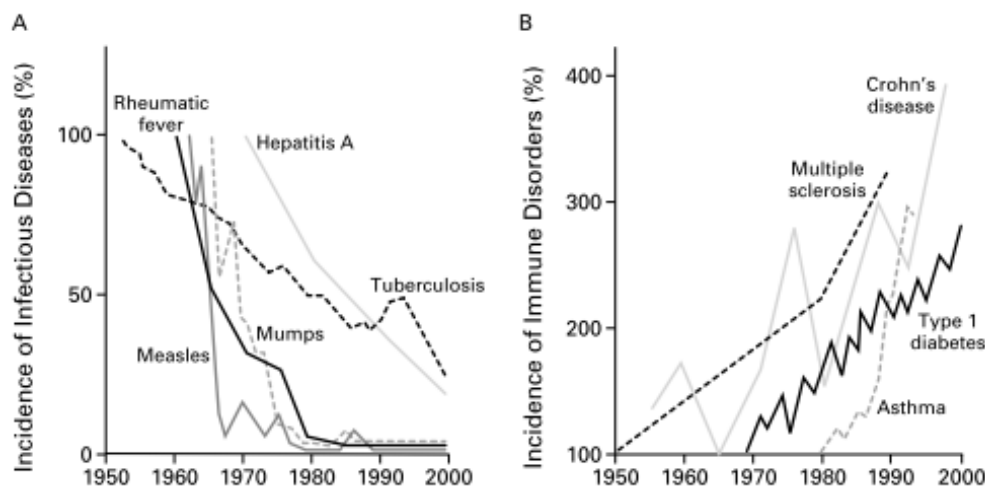


Fig 1.2: Infectious disease and autoimmune disorder: Epidemiological data indicate an inverse relation between the Incidence Infectious Diseases **(A)** and the Incidence of Immune Disorders **(B)** from 1950 to 2000 (Bach, 2002).

These data suggest that an environmental factor, like infection in childhood is important, to determine MS risk. The possible mechanism behind this protection might be that, infection may shift the immunological balance toward a more immune-suppressed state or infection may cause hyper activation of auto reactive cells which possibly lead to clonal deletion and ultimately reduces the number of aggressive cells. Recent epidemiological investigations with *Trichuris trichiura*, a common human pathogen, indicate that MS prevalence appears to fall sharply once a critical threshold of *Trichuris trichiura* is exceeded (Fleming and Cook, 2006). These studies in humans are validated by investigating the impact of some other parasites in animal models of MS. In one such study animals were pre-infected with the ova of *Schistosoma mansoni* which results in either attenuation or delayed onset of EAE (La Flamme *et al.*, 2003). Studies also indicate that the infection with the ova of *Schistosoma mansoni*

significantly down regulates the induction of some pro-inflammatory cytokine such as IL-12, TNF-alpha (TNF- α), IFN-gamma and express more anti-inflammatory cytokine like IL-10 and TGF-beta. Moreover, these studies also indicate the role of regulatory T- and B-cells in the disease onset. Although lots of research are ongoing on the parasitic infection and viral infection to their relation to the autoimmune diseases like MS, but eventually the role of bacterial infection and their relation to the MS still need to be explored.

1.4: Diagnosis and therapy

A careful medical history and a thorough neurological examination form the basis for treatment of MS patients. There are some defined criteria made in the diagnosis of MS which is changed and modified over time. First diagnostic criteria used for MS was proposed by Schumacher in 1956 (Ross *et al.*, 1965) which were updated by Poser criteria in early 1980s. Poser criteria first emphasized the use of MRI for diagnosis of MS patient. This criteria also included the analysis of cerebro-spinal fluid analysis (CSF) examination (Poser *et al.*, 1983). The Poser criteria was used for several years and updated by "McDonald Criteria" in 2001 (McDonald *et al.*, 2001) and revised time to time. The latest revision of McDonald criteria was made in 2010 by (Polman *et al.*, 2011). According to the latest revision of this criteria if a patient has had more than two attacks, or having more than two lesions, or one lesion but with sufficient evidence of a prior attack, can be diagnosed, if the patient does not fulfill these criteria the further attack is needed for the secure diagnosis (Polman *et al.*, 2011). The McDonald criteria make use of advancement in MRI techniques which allows the diagnosis to be accurate at earlier stage of disease, and therefore offer the potential for earlier treatment. The expanded disability status scale (EDSS) is used worldwide in clinics to evaluate the disease progression in MS, where higher EDSS scores indicate more disability. Since 2001 McDonald criteria made the foundation to diagnose MS in most of the countries.

Over the last 20 year, several therapeutic agents have been studied and tested for the MS treatment. However, immune suppressive therapy becomes the first choice for the attenuation the disease progression probably due to inflammatory nature of this

disease. The first line treatment consists of interferon- β , which is either IFN β -1a or IFN β -1b and glatiramer acetate (GA) for the RRMS.

IFN- β treatment is administered sub-cutaneously showed a reduction in the relapse rate by 30% and reduce lesion load (Jacobs *et al.*, 1996; Jacobs *et al.*, 1995). Although the exact mechanism of action is not clearly understood until now, but it was suggested from several studies that INF- β therapy reduces the expression of MHC class II on APCs, reduces proliferation of T-lymphocytes and the production of anti-inflammatory cytokines (Dhib-Jalbut and Marks, 2010; Genc *et al.*, 1997; Jiang *et al.*, 1995; Teleshova *et al.*, 2000), increases cytotoxic T-lymphocyte and Fas surface molecule on CD4⁺ cells and promote apoptosis of aggressive cells (Hallal-Longo *et al.*, 2007). Other disease modifying drugs heavily in use is glatiramer acetate (GA), a four-amino acid synthetic copolymer administered sub-cutaneously and have been shown to reduce the relapse rate by 29% (Johnson *et al.*, 1995). The precise mechanism of action of GA is not clearly known, but several studies indicate that GA leads to an inhibition of the antigen-specific T-lymphocyte activation by binds directly to the portion of the APC. Due to lower efficacy of these drugs, escalation therapy for the treatment MS was introduced which primarily includes natalizumab (humanized monoclonal antibody) and fingolimod (sphingosine-1 phosphate receptor agonist). Natalizumab targets a component of the VLA-4, α 4-integrin molecule which is present on lymphocytes thus prevent the interaction with VCAM-1 found on endothelial surfaces and subsequently blocks the infiltration of these lymphocytes across the BBB which results in reduced inflammation of the CNS. Due to a very high efficacy (Miller *et al.*, 2007; Polman *et al.*, 2006) and severe side effect of natalizumab (Bloomgren *et al.*, 2012; Clifford *et al.*, 2010), several other monoclonal antibody directed therapy (Alemtuzumab, Ocrelizumab) is under trial for MS treatment. A sphingosine-1 phosphate receptor agonist, Fingolimod (FTY720), which blocks leukocyte trafficking in lymph nodes, is the first oral disease-modifying treatment available for relapsing MS and reduces the relapse rate by 54% and shows a significant decline in MRI lesion (Kappos *et al.*, 2010). Mitoxantrone an anthracenedione cytotoxic agent can cross the disrupted BBB and inhibits DNA replication and reduces lymphocyte proliferation. Although mitoxantrone is effective in MS treatment, it remains second line for RRMS due to serious adverse

effects. The role of B-cell has been explored in the MS and several therapies are on trial now a day's targeting B-cell populations and such hypothesis leads to the development of Rituximab and Ocrelizumab (van Oers *et al.*, 2010; Zhou *et al.*, 2008).

Although in the last few years there is a huge intensification of MS therapy and have shown very high efficacy, especially in the RR form of MS but none of the marketed anti-inflammatory therapies are approved for PPMS or SPMS (Cree, 2014). Moreover, there is no treatment option available, which makes the neurological impairment reversed. More studies are needed to develop therapies which could control neurodegeneration, which is the main cause of permanent neurological disability.

1.5: Experimental autoimmune encephalomyelitis

Since the CNS is hardly accessible in humans for molecular and biochemical studies, therefore to assess the impact of a treatment or infection on MS various animal models have been used so far. These experimental animal models have provided important information in our understanding of various human autoimmune diseases which allow us to investigate the influence environmental and genetic factors associated with the disease progression. These animal models also provide a different experimental therapeutic approach to test the impact of a particular drug before going to trial in human.

Although none of the animal models replicates all the immunological and histopathological aspects of MS. However, experimental autoimmune encephalomyelitis (EAE) is one of the most commonly used models of MS (Wekerle *et al.*, 1994) and shows several immunological and histopathological aspects of MS. EAE is an inflammatory autoimmune disease of CNS clinically manifested by the development of a tail and limb paralysis which reflect the histopathological resemblance to MS (Goverman *et al.*, 1993; Swanborg, 1995). Depending on the strain of the animals an EAE can be induced in animals models by immunization with various myelin proteins like proteolipid protein (PLP), myelin oligodendrocyte glycoprotein and myelin basic protein (MBP) along with the adjuvants (Gold *et al.*, 2006). The most commonly used adjuvant is Freund's adjuvant which contains mineral oil together with heat killed *Mycobacterium*

tuberculosis. To induce EAE, pertussis toxin is also used in combination with myelin proteins several animal model. The EAE can also be induced by adoptive transfer of myelin specific activated T-cells (Baxter, 2007; Zamvil and Steinman, 1990).

MOG is a myelin surface antigen, highly conserved between species (Gardinier *et al.*, 1992; Kroepfl *et al.*, 1996) and present on the outer lamella of the myelin sheath and is recognized by activated T-lymphocytes and demyelinating antibodies. The precise biological function of MOG is not known, although it has been speculated that it has an adhesive function between myelin sheets and provides microtubule stability in oligodendrocytes. Immunization with recombinant myelin oligodendrocyte glycoprotein leads to the activation of MOG specific T-cells, which further reaches to the secondary lymph nodes where these cells get proliferated and migrated to the CNS by crossing the BBB (Cannella *et al.*, 1990). Migration of these activated T-cells cause autoimmune inflammation of the CNS and release of pro-inflammatory cytokine which induces more damage to the BBB. The breakage of BBB allows other peripheral blood cells like B-cells, monocytes and macrophages to enter into the CNS, which primarily attack the myelin sheath of neuron (Traugott *et al.*, 1986).

In this thesis dissertation, we have used MOG (extracellular domain of rat MOG amino acid sequence 1-125) immunized female Brown Norway (BN) rats as an animal model. After an active immunization with MOG this animal model shows severe optic neuritis with acute axonal degeneration of the optic nerve (ON) and consecutive apoptosis of retinal ganglion cells (RGCs) in more than 90% of the rat regardless of a clinical manifestation of the disease (Hobom *et al.*, 2004; Maier *et al.*, 2006; Meyer *et al.*, 2001). Therefore, this EAE model provides a unique tool to investigate the morphological changes in neuronal cell bodies and in axons.

1.6: Infection models

Epidemiological studies suggest that MS is greatly influenced by environmental factors like infection. The role of viruses in MS etiology is highly convincing. Moreover, it is also shown that bacterial components like enterotoxins, LPS and peptidoglycans are able to influence the course of EAE (Moreno *et al.*, 2011; Schiffenbauer *et al.*, 1993; Soos *et*

al., 1995). But the role of common bacterial infections has hardly been explored which reflect the true clinical situation. Although in most studies, the effects of acute infection were examined, but in the best of my knowledge the impact of chronic bacterial infection was never been evaluated which more closely mimic the clinical situation in humans. Therefore in this thesis dissertation we evaluated the impact of both acute and chronic infections in MOG immunized female BN rat.

1.6.1: Acute infection model

Despite of strong stimulatory effect of *S. aureus* enterotoxins on human (Langford *et al.*, 1978; Peavy *et al.*, 1970) and mouse (Janeway *et al.*, 1989; White *et al.*, 1989) T-lymphocyte subsets, it was unable to stimulate proliferative responses of rat T-lymphocytes in a dose dependent manner (Ben-Nun and Yossefi, 1992). The studies with these bacterial components induce different responses towards different animal models. Such studies cannot be correlated with the normal infectious condition in MS or EAE.

Du and coworkers (2002) showed in mice that intraperitoneal infection with *C. pneumoniae* can aggravate symptoms of EAE. This effect is likely to occur, since infection of the CNS increases the auto-reactive lymphocyte pool, hence influenced the development of autoimmune disease. However, in the same study infection with *Chlamydia trachomatis* did not influence the clinical course of disease. This study indicates that a CNS infection would thus essential for a worsening of EAE and it is also likely possible that the impact of infection on EAE varies with infection with different microorganisms. Hermann and colleagues in 2006 did an experiment where they infected the animals with *Streptococcus pneumoniae* at different time point after immunization in EAE. They observed that infection was able to exacerbate EAE only in case when the infection was made on day 7 post immunization (early asymptomatic phase). However, they did not observe any influence of infection on EAE when infection was made on 2 days prior to immunization or during the late phase of the disease (day 21 pi). This study not only indicates the influences of infection on EAE rather the importance of infection induced at different time points during the course of the disease

are equally important. Furthermore, they also observed that bacterial infection enhances the release of pro-inflammatory cytokines in peripheral blood circulation. As indicated in previous studies that TNF- α (Murphy *et al.*, 2002; Ruddle *et al.*, 1990) alpha and IL-6 (Samoilova *et al.*, 1998) plays an important role in worsening of EAE. The increase in the level of TNF- α and IL-6 was also observed in the study of Herman *et al.*

Research evidences over a period of time also suggested that infectious agents may play a protective role in autoimmune diseases like MS. The inhibitory effect of bacterial infection in EAE and MS has also been observed (Ben-Nun *et al.*, 1995; Ben-Nun and Yossefi, 1992). Ben-Nun and colleagues has shown that the SJL/J mice pre-exposed to *Mycobacterium tuberculosis* (Mt) and some other bacterium, developed long-lasting resistance to EAE and also identified a 12-kDa protein fraction, belongs to heat shock protein family, to be responsible for this protective effect. They also speculated that the protection in these mice from EAE arises due to the result of similar T-cell epitopes between mycobacterial heat shock protein and different myelin proteins. Moreover, some role of parasitic infection also has proven protective in EAE, which generally shift the immunological balance towards Th₂-cell type. From the above research evidences it could be speculated that infections increases the natural propensity of these animal models, hence they developed resistant towards an autoimmune disease.

To induce a mild infection and to increase the survival of animals after infection, they are generally treated with antibiotics. Antibiotic treatment seems to affect the EAE in both ways, some antibiotics seems to be protective in EAE while some are not (Wang *et al.*, 2009). The precise mechanism of how antibiotic treatment influences EAE is not clear so far. Through a short term treatment in any case seems to have no direct neuroprotective effect in the EAE (Herrmann *et al.*, 2006). However, antibiotic treatment like ceftriaxone may cause rapid degradation of bacterial components which may probably do not reflect the normal infectious condition. Therefore the impact of infection in animal models should be evaluated without intervention of any antibiotic treatment. Hence for that reasons, chronic infection model without any antibiotic treatment would be more appropriate to evaluated impact of infection in EAE.

1.6.2: Chronic infection model

Infections in prostheses lead to serious complications. This is due to the formation of a bacterial biofilm, antibiotic tolerance and an altered activity of phagocytic cells associated with the foreign body. The main causative agent for this is *S. aureus*. Therefore, by taking advantage of this microorganism Werner Zimmerli and co-workers in 1982 established an experimental model chronic foreign body infection in guinea pig to study pathogenesis of foreign body Infection (Zimmerli *et al.*, 1982). Later on Lucet *et al* established this model in Wistar rat. A chronic infection is achieved by implantation of a foreign body, in which the bacteria can adhere (Lucet *et al.*, 1990; Murillo *et al.*, 2006). In the study of Lucet *et al* tissue cages were implanted under the skin and infected with *S. aureus* to test the effect of different antibiotics. The inoculum dosage of 10^5 - 10^6 colony forming unit (CFU) was used to establish this infection model. However, this inoculum dosage may vary with the depending on the strain of the rat.

Shapira and colleagues (2002) implanted tissue cages made of titanium wire in mice and immunized them 2 weeks later with mouse spinal cord homogenate (MSCH). Five days after immunization, animals were infected with heat killed *Porphyromonas gingivalis*, which resulted in a more severe EAE (Shapira *et al.*, 2002). They concluded that this effect is due to more peripheral inflammation. However, infection with heat killed bacterium does not represent the true infectious condition and does this infection leads to the chronic systemic inflammation was also not clear. Hence in best of my knowledge, there is no such study with the true chronic bacterial infection on an animal model of MS has been performed until now.

2. AIM OF THIS WORK

The course of Multiple Sclerosis, a chronic autoimmune disease, can be influenced by systemic infections. The common notion is that, bacterial infections worsen disease symptoms and to lead to enhanced neurodegeneration. However, the underlying biological mechanisms by which bacterial infections influence the disease course and neurodegeneration in MS are complex and poorly understood. Most of the previous experimental studies on animal model used the bacterial component to induce the systemic inflammation to mimic the situation of viable bacterial infection. Further, the Inflammation induced by the bacterial components or by a bacterial infection induces similar effects on the course of the disease has not yet proven. Furthermore, the kind of infection (acute and chronic) even more the type of bacteria used may also have varying impact on the course of disease and neurodegeneration.

So our objective in this study was

1. To investigate the influence of acute *Escherichia coli* (*E. coli*) infection on the clinical course and the extent of neurodegeneration in experimental autoimmune encephalomyelitis, when infection was induced during preclinical and clinical phase of disease
2. Establish a chronic infection model in female BN rats
3. To investigate the influence of chronic *Staphylococcus aureus* (*S. aureus*) on the clinical course and the extent of neurodegeneration in experimental autoimmune encephalomyelitis, when infection was induced in the preclinical phase of disease

3. MATERIALS AND METHODS

3.1: Materials: - Materials used in this thesis are listed in the following tables. Unless otherwise stated, materials were purchased from companies located in Germany.

3.1.1: Instruments

Cell counting chamber	
Neubauer cell chamber	Superior-Marienfeld
Centrifuges	
Allegra X 22R	Allegra
5415C	Eppendorf
5425 R	Eppendorf
Cryotome	
CM 30505	Leica
Luminex	
Bio-Plex 200	Bio-Rad
Fridge and freezers	
GNP 3656 (4°C)	Liebherr
KDR40420GB/01 (4°C)	Bosch
GSS 3626 (-20°C)	Liebherr
KSD 3142 Premium (-20°C)	Liebherr
GNP 3056 (-80°C)	Liebherr
Flat cooler	
TUC2	Pathhisto
Flow cytometer	
BD FACSCalibur	BD Biosciences

High thermal flattening table

HI 1220	Leica
Hood	
Laminar air hood	Thermo Scientific
Magnetic stirrer	
RH basic	IKA
IKAmag reo	IKA
Microscope	
Axiopaln 2	Carl Zeiss
axiovert 200	Carl Zeiss
Axiostar plus	Carl Zeiss
Stemi 2000-C	Carl Zeiss
Examiner Z1	Carl Zeiss
Microtome	
SM2000R	Leica
Pipette	
2.5 µl, 10 µl, 100 µl, 200 µl, 1 ml, 5 ml	Eppendorf
2.5 µl, 10 µl, 100 µl, 200 µl, 1ml	Gilson
50 µl, 200 µl (Multichannel)	Eppendorf
pH Meter	
PB-20	Sartorius
Vortex	
MS 3 basic	IKA
Vortex-Genie 2	IKA
Weighing balance	
BL2105	Sartorius
BL 3100	Sartorius

3.1.2: Consumables

Name	Company
Aluminium foil	Carl Roth
Blood agar plates	Department of Microbiology Göttingen
Centrifuge tubes (0.5-, 1.5-, 2-ml)	Eppendorf
Centrifuge tubes (15-50 ml)	BD Falcon
Cover slip (24*48, 24*60)	Menzel-glaser
Cuvette	Carl Roth
Filter tip (10-, 200-, 1000-,1250- μ l)	Greiner Bio One, Sarstedt
Gloves	Meditrade
Microscope slides (Superfrost plus)	Thermo Scientific
Microtome blade	Feather
Needles (28G, 23G, 27G)	BD Eclipse
Nitrile gloves	Meditrade
Nunc 96 well plates	Apogent
Para film	Pechiney Plastic Packaging
Pasture pipette bulb	Carl Roth
Petri dish 15 mm	BD Biosciences
Pipette tips (10-, 100-, 200-, 1000- μ l)	Sarstedt
Plastic wares	Vitlab
PMMA cover slip for implant (Self made)	Carl Roth
Safety needle (26G)	Sarstedt
Syringes (2 ml, 5 ml,10 ml)	BD Biosciences

3.1.3: Chemicals, miscellaneous reagents and commercial kits.

Name	Molecular formula / Abbreviations	Company
ABC-peroxidase solution		Vector Labs
Agar		MERCK
Albumin bovine serum		Sigma-Aldrich
Ammonium chloride	NH ₄ Cl	Sigma-Aldrich
Ammonia		Merck
Bio-Plex Pro™ Rat Cytokine Th1/Th2 Assay #171-K1002M		Bio-Rad
Brain-heart infusion medium		Department of Microbiology Goettingen
Bromphenol blue	C ₁₉ H ₉ Br ₄ NaO ₅ S	Fluka
Chloral hydrate	C ₂ H ₃ Cl ₃ O ₂	Fluka
Citric acid		Merk
DAB substrate Kit		Vector Labs
Disodium hydrogen phosphate	Na ₂ HPO ₄ *2H ₂ O	MERCK
Ethyl alcohol 99% pure	C ₂ H ₅ OH	Chemie-Vertrieb GmbH
Ethyl alcohol ultra pure	C ₂ H ₅ OH	Merk
Ethylenediaminetetraacetic acid	EDTA	Carl Roth
Formalin		Merck
Fluorogold		Fluorochrome
Glacial acetic acid	C ₂ H ₄ O ₂	Merk
Glucose 5%		B. Braun
Glycerin	C ₃ H ₈ O ₃	MERCK
HCl-ethanol		Merk
Mycobacterium tuberculosis (Heat inactivated) (strain H 37 RA)		Difco Laboratories
Hydrochloric acid (32%)	HCl	Carl Roth

Hydrogen peroxide 30%	H_2O_2	MERCK
Immersion oil for microscopy		Zeiss
Isopropanol	C_3H_8O	Carl Roth
Luria Broth		Fluka
Lithium carbonate	Li_2CO_3	Fluka
Myelin oligodendrocyte glycoprotein	MOG ^{lgd}	Institute of Neuropathology, Göttingen
Mount medium (IF)		Thermo Scientific
Mount medium (IHC)		Carl Roth
Nitric acid	HNO_3	J.T.Baker
Paraformaldehyde	PFA	Carl Roth
Periodic acid		Sigma-Aldrich
Potassium phosphate	K_2HPO_4	Sigma-Aldrich
Potassium chloride	KCl	MERCK
Potassium phosphate	KH_2PO_4	Sigma-Aldrich
Schiff's reagent		Sigma-Aldrich
Silver nitrate	$AgNO_3$	Carl Roth
Sodium azide	N_3Na	Sigma-Aldrich
Sodium chloride (0.9%)		B. Braun
Sodium citrate	$Na_3C_6H_5O_7 \cdot H_2O$	MERCK
Sodium thiosulfate	$Na_2O_3S_2$	Fluka
Sodium chloride	NaCl	MERCK
Sodium phosphate dibasic	Na_2HPO_4	Sigma-Aldrich
Solvent blue 38		Sigma-Aldrich
Sucrose	$C_{12}H_{22}O_{11}$	Fluka
Triton X-100		Sigma-Aldrich
Vaseline		Lever Faberge

3.1.4: Surgical instruments

Instruments	Company
Cauterizer	Fine Science Tools
Forceps	Fine Science Tools
Hamilton syringe	Hamilton Messtechnik GmbH
Hemostatic clamp	Fine Science Tools
Infuse/withdraw syringe pump (KDS 310)	KD Scientific
Light source microscope KL 1500 LCD	Schott
Micro motor (FM3545)	Freedom
Rongeurs	Fine Science Tools
Scalpel	Fine Science Tools
Scissors	Fine Science Tools
Steriotactic frame (Model 963, Ultra Precise)	Kopf
Suture	Ethicon
Teflon tube	Carl Roth

3.1.5: Pharmacological products

Product	Company	Application
Ceftriaxone	Hikmapharma	Antibiotic
Desitin	DesitinArzneimittel GmbH	Wound cream
Glucose 5%	B. Braun Melsungen	Infusion (Energy)
Isoflurane	Abbott	Inhalation Anesthesia
Ketamine	Atarost	Anesthesia
Metapyrin	Serum-Werk	Pain killer
Rimadyl	Pfizer	Painkiller
Xylazine (2%)	Albrecht	Anesthesia

3.1.6: List of primary antibodies

Antibody	Conjugate	Company	Application
CD134 (OX-40)	FITC	Biolegend	Flow-cytometry
CD3	Alexa fluor 647	Biolegend	Flow-cytometry
CD3	Biotin	Biozol	IHC
CD4	PE-Cy5	Biolegend	Flow-cytometry
CD45-RA	PE	Biolegend	Flow-cytometry
CD8	PE	Biolegend	Flow-cytometry
CD 68 (ED1)	Biotin	AbDserotec	IHC

3.1.6: List of secondary antibodies and purified protein

Antibody (species)	Company	Conjugate	Application
Goat anti-mouse IgG	Vector Lab	HRP	IHC
Goat anti-rabbit IgG	Vector Lab	HRP	IHC
Goat anti-mouse IgM	Vector Lab	HRP	IHC
Streptavidin (for biotinylated primary Ab)	Biolegend	PE-Cy5	Flow-cytometry

3.1.7: List of computer softwares

Program	Company	Application
Adobe Illustrator CS2	Adobe Systems	Image processing
Adobe Photoshop 4.0	Adobe Systems	Image processing
AxioVision	Carl Zeiss	Image processing
Definiens Developer XD software	Definiens	Image processing
FACSCalibur	BD bioscience	Flowcytometric analysis
Bio-Plex Manager 6.0	Biorad	Cytokine analysis
Office Excel	Microsoft office 2007	Data analysis
Prism	GraphPad	Data analysis
SPSS	IBM	Data analysis

3.1.8: Buffers and other stock solutions**3.1.8. A: Phosphate buffer 10x for 1liter**

Chemicals		Weight
1	Sodium chloride	80 g
2	Potassium chloride	2 g
3	Disodium hydrogen phosphate	21.6 g
4	Potassium phosphate	2 g

All the components were mixed in one liter of distilled water and pH (7.4) was adjusted by using 1 molar NaOH and HCl.

3.1.8. B: Citrate buffer

6 g Sodium citrate was added in three liter of water and pH was adjusted to 6.0.

3.1.8. C: Developer stock solution for Bielschowsky silver staining

Chemicals		Weight / Volume
1	Formalin	10 ml
2	Citric acid	0.25 g
3	6% nitric acid	2 drops
4	Distilled water	50 ml

3.1.8. D: RBC lysis buffer

5.349 grams of ammonium chloride mixed in 1 liter of PBS, pH was adjusted to 7.4.

3.1.8. E: FACS buffer

Chemicals		Weight / Volume
1	Bovine serum albumin	10 g
2	Sodium azide	1 g
3	PBS	1 liter

3.2: Methods

3.2.1: Rats

Female brown Norway rats, 8 to 10 weeks of age, weighing between 150-175 grams were used in all experiments. They were obtained from Charles River (Sulzfeld; Germany) and kept under environmentally controlled and pathogen-free conditions. They were given water and standard rodent chow ad libitum. In case animals had severe EAE they were given 5 ml of glucose and sterofudin (1:1) and softened food inside side the cage during the experiment. All experiments involving animal use were performed in accordance with the relevant laws and institutional guidelines of University Medicine Göttingen. These experiments have been approved by the local authorities of Braunschweig, Germany.

3.2.2: Immunogen

Recombinant rat MOG^{Igd}, corresponding to the N-terminal sequence of rat MOG (aa 1-125), was expressed in *E. coli* and purified to homogeneity by chelate chromatography (Weissert *et al.*, 1998). The purified protein was then dialyzed against 0.01 M sodium acetate (pH 3) to obtain a preparation that was stored at -80°C for further use.

3.2.3: Bacterial inoculum preparation

3.2.3.1: Escherichia coli

The *E. coli* (Strain k1, originally isolated from the CSF of a child with meningitis) inoculum was provided by Dr. Roland Nau, Institute of Neuropathology, University Medicine Göttingen. A single colony of *E. coli* was inoculated into Luria Broth medium. Bacteria were harvested after 4 hours of incubation at 37°C. To determine the exact concentration of bacteria, the inoculum was serially diluted in 0.9% NaCl and plated on blood agar plate (Provided by Medical Microbiology, University Medical Center Göttingen) and incubated for 24 h at 37°C. Bacterial concentrations were determined by quantitative plating on blood agar plates and aliquots were kept at -80°C until further use.

3.2.3.2: *Staphylococcus aureus*

The *S. aureus* strains ATCC 29213 (kindly provided by Raimund Lugert from the University Medicine Göttingen), Newman ATCC 25904 and the extracellular adherence protein (Eap) deficient strain of ATCC 25904, AH12 (kindly provided by Markus Bischoff from the Institute for Medical Microbiology and Hygiene, University of Saarland; Germany) were used in these experiments. A single colony of the respective *S. aureus* strain was inoculated into brain heart infusion medium and incubated for 20 hours at 37°C in a shaking incubator. Afterwards, 50 µl of the bacterial culture grown for 20 hours were inoculated into 10 ml of fresh brain heart infusion medium and again incubated at 37°C in a shaking incubator. Bacteria were harvested after 6 hours of incubation at the time when the bacterial culture reached the log phase of growth. Bacterial concentrations were determined by quantitative plating on blood agar plates, and aliquots were kept at -80°C until further use.

3.2.4: Retrograde labelling of RGCs

By retrograde axonal transport, the RGCs were selectively labeled and visualized for fluorescence microscopy. Retrograde labelling of retinal ganglion cells (RGCs) and tissue cage implantations were done during a single anaesthesia 2 to 3 weeks before the MOG-immunization. Rats were anesthetized by intraperitoneal injection of ketamine (0.95 ml/kg) and xylazine 2% (0.25 ml/kg) and positioned in a stereotaxic frame. The skin was incised mediosagittally, and holes were drilled into the skull above each superior colliculus (6.8 mm dorsal and 2 mm lateral from bregma). 2 µl of fluorescent dye fluorogold (5% in distilled water) were injected into both superior colliculi at a rate of 0.50 µl per min. For pain relief, animals received metapyrin in drinking water (3ml/L) 2 days before the start of surgical procedure, which were continued until day 8 of post surgical procedure.

3.2.5: Tissue cage preparation and implantation

Rigid polytetrafluoroethylene (Teflon) tissue cages (internal and external diameter of 8 and 10 mm respectively, length 25 mm) perforated by around 100 regularly spaced holes (diameter 1 mm) containing two polymethylmethacrylate (PMMA) cover slips per

tissue cage sealed at both ends with a suture, were used as a foreign body implant. A cutaneous incision in the left flank was made under anaesthesia, and one autoclaved tissue cage was subcutaneously implanted under sterile conditions into each animal. After fluorogold (FG) injection and tissue cage implantation, the wounds were sutured at place of implantation and given 6ml of a glucose 5% / Sterofundin solution (1:1) (1/3 intraperitoneally and 2/3 subcutaneously). After tissue cage implantation and colliculus injection animals were treated with Rimadyl 10µl / 100g / animals (analgesia) followed by metapyrin (3ml/L) in drinking water until 8 days post surgical procedure.

3.2.6: Induction of acute and chronic infection

To study the effect of acute infection in EAE animals were infected intraperitoneally with 10^6 CFU of *E. coli* in 200 µl of 0.9% saline. Control rats received an equal volume of saline. The chronic infection with *S. aureus* was induced by injection of 200 µl of bacterial culture diluted in 0.9% saline containing 2×10^7 colony forming units (CFU) into the agar of the tissue cage. Control animals received 200 µl saline into the tissue cage.

3.2.7: Experimental setup and timeline

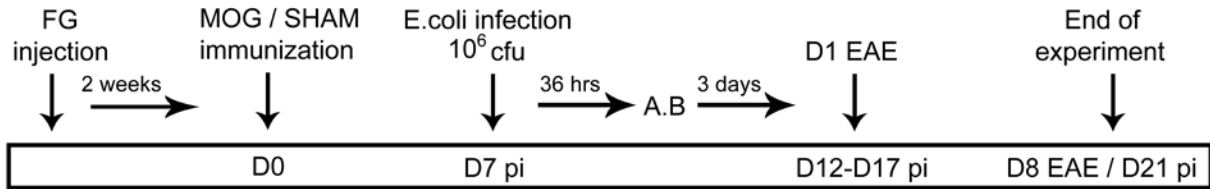
3.2.7.1: Experimental design for acute infection model

Number of animals were used in these experiments was 57. Immunized animals were randomly assigned to four different groups. Animals were infected intraperitoneally with *E. coli* either on day 7 post immunization (early group; Fig 3.2.1.A) or on day 1 of clinical manifestation of EAE (late group; Fig 3.2.1. B). Control rats received an equal volume of saline. To elucidate whether infection could shift the time point of disease onset in MOG-EAE infection was performed during asymptomatic phase of the disease (day 7 post immunization). Number of animals used was 28 (n=15, early infection; n=13, control) excluding the animals which died after *E. coli* infection or were sacrificed for ethical reasons.

To reflect the clinical situation in MS patients, animals were infected on day 1 of EAE (late group). Here we addressed the question whether the infection could aggravate the clinical symptoms of EAE. Number of animals used for this experiment

was 29 (n=15, late infection; n=14, control). Animals which either did not show clinical signs of EAE or died after *E. coli* infection were not included in the analysis of late infection group.

A. Early infection group



B. Late infection group

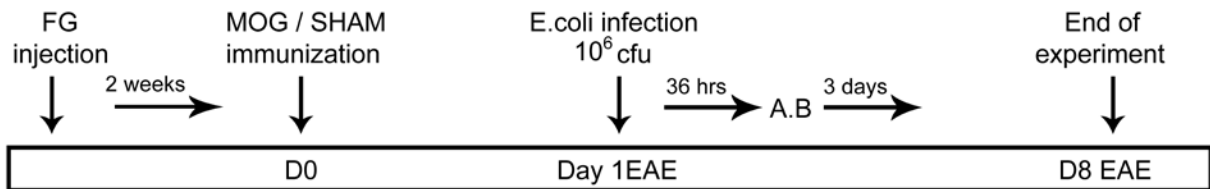


Fig 3.2.1: Experimental design. Fluorogold (FG) injection was performed 2 weeks prior to MOG immunization. **A;** In early infection group, intraperitoneal injection of *E. coli* or saline was performed on day seven post immunization followed by antibiotic treatment for three consecutive days. Day12 - day17 was the usual time point of clinical onset of disease. Animals were followed until day 8 of EAE or day 21 post immunization in case of clinically healthy animals. **B;** In late infection group intraperitoneal injection of *E. coli* or saline was performed on the day of clinical manifestation of the disease. Animals were followed until day 8 of EAE. At the end of experiment optic nerve was taken out for histopathological analysis, the retina was flat-mounted for quantification of retinal ganglion cells.

To confirm the systemic infection, blood samples were collected from the tail vein under inhalation anaesthesia with isoflurane (n=8 for each group) 24 hours after the infection in the late infection group. Plating of blood samples was performed on blood agar plates under sterile conditions. Animals were followed until day 8 after the clinical manifestation of the disease (EAE d8), or day 21 post immunization in case of absence of EAE symptoms. To ensure the survival of *E. coli* infected animals, antibiotic treatment was started 36 hours after the infection and was followed for three consecutive days at a difference of 12 hours in both the groups.

3.2.7.2: Experimental design for chronic infection model and timeline

Previously Lucet and co-workers established an experimental model for chronic foreign body infection by inoculation of 10^5 - 10^6 CFU of *S. aureus* into the implanted tissue cages of male Wistar rats (Lucet *et al.*, 1990). However, due to variation in the strain of the rat (female BN rat), with the similar setup, 10^9 CFU of *S. aureus* was insufficient to induce a chronic infection.

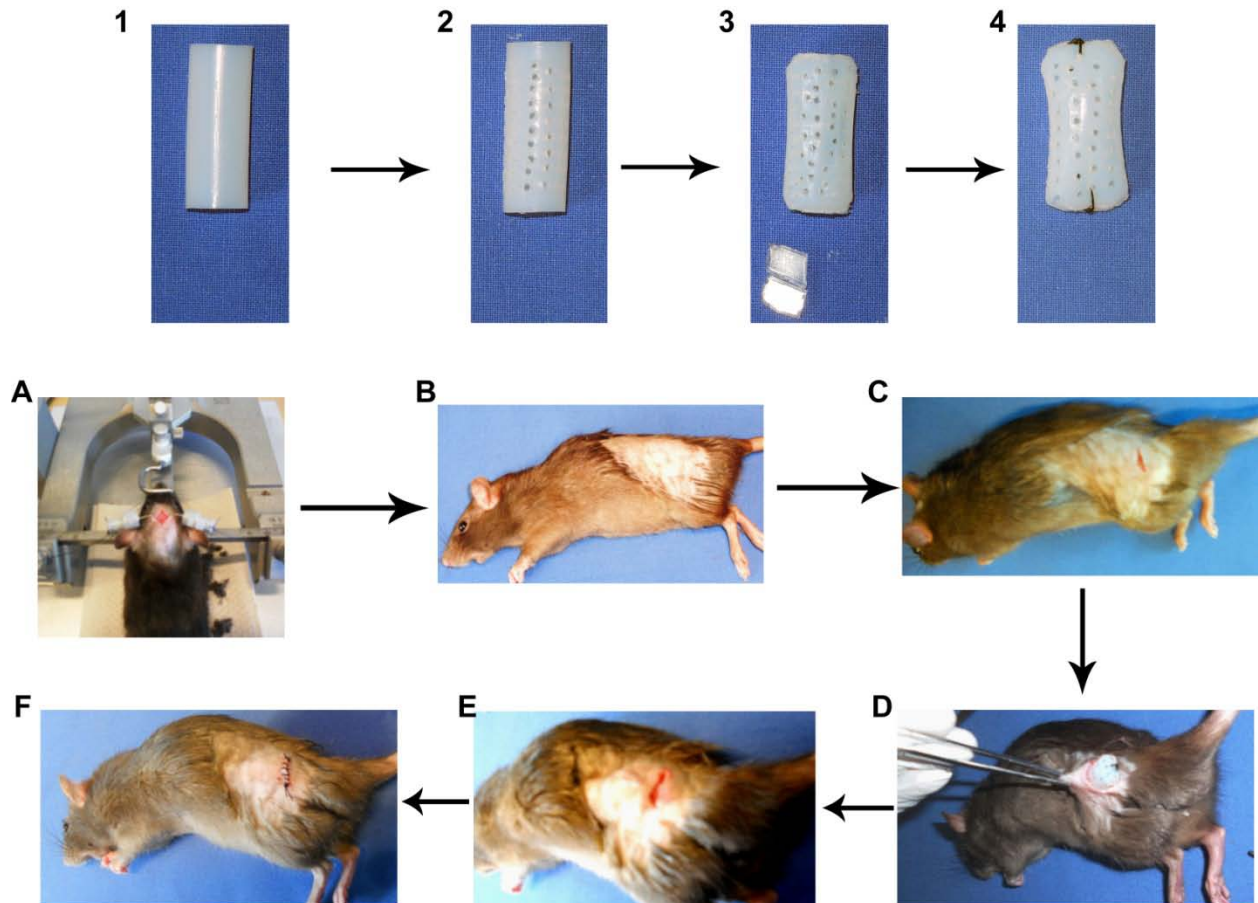


Fig 3.2.2: Experimental setup. Tissue cages were made by using a piece of Teflon plastic (1). Around hundreds of drills were made (2) and 2 PMMA cover slip was placed inside the tissue cage (3) stitched at both ends (4). Animals were anaesthetised and fluorogold was injected stereotactically into the superior colliculus (A). Hair was removed (B) and an incision was made in the flank of the animals (C). One tissue cage per animal was placed in the incised region (D). Stitching was done (F) and animals were left 3 weeks for complete recovery.

Agar is an excellent substrate for the growth of the microorganism and indigestible for many micro-organisms and for animals. Thus to provide more surface area for the attachment of bacteria we injected 400 μ l of 2.3% semi-solid agar into the implanted tissue cage using a 27G needle 6 days prior to the infection (corresponding to 2 days prior to immunization). In such optimized condition animals showed a chronic infection even 3 weeks after inoculation of bacteria in the tissue cages of all the animals. Inoculum dosage was also optimized between inoculum concentrations of 10^5 to 10^9 CFU to establish the chronic infection in this animal model. However, in the absence of agar injection in to the tissue cage, 10^9 CFU were non-infective. Before the agar injection, tissue cage fluid (TCF) was obtained percutaneously from all animals and checked for the sterility by plating on blood agar plates. Animals were excluded from the experiment if they had contaminated TCF.

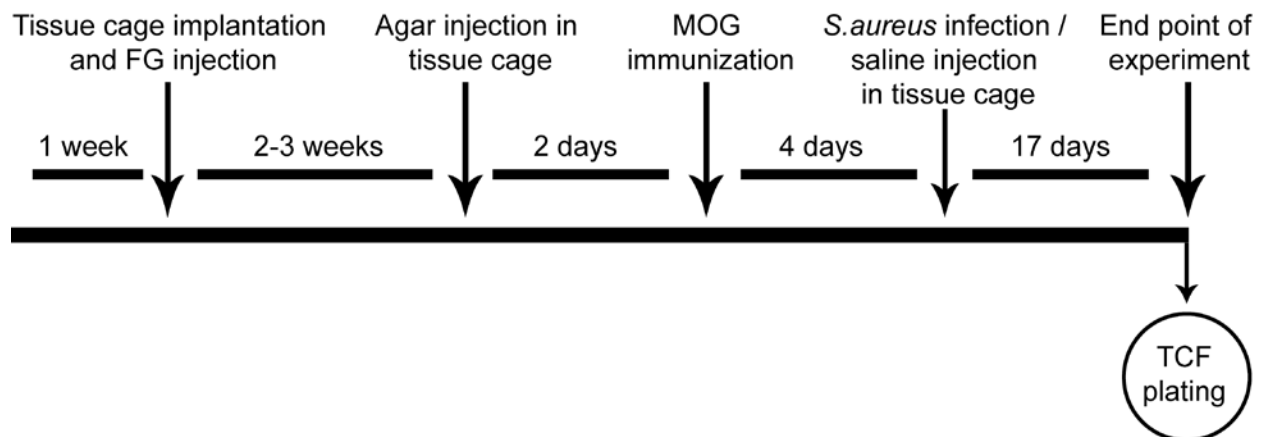


Fig 3.2.3: Experimental timeline. Tissue cage (TC) implantation and fluorogold (FG) injection was done 2 to 3 weeks prior to myelin oligodendrocyte glycoprotein (MOG) immunization. Agar was injected into the TC 2 days before immunization. Injection of *Staphylococcus aureus* (*S. aureus*) or saline was performed on day 4 post immunization (pi). Animals were followed until day 8 of experimental autoimmune encephalomyelitis (EAE) or day 21 pi in case of clinically healthy animals. To monitor the infection, blood samples were taken throughout the whole experiment. Tissue was harvested from the optic nerve for histopathological analysis; retina was flat-mounted for quantification of retinal ganglion cells. Tissue cage fluid (TCF) was checked for the presence of bacteria.

Two days after the agar injection in tissue cage, immunization was done and considered day zero of immunization. Infection was performed on day 4 post immunization (pi). This infection time point was chosen to elucidate whether infection modulates the course of EAE. In the first set of experiment (n=30), one animal group was infected with a wild-type strain of *S. aureus* (ATCC 29213). The corresponding control group received saline. To prove our hypothesis of anti-inflammatory effect of extracellular adherence protein (Eap), animals (n=26) were again randomized into the three different groups. One group was infected with the wild-type *S. aureus* strain (ATCC 25904). A second group was infected with *S. aureus* strain deficient for Eap (AH12), which is an isogenic mutant of the wild-type Newman strain of *S. aureus* (ATCC 25904). The corresponding control group received saline. Animals were followed until day 8 after the clinical manifestation of the disease (EAE_{d8}) or day 21 post immunization (pi) in case of clinically healthy animals. At the end of the experiment, TCF was obtained percutaneously from all (infected and noninfected) animals. TCF was checked for the presence of bacterial colonies by plating on blood agar plates.

3.2.8: Induction and evaluation of EAE

Rats were anesthetized by inhalation anaesthesia with isoflurane and injected intradermally at the base of the tail with a total volume of 200 µl inoculum, containing 50 µg recombinant rat MOG^{IgD} (kindly provided by C. Stadelmann, Institute of Neuropathology, University Medicine Göttingen) in saline emulsified (1:1) with complete Freund's adjuvant containing 200 µg heat-inactivated *Mycobacterium tuberculosis*. Rats were scored for clinical signs of EAE and were weighed daily. The signs were scored as follows: grade 0, no symptoms; grade 0.5, distal paresis of the tail; grade 1, complete tail paralysis; grade 1.5, paresis of the tail and mild hind limb paresis; grade 2.0, unilateral severe hind limb paresis; grade 2.5, bilateral severe hind limb paresis; grade 3.0, complete bilateral hind limb paralysis; grade 3.5, complete bilateral hind limb paralysis and paresis of one front limb; grade 4, complete paralysis (tetraplegia), moribund state, or death.

3.2.9: Blood leukocyte counts

To monitor the local application of *S. aureus* in the tissue cage induces the systemic inflammation, leukocyte counts were performed in peripheral blood every second day until day 8 after MOG immunization and then at an interval of 3 days until day 20 pi. Blood samples were taken sublingually after inhalation anaesthesia with isoflurane. A total of 10 µl of blood sample was diluted in 190 µl of 4% glacial acetic acid, and leukocyte counting was performed in a Neubauer haemocytometer.

3.2.10: Lymphocytes count after *S. aureus* infection

Flow cytometry for lymphocyte counting was performed in blood on day 4 post immunization (pi) prior to infection and 2 days after infection at the time when the leukocyte counts reached their peak. In brief, 100 µl blood taken sublingually was collected in 250 µl of 5 mM EDTA prepared in isotonic PBS. Samples were washed once with PBS, and RBC lysis buffer (0.1 M ammonium chloride in PBS) was added in each sample. Samples were left at 4°C for 10 minutes and washed once with PBS. Samples were divided in two equal parts. In first set of staining a total of 100 µl of FACS buffer (PBS + 1% BSA + 0.1% NaN₃) containing florescent tagged anti rat-CD45RA (BioLegend Cat# 202307) and anti rat-CD3 (BioLegend Cat# 201408) antibodies along with the beads were added in each samples at a dilution of 1:200. Similarly in the second set of staining a total of 100 µl of FACS buffer containing florescent tagged anti rat-CD3 (BioLegend Cat# 201408), anti rat-CD8a-PE (BioLegend Cat# 201705), anti rat-CD134 (OX-40) (BioLegend Cat# 204503) and anti rat-CD4-AF647 (BioLegend Cat# 201513) antibodies were added in each samples at the similar dilution of 1:200. Incubation was done for 45 minutes at 4°C in the dark. The cells were washed once with PBS and re-suspended in 200 µl of FACS buffer prior to flow cytometric analysis. Analysis was performed by four-color flow cytometry using a FACSCalibur and FacScan software (BD; Goettingen; Germany).

3.2.11: Cytokine multiplex assay

Blood samples were collected at the end of the experiment by puncturing the heart with an 18G syringe before the perfusion of animals and kept on ice for 30 minutes. Serum

was collected by centrifuging all the samples at 3000 RPM for 5 minutes at 4°C. Aliquots were kept at -80°C until further use. A Luminex assay kit (Bio-Plex Pro™ Rat Cytokine Th1/Th2 Assay #171-K1002M), containing premixed coupled magnetic beads and detection antibodies for detecting 12 rat cytokines (IL-1 alpha, IL-1 beta, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-alpha, INF-gamma, GM-CSF) was used for measurement of cytokine concentrations in serum samples collected at the end of the experiment. The total number of animals used in this cytokine assay was 25 (n = 11 infected with wild-type strain of *S.aureus* (ATCC 25904); n = 9 infected with Eap-deficient of *S.aureus* (AH12); n = 5 noninfected control). Quantification of cytokines was performed according to the manufacturer's instructions. Measurement was performed by using a Bioplex 200-system based on the Luminex xMAP-technology (Bio-Rad, Hercules, CA, USA). The signal detection occurred using the Bioplex 200-system and the Bioplex Manager Software version 6.0.

3.2.12: Histopathology and immunohistochemistry

Retinas were removed for counting of RGCs; paraformaldehyde (PFA)-fixed paraffin-embedded optic nerves (ONs) were taken for histopathological evaluation. After perfusion of rats with 4% PFA, ONs were post-fixed in 4% PFA for 24 h and embedded in paraffin. All histopathological analysis was performed on paraffin embedded ON cross sections representing three different levels of an ON.

3.2.12.1: Staining and quantification of demyelination

Luxol Fast Blue staining was used to evaluate demyelination on 2 µm thick optic nerve s following standard protocol was used for staining

- Sections were deparaffinized in xylene (2 times for 10 minutes), followed by rehydration in descending alcohol series (100%-, 2 times for 3 minutes; 96%-, alcohol) for 3 minute each.
- Thereafter, the sections were incubated in LFB solution overnight at 60°C
- Sections were serially dipped in 90% alcohol, 0.05% lithium carbonate solutions, and in 70% ethanol for 1 minute each.

- Sections were incubated in 1% periodic solutions for 5 minutes and followed by a wash in running tap water for 5 minutes and further dipped in distilled water.
- Then sections were incubated in Schiff's reagent for 30 minutes at 4°C followed by a washing in running tap water for 5 minutes.
- Sections were counter stained with hematoxylin solution for 7 minutes and dipped in distilled water and HCl-ethanol for few seconds.
- Sections were washed in running tap water for 10 minutes and dipped in distilled water followed by dehydration in ascending ethanol series (50%-, 70%-, 90%-, 96%- ethanol briefly, 100%- 2 times for 1 minute, xylene 2 times for 3 minutes).
- Slides were mounted and cover slipped and used for quantification.

3.2.12.1.1: Quantification of demyelination

Photos of vertical sections were taken using an AxioCam MR Microscopy camera (Zeiss, Göttingen; Germany). The images were processed using Zeiss AxioVision 4.2 software to evaluate the demyelinated area as a percentage of the whole ON cross sections.

3.2.12.2: Axonal count and data evaluation

To illustrate the axonal counts in the optic nerve, the paraffin sections were stained by the Bielschowsky silver staining. An evaluation was performed on paraffin embedded 0.5 µm thick sections of the optic nerve.

Following protocol was used for the staining and data evaluation:

- Sections were deparaffinized in xylene (2 times, 10 minutes each), followed by rehydration in descending alcohol series (100%, 2 times, 3 minutes; 96%-, 90%-, 70%-, 50%- alcohol) for 3 minute each.
- Thereafter, the sections were incubated for 20 minutes in 20% silver nitrate solution.
- Dipped in water for a short time.

- Ammonia was added in 20% silver nitrate solution drop wise until solution turns from bright to dark and turned bright again.
- The optic nerve sections were then transferred back into the silver nitrate - ammonium solution and incubated for 15 minutes in the dark.
- 6 - 8 drops of a developer solution were added.
- Few slides were incubated in silver ammonia solution and looked for dark brown coloration of optic nerve microscopically.
- Slides were washed in distilled water and incubated in 2% sodium thiosulphate for 2 minutes.
- Slides were washed in running tap water and dehydrated in ascending ethanol series (50%-, 70%-, 90%-, 96%- ethanol briefly, 100%- 2 times for 1 minute, xylene 2 times for 3 minute each), mounted and cover slipped.

3.2.12.2.1: Quantification of axonal densities in optic nerve

To quantify the axonal density, 0.5 μm thick slices of ONs were stained by Bielschowsky silver impregnation staining to visualize axonal structures. Automated quantification of axonal densities was performed on high magnification pictures (1000x) applying a custom-programmed script in *Cognition Network Language* based on the Definiens Cognition Network Technology® platform. Briefly, the programmed script first discriminates between tissue and background (no tissue) by spectral difference detection. Subsequently, the area of detected tissue (region of interest, ROI) is calculated and “axonal structures” within this ROI are detected based on their dark black color. To discriminate between nucleoli of inflammatory cells and axons (which both structures are stained black in Bielschowsky silver impregnation) only black structures without a faint brown-stained circumference (nucleus) were further classified as axonal structures. In a next step, multiple axons in direct contact with each other were further split into the corresponding individual axons if the splitting improved the elliptic shape of an axon. Within each area ROI, the total number of axons was calculated. The density of axons in each ON measured in at least nine standardized microscopic fields of each ON. The mean axon density was calculated for each ON and expressed as axons/ mm^2 . However, in the experiment with acute infection model

infected with *E. coli* group and respective control group, axonal density was measured semi-quantitatively by Bielschowsky silver impregnation staining on 2 μm thick vertical section of ONs in three standardized microscopic field per . In brief axonal density was determined in vertical sections of the ON by point sampling using a 25-point Zeiss eye - piece. The number of points crossing axons was measured as a fraction of the total number of points of the stereological grid.

3.2.12.3: Immunohistochemistry

Immunohistochemistry was performed in serial sections of paraffin-embedded ONs. ED1 macrophages/ activated microglia and CD3-positive cells were detected in cross sections using biotin-avidin detection. Spleen sections served as positive control for ED1 staining. The following standard protocol was followed for immunohistochemical analysis;

- The paraffin embedded slides were deparaffinised by placing twice in xylol for 10 minute each.
- Sections were rehydrated in descending ethanol concentration for 3 minute each (100% 2 times, 96%-, 90%-, 70%-, 50%- ethanol) followed by placing them in water.
- Antigen retrieval was performed by heating the slides into microwave in citrate buffer for 10 minutes at 800 watt and 5 minutes for 560 watt.
- Slides were allowed to cool down for 20 minutes at room temperature.
- Quenching of endogenous peroxidise was done by dipping the slides into 3% H_2O_2 for 10 minutes at room temperature.
- Slides were washed with PBS (3 times for 5 minutes).
- Blocking was done for 1h at room temperature with 10% serum (ED1) or in 10% serum+ 2% BSA (CD3).
- Primary antibodies were applied in appropriate dilutions (1/500 for ED1 and 1/200 for CD3).
- Slides were incubated at 4⁰C (For ED1, 12-16 h; for CD3, 20-24 h).

- Washing was done with PBS (3 times for 5 minutes) followed by incubation with secondary antibody 1 h at room temperature.
- Washing with PBS (3 times for 5 minutes) was done.
- Sections were incubated with VECTASTAIN® ABC Kit (Vector Burlingame; USA) for 1 h at RT followed by washing with PBS (3 times for 5 minutes).
- Development was done by using DAB kit.

3.2.12.3.1: Quantification of ED1 positive cells

The evaluation of ED1 positive cells was performed using the following score: 0, no labelled cells; 1, a few ED1 positive cells (number countable) in at least one of the three cross sections of an ON; 2 infiltration of less than 10% of the ON area with ED1 positive cells (number not countable) in at least one of the three cross sections of an ON; 3, infiltration of 10-50% of the ON area with labelled ED1 positive cells in at least one of the three cross sections of an ON; 4, infiltration of 80% of the ON area; 5, infiltration of more than 80% of the ON area in at least one of the three cross sections of an ON.

3.2.12.3.2: Quantification of T-cell infiltrates in the optic nerve

CD3 staining for assessment of infiltration of CD3 positive cells across the BBB was performed on 0.5 µm thick slices of paraffin-embedded ONs using anti-CD3 antibody. CD3 positive cells were detected by biotin-avidin detection. Spleen sections served as positive control for CD3 staining. To quantify T-cell infiltrates in the ON, we used tissue studio that is based on *Cognition Network Language* (Definiens Cognition Network Technology; Definiens Developer XD software, Munich; Germany) In brief, CD3 stained sections were scanned using the 3D Histech 250 Flash slide scanner at 200 fold magnification. The area of interest (optic nerve) was first detected from the background by the colour spectrum. After segmentation, nuclei and CD3-positive cytoplasm were discriminated. Within each area of interest, the total number of CD3-positive cells was calculated and expressed as cells/mm².

3.2.13: Quantification of RGC density

Eyes were enucleated from the rat before perfusion and kept in PFA for 2 minutes and transferred to PBS. An incision was made by using a 23G syringe on the junction of Sclera and the cornea. Frontal part was removed along with the lens. Then vitreous humour present on the retinal surface was taken out carefully. Retinae were dissected, flat-mounted on glass slides, and examined by fluorescence microscopy (Zeiss, Axioplan 2) using a DAPI filter (315/395 nm). Retinal ganglion cell densities were determined by counting labelled cells in three areas (62,500 μm^2 each) per retinal quadrant at three different eccentricities of 1/6, 3/6, and 5/6 of the retinal radius. Cell counts were performed following a blinded protocol.

3.2.14: Statistical analysis

Data are presented as means \pm SEM. Student's t-test was used to analyze differences between two animal groups. Multiple comparisons were statistically evaluated by one-way ANOVA using Prism 5 (GraphPad software). Mann-Whitney U test was used for the analysis of the EAE score (SPSS, IBM). Data presented as means \pm S.E.M. of the daily score. A p-value (P) of < 0.05 was considered to be statistically significant and indicated as follows *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. "n" indicate the number of animals. However, for RGCs counting n indicate the number of eyes used for counting.

4. RESULTS

4.1: Acute *E. coli* infection and EAE

Evidences from experimental and clinical studies indicated that the progression of multiple sclerosis (MS) is influenced by systemic infection caused by bacteria. In most of the previous experimental studies only bacterial components were used to mimic the situation of the bacterial infection that probably might not be the real clinical condition. Therefore to address the impact of a viable bacterial infection in MS, we infected an animal model of MS with *E. coli*, which causes the vast majority of urinary tract infections. Moreover, we infected the animals at two different time points to address the question that does the time course of infection have any impact on the onset of the clinical disease symptoms, when infection was performed in the preclinical phase of disease (day 7 post immunization) “early infection group” and on the severity and aggravation of clinical disease course, when the infection was performed on day 1 of clinical manifestation of the disease (day 1EAE) “late infection group”.

4.1.1: Intraperitoneal infection with *E. coli* induces systemic inflammation

The common notion is that a bacterial infection induces changes termed as a sickness behavior, which reflects in animals in form of weight loss, rise in body temperature, increase in heartbeat counts, sepsis and sometimes as the death of the animal.

In the early infection group, the animals were infected with *E. coli* on day 7 after MOG-immunization, whereas late infection group reflects the condition when infection was induced on the day of clinical onset of disease (day 1EAE). In the early infection group (Fig; 4.1.1 A) the animals infected with *E. coli* (n=15) showed significant weight loss after the infection compared to the control group (n=13; $P < 0.01$). This loss in weight of animals indicated a general sign of sickness behavior. These animals recovered their weight loss after antibiotic treatment. In contrast, in the late infection group (infected on day 1 EAE; Fig 4.1.1 B) the animals infected with *E. coli* (n=15) did not show any significant difference in weight loss compared to the control group (n=14; B; $P = 0.69$).

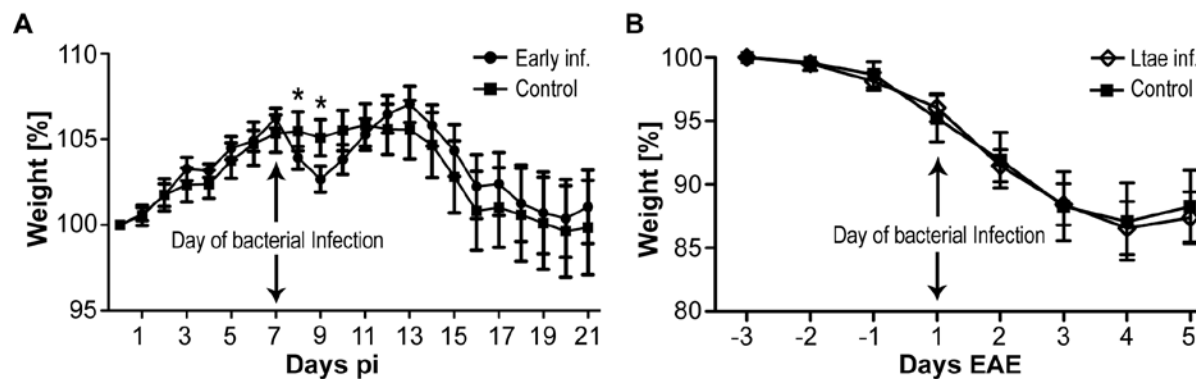


Fig 4.1.1: Weight loss: Animals infected with *E. coli* on day 7 post immunization (**A**) showed a significant weight loss compared to the control group. No significant weight loss was observed in late infection group (**B**) compared to control.

The loss in the weight was probably due to the fact that during the clinical disease course of EAE both infected and control groups lost their weight rapidly. Therefore, to confirm the systemic infection in a late infection group of animals, 24 hours after the infection, blood samples were collected from the tail vein of animals infected with *E. coli* and from the respective control group ($n = 8$ in each group) under sterile conditions. Plating of blood samples was performed on blood agar plates, which showed a mean bacterial count ($\text{Log}_{10} 3.64 \pm 3.32$; $P = 0.04$) only in the animals infected with *E. coli*. No bacterial counts were detected in saline treated control animals. The bacterial detection limit was 100 CFU/ml. We also observed a mortality rate around 20% due to *E. coli* infection in both early and late infection group. However, the data from these animals were not included in the analysis of the results.

4.1.2: The influence of *E. coli* infection on clinical course of MOG-EAE

The “early infection” group infected animals did not show any significant difference in the mean cumulative clinical score in comparison to the control animals (0.9 ± 0.35 , $n = 15$ vs. 1.038 ± 0.39 , $n = 13$; $P = 0.8$; Fig 4.1.2 A). Also the onset of disease was similar in both the groups (13 days post immunization in the infection and 12 days post immunization in the control group).

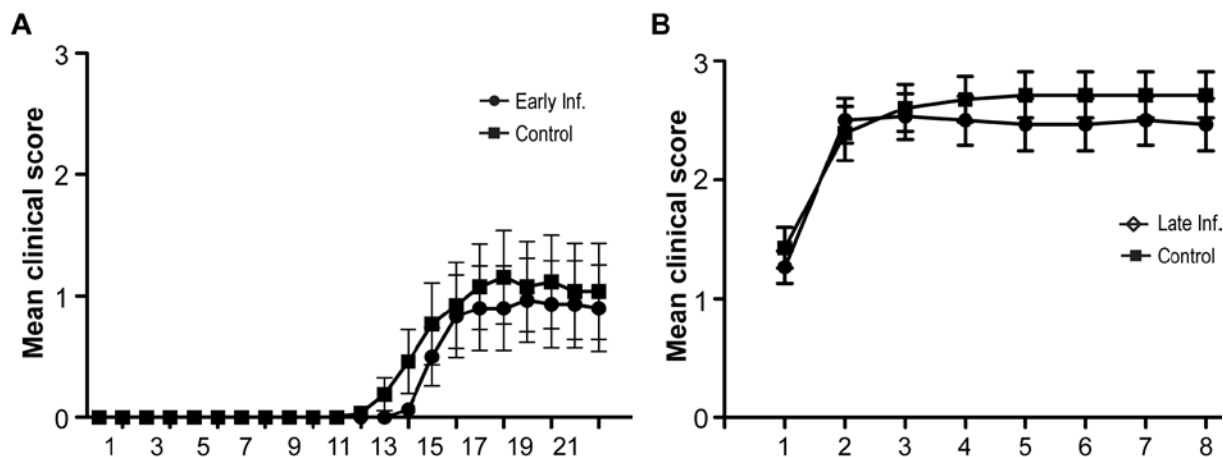


Fig 4.1.2: Mean clinical score. Infection with *E. coli* on day 7 post immunization (**early infection group; A**) had no impact on the time of clinical onset or severity of the disease in MOG-EAE. Infection with *E. coli* on day 1 EAE (**late infection group, B**) did not show any significant impact on the severity of the disease compared to the control group. Data are shown as means \pm S.E.M. of the daily score.

In the second set of experiments, infection with *E. coli* was induced on the day when the animals showed first clinical symptoms (late infection group). *E. coli* infection did not substantially modulate the course of EAE. Here, the mean cumulative score was not significantly differed between the group infected with *E. coli* and the respective control group (2.47 ± 0.22 n =15 vs. 2.71 ± 0.19 n =14; $P = 0.51$; Fig 4.1.2 B) at the end of the experiment.

4.1.3: Infection with *E. coli* and neuropathological damage of the optic nerve

Animals were sacrificed on either day 8 of clinical manifestation of disease or at day 21 after immunization in the absence of clinically manifest disease. Histopathological analysis was performed on paraffin embedded optic nerve s. We determined the extent of demyelination of the ONs by Luxol Fast Blue staining. In the early infection group we did not observe any significant difference in demyelination of *E. coli* infected animals ($62.90 \pm 9.43\%$; Fig 4.1.3 A, G; n=15) compared to control group ($79.18 \pm 8.06\%$; $P = 0.20$; Fig 4.1.3 B, G; n=13). Analysis of inflammatory infiltrates by ED1 staining revealed no significant difference in the animals infected with *E. coli* on day 7 post immunization (2.84 ± 0.39 ; Fig 4.1.3 C, H; n=15) compared to the saline treated control

animals (3.45 ± 0.3 ; $P = 0.50$; Fig 4.1.3 D, H; $n=13$). Bielschowsky silver impregnation was performed to access the axonal loss of the ON. At day 8 of MOG-EAE, we observed a loss of ($66 \pm 7.64\%$; Fig 4.1.3 E, I; $n=15$) in the animals infected with *E. coli*. In the control group, the axonal loss did not differ significantly ($82.19 \pm 5.69\%$; $P = 0.1$; Fig 4.1.3 F, I $n=13$).

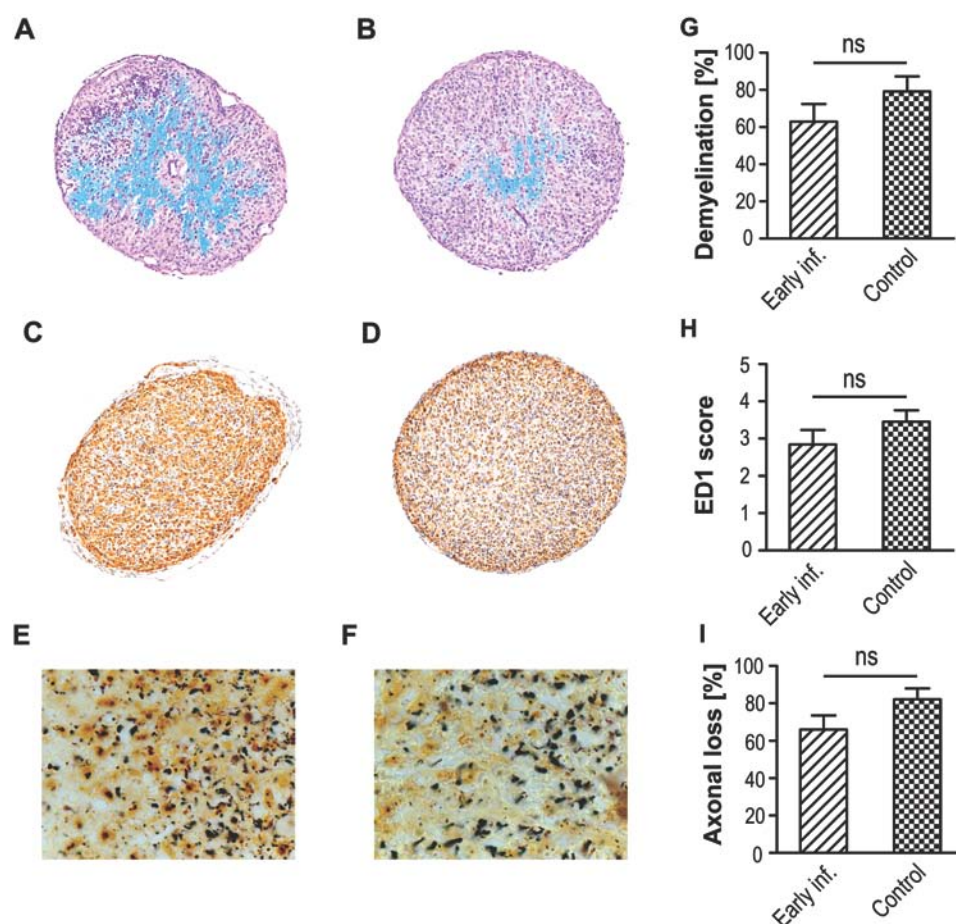


Fig 4.1.3: Optic nerve (ON) histopathology for early group: **A** and **B**: Representative Luxol Fast Blue-stained sections of ONs of an animal infected with *E. coli* (**A**) and an animal from respective control group (**B**). Representative examples for number of infiltrating ED1 macrophages / activated microglia detected in the ON of an *E. coli* infected animal (**C**) and an animal from noninfected control group (**D**). **E** and **F**: Bielschowsky's silver impregnation of an ON revealed no differences in axonal densities of MOG immunized animal infected on day 7 post immunization (**E**) compared to noninfected control animal (**F**). **G**, **H** and **I** represent the quantitative data for demyelination, inflammatory infiltrates and for axonal counts in both *E. coli* infected and control group.

In the experiment performed with “late infection” a similar scenario was observed. Here the extent of demyelination ($77.06 \pm 5.77\%$; $n=15$; for infected group; $72.87 \pm 6.46\%$; $n=14$; for the control group; $P = 0.63$; Fig 4.1.4, A) and number of inflammatory infiltrates (3.944 ± 0.24 ; $n=15$; for infected group; 3.827 ± 0.26 ; $n=14$; for the control group; $P = 0.74$; Fig 4.1.4, B) did not show any significant difference between the group infected with *E. coli* and the saline treated control group. Moreover, we did not find any effect of *E. coli* infection on axonal loss as quantified by Bielschowsky silver impregnation ($71.84 \pm 5.03\%$; $n=15$; for infected group and $70.07 \pm 5.39\%$; $n=14$; for the control group; $P = 0.81$; Fig 4.1.4, C).

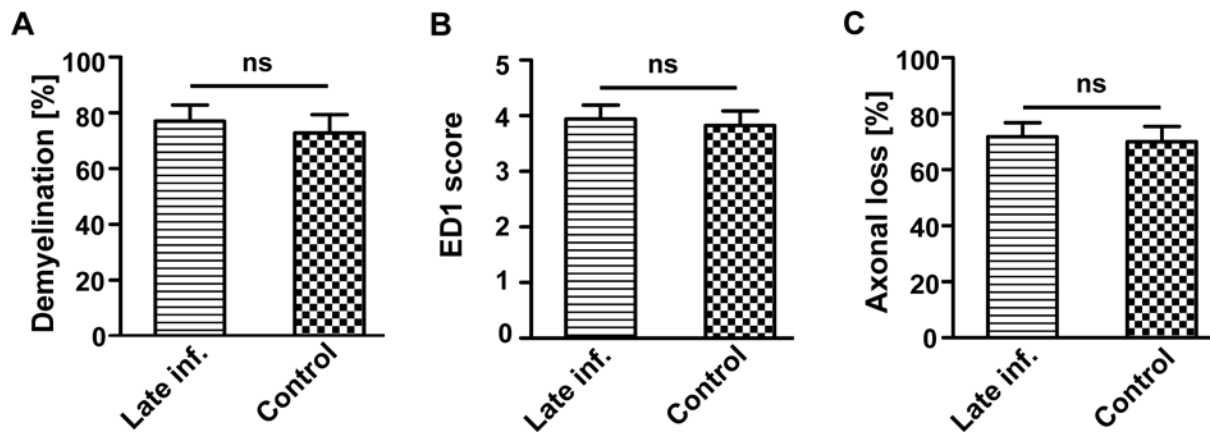


Fig 4.1.4: Optic nerve (ON) histopathology for late infection group: No significant difference was observed in quantitative data analysis for demyelination (A), inflammatory infiltrates (B) and for axonal counts (C) between the groups infected with *E. coli* and the respective control.

In summary these results from the histopathological analysis performed on optic nerve cross sections indicated that the *E. coli* infection did not have any influence on demyelination, infiltration of inflammatory cells or on axonal damage.

4.1.4: Impact of *E. coli* infection on loss of RGCs

It is presumed that bacterial infections lead to enhanced neurodegeneration. Therefore to evaluate the impact of infection on neuronal damage in MOG-EAE after *E. coli* infection, numbers of surviving RGCs were counted on fluorogold labeled retinal flat mount. In the early infection group (Fig 4.1.5 A; $n=25$ each) RGCs density did not reveal

any significant difference between *E. coli* infected group ($1411 \pm 87.57/\text{mm}^2$) compared to corresponding control group ($1248 \pm 68.99/\text{mm}^2$; $P = 0.15$).

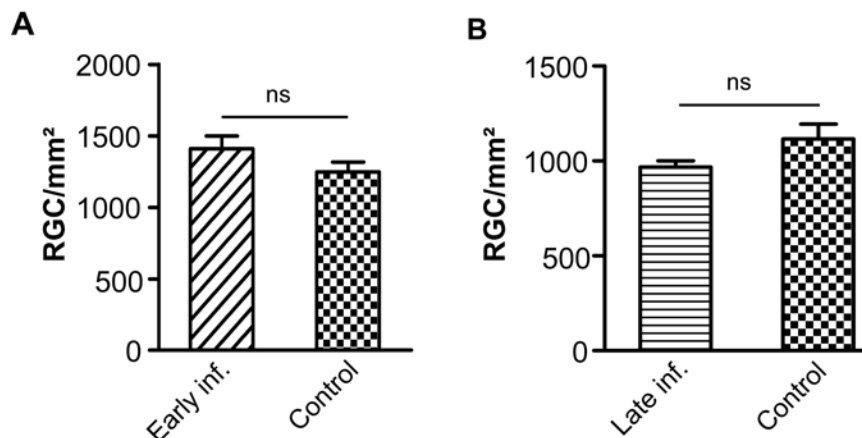


Fig 4.1.5: RGCs counting: No significant differences were observed in a number of surviving retinal ganglion cells (RGCs) of the animals infected with *E. coli* either in preclinical phase of disease (A) or in clinical phase of disease (B).

Furthermore, to know the impact of infection during the clinical course of the disease the RGCs counting performed in the late infection group of animals which (Fig 4.1.5 B; $n=16$ each group) did not reveal any significant difference between the group infected with *E. coli* ($967 \pm 33/\text{mm}^2$) compared to the corresponding control group ($1116 \pm 78/\text{mm}^2$). However, the group infected with *E. coli* on day of onset of the disease showed a tendency towards more neuronal cell loss compared to the respective control group ($P = 0.08$).

4.2: Chronic *S. aureus* infection and EAE

Several acute infection models have been used to study the impact of infection on the course of EAE and mostly showed a deleterious effect on severity of disease. However, most of these studies also used an immediate antibiotic treatment to ensure the survival of animals after the infection. These situations mask the impact of the infection alone since the modulatory role of these antibiotics in the progression of EAE has been proven (Melzer *et al.*, 2008; Mor and Cohen, 2013). Moreover, there is no direct evidence of *S. aureus* or any other chronic infection causes modulation of the EAE. In this study to ensure the chronic infection, to achieve this tissue cage was implanted under the skin and infected with *S. aureus* which leads to the chronic infectious condition in this animal model. No antibiotic treatment was performed during the course of the experiment.

4.2.1: Local infection with *S. aureus* leads to persistent systemic inflammation

The animals were infected with *S. aureus* on day 4 post immunization (pi) into the previously implanted tissue cage. After infection we did not observe any significant weight loss in the animal infected with *S. aureus* (Fig 4.2.1). However, animals from the control group lost their weight once they start showing clinical sign of EAE.

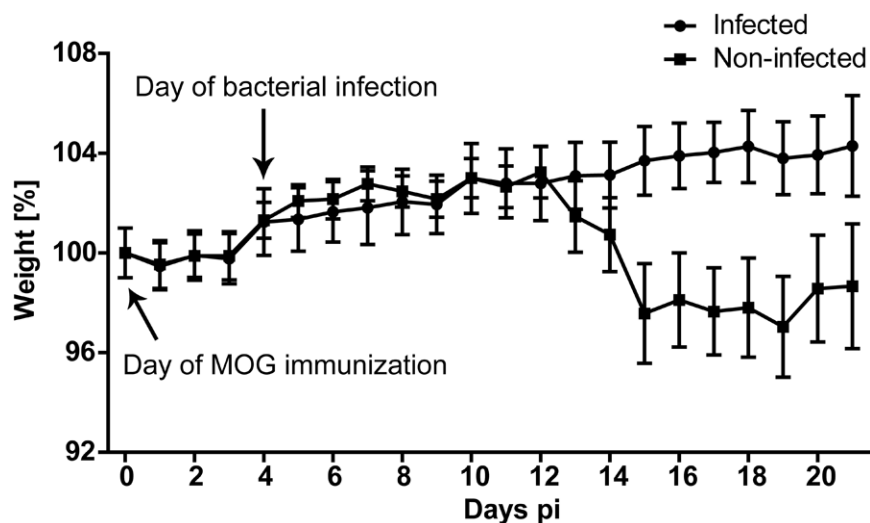


Fig 4.2.1: Weight loss: After infection no significant weight loss was observed in animals infected with *S. aureus*.

Therefore to confirm the persistent local infection in the animals we collected TCF (tissue cage fluid) from all animals under sterile conditions at the end of the experiment and plated the TCF on the blood agar plates. TCF obtained from infected animals contained a high number of bacteria (*S. aureus*), whereas no bacterial growth was observed in TCF from noninfected control animals. Due to the semi-solid consistency of TCF, which is probably due to the presence of agar, dilutions for a more exact determination of bacterial numbers were not possible. In this animal model the inoculation with 10^7 CFU was optimal to induce the chronic infection.

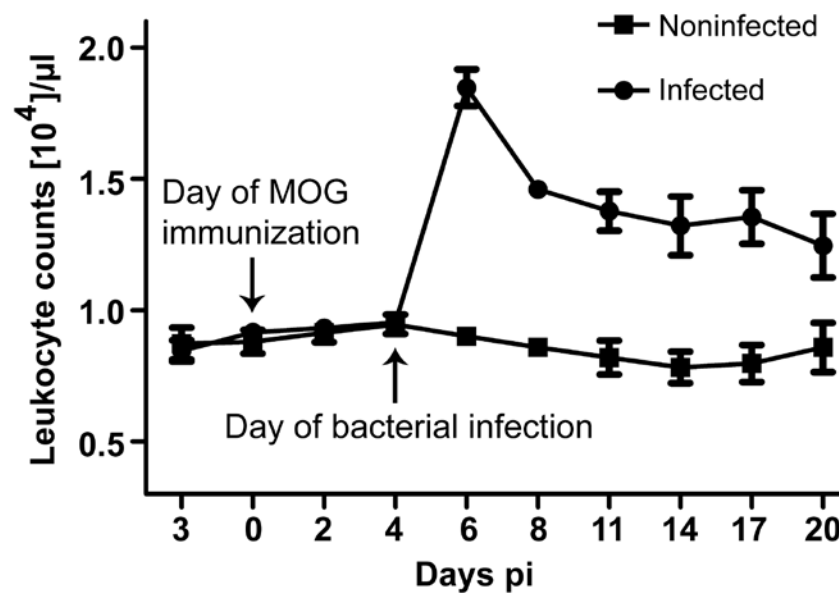


Fig 4.2.2: Blood leukocyte counts: Leukocyte counts were performed in peripheral blood of the animals infected with *S. aureus* wild type and noninfected myelin oligodendrocyte glycoprotein (MOG)-immunized control animals. The leukocyte counts were found to be significantly higher in the group infected with *S. aureus*.

During the course of the experiment we did not observe the presence of bacteria in the peripheral blood of animals. Therefore to monitor the local application of bacteria induced systemic inflammation, blood leukocyte counts were performed in peripheral blood samples until day 20 pi (corresponding to day 16 post infection). Here, we observed that the leukocyte counts in the *S. aureus* infected animals ($18475 \pm 685/\mu\text{l}$; $n=12$; Fig 4.2.2) were significantly elevated two days after infection compared to noninfected control animals ($9000 \pm 305/\mu\text{l}$; $n=11$; $P < 0.001$; Fig 4.2.2). Afterwards,

leukocyte counts decreased in the *S. aureus* infected animals but, remained significantly elevated compared to the control animals until day 20 pi ($12457 \pm 1219/\mu\text{l}$ versus $8583 \pm 936/\mu\text{l}$, $P = 0.03$; Fig 4.2.2). This increase in leukocyte counts in animals after *S. aureus* infection confirmed a strong chronic systemic inflammation.

4.2.2: Infection with *S. aureus* enhanced lymphocyte counts in peripheral blood

Flow cytometry was performed to detect the lymphocyte counts in peripheral blood on day 4 post immunization (pi) (directly prior to infection) and day 6 post immunization (corresponding to day 2 after the infection).

B-cell counts were significantly increased in the *S. aureus* infected group ($67 \pm 10.55\%$; $P = 0.002$; Fig 4.2.3 B, D; E; $n=4$) on day 6 pi compared to day 4 pi. In contrast, no significant change in B-cell counts was observed in noninfected animals on day 6 pi compared to day 4 pi (Fig 4.2.3 A, C; E). T-cell counts increased significantly in both the groups on day 6 pi compared to day 4 pi, however, the increase was more prominent in infected than in noninfected animals ($117 \pm 19.38\%$ (Fig 4.2.3 D, F; versus $45 \pm 8.32\%$; $P = 0.013$; Fig 4.2.3 C; F; $n=4$).

Subsequently, T cell subpopulations were also counted and we observed a significant increase in Th- and Tc-cell count in the animals infected with *S. aureus* on day 6 pi compared to day 4 pi (Fig 4.2.3 G, H). However, the Th-cell count was also increased significantly in the control group (Fig 4.2.3 G) and showed an increasing trend in Tc-cell counts (Fig 4.2.3 H). Moreover, CD134 (Ox40) an activation marker for T-cells counts showed an increasing trend in both the infected and noninfected group (Fig 4.2.3 I) on day 6 pi corresponding to day 2 post infection. These results indicated that infection with *S. aureus* induces both B-cell and T-cell response in the periphery without any significant difference in the number of activated T-cell counts.

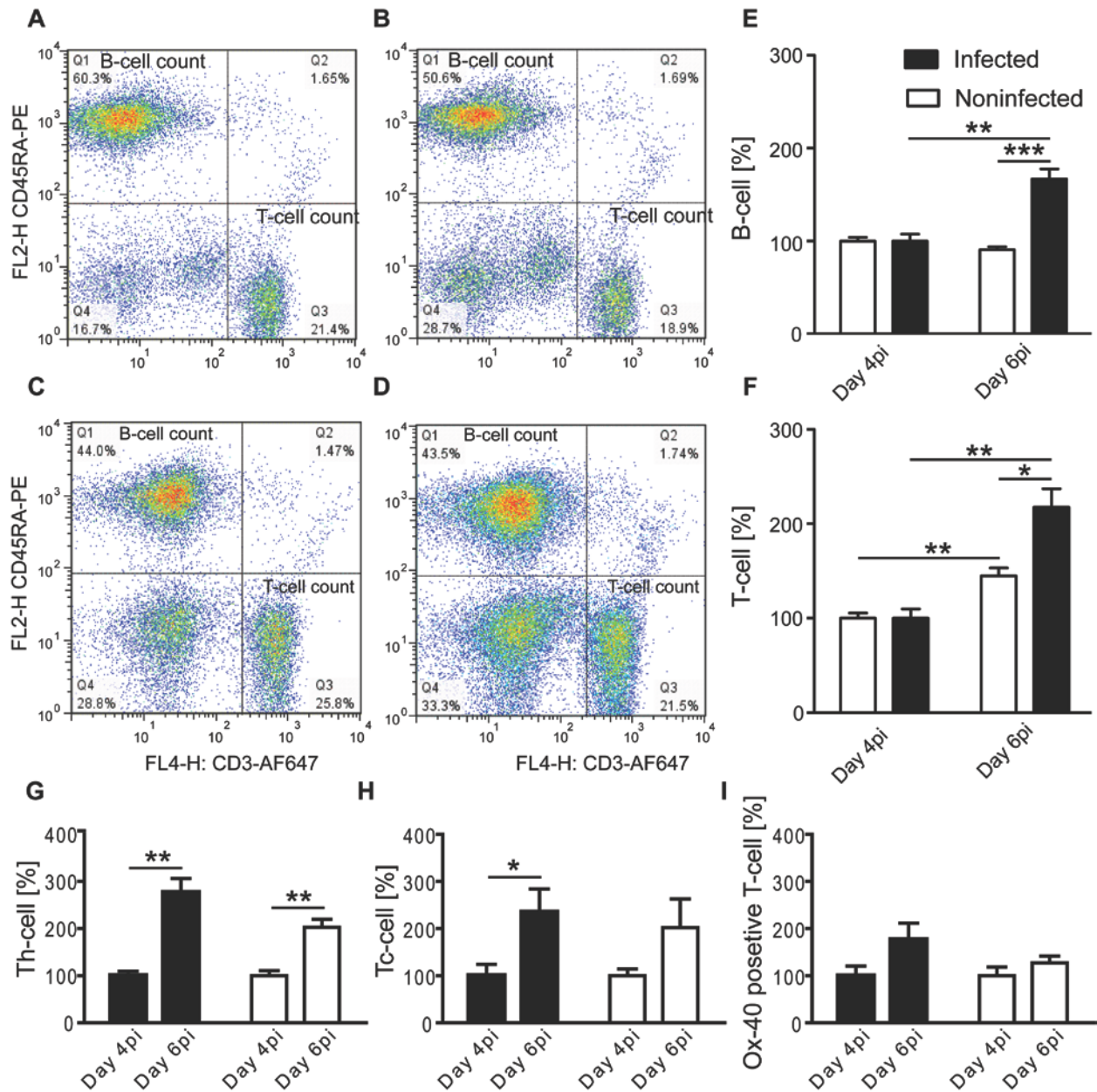


Fig 4.2.3: Flow cytometry: Flow cytometry was performed in peripheral blood to assess lymphocyte populations in animals infected with *S. aureus* (**B**, day 4; **D**, day 6) and noninfected control group (**A**, day 4; **C**, day 6) on day 4 post immunization (prior to infection) and on day 6 post immunization (corresponding to day 2 post infection). **E** and **F** represent the quantitative data for B- and T-cell counts, which were substantially increased in infected animals two days after infection. **G**, **H** and **I** represent the quantitative data for Th-, Tc- and the number of activated T-cells on day 6 compared to day 4 post immunization.

4.2.3: Chronic *S. aureus* infection and disease activity in MOG-EAE

There is a possibility that infection may interfere with the immunization process. Therefore to avoid interference with the initiation of the autoimmune response animals were infected with *S. aureus* on day 4 after immunization

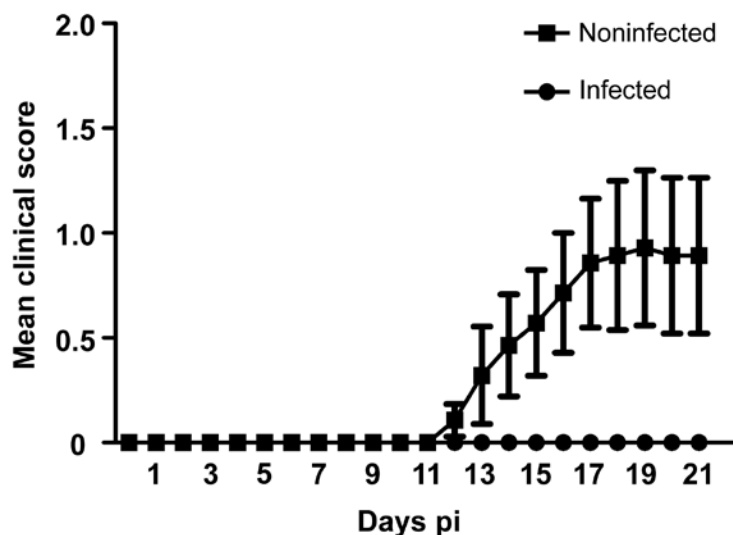


Fig 4.2.4: Clinical score: Infection with *S. aureus* on day 4 pi prevented disease onset in MOG-EAE. Data presented as means \pm S.E.M. of the daily score.

Around day 13 after immunization, 6 of the 14 placebo-infected animals developed clinical symptoms while infection with *S. aureus* prevented the clinical manifestation of EAE in all the infected animals. At the end of the experiment the mean cumulative score differed significantly between the group infected with *S. aureus* and the respective control group (no score vs 0.89 ± 0.4 ; $P = 0.047$; Fig 4.2.4).

4.2.4: Effect of *S. aureus* infection on neurodegeneration

At day 8 of clinical disease or at day 21 after immunization in the absence of manifest disease, we determined the extent of demyelination of the optic nerves (ONs) by Luxol Fast Blue staining. Additionally, we performed immunohistochemistry for ED1 macrophages/microglia and CD3⁺ T-lymphocytes to assess the extent of inflammatory infiltration. The chronic axonal damage was assessed by Bielschowsky's silver impregnation. For each staining, ON cross sections of three different levels were evaluated.

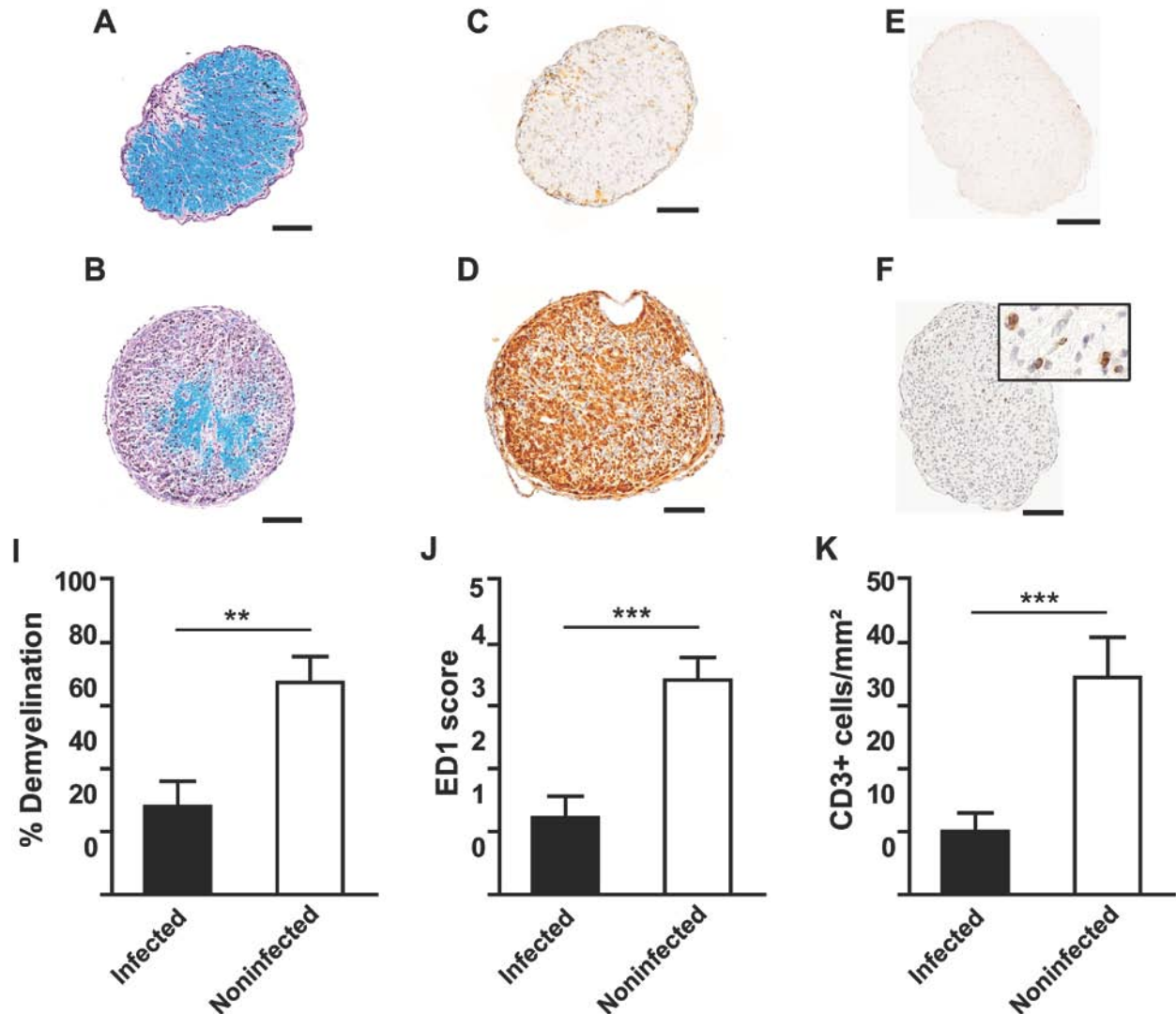


Fig 4.2.5: Optic nerve (ON) histopathology: **A** and **B**: Representative Luxol Fast Blue-stained cross sections of ONs of a wild-type *S. aureus* infected rat (**A**) showed only small areas of demyelination, with mainly intact myelin (blue) ; in contrast, a from a noninfected control animal (**B**) showed extensively demyelinated areas (purple). **C** and **D**: Representative examples of the substantially higher number of ED1 macrophages / activated microglia detected in the ON of a noninfected control animal (**D**), compared with an animal infected with wild-type *S. aureus* (**C**). **E** and **F**: representative images for CD3-positive T-Lymphocyte counts, which were noted higher in the optic nerve of the animals from the control group (**F**) whereas CD3-positive cells were hardly present in the optic nerve of the group infected with bacteria (**E**). **I**, **J**, and **K** represent the quantitative data for demyelination, ED1 score and CD3-positive cells respectively. Bar length = 100µm.

In the animals infected with the *S. aureus* wild-type strain we observed significantly less demyelination in ON cross sections ($28.08 \pm 7.94\%$; Fig 4.2.5 A, I; $n=16$) compared to noninfected control animals ($67.41 \pm 8.17\%$; $P = 0.0011$; Fig 4.2.5 B, I; $n=14$). Analysis of inflammatory infiltrates with ED1 staining revealed a substantially lower infiltration by macrophages and activated microglia in the infected group (1.22 ± 0.33 ; Fig 4.2.5 C, J; $n=16$) compared to the control group (3.41 ± 0.35 ; $P < 0.001$; Fig 4.2.5 D, J; $n=14$). Quantification of $CD3^+$ cells revealed that only the minority of infiltrating cells were $CD3^+$ T-lymphocytes. At day 21 after immunization, numbers of $CD3^+$ cells were found to be significantly lower in the *S. aureus* infected group ($10.09 \pm 2.88/\text{mm}^2$; Fig 4.2.5 E, K; $n=16$) in comparison to the noninfected control group ($34.46 \pm 6.39/\text{mm}^2$; $P < 0.001$; Fig 4.2.5 F, K; $n=14$).

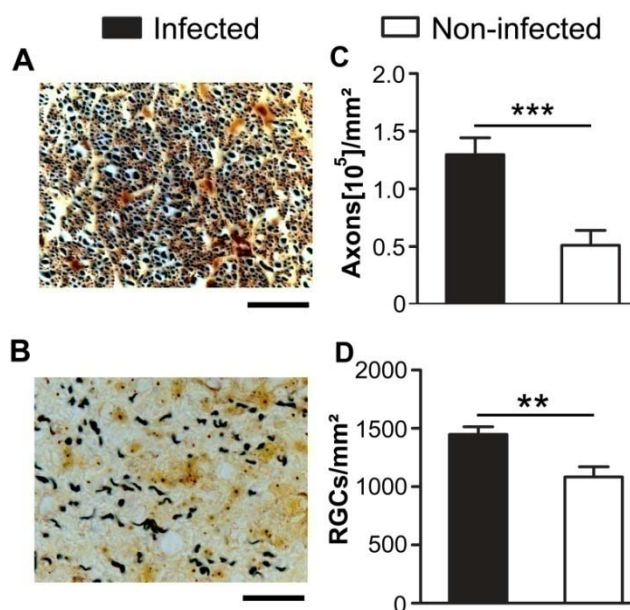


Fig 4.2.6: Axonal loss and RGCs counts: Representative Bielschowsky's silver impregnation of ON cross sections revealed a higher density of axons on EAE day 8 in wild-type infected animals (A) compared to noninfected control animals (B). C; shows the quantitative data for axonal loss between the group of animal infected with *S. aureus* and the noninfected group. Retrogradely labeled RGCs counting revealed a higher number of RGCs in the *S. aureus* infected group (D). Bar length = 20 μm .

Using Bielschowsky's silver impregnation we observed a higher axonal density in *S. aureus* infected animals ($129700 \pm 14580/\text{mm}^2$; Fig 4.2.6: A, C; $n=16$) compared to

noninfected control animals ($51260 \pm 12810/\text{mm}^2$; $P < 0.001$; Fig 4.2.6: B, C; $n=14$). To evaluate the impact of infection on neurodegeneration, numbers of surviving RGCs, the neurons that form the axons of the ON, were counted on day 8 of MOG-EAE. Previously, we have shown that in healthy BN rats sham-immunized with complete Freund's adjuvant (CFA), 2730 ± 145 RGCs/ mm^2 were detectable (mean \pm SEM; $n=8$) (Meyer *et al.*, 2001). In our present study, the mean RGC density in noninfected control animals at day 8 of MOG-EAE dropped to $1085 \pm 85.7/\text{mm}^2$ (Fig 4.2.6: D, $n=14$). In contrast, the RGCs density of infected animals was significantly higher ($1449 \pm 64.60/\text{mm}^2$, $P = 0.002$; Fig 4.2.6: D, $n=20$).

The results from clinical studies indicated a protective effect of *S. aureus* infection on course of disease and at the same time *S. aureus* infection also improved neuropathological damaged induced by MOG-immunization by reducing anti-inflammatory activity in the CNS.

4.3: Effect of Eap-deficient strain of *S. aureus* on EAE

These results were surprising because, despite of a strong systemic inflammation induced by locally applied chronic *S. aureus* infection the animal did not show any clinical sign of EAE and showed reduction in number of inflammatory infiltrate in the optic nerve.

It is well known that *S. aureus* secrete various factors which could exert an anti-inflammatory effect during the infection. An extracellular adhesion protein secreted by this bacterium has anti-inflammatory property which has been found to be present in 97.9% of clinical isolates (Hussian *et al.*, 2001). Furthermore, anti-inflammatory effect of Eap secreted by *S. aureus* was demonstrated in chronic infections as well as in autoimmune disease models (Chavakis *et al.*, 2002; Lee *et al.*, 2002; Wang *et al.*, 2010). Therefore, we hypothesize that this protein to be responsible for the protective effect of *S. aureus* infection in EAE. To prove our hypothesis a second set of experiments was performed by including a group of animals infected with *S. aureus* strain deficient for Eap (AH12), which was an isogenic mutant of wild-type Newman strain of *S. aureus* (ATCC 25904).

4.3.1 Infection with *S. aureus* strain deficient for Eap and systemic inflammation

In this experiment leukocyte counts were significantly elevated two days after infection in the animals infected with a wild-type strain ($17236 \pm 742/\mu\text{l}$; $n=11$; $P < 0.001$) and in the animals infected with an Eap-deficient strain ($18575 \pm 585/\mu\text{l}$; $n=10$; $P < 0.001$) in contrast to the control group ($9940 \pm 527/\mu\text{l}$; $n=5$). Moreover, TCF plating revealed the presence and absence of bacteria in tissue cage fluid of both the infected and control group respectively. Although the exact determination of bacterial count was not possible due to the semi-solid consistency of TCF obtained from both the infected groups. However, we did not observe any significant difference between the leukocyte counts from both the infection groups at any time point during the whole course of the experiment. These results indicated that the level of infection in both the bacterial infected groups was similar and that should not be a region for any discrepancies in the results obtained from the group of animals infected with either wild-type *S.aureus*

(ATCC 25904) or the animals infected with strain of the *S. aureus* deficient for Eap (AH12).

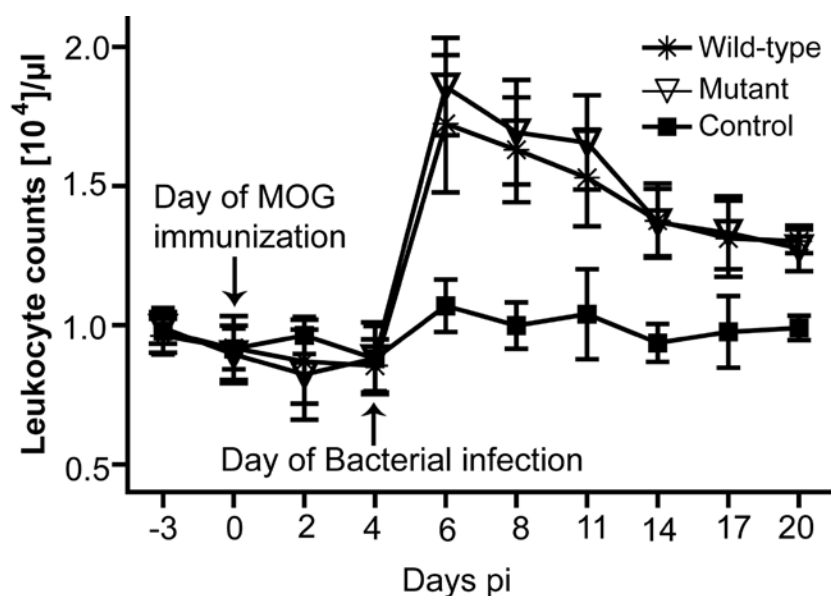


Fig 4.3.1: Blood leukocyte counts: - Leukocyte counts performed in peripheral blood of animals infected with, Eap-deficient bacterium (Mutant), *S. aureus* wild-type bacterium (Wild-type), and in saline injected myelin oligodendrocyte glycoprotein- (MOG) immunized animals (Control). The leukocyte counts were found to be significantly higher in both the infected group 2 days after the infection. Data are presented as means \pm SEM of all animals from each group.

4.3.2: Infection with *S. aureus* and cytokines release in the systemic circulation

In spite of Eap exhibit anti-inflammatory activity during infections and do not resemble the structural homology to superantigens (Hagggar et. al., 2010), the binding of Eap to ICAM-1 on the surface of peripheral blood mononucleocytes triggers the release of pro-inflammatory cytokines like TNF- α and IL-6 (Scriba et.al., 2008). However, determinations of cytokines from in vivo is rather difficult, but in this experiment concentration of 12 cytokines were measured simultaneously by using the Bioplex 200-system in the serum samples collected at the end of the experiment. These results indicated that animals infected with the wild-type strain of *S. aureus* showed a significantly higher level of IFN-gamma, IL-6 (Fig 4.3.2). The quantitative levels of the cytokines measured in the serum samples are indicated in table 4.3.1.

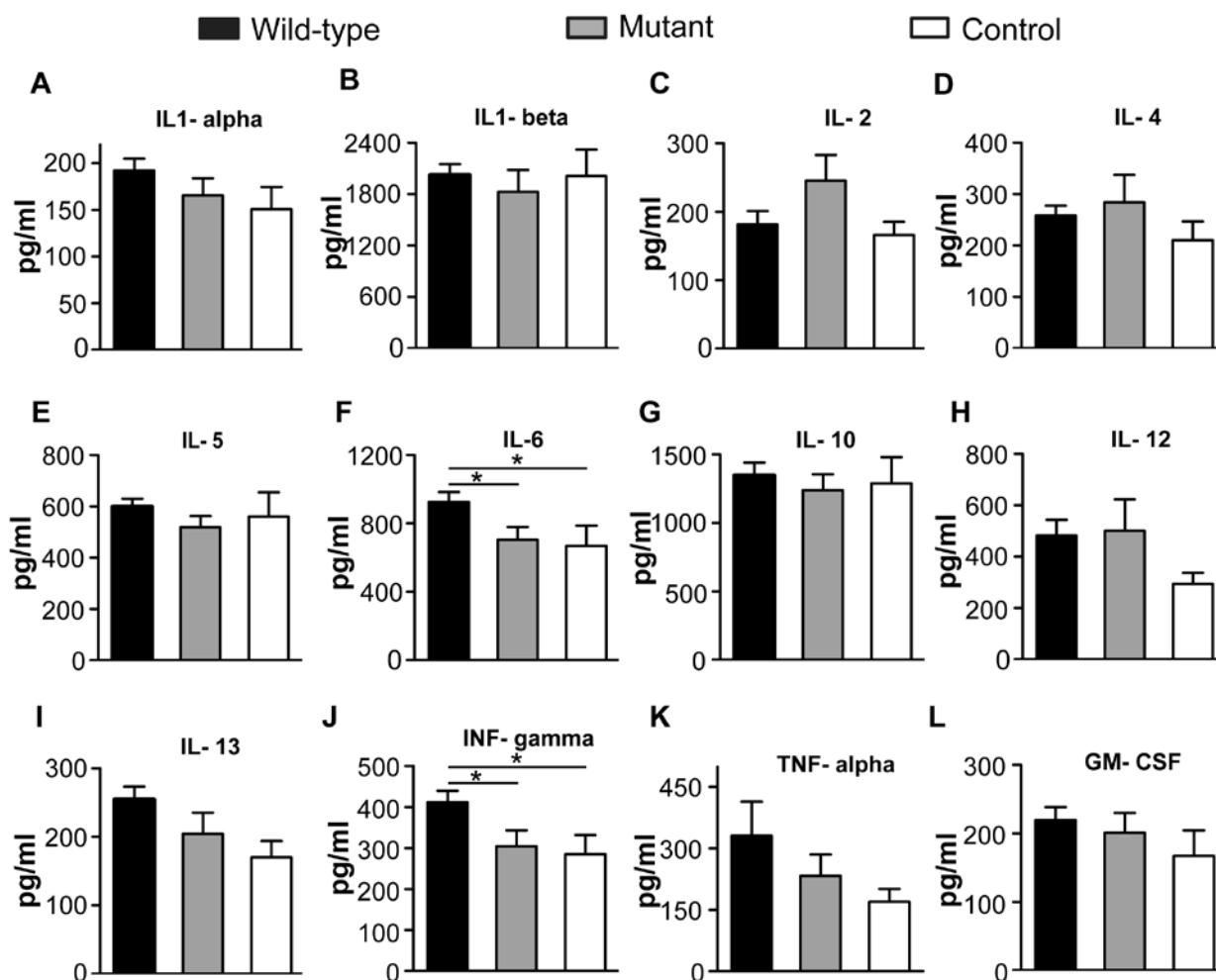


Fig 4.3.2: Luminex assays. Luminex detection assays indicated a significant increase of IL-6 (F) and IFN-gamma (J) and in the blood of animals infected with *S. aureus* wild-type strain.

More detailed, levels of IFN-gamma (Fig 4.3.2 J) was significantly increased in animals infected with the wild-type bacteria (411.4 ± 28.37 pg/ml) compared to animals infected with the Eap-deficient strain (303.9 ± 39.08 pg/ml; $P = 0.035$) and compared to noninfected control animals (284.9 ± 46.49 pg/ml; $P = 0.029$). Serum levels of IL-6 (Fig 4.3.2 F) were also significantly elevated in the group infected with the wild-type *S. aureus* bacterium (926.6 ± 57.27 pg/ml) compared to the group infected with the Eap-deficient bacterium (704.4 ± 75.48 pg/ml; $P = 0.028$) and the control group (668.7 ± 119.1 pg/ml; $P = 0.042$). A tendency towards higher serum levels of TNF- α (Fig 4.3.2 K) and IL-13, an anti-inflammatory cytokine (Fig 4.3.2 I) was observed in the animals

infected with wild-type *S. aureus* bacteria compared to the group infected with Eap-deficient *S. aureus* and the noninfected control group.

Cytokines	Wild-type group (pg/ml)	Eap-deficient group (pg/ml)	Control (pg/ml)
IL1 -alpha	192.3 ± 12.70	165.5 ± 18.54	150.7 ± 23.82
IL-1 beta	2025 ± 125.9	1827 ± 256.3	2012 ± 308.1
IL-2	181.7 ± 19.44	245.7 ± 37.52	166.1 ± 19.57
IL-4	258.6 ± 18.98	284.1 ± 53.28	210.6 ± 36.45
IL-5	599.9 ± 29.66	519.9 ± 42.49	560.6 ± 94.28
IL-6	926.6 ± 57.27	704.4 ± 75.48	668.7 ± 119.1
IL-10	1348 ± 92.67	1239 ± 117.0	1290 ± 191.8
IL-12	483.3 ± 60.40	500.9 ± 122.7	293.4 ± 43.98
IL-13	254.8 ± 18.78	204.4 ± 31.00	170.2 ± 23.76
TNF- alpha	330.7 ± 82.96	233.2 ± 51.44	169.9 ± 30.89
IFN- gamma	411.4 ± 28.37	303.9 ± 39.08	284.9 ± 46.49
GM- CSF	219.4 ± 19.10	201.2 ± 28.80	167.1 ± 37.49

Table 4.3.1: Quantitative data for cytokine analysis: Table represents the quantity of cytokine concentration in the serum sample collected at the end of the experiment.

Although the results were not significant but the group of animals infected with Eap-deficient bacteria also showed a tendency towards higher of proinflammatory cytokines such as IL2 and IL12. Therefore these data showed that the pro- and anti-inflammatory

activity in the periphery is ongoing together, indicating that the variation in the cytokines profile could have influenced on disease severity.

4.3.3: *S. aureus* infection and its impact on clinical manifestation of the disease

Concerning the clinical manifestation of the disease, no clinical signs of EAE were observed in the group infected with *S. aureus* wild-type as well as in the group infected with the Eap-deficient strain. In contrast, noninfected control animals were severely affected with an EAE score of 2.4 ± 0.36 ($n=5$; $P < 0.001$) at the end of the experiment. Although no clinical signs of EAE were observed in the group infected with *S. aureus* or in the group infected with the Eap-deficient strain of *S. aureus*, animals of the group infected with Eap-deficient *S. aureus* displayed higher level of histopathological abnormalities (see below).

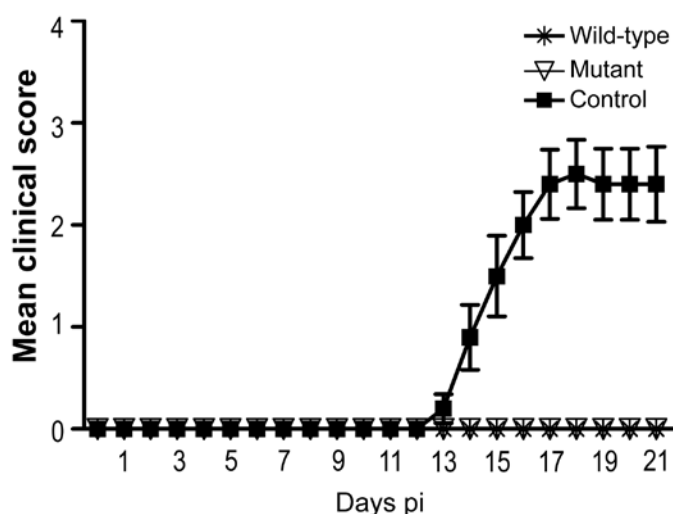


Fig 4.3.3: Clinical manifestation of disease: Clinical manifestation of disease was abolished completely in animals infected with *S. aureus* bacteria.

4.3.4: Neuropathological damage and RGCs survival after Infection with Eap-deficient strain of *S. aureus*

Histopathological analysis performed on the optic nerve s revealed that the protective effect of wild-type *S. aureus* infection on optic neuritis is reduced in the group infected with respective Eap-deficient strain.

In more detailed, we observed significantly less demyelination (Fig 4.3.4 A) in wild-type *S. aureus* infected animals ($22.77 \pm 8.46\%$; $n=11$) in comparison to both the animals groups infected either with *S. aureus* deficient for Eap ($53.53 \pm 10.56\%$; $P = 0.027$; $n=10$) or the control animals ($100 \pm 0\%$; $P < 0.001$; $n=4$). Accordingly, the extent of inflammation accessed by ED1 staining (Fig 4.3.4 B) was significantly lower in the group infected with wild-type *S. aureus* bacteria (1.24 ± 0.39 ; $n=11$) in comparison to the group infected with Eap-deficient bacteria (2.73 ± 0.40 ; $P = 0.012$; $n=10$) and the noninfected control group (4.66 ± 0.15 ; $P < 0.001$; $n=4$).

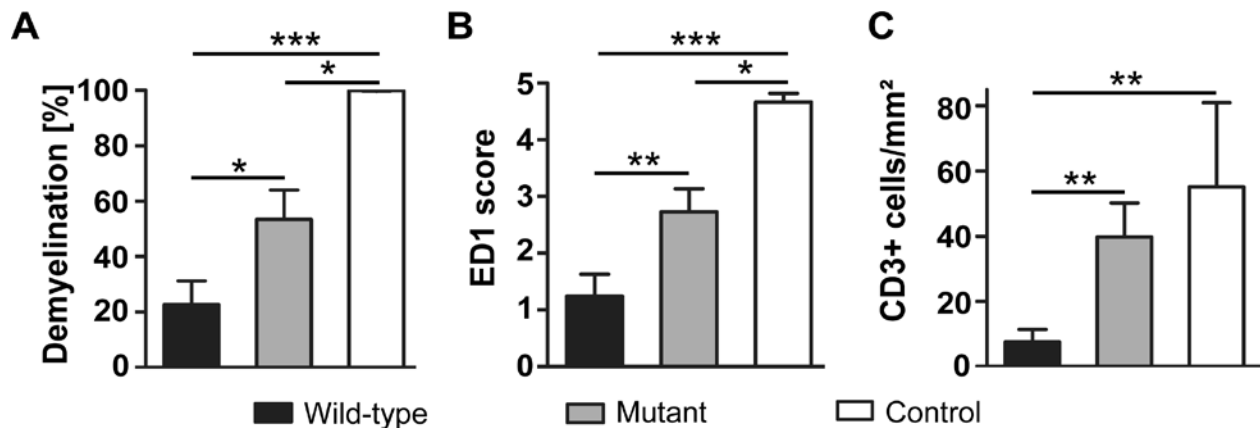


Fig 4.3.4: Optic nerve (ON) histopathology after infection with wild type *S. aureus* along with the strain deficient for Eap: The positive effect of *S. aureus* on optic neuritis was reverted in the Eap-deficient strain. **A**, **B** and **C** represents the quantitative data for extent of demyelination, ED1 score and CD3⁺ cells respectively.

CD3⁺ T-Lymphocyte counts revealed significantly lower numbers of CD3⁺ cells (Fig 4.3.4 C) in animals infected with wild-type *S. aureus* ($7.6 \pm 3.75/\text{mm}^2$; $n=11$) in comparison to the animals infected with Eap-deficient bacteria ($39.81 \pm 10.37/\text{mm}^2$; $P = 0.004$; $n=10$) and the control group ($55.2 \pm 25.8/\text{mm}^2$; $P = 0.007$; $n=4$). No significant difference was observed in CD3⁺ cell counts between the animals infected with Eap-deficient *S. aureus* bacteria and the noninfected control group ($P = 0.5$).

Bielschowsky's silver impregnation was performed to assess the axonal pathology of the optic nerve (Fig 4.3.5 A) and revealed that the animals infected with wild-type bacteria showed a higher number of axonal counts ($173500 \pm 18220/\text{mm}^2$;

n=11) in comparison to the group infected with Eap-deficient bacteria ($73953 \pm 14500/\text{mm}^2$; $P < 0.001$; n=10) and noninfected control animals ($16670 \pm 1938/\text{mm}^2$; $P < 0.001$; n=4).

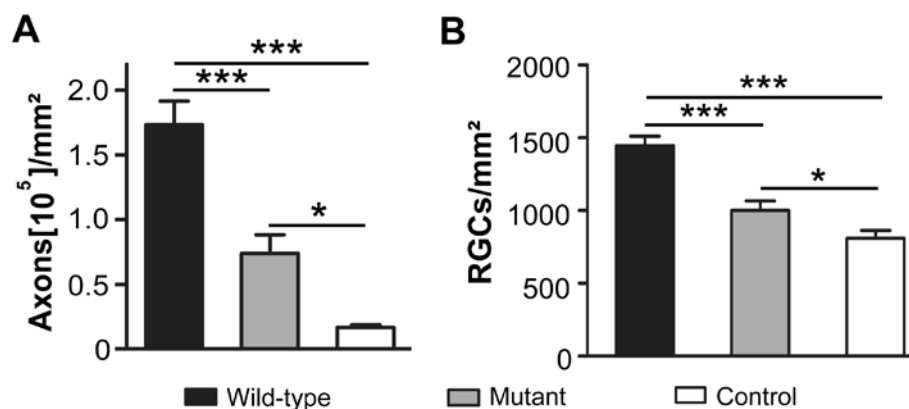


Fig 4.3.5: Axonal loss and RGCs count after infection with wild type *S. aureus* along with the strain deficient for Eap (mutant): Bielschowsky's silver impregnation (A) of ON cross sections revealed a significantly higher density of axons on EAE day 8 in wild-type infected animals. Retrogradely labeled RGCs counting (B) showed a higher number of RGCs in the group of animals infected with wild-type *S. aureus* bacterium.

The RGCs counts were performed on retinal flat mount which revealed a higher RGC density in the animal group infected with the wild-type strain ($1447 \pm 66/\text{mm}^2$) compared to animals infected with the Eap-deficient strain ($1001 \pm 65/\text{mm}^2$; $P < 0.001$; Fig 4.3.5) and the noninfected control group ($810 \pm 52/\text{mm}^2$, $P < 0.001$; Fig 4.3.5).

Although the infection with Eap-deficient *S. aureus* bacterium has a protective effect on the clinical course of disease but these results also indicated that the infection with wild-type bacterium is more protective in terms of optic nerve injury and RGCs counts compared to the animals infected with bacterium deficient for Eap or the control animals.

5. DISCUSSION

There is an extensive clinical literature describing the effect of infections on clinical course of MS, mostly on exacerbation of the disease course, but in some instances reducing relapses especially in case of parasitic infection (Correale and Farez, 2011; Correale and Farez, 2013; Fleming and Cook, 2006). In the present work we investigated the extent to which an acute infection with *E. coli* and chronic infection with *S. aureus* influences the course and severity of MOG induced EAE in female Brown Norway rats.

5.1: Infection and MS

There is a growing body of evidence that the progression of MS is influenced by systemic infection caused by bacteria or viruses. Moreover, components associated with certain bacteria and viruses have been shown to be a potential modifier in the progression of autoimmune diseases (Hamada *et al.*, 1989; Nogai *et al.*, 2005; Soos *et al.*, 1995; Xie *et al.*, 2006). However, only few studies have investigated the direct impact of infection with viable bacteria in the animal models of MS, which reflect more likely a clinical situation. The results of MRI on the influence of peripheral infection and the number of contrast-enhancing MS lesions as a marker of disease activity are inconsistent (Edwards *et al.*, 1998; Kalkers *et al.*, 2002). Moreover, reliable markers for neurodegeneration in MS patients do not exist. For this reason, much of the knowledge about the interaction of systemic infection and autoimmunity in MS is derived from studies in EAE models. Previous studies in EAE models mainly focused on analyzing the clinical course of the disease. Furthermore, in most experiments solely bacterial compounds were used to induce systemic inflammation (e.g. LPS, enterotoxins) (Moreno *et al.*, 2011; Schiffenbauer *et al.*, 1993; Soos *et al.*, 1995) instead of whole viable bacteria, therefore these studies showed the limited value concerning the reflection of the clinical situation in MS-patients.

5.1.1: Acute infection with *E. coli* and EAE

Clinical as well as experimental data indicated that the preclinical phase prior to the disease manifestation to be a period with the highest susceptibility to infections (Herrmann *et al.*, 2006). However, during the disease course of MS, infections with common bacteria are also observed, which could have the potential to modulate the disease severity. Therefore, to elucidate the impact of infections in our study, two different infection time points were selected. In the first group, animals were infected at day 7 post immunization corresponding to the preclinical phase of the disease. Furthermore, to reflect the clinical situation a second group of animals was infected on day of clinical onset of disease. To increase the survival of animals after the infection, they were treated with ceftriaxone, a beta lactam antibiotic used for the cure of bacterial infection. The effect of beta lactam antibiotic in an animal model of MS remains controversial. In the study of Nico Melzer and coworkers, it was shown that the ceftriaxone treatment ameliorated the clinical course of MOG-induced EAE in mice by modulating cellular antigen-presentation and impairment of antigen-specific T-cell migration into the CNS (Melzer *et al.*, 2008). In contrast to this finding Felix Mor and colleagues showed that adoptive transfer of BP10 cell line incubated with ceftriaxone shown more severe EAE symptoms (Mor and Cohen, 2013). Moreover, they also showed that cefuroxime, another beta lactam antibiotic enhanced adjuvant-induced arthritis and experimental autoimmune encephalomyelitis (EAE) in both active and passive forms. However, to rule out the side effect of ceftriaxone in our study all the animals from infected and noninfected group was treated with ceftriaxone during the experimental phase of disease.

Viral and bacterial infections can aggravate the disease symptoms in MS patients (Buljevac *et al.*, 2002). Moreover, in the previous experimental studies in EAE it has been shown that the, bacteria like *Chlamydia pneumoniae* and *Streptococcus pneumoniae* could exacerbate the disease symptoms (Du *et al.*, 2002; Herrmann *et al.*, 2006). Here, in our study, in spite of a systemic infection with *E. coli* in the preclinical phase of disease induced weight loss in EAE animals considering as a sign of general sickness behavior, it has no influence neither on the time point of onset of clinical EAE

symptoms nor on the severity of the disease. Moreover, to reflect the clinical situation, we infected the animals on the clinical onset of EAE. Despite the presence of bacteria in the systemic circulation of *E. coli* infected animals next days after clinical onset of disease it did not influence the disease severity. Further, histopathological analysis performed on the optic nerves of animals did not reveal any significant increase in inflammatory infiltrates in the animals infected with *E. coli* either on day 7 post immunization or on day 1 EAE compared the respective control. Further, in this study, we did not observe any significant difference in other histopathological correlates like demyelination and axonal counts on the optic nerves in the group infected with *E. coli* compared to the control.

Results obtained from the acute infection with *E. coli* in this animal model, did not reflect the previous findings, those which were performed with the bacterial components and induce a robust, but short lasting systemic inflammation. It has been shown that *E. coli* infection increases the permeability of the BBB during sepsis, but unable to cross the BBB (Tsao *et al.*, 2001). Moreover, it has also been suggested that infection of CNS is necessary for worsening of EAE (Du *et al.*, 2002). These experimental data could possibly explain the discrepancies in our finding compared to the previous experimental studies. However, other possible reasons for such discrepancies in our result might be due to balance between Th1/Th2 cytokine profile, molecular mimicry and the role of Toll like receptors (TLRs). These finding also suggested that the kind of micro-organism also has influence on exacerbation of EAE relapse and their histopathological changes.

5.1.2: Chronic infection model

Foreign body infections are characterized by the presence of adherent bacteria on the surface of the synthetic prosthetic materials for a very long time and these bacteria supposed to escape host dependent phagocytic mechanisms. *S. aureus* is a common pathogen in both hospital- and community-associated infections (Grady and Cullen, 2003) and cause serious complications in prostheses by formation of biofilm and developing tolerance towards an antibiotic treatment (Murillo *et al.*, 2006). *S. aureus* contains or secretes around 200 products, which may interfere with immune-

inflammatory reaction and can confer virulence of the host. Moreover, the involvement of some of the bacterial components, isolated from *S. aureus*, in the modulation of EAE was demonstrated in previous experimental studies (Schiffenbauer *et al.*, 1993; Soos *et al.*, 1995; Xie *et al.*, 2006). Therefore, these observations indicated, *S. aureus* to be the ideal microorganism to study the effect of chronic infection *in vivo*. Lucet and co-workers established an experimental model of chronic foreign body infection by inoculating 10^5 - 10^6 CFU of *S. aureus* in the implanted tissue cages containing two PMMA cover slips in Wister rat (Lucet *et al.*, 1990).

However, in our study tissue cages implant with PMMA cover slips was unable to induce the chronic infection even with a high inoculum dosage of 10^9 CFU of *S. aureus* in BN rat. To overcome this problem semi-solid agar, an excellent medium for the growth of bacteria, was injected directly into the implanted tissue cages of the animals prior to infection. That further leads to a persistent chronic infection in this animal model. The advantage of this chronic infection model is that, it induces mild infection and even without antibiotic treatment, it does not lead to death of the animal. A major advantage of agar injection into the implant was that, it reduces the inoculum dosage of *S. aureus* to induce the chronic infection, which allow us to stabilize the implant in the body of the animals for longer time duration.

5.1.3: Chronic infection with *S. aureus*, induces systemic inflammation

Infection with bacteria induces the sign of sickness behavior, which reflects in the form of weight loss (Perry, 2010). Interestingly, animals infected with *S. aureus* did not show any weight loss after the infection. Moreover, during the course of the experiment we did not observe the presence of bacteria in the peripheral blood of animals, when plated on the blood agar plate. However, at the end of the experiments, TCF obtained from the animals infected with *S. aureus* showed a high number of bacterial counts (*S. aureus*) on the blood agar plate, whereas no bacterial growth was observed in TCF obtained from noninfected control animals. The data obtained from TCF, obtained at the end of the experiments, plated on the blood agar plate confirmed the persistent chronic infection in the tissue cage of the animals. Moreover, leukocyte counts in the peripheral

blood from the *S. aureus* infected animals showed a significant increase in the blood leukocyte counts, 2 days after the local application of the bacteria in the tissue cage. Further, flow-cytometric analysis of the peripheral blood circulation also showed a significant increase in the population of B- and T-cell count in the animals infected with *S. aureus*. Furthermore, the flow cytometric analysis also indicated a more pronounced increase in the T-cell subpopulation 2 days after the infection, compared to the control. These results confirm that a chronic infection with *S. aureus* leads to a very strong systemic inflammatory response in the peripheral blood circulation of the animals.

It has been shown that after the infection, Infectious agents can expand the pool of host's immune cells, which also includes auto-reactive T-cells in EAE and thus can contribute to the disease progression (Du *et al.*, 2002; Nogai *et al.*, 2005; Visser *et al.*, 2005). Thus we hypothesized that due to increase in leukocyte counts and a substantial increase of B-, T- and subpopulation of T-cell in peripheral blood after the infection, it will lead to more severe damage to the brain tissue and possibly could exacerbate the symptoms of EAE. Unexpectedly, the infection with *S. aureus* prevented the development of clinical EAE. Moreover, it significantly decreased the extent of inflammatory infiltrates and demyelination of the optic nerve. However, these results can be explained on the basis of hygiene hypothesis which states that infections protect rather than induce/accelerate autoimmune diseases like MS (Bach, 2002). The hygiene hypothesis proved to be protective in several clinical and experimental studies (Correale and Farez, 2011). Moreover, hygiene hypothesis also state that due to hyper-activation of auto-reactive cells with further activation leads to cell death and may reduce the number of aggressive cell number. Moreover, the competitive mechanisms between the infectious agent and the body own immune cells for the antigen binding over MHC molecules and antigen processing by phagocytic cells could also play an important role in this protective function. In the previous finding it has been shown that the induction of superantigens produced by different infectious agents can exacerbate the EAE (Schiffenbauer *et al.*, 1993). However, these findings could have a different prospective as well. It is well known that after the antigen presentation on MHC groove it leads to the activation of specific T-cell population bearing particular T-cell-receptor in variable regions of the beta chain. However, the presence of superantigen induces polyclonal T-

cell activation (Fraser and Proft, 2008). Hence, in my opinion of superantigen might cause a reduction of activated T-cell number expressing a particular TCR by modulation in the variable region of beta chain (Correale and Farez, 2011).

5.1.4: Chronic infection with *S. aureus* and cytokine release

It is well known fact that infection induces cytokine release in the periphery, which is crucial for fighting against infections. However, sometimes they can become dysregulated during inflammation, trauma, and sepsis (Dinarello, 2000). In multiple sclerosis how the regulations of different cytokine takes place are not clearly known. Experimental studies indicated that in EAE, the release of pro-inflammatory cytokines worsen the disease, whereas anti-inflammatory cytokines reduce inflammation and promote healing. Moreover, it has been shown that the pro-inflammatory cytokines like IL-1, IL-6, IL-12, IL-17, IL-23, TNF- α , and IFN-gamma seems to increase the severity of the disease in EAE (Komiya *et al.*, 2006; Langrish *et al.*, 2005; Samoilova *et al.*, 1998). In contrast to this several experimental studies indicated the protective role of IFN-gamma and TNF- α in EAE (Furlan *et al.*, 2001; Willenborg *et al.*, 1996; Zhang *et al.*, 2012).

In spite of anti-inflammatory activity of Eap during infection, in our study, we detected a significant increase in the level of the IL-6 and IFN-gamma levels at the end of the experiment in the group infected with *S. aureus* wild-type strains. However, such a pro-inflammatory cytokine pattern was not detected in animals infected with the Eap-deficient strain. Our findings correspond well to in vitro data showing increased secretion of IL-6 and TNF- α in murine cells and in human blood samples after stimulation with Eap (Edwards *et al.*, 2012; Scriba *et al.*, 2008) and indicate pro-inflammatory properties of this protein in the systemic circulation. However, Scriba and coworkers also showed that this pro-inflammatory cytokine release is taking place through monocytes and macrophages but not by T-cell populations. Intriguingly, in spite of ongoing inflammation in the periphery the infection with *S. aureus* prevented the onset of clinical EAE symptoms and decreased the severity of autoimmune optic neuritis. According to our hypothesis secretion of Eap blocks the migration of

inflammatory cells into the CNS, which in turns protects the integrity of the BBB. However, less infiltration of T- and B-cells in the animals infected with *S. aureus* restrict the innate release of pro-inflammatory cytokines from these cells into the CNS. When the BBB is intact, the majority of the blood borne cytokines enters into the CNS by the saturable transport system (Banks, 2005; Banks *et al.*, 1991), which facilitates the selective movement these cytokines across the BBB. However, during EAE the alteration in the permeability of the BBB allows an unrestricted movement of these cytokines into the CNS, which probably not the case in the animals infected with *S. aureus*. This could be a possible reason why ongoing inflammation in the periphery has no deleterious effect on the course of this disease.

5.1.5: The role of adhesion molecules in transendothelial migration

The entry of leukocytes into the CNS is considered as an early event in MS. That includes the involvement of cytokines, chemokines and adhesion molecules expressed by both the activated endothelium and the immune cells, which further leads to the breakdown of the BBB and inflammation of the CNS. The relevance of VLA-4 - VCAM-1 for migration of lymphocytes across the BBB during EAE has been shown in several experimental studies (Archelos *et al.*, 1993; Gordon *et al.*, 1995; Yednock *et al.*, 1992). Moreover, several genetics studies also indicated the relevance of adhesion molecules in the susceptibility of the disease (Damotte *et al.*, 2014). The co-ordinated sequential events of inflammatory cells migration into the CNS, especially, involve the VLA-4 - VCAM-1 and the LFA-1 - ICAM-1 adhesion systems (Engelhardt and Wolburg, 2004; Laschinger and Engelhardt, 2000; Lee and Imhof, 2008; Wittchen, 2009). However, there is increasing evidence that both systems may have a distinct contribution in the recruitment of myelin activated T-cells. Experimental studies also indicated the organ specific expression of such molecules on their endothelium during autoimmune inflammation. Moreover, the subtype of T-cells that are involved in the pathology may vary in patients (Bitsch and Bruck, 2002; Metz *et al.*, 2014). It has been shown, that Th1- and Th17-cells filtration in the spinal cord is dependent on a VCAM-VLA-4 interaction (Rothhammer *et al.*, 2011). In contrast to this finding Bharadwaj and coworkers showed that the vascular endothelial migration of Th1- and Th17-cells was

mediated by ICAM instead of VCAM expression (Bharadwaj *et al.*, 2013). Hence it can be speculated from a previous finding that different subtypes of activated T-cells will be recruited on various adhesion molecules and crosses the BBB.

Humanized monoclonal antibody natalizumab is in therapeutic use for MS. In a clinical study it was observed that natalizumab treatment reduced the MRI activity by 92%, which showed the highest efficacy for any drugs available for treatment of MS (Miller *et al.*, 2007; Polman *et al.*, 2006). Natalizumab treatment targets the α 4-integrin molecule, a component of the VLA-4 present of lymphocytes thus prevents the interaction with VCAM found on endothelial surfaces and subsequently blocks the migration of the lymphocytes to the CNS, which resulted in the reduction of the inflammation in the CNS and disease severity of the disease. However, some patients do not respond to the natalizumab treatment, especially in patients with progressive forms of MS. This clearly indicates the heterogeneity of mechanism involve in the disease pathogenesis of MS. Another concerning factor for natalizumab treatment is the incidence of PML (2.1/1000 patients) which increases with the length of treatment (Bloomgren *et al.*, 2012; Clifford *et al.*, 2010). Moreover, the patient becomes more susceptible if they were treated with an immunosuppressant before receiving natalizumab (Kappos *et al.*, 2011). In contrast to the VLA-4 - VCAM-1 interaction several studies indicated the role of ICAM in pathogenesis of MS (Bullard *et al.*, 2007; Damotte *et al.*, 2014; Steiner *et al.*, 2010). Furthermore, the expression of ICAM-1 has been shown to be up-regulated in MS. Eventually there is no current therapy targeting the LFA-1 - ICAM-1 adhesion pathway.

Staphylococcal enterotoxins (SEs) are one member of a unique group of molecules called super-antigens which could induce a very strong immune response in the animals. Moreover, it has been also shown that super antigens may trigger EAE by stimulation of autoreactive T-cells from the variable region of beta chain (Schiffenbauer *et al.*, 1993; Soos *et al.*, 1995). In contrast to this, *S. aureus* also secrete several anti-inflammatory and anti-adhesive factors that may interfere with every step of inflammatory cells recruitment of the host (Chavakis *et al.*, 2007) and help them for an efficient colonization within the host (Chavakis *et al.*, 2005; Foster and Hook, 1998).

S. aureus secrete a group of anti-adhesive protein consist of 5 different structurally unrelated secreted adhesion molecule named as “secretable expanded repertoire adhesive molecules” (SERAM), and one of them is Eap (Chavakis *et al.*, 2007). Eap, also called as Map, “MHC class II analogous protein”, which has been shown to be present in 97% of clinical isolates (Harraghy *et al.*, 2003). The Eap not only help bacteria to get adhere but also interferes with host defense mechanisms. It has been shown that Eap specifically interacts with endothelial ICAM-1 and inhibit the recruitment of neutrophil at the site of infection. Furthermore, Eap also interfere with T-cell migration by blocking the interaction between LFA-1 and ICAM-1 (Athanasopoulos *et al.*, 2006; Chavakis *et al.*, 2002; Hagggar *et al.*, 2004). Moreover, an anti-inflammatory effect of Eap in autoimmune diseases has been found in EAE and an animal model of psoriasis (Wang *et al.*, 2010; Xie *et al.*, 2006).

Despite of a significant increase in T-cells count in the animals infected with *S. aureus* it showed a significantly less infiltration of T-cells on the optic nerve of the animals. These findings strongly suggested the involvement of a factor which, probably involved in the trans-migration of myelin activated T-cells across the BBB. Based on these observations, we hypothesized that the anti-inflammatory effects of *S. aureus* infection in MOG-EAE are mediated through the secretion of Eap. We infected the animals with the *S. aureus* strain deficient for Eap. In our study, the infection with the Eap-deficient *S. aureus* strain led to increased inflammation, demyelination and axonal damage in the optic nerve compared to the animals infected with the wild-type *S. aureus* strain. However, histopathological analysis of the optic nerve revealed that the anti-inflammatory property of *S. aureus* infection was not completely abolished by lacking Eap. Moreover, the infection with the Eap-deficient strain prevented the clinical manifestation of the disease, indicating that additional mechanisms are involved in the anti-inflammatory effects of *S. aureus*. Given the importance of ICAM-1 antagonism in different autoimmune diseases (Kaneider *et al.*, 2006), Eap is considered to be the most potent factor for inhibition of T-cell migration into the CNS. This hypothesis is supported by our histopathological evaluations revealing a substantial decrease of lymphocyte count in inflammatory infiltrates of the optic nerve in wild-type *S. aureus* infected animals.

5.2: Infection and neurodegeneration

Epidemiological data implicated both genetic and environmental factors in the etiology of MS. The association of systemic infection and risk of MS relapse has been proposed on the basis of epidemiological data (Handel *et al.*, 2010) from several clinical studies (Correale *et al.*, 2006; Sibley *et al.*, 1985). However, whether the infection leads to the increased neurodegeneration, a major hallmark of disability in MS patients is not fully understood.

Microglia are the innate immune cells of the brain and play a very important role in the maintenance of the microenvironment of the CNS. In the presence of external stimulus these cells can become activated which is crucial for host defense mechanism. However, over-activation of microglia may cause neurotoxicity (McGeer *et al.*, 2005; Polazzi and Contestabile, 2002). Peripheral inflammation, which leads to the activation of Microglial cells is a common phenomenon of several neurodegenerative diseases like Parkinson's disease, Alzheimer's disease, Multiple sclerosis and Amyotrophic Lateral Sclerosis (Nguyen *et al.*, 2002). It is now well established, that in chronic neurodegenerative diseases of the CNS, such as Parkinson and Alzheimer diseases the resident microglia are primed by prior pathology to respond more vigorously to inflammatory stimulation. In this context, the signals from systemic infection or inflammation lead to a switch of microglia/macrophages towards a pro-inflammatory phenotype (Cunningham, 2013; Perry *et al.*, 2007) and this switch contributes to progression of neurodegeneration (Ferrari *et al.*, 2006; Nguyen *et al.*, 2004).

The majority of studies on studying the interaction of systemic infection and autoimmunity have been performed on EAE animals using a single challenge with lipopolysaccharide (LPS), which is present on the outer membrane of Gram-negative bacteria. Systemic insult with LPS leads to a switch in the macrophage/microglia phenotype in the rat EAE model. This switch was accompanied by prolonged pro-inflammatory response by releasing inducible nitric oxide synthase (iNOS) and expression of pro-inflammatory cytokines, which in turn lead to the more pronounced neuronal damage (Moreno *et al.*, 2011). But whether these bacterial components (LPS, enterotoxins, or teichoic acid, etc.), which are commonly used to mimic the situation of

bacterial infections does have the similar effect on neurodegeneration is not clearly known. Thus, the systemic inflammation induced by bacterial infections has to be addressed, particularly within the experimental models of MS.

Role of TLRs in infection and EAE have been studied which are expressed on several types of cells, including peripheral lymphocytes, microglia cells and neurons (Kawai and Akira, 2007). Extracellular leucine-rich repeat domains of these receptors have been shown to be involved in specific pathogen recognition (Akira *et al.*, 2006). However, the cytoplasmic domain is involved in signaling pathways. Upon sensing ligands, recruitment of adaptor proteins (MyD88 except for TLR3) takes place which in turns initiate the signal transduction pathway and leading to activation of NF- κ B and induced the production of pro-inflammatory cytokines like TNF- α , IL-6, and I L-1b etc. It is also observed that MyD88 pathway plays a crucial role in CNS infection and optic nerve injury (Zheng *et al.*, 2012). In the previous study it has been shown that *Chlamydia pneumoniae* infection exacerbates EAE in mice. Moreover, from the mechanistic point of view Herrmann and coworkers showed that *Streptococcus pneumoniae*, a gram negative bacterium causes respiratory tract infection, can aggravate EAE via TLR2 without affecting the level of pro-inflammatory cytokines and T-cell proliferation. However, in both of these studies there was no evidence for the neuronal cell loss. These animal studies indicate the role of systemic infection on CNS, but whether these infections lead to the more pronounced neurodegeneration is not clearly known. In this study, we address the impact of infection on neuronal damage after the infection in acute as well as in chronic Infectious conditions. In our study despite of presence of *E. coli* in the peripheral blood circulation 24 h after the infection we did not observe any significant difference in the loss of RGCs between the animals infected with *E. coli* and the control during clinical and preclinical phase of disease. However, the loss of RGCs showed a trend for more pronounced RGCs loss of in the animals infected on day 1 EAE compare to its control. Thus, acute *E. coli* infection could possibly have long-term consequences in regards to neuronal degeneration.

We know from previous study that neuro-inflammation caused by external stimulus, which leads to the activation of microglial cells is not always deleterious

(Glezer *et al.*, 2006; Simard *et al.*, 2006). However, in our study with the chronic infection model we infected the animals with *S. aureus* during the preclinical phase of disease, which induces a strong inflammatory response in the periphery showed by increased leukocyte counts. Surprisingly, we observed an increased neuronal survival in the animals infected with *S. aureus* compared to its control. These results indicated that the importance of BBB, that probably does not allow the inflammatory cells to enter into the CNS and induce the inflammatory signal across it. Moreover, by introducing Eap mutant strain in this study, we observed a higher neuronal survival in animals infected with wild-type bacterium compared to the animals infected with bacterium deficient for Eap. In our previous EAE studies with the same animal model, we observed that strong anti-inflammatory treatment is not always sufficient to prevent neurodegeneration (Diem *et al.*, 2003; Maier *et al.*, 2006; Rau *et al.*, 2011). Even though we see a more neuronal survival in the animals infected with wild-type bacterium compared to the animals infected with bacterium deficient for Eap we cannot rule out a direct neuroprotective effect of Eap. Further studies are needed to clarify this issue. Nevertheless, by suppression of LFA-1 and ICAM-1 mediated transendothelial migration of immune cells into the CNS with consecutive neuroprotection, Eap has potential to serve as a new therapeutic for the treatment of MS.

5.3: A clear distinction between the animal model of MS is important

In the previous experimental studies it has been shown that the systemic inflammation induced by bacterial components lead to exacerbation of the disease course in EAE (Moreno *et al.*, 2011; Schiffenbauer *et al.*, 1993; Soos *et al.*, 1995). Although in our studies we found that the inflammatory stimulus induce by a viable bacterium was more pronounced, it does not seem to have the similar effect as found in the previous finding performed by the use of solely bacterial components. Moreover, data from the acute infection model induced by *E. coli* also differed from the results obtained from the chronic infection model induced by the *S. aureus*. Nevertheless, the time point of infection as shown by Herrmann *et al* could also play determining role in the disease pathogenesis. From the previous and our current finding we conclude that the robust, but short lasting defined inflammatory stimulation induced by the bacterial components

like LPS or enterotoxins felt adequately to reproduce the full spectrum of inflammatory changes a real systemic infection (Remick and Ward, 2005). Therefore, this is important to draw a clear distinction between these models of systemic inflammation to study various autoimmune diseases like MS.

6: CONCLUSIONS

To address the question how does the bacterial infections (acute and chronic) influence the severity of disease in an experimental model of MS, we investigated the extent to which an acute infection with *E. coli* and chronic infection with *S. aureus* influences the disease course of MOG induced EAE in female Brown Norway rats. Moreover, we also investigated the effect of the acute and chronic bacterial infection on neurodegeneration.

In the current study, viable *E. coli* infection in female BN rats did not influence the clinical course of disease in EAE. Moreover, we did not observe any significant histopathological changes, performed in the optic nerve s as well as on the survival of RGCs, in the group infected with *E. coli* compared to the control. Findings from these studies indicated that the systemic insult with bacterial components (Lipopolysaccharides, superantigens) do not reflect the true inflammatory conditions induced by a viable bacterial infection. Therefore this is important to draw a clear distinction between these two models of systemic inflammation.

In contrast to the acute infection, chronic infection induced by inoculating *S. aureus* into the agar of the implanted tissue cage showed a chronic systemic inflammatory response with increased in T- and B-cell counts as well as systemic production of pro-inflammatory cytokines. Unexpectedly, the chronic infection with *S. aureus* prevented the development of clinical EAE and markedly decreased the extent of inflammatory infiltrates and demyelination of the optic nerve. Moreover, using a *S. aureus* strain deficient for the extracellular adherence protein we showed the beneficial effect of a chronic bacterial infection on disease modulating effect in EAE is due to the secretion of this protein. Furthermore, infection with *S. aureus* also increased the number of surviving RGCs and axonal counts that seems to be related to the anti-inflammatory properties of Eap. Further studies are needed to clarify the direct neuroprotective effect of Eap.

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List of abbreviations

°C	Degree Celsius
µl	Microliter
APC	Antigen-presenting cell
ATCC	American Type Culture Collection
BBB	Blood brain barrier
BN	Brown Norway
CD	Cluster of differentiation
CFU	Colony forming units
CNS	Central nervous system
CSF	Cerebro spinal fluid
Cy	Cyanine
DNA	Deoxyribonucleic acid
<i>E. coli</i>	Escherichia coli
EAE	Experimental autoimmune encephalomyelitis
Eap	Extracellular adherence protein
EBV	Epstein Barr virus
EDSS	Expanded Disability Status Scale
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FG	Fluorogold
FITC	Fluorescein isothiocyanate
FPRL-1	Formyl peptide receptor-like 1
FTY	Fingolimod
G	Gram
GA	Glatiramer acetate
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
H	Hour

ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL	Interleukin
Ip	Intraperitoneal
Kg	Kilogram
L	Liter
LFA-1	Lymphocyte function-associated antigen-1
LFB	Luxol fast blue stain
LPS	Lipopolysaccharides
MAG	Myelin-associated glycoprotein
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MI	Milliliters
Mm	Millimeters
MOG	Myelin oligodendrocyte glycoprotein
MOMP	Major outer membrane protein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSCH	Mouse spinal cord homogenate
Mt	Mycobacterium tuberculosis
NaCl	Sodium chloride
ON	Optic nerve
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
Pi	Post immunization
PLP	Proteolipid protein
PML	Progressive Multifocal Leukoencephalopathy
PMMA	Polymethylmethacrylate

PMN	Polymorphonuclear leukocytes
PPMS	Primary progressive multiple sclerosis
PSGL-1	P-selectin glycoprotein ligand-1
RGC	Retinal ganglion cells
ROI	Regions of interest
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
<i>S. aureus</i>	Staphylococcus aureus
S1P	Sphingosine-1 phosphate
SERAM	Secretable expanded repertoire adhesive molecules
SPMS	Secondary progressive multiple sclerosis
TCF	Tissue cage fluid
TGF- β	Transforming growth factor beta
TLR	Toll-like receptors
TNF	Tumor necrosis factors
V β	Variable region
VCAM-1	Vascular cell adhesion molecule 1
VLA-4	Very late Intigen-4

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Sabine Herold[#], **Prateek Kumar[#]**, Sven P. Wichert, Benedikt Kretzschmar, Mathias Bähr, Moritz J. Rossner, Katharina Hein Neurodegeneration in auto-immune optic neuritis is associated with altered APP cleavage in neurons and transcriptional up-regulation of p53. Submitted (Under review)

Prateek Kumar, Catherine Friebe, Rieka Schallorn, Roland Nau, Sandra Schütze, Mathias Bähr, Katharina Hein, Acute Escherichia coli infection has no influence clinical course and the extent of neurodegeneration in animal model of multiple sclerosis. Submitted (Under review)

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