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Biopsychological interactions in autoimmune models of CNS inflammation

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To my parents

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Abbreviations

Aβ	Amyloid-β
BDNF	Brain-derived neurotrophic factor
cAMP	Cyclic adenosyl monophosphate
Ccl5	Chemokine (C-C motif) ligand 5
CFA	Complete Freund's adjuvant
COX-2	Cyclooxygenase-2
CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFAP	Glial fibrillary acidic protein
IDO	indolamine 2,3 dioxygenase
IFN-γ	Interferon gamma
IL-1β	Interleukin 1beta
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-17	Interleukin 17
KO	Knockout
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
P.i.	Postimmunization
PLP	Proteolipid protein

rtPCR	Real-time polymerase chain reaction
S.c.	subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
TLR	Toll-like receptor
TNF	Tumor necrosis factor

Abstract

Inflammatory processes are known to impair psychological functioning in several species by the induction of various proinflammatory molecules in the CNS. In the present thesis, these biopsychoimmunological interactions were investigated in a multidimensional approach.

The immunomodulatory properties of the antidepressant venlafaxine were examined in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. In EAE, oral treatment with venlafaxine significantly ameliorated the clinical symptoms and neuropathological manifestation of the disease compared to vehicle during both preventive and therapeutic intervention. Venlafaxine suppressed the generation of proinflammatory cytokines in encephalitogenic T cells and peritoneal macrophages *in vitro*. In an astroglia-microglia co-culture model, venlafaxine significantly changed the microglial phenotype from activated to resting morphology. To further identify the impact of inflammatory processes on biopsychological functions, an autoimmune model of cognitive and behavioral impairment was established by active immunization with amyloid-β 1-42 (Aβ1-42), a peptide implicated in the pathogenesis of Alzheimer's disease. In C57BL/6 mice, active immunization with Aβ1-42 impaired locomotor activity, habituational learning and spatial-learning abilities compared to mice immunized with a myelin peptide or adjuvant alone. A disseminated, non-focal immune cell infiltrate mainly consisting of macrophages was identified in the CNS of Aβ1-42-immunized animals. These findings taken together strongly indicate that neurocognitive impairment is induced by the activation of the innate immune system after immunization with Aβ1-42.

In conclusion, the results of the present thesis might have direct clinical implications regarding the future therapy of neuroinflammatory and neurodegenerative diseases in humans.

Zusammenfassung

Entzündliche Prozesse und die damit einhergehende Sekretion proinflammatorischer Moleküle können psychologische Prozesse nachhaltig beeinflussen. Die Interaktion inflammatorischer und psychologischer Prozesse war Gegenstand der vorliegenden Arbeit und wurde aus verschiedenen Perspektiven beleuchtet.

Zunächst wurden die antiinflammatorischen Eigenschaften des Antidepressivums Venlafaxin im Mausmodell der Multiplen Sklerose, der experimentellen autoimmunen Enzephalomyelitis (EAE), untersucht. In diesen EAE Studien konnten wir zeigen, dass die orale Gabe von Venlafaxin die klinische und neuropathologische Manifestation der Erkrankung sowohl in präventiven als auch therapeutischen Interventionen im Vergleich zum Vehikel signifikant milderte. *In vitro* unterdrückte Venlafaxin die Produktion von proinflammatorischen Zytokinen in T Zellen und peritonealen Makrophagen. In einem Astroglia-Mikroglia Ko-Kultur Modell konnte Venlafaxin zudem die Aktivierung von Mikroglia Zellen verhindern. Die antiinflammatorische Wirkung des Antidepressivums liefert so neue Erkenntnisse über die Interaktion von entzündlichen ZNS Prozessen und der Pathogenese affektiver Störungen. Um den Einfluss entzündlicher Aktivität auf biopsychologische Prozesse weiter zu charakterisieren, wurde ein Mausmodell etabliert, welches die Induktion von kognitiven Defiziten durch aktive Immunisierung mit dem neuronalen Autoantigen Amyloid-β 1-42 (Aβ1-42) erlaubt. Aktive Immunisierung mit Aβ1-42 Peptid führte in C57B/6 Mäusen zu reduziertem Lokomotionsverhalten und eingeschränkten Leistungen im visuell-räumlichen Lernen. Die alleinige Gabe des Adjuvans oder EAE Induktion hatte im Vergleich zur Immunisierung mit Aβ1-42 keine Auswirkungen auf kognitive Parameter. Die Immunisierung mit Aβ1-42 führte zu einer profunden Aktivierung des angeborenen Immunsystems, welches über Infiltration von Makrophagen ins ZNS kognitive Defizite verursacht.

Die Ergebnisse dieser Dissertation verdeutlichen die Interaktionen inflammatorischer und biopsychologischer Prozesse und könnten darüber hinaus zukünftige Therapien neuroinflammatorischer und neurodegenerativer Erkrankungen nachhaltig beeinflussen.

Introduction

CNS autoimmunity

Inflammation is apparently associated with complex biological responses of an organism to harmful stimuli such as pathogens. In autoimmune diseases, an organism fails to recognize its own constituent parts as self, which allows an inflammatory response against its own cells and tissues. The most prevalent autoimmune disease affecting the central nervous system (CNS) is multiple sclerosis (MS). In MS, the CNS is infiltrated by immune cells leading to demyelination and axonal damage (Lassmann et al., 2007). Besides neurological deficits, fatigue and depressive episodes appear in the course of this disease in more than 50% of MS patients (Joffe, 2005).

Animal models of CNS autoimmunity

Experimental autoimmune encephalomyelitis (EAE) is the well-known animal model of MS which allows studying inflammation-related damage of CNS tissues. EAE can be induced in several animal strains by immunization with myelin components or by adoptive transfer of myelin-specific T cells (’t Hart and Amor, 2003). The disease is clinically characterized by neurological deficits, mainly paresis, and histopathologically by perivascular infiltrates in the spinal cord and brainstem. In most EAE models, the disease is initiated by CD4+, Major histocompatibility complex (MHC) class II-restricted Th1 and Th17 cells (Stromnes et al., 2008). Activated CD4+ T cells can cross the blood–brain barrier, infiltrate the CNS and secrete chemokines and proinflammatory cytokines upon rechallenge by microglial cells and autoantigen. The secreted chemokines and cytokines will attract macrophages to the lesion and activate microglial cells which both significantly contribute to CNS tissue damage by secreting inflammatory molecules. Furthermore, astroglial cells proliferate within demyelinating lesions of MS and EAE (Holley et al., 2003; Tani et al., 1996) and promote inflammation, oligodendrocyte damage and glial scarring (Ambrosini et al., 2005).

CNS inflammation and affective disorders

At first view, affective disorders and autoimmune diseases such as MS or EAE are different diseases with distinct clinical phenotypes. However, studying the immunological basis of MS and affective disorders may shed light on the interaction of both diseases. The role of cytokines in the pathogenesis of mood disorders has received considerable attention during the last decade (e.g. Besedovsky and Rey, 2007). In particular, increased levels of Interleukin 6 (IL-6), Interleukin 1β (IL-1β) and tumor necrosis factor (TNF) in stimulated peripheral blood mononuclear cells of depressed patients were reported (Cyranowski et al., 2007).

In MS, a number of proinflammatory cytokines [e.g. Interleukin 17 (IL-17), Interferon-γ (IFN-γ), TNF] are found in the cerebrospinal fluid (Ishizu et al., 2005) or in lesions during acute MS relapses (Lassmann et al., 2007), whereas antiinflammatory cytokines such as Interleukin 10 (IL-10) and transforming growth factor-β (Carrieri et al., 1998) are detected during remission, suggesting an imbalance of pro- and antiinflammatory cytokines in this disease. Interestingly, autoreactive T cells from MS patients with concomitant depression revealed a reduced IFN-γ production during antidepressant therapy with sertraline (Mohr et al., 2001).

Antidepressants and inflammation

In the past years, several studies uncovered immunoregulatory effects of antidepressant agents (e.g. Maes, 2001). Venlafaxine, fluoxetine and imipramine were found to have negative immunoregulatory effects by reducing the IFN-γ and elevating the IL-10 production in whole blood cells (Kubera et al., 2001). Further studies reported reduced levels of proinflammatory and increased levels of antiinflammatory cytokines (Kenis and Maes, 2002; Obuchowicz et al., 2005; Xia et al., 1996) during antidepressant treatment. To investigate the close interaction of CNS inflammation and affective disorders, we examined the effects of the antidepressant venlafaxine in murine EAE.

Psychological and cognitive effects in EAE

Whereas MS is known to impact on cognitive functioning (Tiemann et al., 2010) and to cause neuropsychiatric symptoms (Chiaravalloti and DeLuca, 2008), EAE-induced animals only show mild biopsychological impairment. Pollak et al. (2002) reported an 'EAE-associated behavioral syndrome' which is related to human major depression in terms of body weight reduction, changes in food and sucrose intake and a decrease in social exploration. However, these effects are rather mild compared to the extent of neurological symptoms in murine EAE. To provide a suitable model of autoimmune-mediated cognitive impairment reflecting the same severity as in humans, a new murine model was established. In this model, Aβ1-42 was used as target antigen, which is ubiquitously expressed in various body compartments but strongly related to neuronal functioning and neurodegenerative diseases.

Amyloid-β and autoimmunity

Several therapeutic strategies have been developed to eliminate or reduce Aβ deposits within the CNS. Active immunization in which Aβ peptide is combined with an adjuvant to stimulate an antibody response against Aβ was shown to lower brain Aβ burden in animal models (Schenk et al., 1999; Bard et al., 2000). These promising observations led to a clinical trial with active immunization using synthetic Aβ1-42. However, the trial was discontinued due to the occurrence of meningoencephalitis in 6% of the patients without a clear correlation to the strength of the anti-Aβ1-42 antibody response (Orgogozo et al., 2003). Other studies on wild-type mice found an induction of mild autoimmune encephalomyelitis by active Aβ immunization with macrophage, B cell and T cell infiltrates in the CNS (Furlan et al., 2003). Yet, the mechanism how Aβ immunization affects the immune system and cognition in healthy individuals is poorly understood.

Objectives

The present thesis intends to unravel the reciprocity of autoimmune-related inflammation and its biopsychological manifestation. To demonstrate the close interaction of CNS inflammation and affective disorders, the effects of the antidepressant venlafaxine on the clinical manifestation of EAE were investigated. To further identify the impact of inflammatory processes on biopsychological functions, a mouse model of autoimmune-mediated cognitive and behavioral impairment was established by active immunization with a neuronal peptide. Thus, the aim of the present thesis is to dissect the mechanisms involved in inflammation-related cognitive and affective alterations and to provide guidance for the development of future therapies of neuroinflammatory and neurodegenerative diseases in humans.

Material and Methods

Animals

Female C57BL/6 and SJL/J mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and were used in experimental paradigms at the age of 6–8 weeks. Toll-like receptor 2/4 (TLR2/4)-deficient mice on the C57BL/6 background were provided by C. Kirschning (Institute of Medical Microbiology, Technische Universität München, Germany). All procedures were conducted in compliance with the local guidelines for animal experimentation.

Immunization

To investigate autoimmune-mediated cognitive impairment, animals were immunized subcutaneously (s.c.) with 100 mg per animal human Aβ1–42 peptide (American Peptide Company, Sunnyvale, CA; EZBiolab, Carmel, CA) emulsified in complete Freund's adjuvant (CFA) containing 5 mg/ml Mycobacterium tuberculosis extract (strain H37Ra, DIFCO Laboratories, Detroit, MI). EAE induction was performed by subcutaneous (s.c.) injection of 100 mg/animal of myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (Jerini, Berlin, Germany) emulsified in CFA. Control animals received CFA with phosphate buffered saline (PBS). On days 0 and 2, all animals were injected with 500 ng/animal pertussis toxin (Sigma-Aldrich, Munich, Germany) intraperitoneally.

Adoptive transfer EAE

To investigate therapeutic effects of venlafaxine, relapsing-remitting EAE was induced by the adoptive transfer of myelin-specific cells (Figure 1). Briefly, SJL/J mice were s.c. immunized with 200 μg per animal Proteolipid protein (PLP) 139–151 (HSLGKWLGHPDKF, single letter code, Jerini, Berlin) emulsified in CFA. The draining lymph nodes were removed 11

days later and single cell suspensions were made. After *in-vitro* restimulation with 10 µg/ml PLP139–151 for four days, 5 x 10^6 to 2 x 10^7 cells were injected i.p. into syngenic recipients. Clinical signs of EAE were ranked with an established score from 0–5: 0 (normal); 1 (tail limpness), 2 (paraparesis with clumsy gait); 3 (hind limb paralysis); 4 (hind limb and forelimb paralysis); and 5 (death). All ratings were done by observers blinded to the treatment.

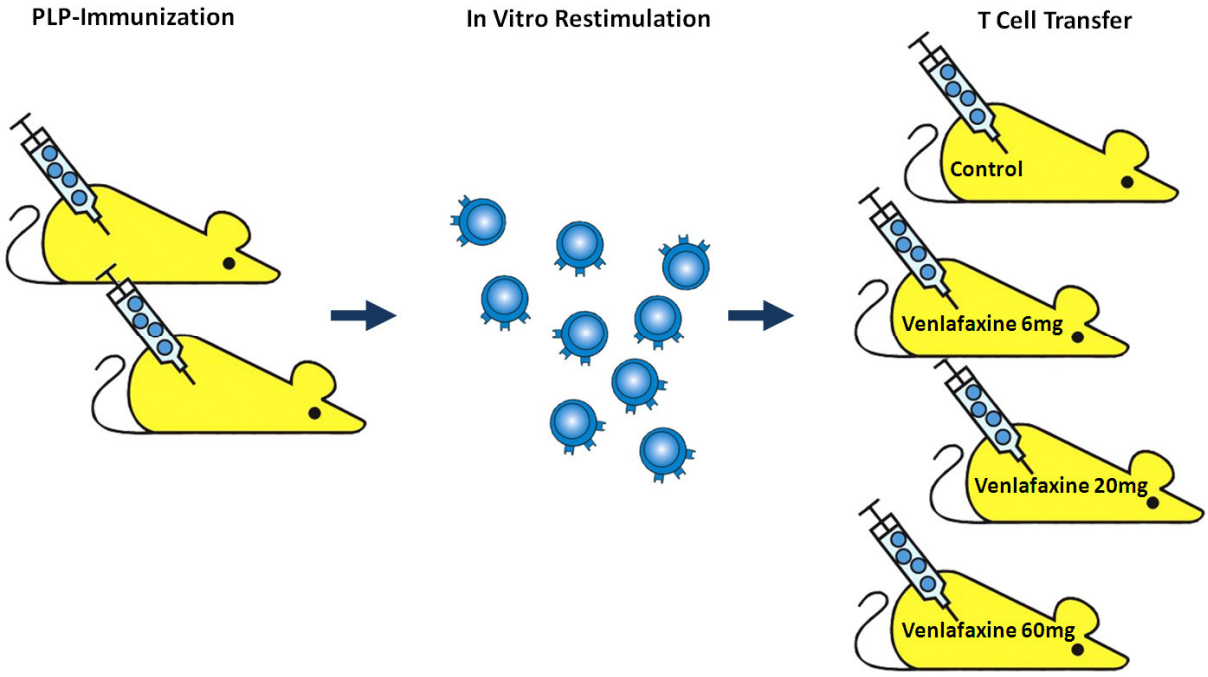

Figure 1. Illustration of the adoptive transfer EAE model.

Behavioral tests

Open Field

For evaluation of habituation and visuospatial learning, mice were observed in the open field. Briefly, the open field was a square arena (30 x 30 x 40 cm) with clear plexiglas walls and a grid square floor composed of nine equal quadrants (Figure 2). At the beginning of the test, mice were placed in the center of the open field and left to freely explore. The total number of

quadrant borders the mice crossed and the number of rearings were counted by a blinded observer during a 10-min observation period. Baseline values were assessed prior to immunization. To asses a habituation learning measure (habituation learning index), the difference of crossed segments in the first and last 150 s of each 10-min observation period was determined. The open field test was repeated every 3 days.

Visuospatial learning task

Visuospatial learning performance was tested in the open field paradigm with slight modifications from published protocols (Dere et al, 2005). For ethical reasons, the water maze paradigm was not applied, as some of the animals in the MOG35–55/CFA-immunized control group developed severe pareses. For 3 consecutive learning days, mice were placed into the open field in which two identical objects (bottles) in terms of height, color, shape, and surface texture were located. Spatial configuration did not change for three training sessions. On day 4, the bottle in the corner was moved to the opposite corner, leaving the configuration and distance of the objects undisturbed. The total exploration time for each object was determined during a 10-min observation period. Object exploration was defined as physical contact with the bottle by mouth, vibrissae, and forepaws. Compassing or sitting inactively next to the objects was not regarded as object exploration. For statistical evaluation, the initial exploration time for each stimulus in the first session was calculated, and the relative change in exploration time of the replaced stimulus in the fourth session was determined.

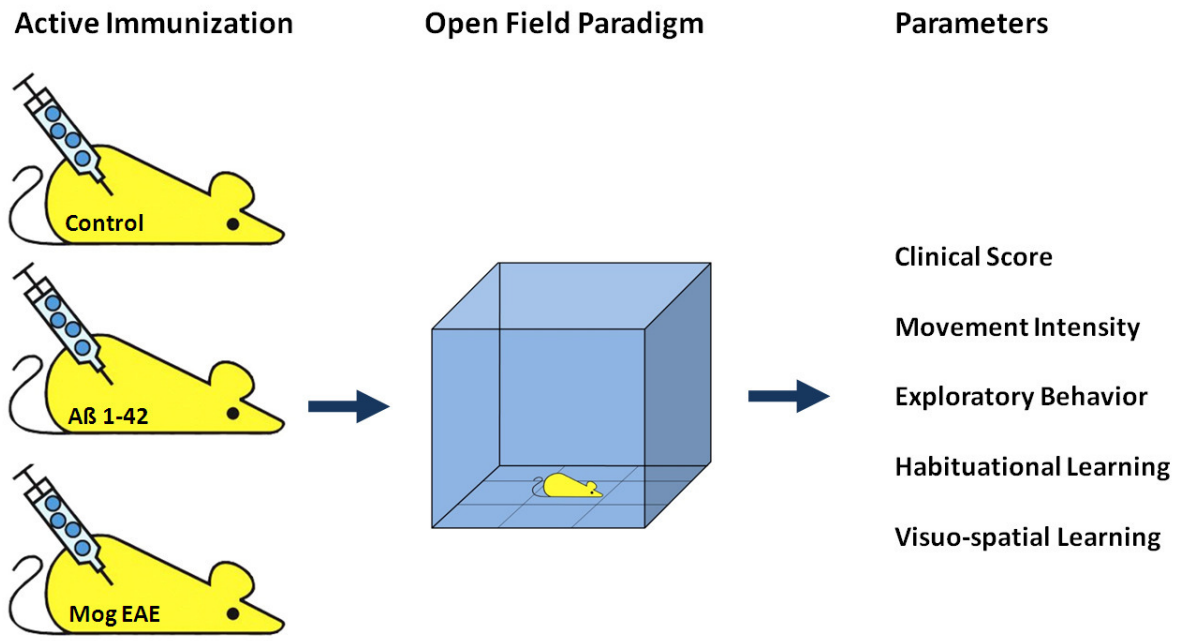

Figure 2. Illustration of the experimental design.

Cell separation

Macrophages and dendritic cells

Cells immunoreactive for CD11b and CD11b/CD11c were isolated from naive mouse spleen tissue by magnetic cell sorting with MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity of cells (ca. 90%) was confirmed by flow cytometry.

Peritoneal macrophages

Primary macrophages were isolated from the peritoneal cavity of mice according to previously published protocols (Ousman et al, 2007). For assessing cytokine production, these cells were cultured (2 x 10^6 cells/ml) for 48 h in culture medium in a humidified incubator at 5% CO_2. For gene expression studies, messenger ribonucleic acid (mRNA) was isolated directly after harvesting the cells from the peritoneal cavity.

Astrocyte-microglia co-culture

Primary cell cultures of glial cells were prepared from hemispheres of postnatal (P0–P2) Wistar rats according to previously published protocols (Faustmann et al., 2003). Depending on the extent of shaking, the fraction of microglial cells remaining in the co-cultures varies between 5% (M5), comparable to the concentration found in healthy adult brain tissue, and 30% (M30) as determined by counting after fixation and immunohistochemical staining with the microglia marker ED1.

In-vitro cytokine production

In-vitro effects of venlafaxine were studied on a MOG35-55 specific encephalitogenic T cell clone, on PLP139-51 specific splenocytes and on peritoneal macrophages activated with LPS. The T cell clone was restimulated with 10 µg/ml MOG35-55 and 4×10^6 /ml irradiated antigen presenting cells for 48 h. Venlafaxine (titrated from 10^{-5} to 10^{-10} mol/l) was added at the time of restimulation. Supernatants were collected after 48 h. Spleens from animals actively immunized with PLP139-151 were removed at day 11 and single cell suspensions were generated. These cells were restimulated with PLP at 10 µg/ml in the presence of 10^{-4} to 10^{-10} mol/l venlafaxine and supernatants were removed after 48 h. Primary macrophages were cultured with media alone for 48 h and then activated with 100 ng/ml of LPS (Sigma-Aldrich) in the presence of 10^{-4} to 10^{-9} mol/l venlafaxine. Supernatants were harvested 24h later.

To study the *in-vitro* effects of Aβ peptide, lyophilized human Aβ1–42 peptide (obtained from American Peptide Company or EZBiolab) was reconstituted with PBS at a concentration of 2 mg/ml. Dissolved peptide was stored at 4°C for up to 48 h. In stimulation experiments, CD11b+ and CD11b+CD11c+ cells ($2x10^6$ cells/ml) were stimulated with different concentrations of Aβ1–42 peptide (0.1–50 mg/ml) or 100 ng/ml lipopolysaccharide (LPS; Sigma-Aldrich) for 48 h at 37°C in culture medium in a humidified incubator at 5% CO_2.

Cytokines

Cytokine levels were determined in culture supernatants. Cell culture supernatants were collected after indicated incubation periods and stored at -80°C until analysis. Cytokine levels were measured by commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

RNA isolation and real-time PCR

Isolation of RNA from fresh CNS tissue or cell material (Rneasy®, Quiagen, Hilden, Germany), its quantification, and the reverse transcription reactions (High-capacity RT Kit®, Applied Biosystems, Darmstadt, Germany) were performed according to established protocols. Expression of mRNA for target genes and the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assessed by real-time PCR (with TaqMan Gene Expression Assay products on a 7500 Fast Real-Time PCR System, Applied Biosystems). The following gene expression assays have been used (Applied Biosystems): BDNF (Mm00432069_m1), CD3 (Mm00599683_m1), CD14 (Mm00438094_g1), GFAP (GFAP; Mm01253033_m1), IFN-γ (Mm00801778_m1), IL-6 (Mm00446190_m1), IL-12 (Mm00434165_m1), S100A8 (Mm00496696_g1), and TNF (Mm00443258_m1).

Expression levels for each gene of interest were calculated by normalizing the quantified mRNA amount to GAPDH. Relative gene expression was determined and used to test significance between different groups.

Histology

Mice were anesthesized with isoflurane and perfused with ice-cold PBS and 4% paraformaldehyde. Brains were dissected and embedded in paraffin. Immunohistochemistry

was performed with a rat antibody against mouse MAC-3 (1:200; clone M3/84, BD Biosciences) and glial fibrillary acidic protein (GFAP; 1:400, clone 6F2, Dako North America). Briefly, tissues were pretreated by microwaving in 10 mM citrate buffer (pH 6) for two cycles of 5 min each. Immunolabeling was detected by the avidin-peroxidase method and visualized with diaminobenzidine by incubation for 5 min. Control sections were incubated in the absence of primary antibody or with nonimmune sera. Slides were counterstained with hematoxylin and coverslipped. Inflammation was assessed by haematoxylin staining. The extent of inflammation is expressed as the mean number of inflammatory infiltrates per spinal cord cross-section (inflammatory index).

Immunocytochemistry

Briefly, the density of astrocytes was determined by immunolabelling of GFAP with a polyclonal antibody (1:100, Sigma G9269). Microglial cells were labelled by using a monoclonal antibody directed to the ED1 epitope (1:250; Serotec MCA 341R, Eching, Germany), which allowed classification of microglia as resting ramified, intermediate and activated, rounded phagocytic phenotypes (Faustmann et al., 2003). For quantification, cells were counter-stained with 4,6-diamidino-2-phenyl-indol (DAPI; 1:2500, Sigma D9542).

Data analysis

For statistical comparisons, a one-way multiple-range ANOVA test or two-tailed Kruskal-Wallis test for multiple comparisons was employed. Unpaired *t* or Mann-Whitney *U* tests were used for comparison of two groups where indicated. Values of $p < 0.05$ were considered significant. Graphs were generated using GraphPad Prism software (GraphPad, San Diego, CA).

Results and Discussion

The antidepressant venlafaxine ameliorates murine EAE

The antidepressant venlafaxine, a selective serotonin-/norepinephrine reuptake inhibitor (SNRI), and its immunomodulatory effects were examined in adoptive transfer EAE (see Figure 1). Mice were orally treated with PBS or different doses of venlafaxine (6 mg/kg/d, 20 mg/kg/d, 60 mg/kg/d) starting at the day of induction or after the onset of clinical symptoms. Early oral treatment with venlafaxine significantly ameliorated EAE when treatment was initiated at the day of disease induction (see Figure 3, a). Whereas all animals in the PBS-treated control group developed signs of EAE the disease incidence in the treatment groups was only 50%. Therapeutic intervention with venlafaxine at the beginning of EAE symptoms showed a dose–response relationship with a significant reduction of EAE symptoms at 60 mg/kg venlafaxine compared to vehicle-treated animals (Figure 3, b). When venlafaxine treatment was started after manifestation of severe clinical symptoms (Figure 3, c) significant amelioration of EAE symptoms could be demonstrated for 20 mg/kg and 60 mg/kg venlafaxine after 2 weeks of therapy.

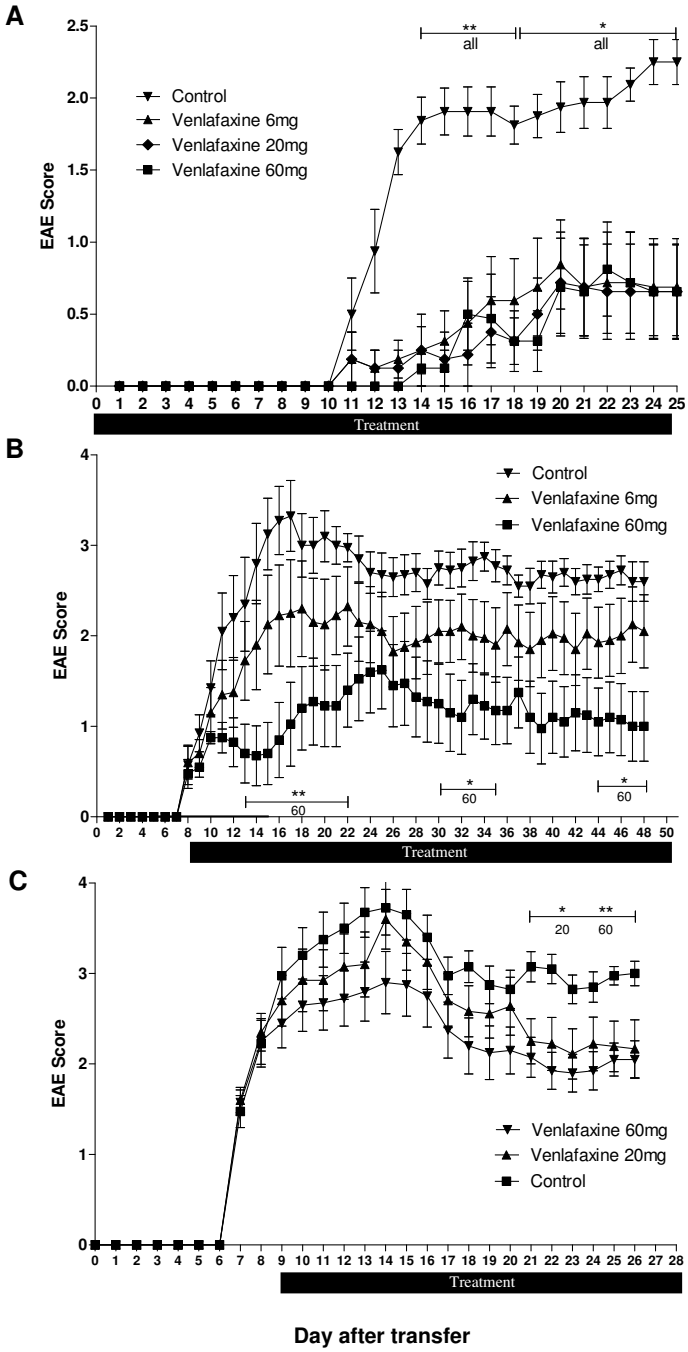

Figure 3 shows mean clinical EAE scores of different groups. Clinical signs of EAE were ranked from 0 (normal), 1 (tail limpness), 2 (paraparesis with clumsy gait), 3 (hindlimb paralysis), 4 (hind- and forelimb paralysis), 5 (death).

Venlafaxine prevents histopathological signs of EAE

Histology of control mice with clinical signs of EAE revealed dense subpial and perivascular infiltrates expanding to the parenchyma (Figure 4, b). Venlafaxine-treated mice showed

markedly reduced CNS inflammation and were largely devoid of inflammatory infiltrates in the brain and spinal cord (Figure 4, a).

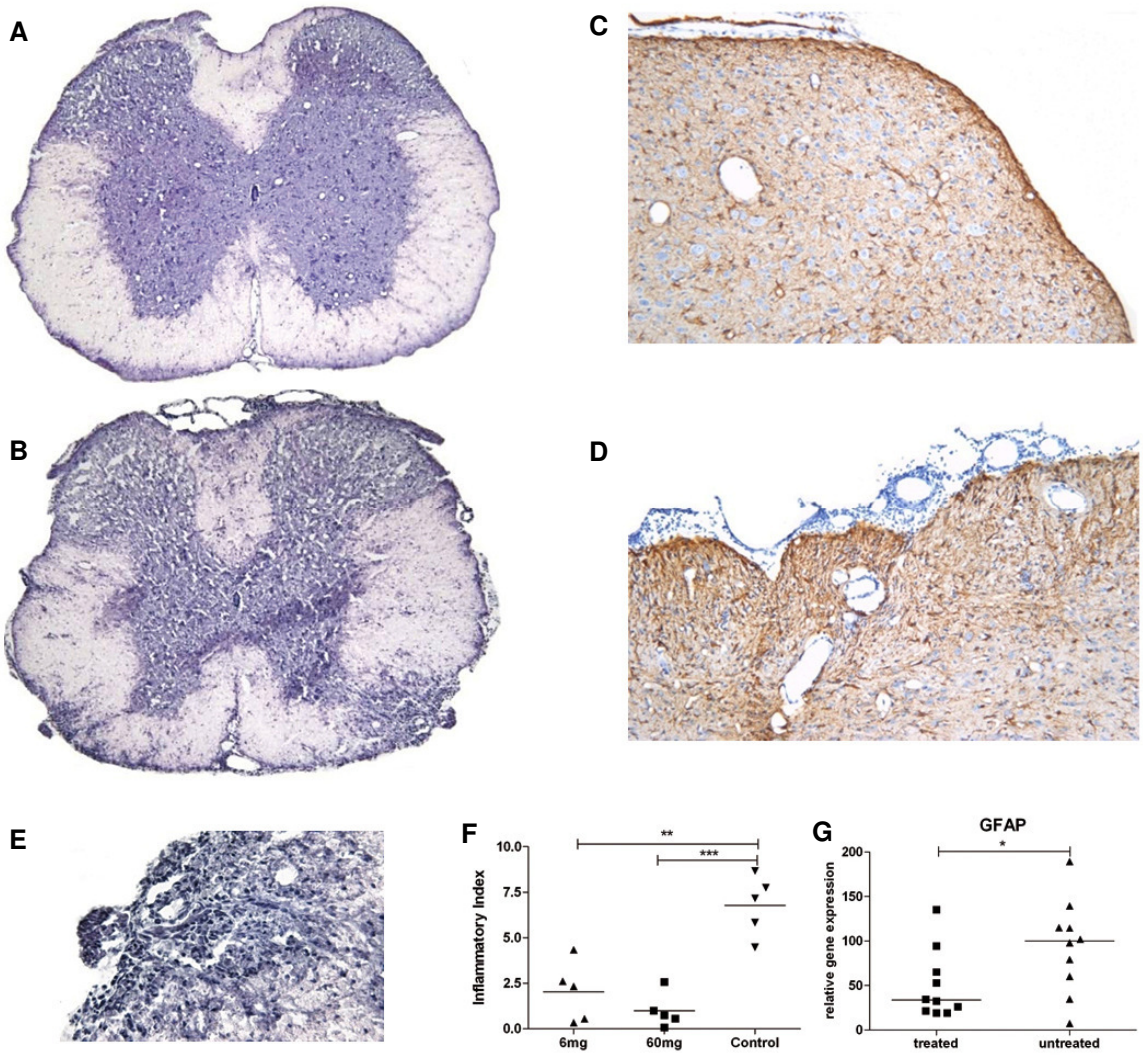

Figure 4. (a) Representative haematoxylin staining (20x original magnification) of the thoracic spinal cord from a venlafaxine-treated animal without inflammatory foci after 3 weeks of adoptive transfer. (b) Illustrates a spinal cord section of a vehicle-treated mouse with considerable amounts of inflammatory foci [(e) 63x magnification]. (f) Shows the mean numbers of inflammatory infiltrates per spinal cord cross-section (inflammatory index). Panels (c) and (d) illustrate reactive gliosis to inflammation in the brainstems of representative untreated [(d) 40x magnification] and treated (c) animals as revealed by GFAP immunostaining and haematoxylin counterstaining after 2 weeks of disease onset. Data were confirmed (g) by quantitative GFAP gene expression analysis.

The average number of inflammatory infiltrates per spinal cord section (Figure 4, f) was significantly higher in untreated animals compared to 6 mg/kg and 60 mg/kg treated mice. In untreated mice, inflammatory cell infiltration evoked severe astrogliosis (Figure 4, d) in the

parenchyma whereas treated mice (Figure 4, c) were almost free of reactive gliosis. Data were confirmed by quantitative GFAP gene expression analysis of CNS material from (Figure 4, g) mice receiving different doses of venlafaxine as preventive treatment.

Venlafaxine reduces the expression of cytokine-related genes in the CNS

Both doses of venlafaxine suppressed the *in-vivo* mRNA expression (Figure 5) of CD3 as marker of T cells. However, the effect was more pronounced on high-dose treatment. Further, the antidepressant significantly reduced the gene expression of the proinflammatory cytokines, Interleukin 12 (IL-12) and TNF whereas the expression of BDNF was significantly increased.

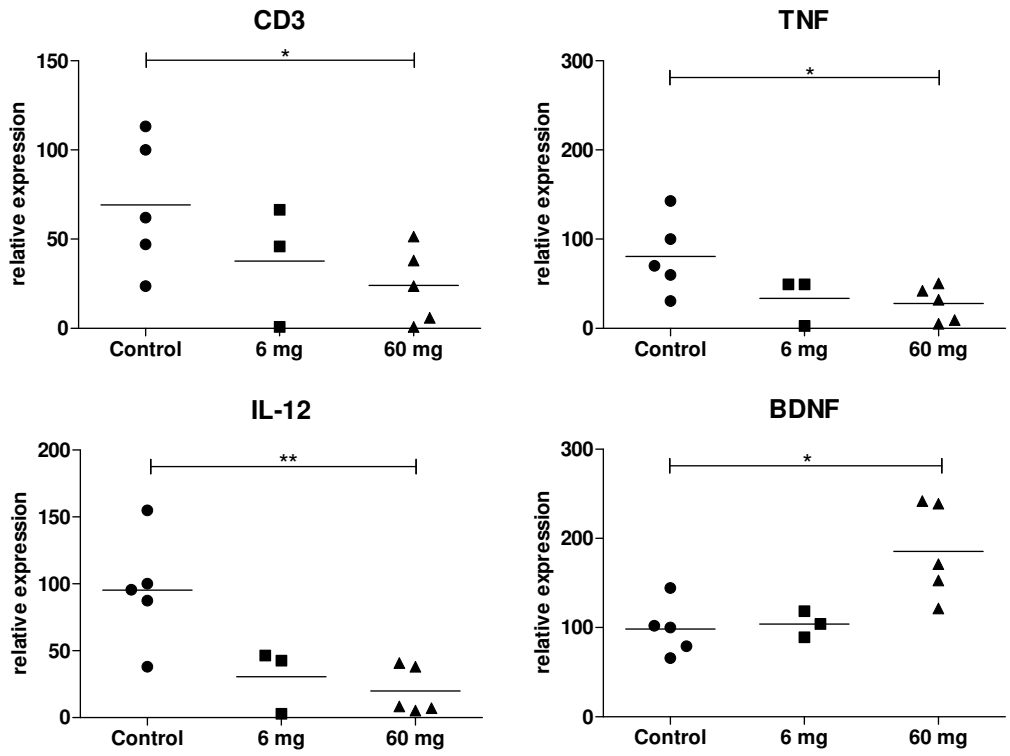

Figure 5. Quantitative mRNA expression of inflammation-related genes in the spinal cord tissue of venlafaxine- and vehicle-treated mice is illustrated. The GAPDH-normalized relative gene expression is shown for single animals.

Mechanisms related to the protective effects of venlafaxine in EAE

Venlafaxine decreases the inflammatory activity of T cells and macrophages

Since we observed a profound clinical effect in the course of venlafaxine treatment we further investigated the antiinflammatory effects *in vitro.* Here, venlafaxine reduced the secretion of proinflammatory cytokines in encephalitogenic PLP-specific T cells (Figure 6, a) and in an encephalitogenic MOG-specific T-cell clone (Figure 6, b). Venlafaxine also attenuated the cytokine production in LPS-stimulated primary peritoneal macrophages (Figure 6, c).

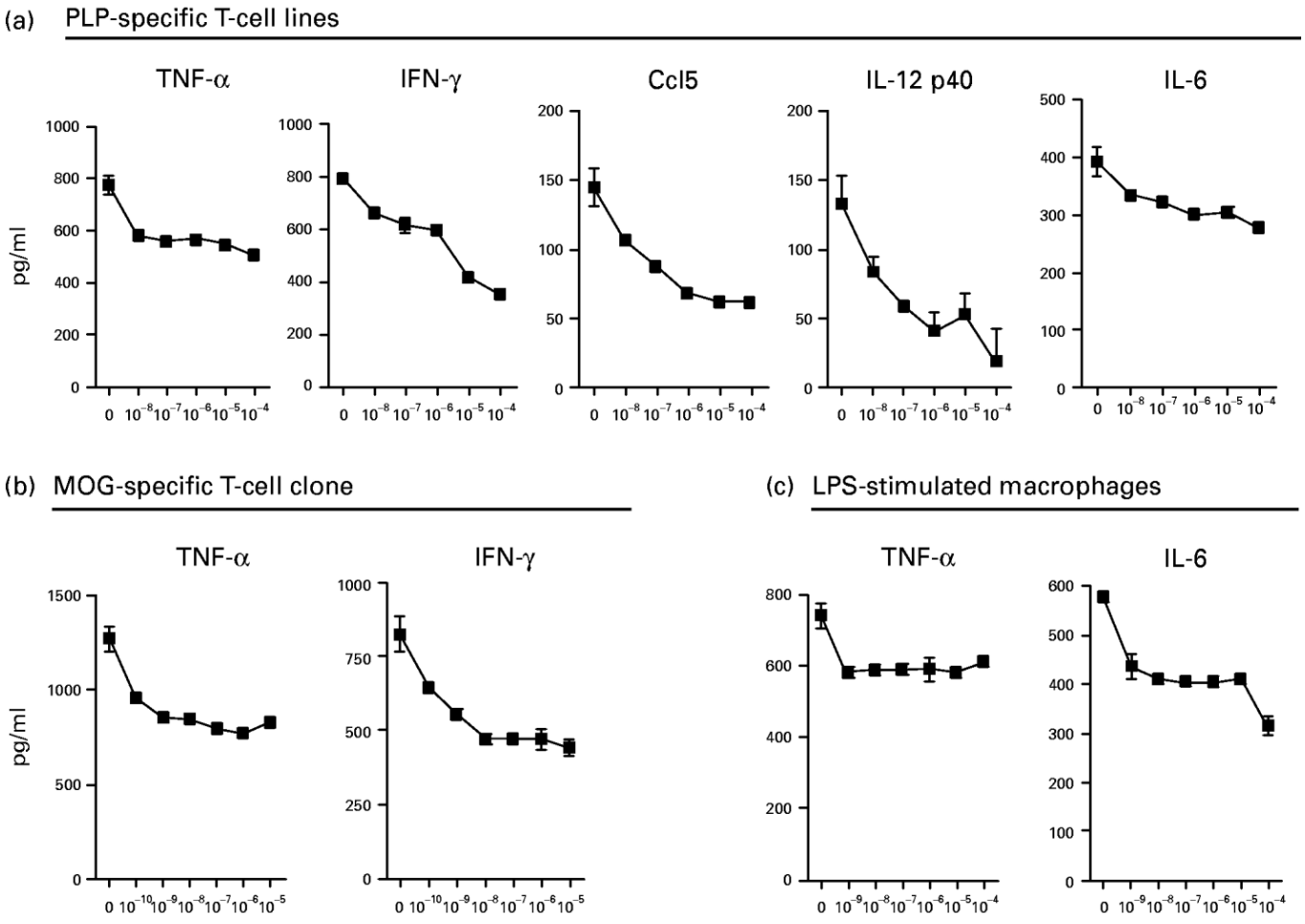

Figure 6. Cytokine production of different immune cells during 48 hr incubation with venlafaxine (concentrations of 10^{-4} to 10^{-10} mol/l). The background cytokine production in the absence of stimulus (LPS or antigen) was subtracted from the stimulated production. All experiments were replicated at least three times.

These data underline venlafaxine's antiinflammatory effects on cells of the peripheral immune system and provide an explanation for the prevention or amelioration of EAE development. Venlafaxine strongly reduced the *in-vitro* secretion of IL-12, which is essential in T cell-mediated autoimmune diseases (Gran et al., 2004). This is based on the strong capacity of IL-12 to induce T cell activation, Th1 cytokine differentiation and macrophage activation (Trinchieri and Scott, 1995).

Venlafaxine inhibits microglia activation in a primary co-culture model

A primary astroglia–microglia co-culture model (Appendix II) was employed to investigate inflammatory conditions in an *in-vitro* bioassay. Especially, the activation of microglia and response of astroglia to microglial activation can be monitored in this assay. Primary astrocyte cultures of newborn rats were cocultured with either 5% (M5) or 30% (M30) microglia (Faustmann et al., 2003). Astroglia/M30 cocultures contained significantly fewer resting microglia and significantly more activated microglia than the M5 cocultures.

Stringent evidence was found (Figure 7) that venlafaxine reversed the inflammatory conditions of M30 cultures in a dose-dependent fashion. Incubation of M30 cultures with venlafaxine was capable of preventing microglial activation and minimizing proinflammatory cytokine secretion. Astrocytes play a crucial role in the pathogenesis of inflammatory diseases of the CNS and represent pharmacological targets of antidepressants (Hertz et al., 2004). Monoamine transporters (Inazu et al., 2003) as well as adrenergic receptors (Hertz et al., 2004), which have been identified on astrocytes might play a key role in mediating antiinflammatory effects by antidepressants.

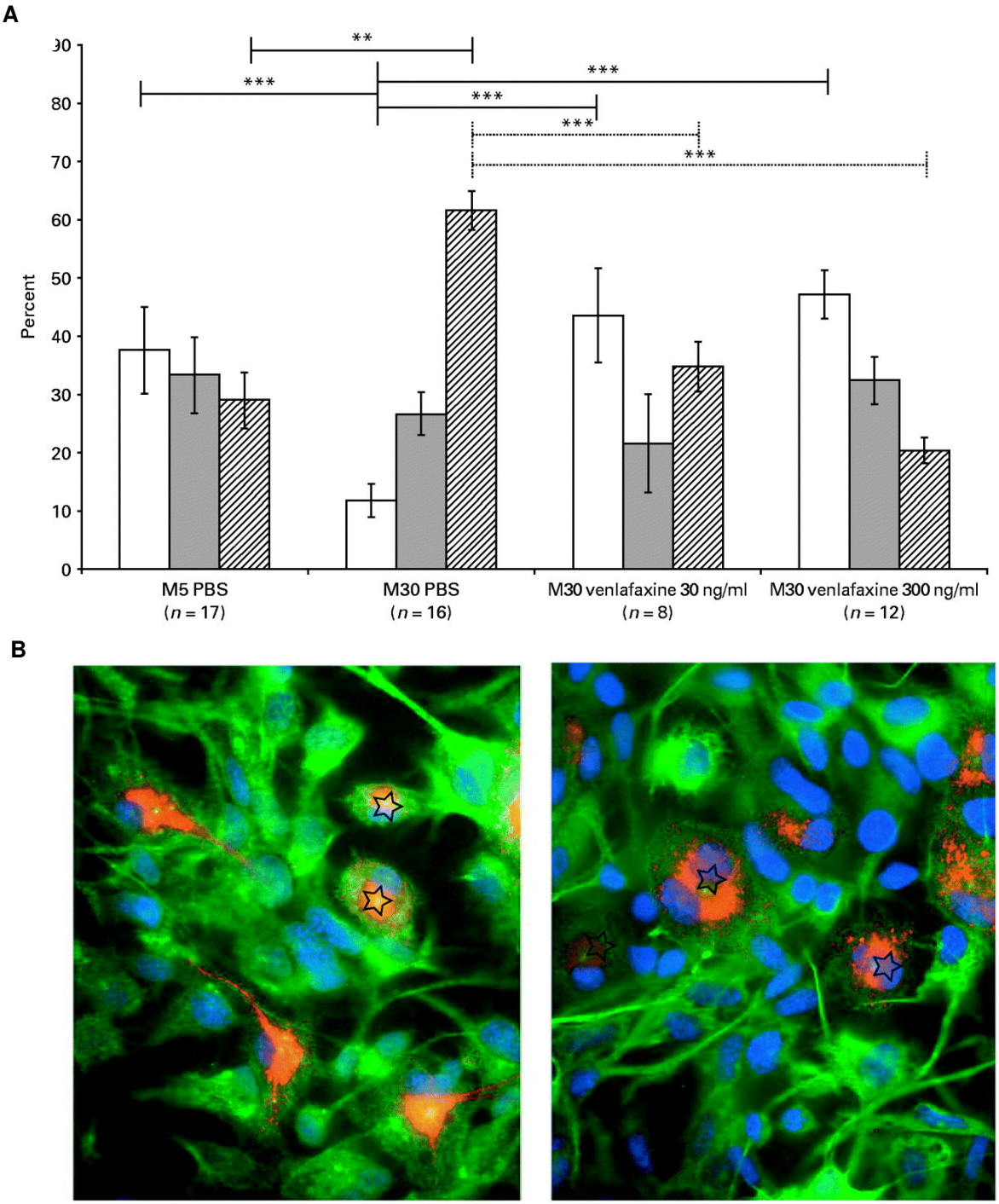

Figure 7 illustrates the microglia phenotype in response to venlafaxine challenge. Each bar (a) represents the mean percentage ± SEM of resting (white), intermediate (grey) or active (dashed) microglial cells in the co-culture after 16 h of incubation with indicated substance concentration or vehicle. Data are from at least four different experiments. In b (63x magnification), the left image displays astrocytes (green) and mainly resting ramified microglial cells (red) after incubation with venlafaxine. In the absence of venlafaxine, microglial cells (right image) largely constitute the round active phagocytic phenotype (indicated by a star).

The mechanisms leading to venlafaxine-mediated reduction of cytokine secretion are still unknown. One putative explanation for this phenomenon might be the increase of

transcription factors (Hindmarch, 2001) such as intracellular cyclic adenosyl monophosphate (cAMP) resulting in activation of neuroprotective proteins, such as BDNF (Xia et al.,1996), which was up-regulated in the spinal cord of venlafaxine-treated animals in this study.

The results are consistent with *in-vitro* findings on the negative immunoregulatory effects of venlafaxine on the IFN-γ/IL-10 production ratio in peripheral blood cells from patients with major depression (Kubera et al., 2001). Further, Hashioka et al. (2007) showed for several antidepressant substances reduced IL-6 and nitric oxide production after IFN-γ activation. Interestingly, studies on antidepressant effects of a cyclooxygenase-2 (COX-2) inhibitor (Müller et al., 2006), which curtails prostaglandin E2 generation and the production of proinflammatory cytokines showed significant improvement in depressive patients under celecoxib add-on therapy. Further, the same COX-2 inhibitor has been found to have preventive effects in EAE through the suppression of proinflammatory cytokine secretion (Miyamoto et al., 2006). COX-2 inhibitors reduce the secretion of IL-12 (Muthian et al., 2006) revealing a mechanism of immunomodulation similar to the one which was identified for venlafaxine. These findings provide further evidence for a neuroimmune interaction and an inflammation-related pathogenesis of affective disorders.

Immunization with Aβ1-42 as model of autoimmune-mediated cognitive impairment

To further identify the impact of inflammatory processes on biopsychological functions, (Appendix III), the cognitive and immunological phenotype of healthy mice challenged with active Aβ1-42 immunization was investigated. Briefly, mice were immunized with CFA and Aβ1-42. Mice immunized with MOG35-55 peptide (classical EAE model) and with CFA alone served as controls.

Immunization with Aβ1–42 is associated with alterations of cognitive performances

Active immunization with Aβ1-42/CFA significantly altered the psychomotor and cognitive phenotype of mice compared to different control groups. Observations in the open field revealed pronounced deficits regarding three cognitive parameters. First, open field testing of Aβ1-42/CFA-immunized mice showed a significant reduction of locomotion (Figure 8, a). Changes in locomotion were detected as early as on day 10 after immunization (vs. MOG35-55/CFA and PBS/CFA) and reduced locomotion persisted over the entire observation period until day 28. Second, reduced rearing behavior was detected already on day 4 (vs. MOG35-55/CFA) and persisted until day 18 (Figure 8, b). Third, a significant decrease in habituational learning ability was observed.

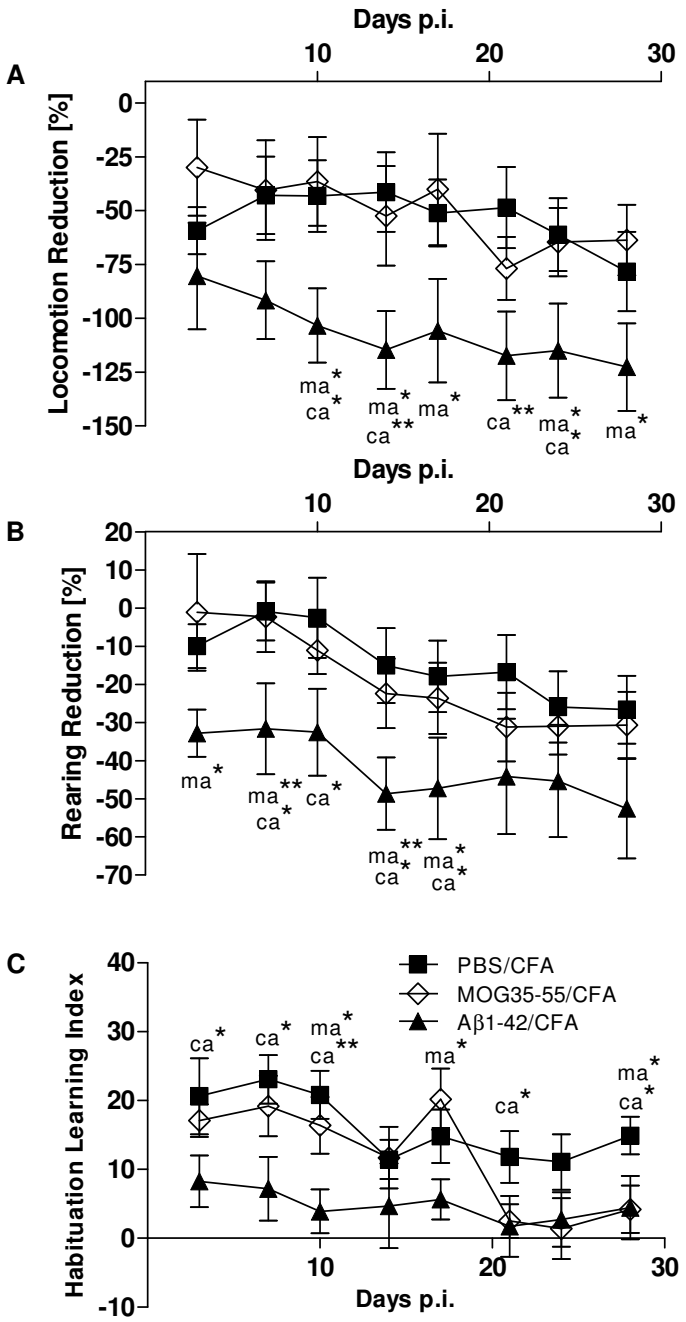

Figure 8. Groups of female C57BL/6 mice (n = 10 per group) were immunized with PBS/CFA, MOG35-55/CFA, or Aβ1-42/CFA plus pertussis toxin and evaluated for locomotion (a) and explorative behavior as measured by rearing events (b) at different time points after immunization. Habituational learning was assessed in a setting that tested the habituation to visuospatial cues and expressed as habituational learning index (c). "ma" and "ca" denote significant differences between the MOG35-55 vs. Aβ1-42 and PBS vs. Aβ1-42 groups, respectively.

Whereas control animals showed habituation to a persisting environment by reduction of exploration over time, Aβ1-42/CFA-immunized mice exhibited a significantly lower

habituational learning index (Figure 8, c) starting on day 3 postimmunization (p.i.). Paralytic disease in the MOG35-55/CFA group started around day 11, but did not mar the specific read-out parameters of the open field tests.

Interference with visuospatial learning

In a complex object recognition task, Aβ1-42/CFA-immunized mice developed profound deficiencies in visuospatial learning both in the acute (observation between days 9-14 p.i.) and chronic (observation between days 23-28 p.i.) phases of disease (Figures 9, a, b).

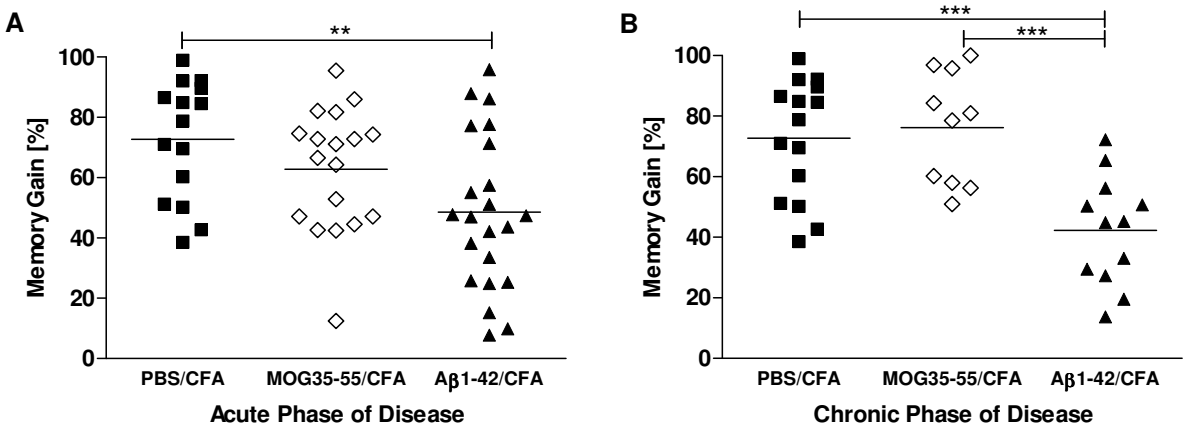

Figure 9. Groups of female C57BL/6 mice were immunized with PBS/CFA, MOG35-55/CFA, or Aβ1-42/CFA plus pertussis toxin and evaluated in a visuospatial object recognition paradigm in the acute (acquisition period between days 9-14 p.i., a) and chronic (acquisition period between days 23-28 p.i., b) phases of disease. Memory gain refers to the relative increase in exploration of a novel stimulus in a habituated environment and is illustrated for each individual mouse.

As compared to controls, Aβ1-42/CFA-immunized animals spent significantly less time to explore a novel stimulus in a known environment (reduced memory gain) both in the acute and chronic phases of disease. Together these behavioral data suggest a profound and persistent decline in motivational and cognitive performance in Aβ1-42/CFA-immunized animals.

Aβ1–42 immunization results in macrophage infiltration in the CNS

To unravel the mechanisms behind this behavioral phenotype, detailed analyses both of CNS tissue from Aβ1-42-immunized and control mice were performed. Immunohistochemistry revealed perivascular and subpial infiltrates of mononuclear cells in the brain and brainstem of Aβ1-42/CFA-immunized mice (Figure 10, b) but not in PBS/CFA controls (Figure 10, a). These infiltrates mainly consisted of macrophages as shown by MAC-3 staining. Infiltrates in Aβ1-42/CFA-immunized mice (Figures 10, b, d) were disseminated and non-focal whereas MOG35-55/CFA controls (Figure 10, c) exhibited EAE-typical focal meningeal and perivascular cell infiltration. Consistent with the immunohistochemical analyses, the expression of CD14 (Figure 10, e) was upregulated in whole brain tissue of Aβ1-42/CFA-immunized animals compared to PBS/CFA and MOG35-55/CFA controls. When comparing the CNS parenchyma between the groups at late stages of the disease (4 weeks after immunization), prominent signs of astrogliosis were found in the Aβ1-42/CFA-immunized mice as determined by a disproportionate upregulation of GFAP mRNA expression in Aβ1-42/CFA-immunized mice (Figure 10, f).

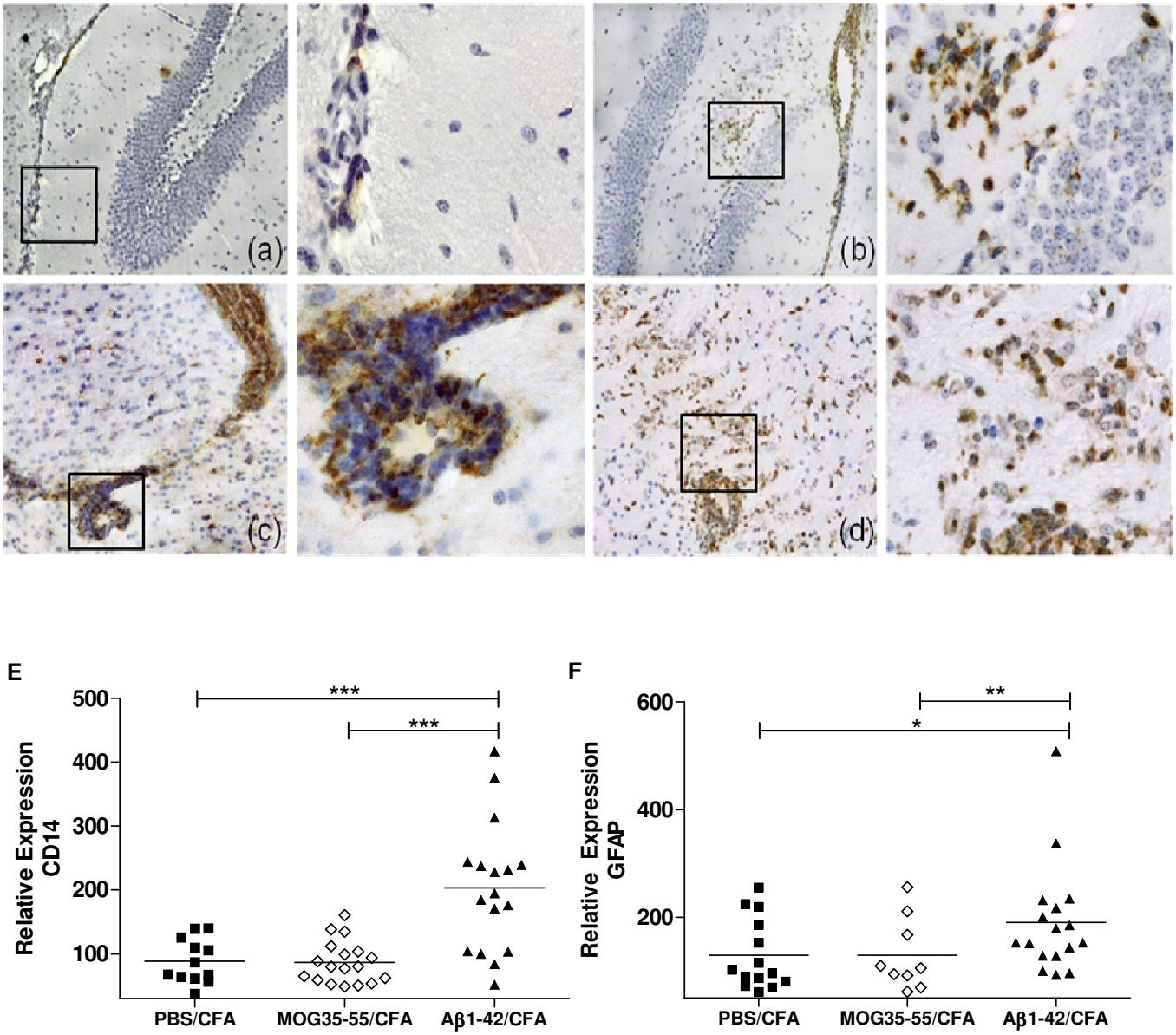

Figure 10. Representative MAC-3 immunostainings (63x original magnification) of coronar sections from the hippocampus region prepared from PBS/CFA (a) and Aβ1-42/CFA-immunized (b) mice are shown. Further, infiltrated vessels (63x original magnification) located in the cerebrum of MOG35-55/CFA (c) and Aβ1-42/CFA-immunized (d) mice are illustrated. Macrophage infiltration was quantified by rtPCR analysis of CD14 gene expression (e) in whole brain tissue. Astrogliosis was confirmed (f) by quantitative GFAP gene expression analysis in whole brain tissue of Aβ1-42/CFA-immunized mice and controls 4 weeks after immunization.

Mechanisms of cognitive impairment induced by Aβ1-42 immunization

Aβ1-42 has stimulatory effects on macrophages and dendritic cells

Since the behavioral observations suggested cognitive changes in Aβ1-42/CFA-immunized mice without focal neurological symptoms, Aβ1-42/CFA immunization might induce a systemic inflammatory response including the systemic release of cytokines. In order to test this hypothesis, possible cellular sources of systemic inflammation were identified. Both the expression of cytokine genes and cytokine production were measured in various cell types of the innate immune system. CD14 transcripts in peritoneal macrophages taken from MOG35-55/CFA-immunized mice (Figure 11, a) were increased 5-fold relative to CFA controls, while cells from Aβ1-42/CFA-immunized mice showed a 12-fold increase in expression. Similarly, IL-1β and IL-6 expression were markedly elevated in mice challenged with Aβ1-42/CFA as compared with MOG35-55/CFA-immunized animals.

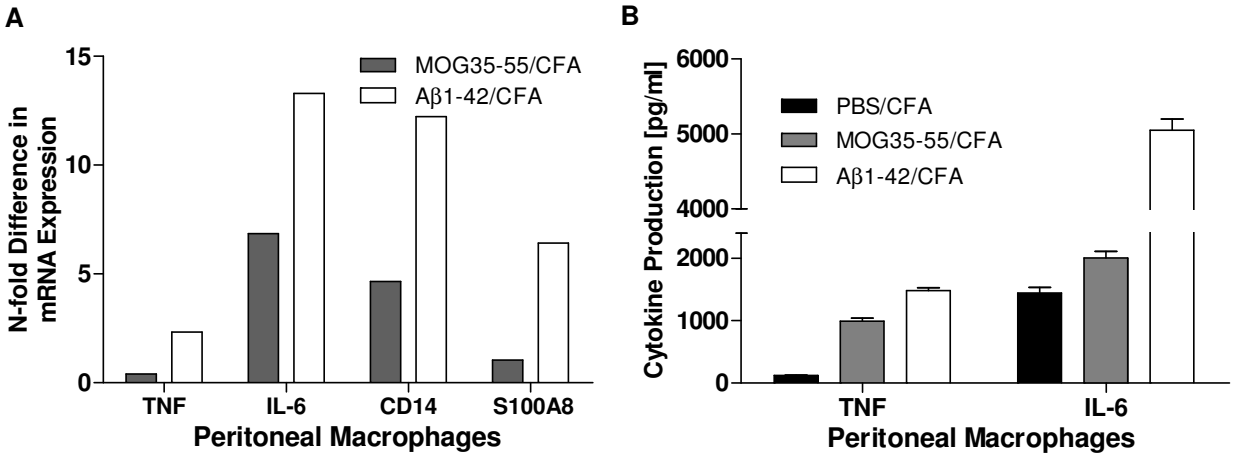

Figure 11. Peritoneal macrophages were isolated from PBS/CFA, MOG35-55/CFA, or Aβ1-42/CFA-immunized mice and tested for gene expression by quantitative rtPCR directly *ex vivo*. The n-fold difference in gene expression of macrophages from Aβ1-42/CFA and MOG35-55/CFA-immunized mice relative to the PBS/CFA group is shown (a). In order to confirm the mRNA data on the protein level, peritoneal macrophages were isolated and cultured without further stimulation for 48 hours. Secretion of IL-6 and TNF in the culture supernatant was measured by ELISA (b). Mean cytokine concentrations plus SD are shown.

The most prominent increase in gene expression was detected for CD14 and IL-6 mRNA. Further, peritoneal macrophages from Aβ1-42/CFA-immunized mice (Figure 11, b) produced 12 times higher levels of TNF compared to PBS/CFA controls and 3 times higher levels of IL-6 as determined in cell culture supernatants.

The stimulatory effects of Aβ1-42 are TLR2/4-dependent

Since we observed a profound activation of the innate immune system after immunization with Aβ1-42, we investigated the stimulatory properties of Aβ peptide *in vitro* and tested the relevance of specific toll-like receptor systems that have been implicated with immunostimulatory effects of Aβ peptide in previous studies. It has been reported that the activation of microglial cells by Aβ peptide requires both TLR2 and TLR4 pathways to activate intracellular signalling (Reed-Geaghan et al., 2009). Here, stimulatory effects of Aβ1-42 on CD11b+ macrophages and CD11b+CD11c+ dendritic cells isolated from naive wild-type and TLR2/4 deficient mice were evaluated *in vitro*. Aβ1-42 induced large amounts of IL-6 and TNF in macrophages (Figure 12, a, b) and IFN-γ in dendritic cells from wild-type mice in a dose-dependent manner (Figure 12, c). In contrast, this effect was not detected in macrophages and dendritic cells derived from TLR 2/4 deficient mice suggesting that either TLR2 or TLR4 or the combined activation of these TLRs mediate the stimulatory effect of Aβ1-42.

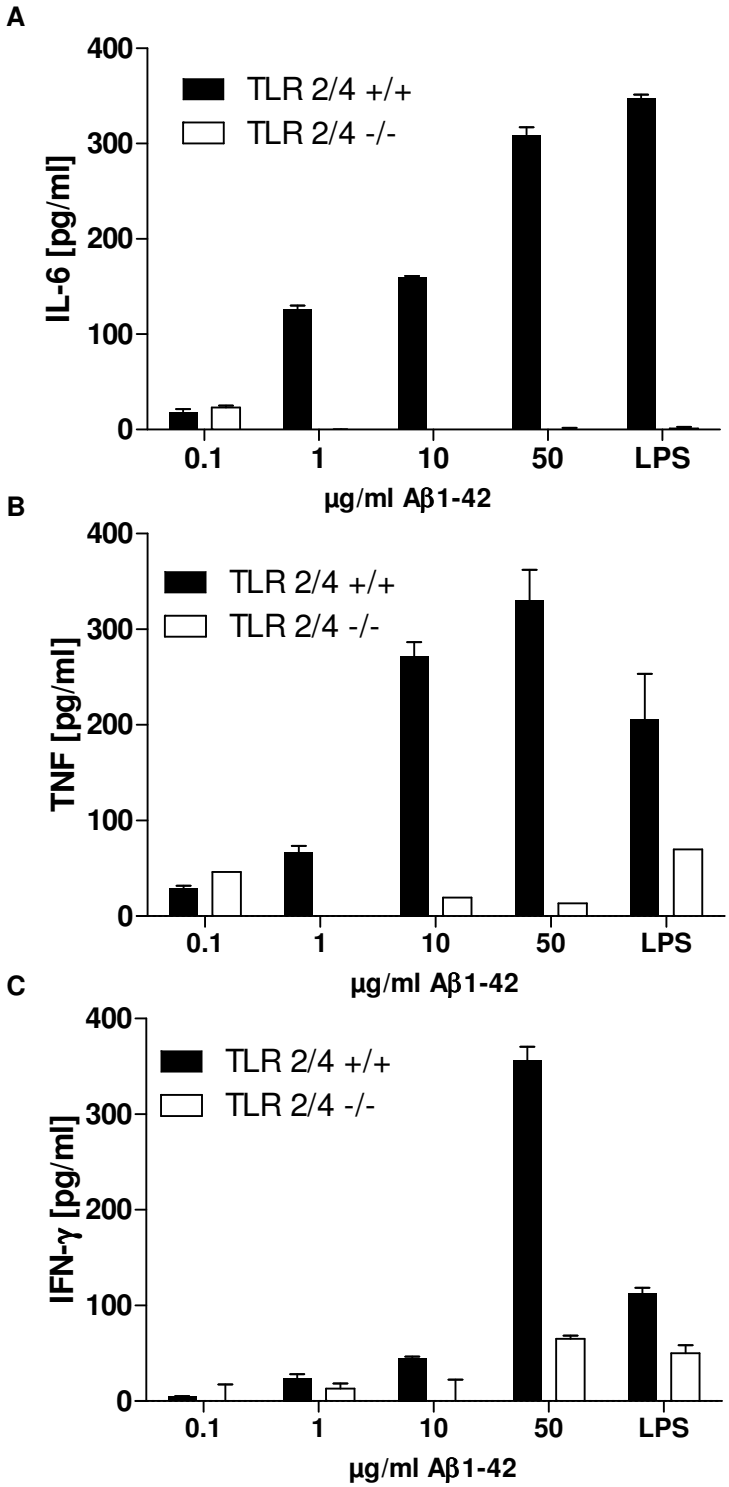

Figure 12. MACS purified CD11b+ cells (macrophages, a, b) and CD11b+CD11c+ cells (dendritic cells, c) from untreated wild-type or TLR2/4 deficient mice were stimulated with increasing concentrations of Aβ1-42 for 48 h. Levels of IL-6, TNF, and IFN-γ were determined in the supernatants by ELISA (a-c). Data are representative of three independent experiments.

To corroborate whether activation of the TLR2/4 pathway by Aβ1-42 was relevant *in vivo*, we immunized TLR2/4 KO animals with Aβ1-42/CFA. Indeed, we determined a significant

decrease in locomotion and rearing in wild-type C57BL/6 mice immunized with Aβ1-42 as compared with immunization with 'CFA only' (Figure 13, a, b).

In contrast, we did not find any additional neurocognitive phenotype (surplus effect) upon immunization with Aβ1-42/CFA as compared with the 'CFA only' condition in TLR2/4 deficient mice. When evaluating the surplus effect induced by Aβ1-42/CFA immunization in wild-type animals vs. TLR2/4 KO mice, the differences were significant as of day 4 p.i. regarding locomotion and as of day 8 with respect to the rearing behavior. Taken together, these data corroborate the critical involvement of the TLR2/4 pathways in the macrophage-induced behavioral changes following active immunization with Aβ1-42 *in vivo*.

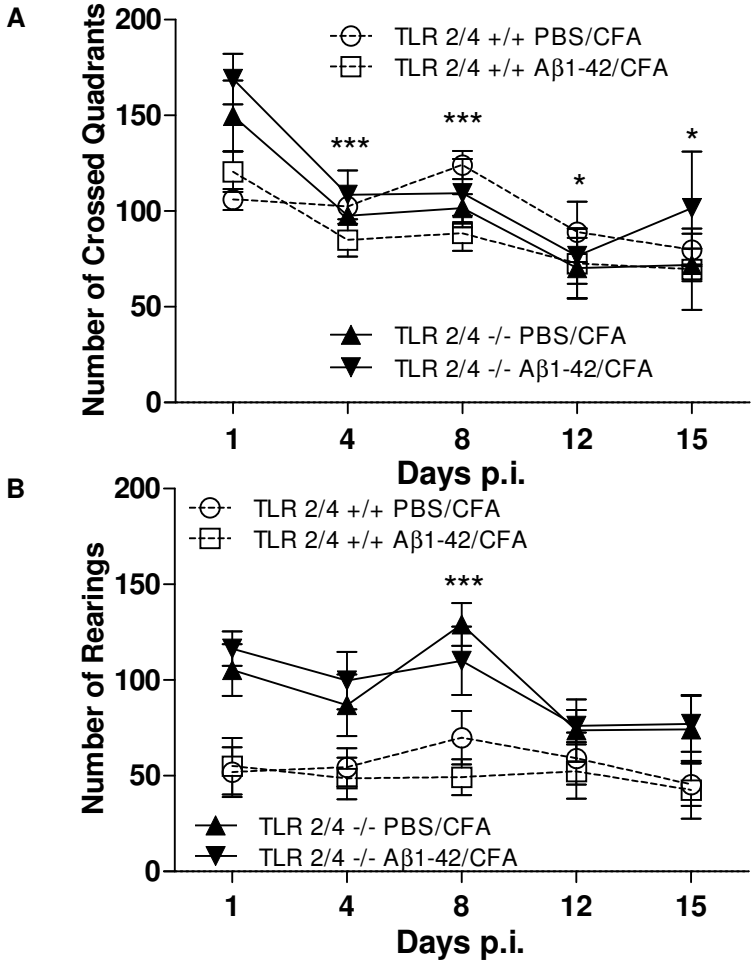

Figure 13. TLR2/4 deficient and wild-type mice (n = 8 per group) were immunized with PBS/CFA or Aβ1-42/CFA and evaluated for locomotion (a) and explorative behavior as measured by the number of crossed quadrants and rearing events (b) at different time points after immunization. The mean performances before and after immunization are summarized for both wild-type and TLR2/4 KO mice upon PBS/CFA or Aβ1-42/CFA challenge. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

The clinical syndrome exhibited by Aβ1-42/CFA-immunized mice was reminiscent of the apathic condition that is the result of a cytokine release syndrome. In fact, deficits in visuospatial tasks were reported in mice injected with LPS. After LPS treatment, mice showed impaired performance in tests of cognition that required animals to effectively integrate new information to complete a spatial task (Chen et al., 2008). A further study in mice (Richwine et al., 2009) found hippocampus-dependent learning and memory impaired after LPS injection. Systemic administration of LPS was reported to induce the (Akashi et al., 2003) secretion of proinflammatory effector cytokines IL-1β, IL-6 and TNF in the CNS (Laye et al., 1994; Gatti at al., 1993; Zhang et al., 2008; Sellner et al., 2009). Further, LPS administration (Dantzer et al. 2008) increases IFN-γ levels in mice and stimulates the indolamine 2,3 dioxygenase (IDO) in the periphery and the brain. IDO activation results in decreased tryptophan levels and increased production of kynurenine promoting depression-like behavior in mice (Lestage et al., 2002). LPS-induced sickness behavior is mainly characterized by systemic inflammation (Dantzer et al., 2008) and increased immunoreactivity of microglial cells (van Dam et al., 1998) in the absence of cell infiltration.

In contrast, in the model of Aβ1-42/CFA immunization, disseminated infiltrates of macrophages in the CNS in addition to a considerable systemic release of proinflammatory cytokines were observed. This systemic inflammation together with the local production of proinflammatory cytokines by infiltrating macrophages is hypothesized to promote the behavioral and neurocognitive disease phenotype in Aβ1-42/CFA-immunized mice. Although the possibility of structural damage to neuronal tissue cannot be excluded, major signs of axonal damage at the end of the observation period have not been identified. Thus, pathogenic effector mechanisms upon immunization with Aβ1-42/CFA are likely distinct from the immuno-pathological scenario evoked in classical EAE models. Aβ1-42 peptide has adjuvant like properties and by this mechanism, induces a profound inflammatory response syndrome.

Aβ1-42/CFA immunization strongly stimulated the production of proinflammatory cytokines in the serum and in peritoneal macrophages. These data suggested that Aβ1-42 acted in a pathogen-associated molecular pattern (PAMP)-like manner on cells of the innate immune system. PAMPs, e.g. LPS, are recognized by pattern recognition receptors such as TLRs triggering the expression of proinflammatory molecules (Mogensen, 2009). It has been demonstrated that Aβ1-42 has the capability to engage TLR2 to transduce intracellular signaling into microglial cells (Jana et al., 2008). Mice transgenic for a chimeric mouse/human APP and the human presenilin-1 gene that are also deficient for TLR2, exhibit increased Aβ deposition in the CNS and accelerated cognitive decline (Richard et al., 2008) due to deficient microglia activation indicating the possibility of a direct interaction of Aβ1-42 with TLRs in the CNS. By activating TLR2, Aβ1-42 induces the secretion of proinflammatory molecules like TNF, IL-6 and IL-1β in mouse primary microglia (Reed-Geaghan et al., 2009). Similarly, both TLR2 and 4 mediate Aβ1-42-induced proinflammatory responses in human monocytic cell lines (Udan et al., 2008).

In contrast, TLR2 and 4 are not required for the induction of EAE by active immunization with myelin antigens emulsified in CFA. In TLR2 deficient mice, the severity of MOG35-55/CFA-induced EAE is similar to wild-type animals (Prinz et al., 2006). TLR4 and TLR9 KO animals are even hypersusceptible to EAE (Marta et al., 2008). Thus, each of TLR2 and TLR4 are dispensable for inducing a paralytic syndrome upon immunization with MOG35-55/CFA suggesting that adjuvant effects of CFA are mediated by other pattern recognition receptors or a combination of these TLRs. However, the neurocognitive phenotype induced by immunization with Aβ1-42/CFA was absolutely dependent on TLR2 and TLR4. Thus, we propose that unique effects of Aβ1-42 were mediated by TLRs and were the molecular basis of the clinical neurocognitive phenotype induced by immunization with Aβ1-42. Since there is also a weak antigen specific T cell response to Aβ1-42 promoting inflammation in tissues

with relevant expression of Aβ (Brown et al., 2007), activated macrophages may subsequently be recruited to the CNS. Here, macrophages were further activated and were induced to release proinflammatory cytokines resulting in clinically manifest psychomotor impairment.

Conclusion

In the present thesis, we investigated biopsychological interactions in autoimmune models of CNS inflammation. We addressed this issue in a manifold approach. The selective SNRI venlafaxine was shown to suppress the clinical and histopathological signs of EAE. In Figure 14, the EAE pathogenesis is summarized to illustrate differential effects of venlafaxine on immunological processes both in the periphery and the CNS. These treatment effects have been confirmed by significant and dose-dependent reductions of *in-vivo* mRNA expression levels of proinflammatory cytokines and immune cell markers in the inflamed CNS tissue.

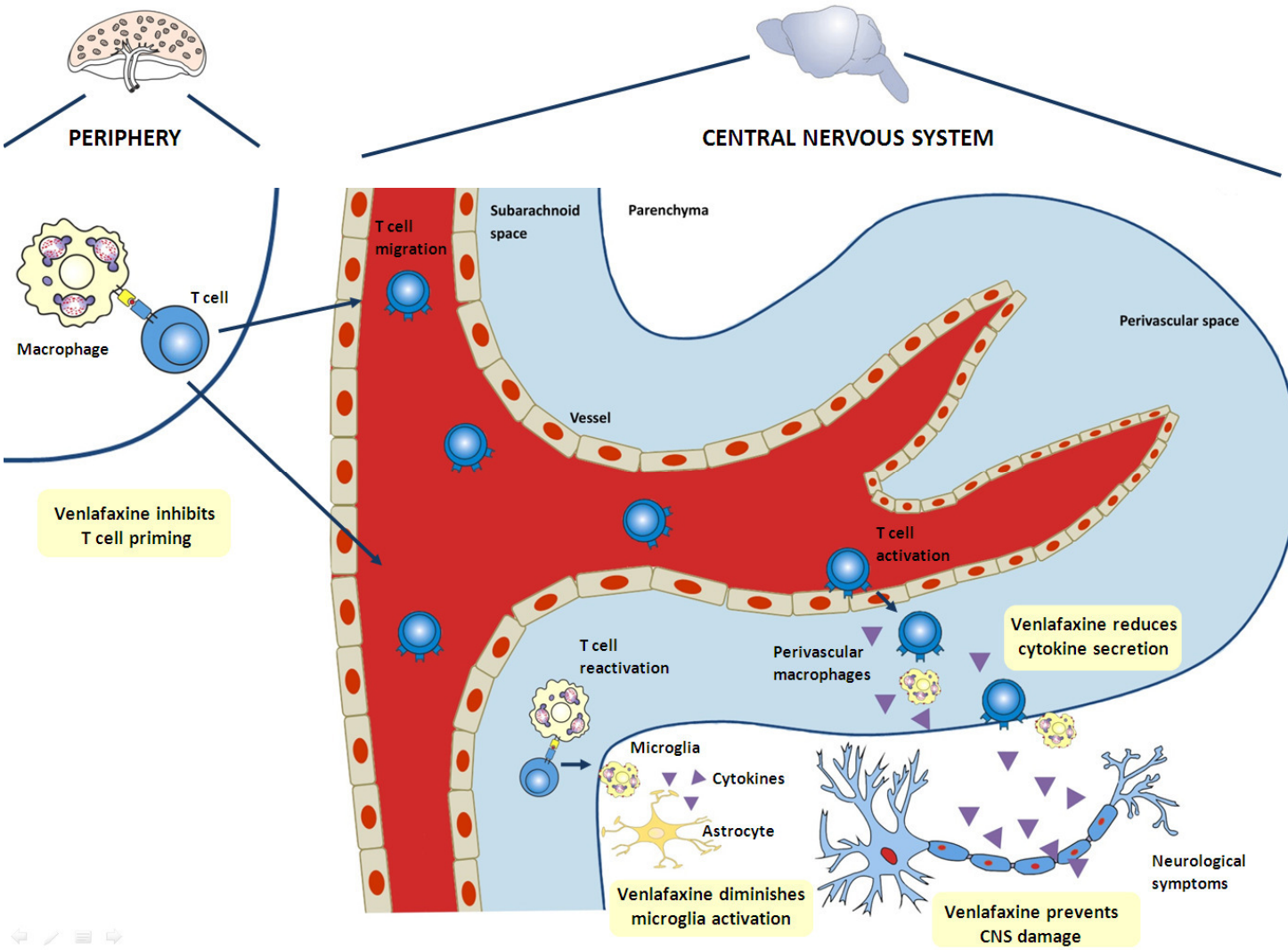

Figure 14. Venlafaxine impacts on different targets both in the periphery and the CNS. Sites of antiinflammatory action are highlighted.

Remarkably, we found venlafaxine, an antidepressant substance, to be highly effective in ameliorating a neurological autoimmune disease indicating that similar mechanisms are relevant for the pathogenesis of both inflammatory and affective disorders/diseases.

To further dissect the mechanisms behind the interaction of inflammation and biopsychological processes, we established an autoimmune model of cognitive and behavioral impairment by active immunization with a peptide related to neuronal functioning. Immunization with Aβ1-42 evoked strong activation of the innate immune system which resulted in cognitive decline through CNS infiltration of macrophages from the peripheral immune compartment. Active immunization with Aβ1-42 induced sustained cognitive and behavioral impairment in wild-type C57BL/6 mice. In histopathological analyses of the CNS, a disseminated, non-focal immune cell infiltration was identified in Aβ1-42/CFA-immunized mice mainly consisting of macrophages. This histopathological pattern is regarded as the morphological substrate of the neurocognitive phenotype of Aβ1-42/CFA-immunized animals. Figure 15 summarizes the effects of active Aβ1-42 immunization.

The findings of the present thesis might have direct implications on the clinical development of substances for the treatment of MS and Alzheimer's disease. This thesis provides the basis for investigating the therapeutic effects of venlafaxine to treat human MS and also adds a key component to the understanding of possible side effects induced by active immunization with Aβ1-42. Here, the effects of immunization even resulted in the impairment of cognitive performance which was assumed to be improved by Aβ immunotherapy. To date, all of the clinical trials investigating Aβ immunotherapy in Alzheimer's disease failed to show beneficial effects on cognitive symptoms in broad patient populations.

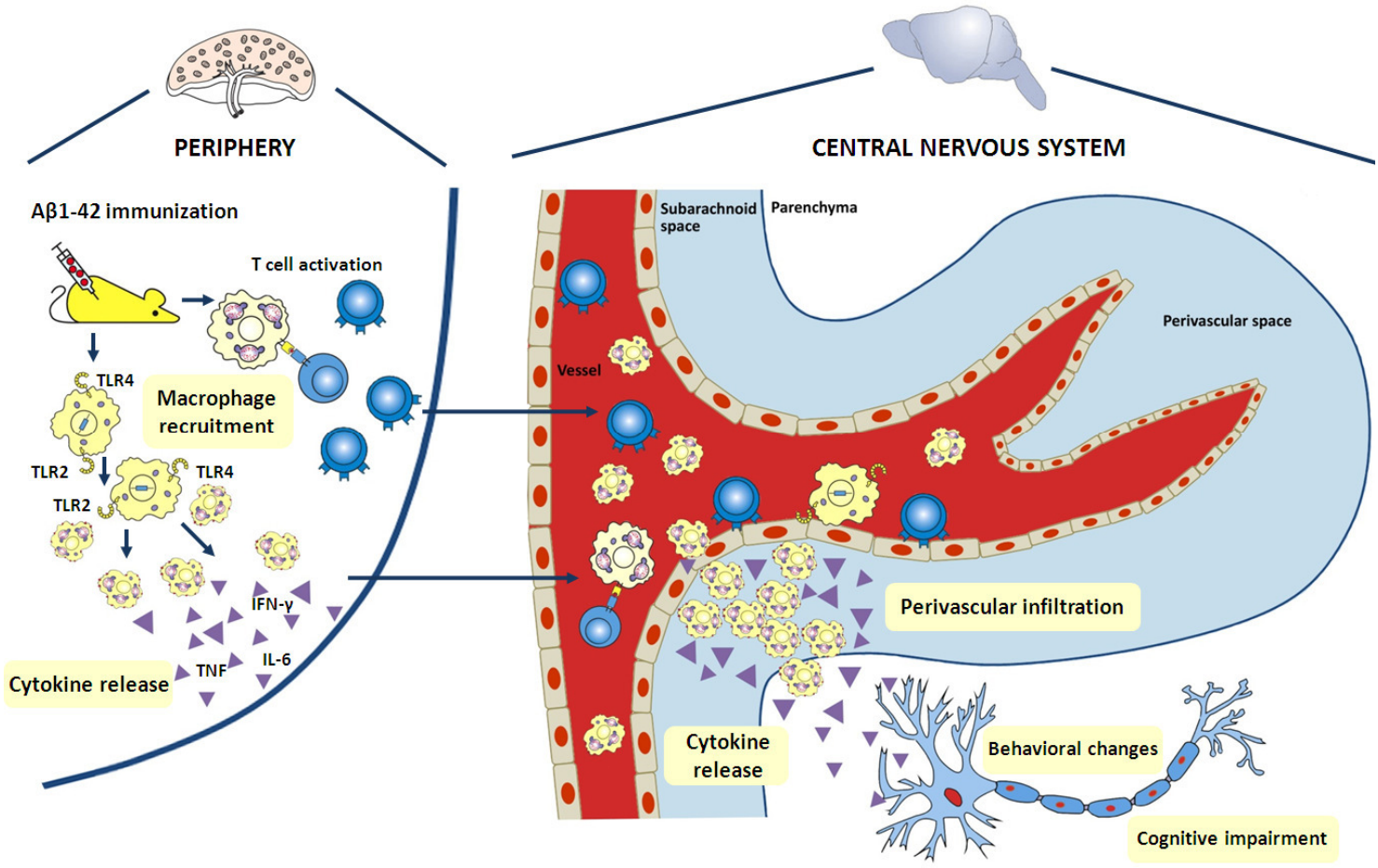

Figure 15. Illustration of different processes in the periphery and the CNS which are affected by active immunization with Aβ1-42.

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Appendix I

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ARTICLE

CINP

The antidepressant venlafaxine ameliorates murine experimental autoimmune encephalomyelitis by suppression of pro-inflammatory cytokines

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Abstract

Antidepressants are known to impact on the immune system. In this study, we examined the immunomodulatory properties of venlafaxine, a selective serotonin/norepinephrine reuptake inhibitor (SNRI), in murine experimental autoimmune encephalomyelitis (EAE), a T-cell-mediated CNS demyelinating disease model of multiple sclerosis. EAE was induced in SJL/J mice by adoptive transfer of myelin-specific T cells. Mice received different doses of venlafaxine before induction and after onset of disease. Sustained daily oral treatment with 6, 20 and 60 mg/kg significantly ameliorated the clinical symptoms of the disease compared to vehicle during both preventive and therapeutic intervention. Venlafaxine suppressed the generation of pro-inflammatory cytokines IL-12 p40, TNF-α and IFN-γ in encephalitogenic T-cell clones, splenocytes and peritoneal macrophages in vitro. It also diminished mRNA expression of a number of inflammatory genes in the inflamed CNS tissue, among them CD3, CD8, Granzyme B, IL-12 p40, IFN-γ, TNF-α and the chemokines Ccl2 and RANTES, whereas the expression of brain-derived neurotrophic factor was increased. These findings demonstrate the strong immunomodulatory property of the selective SNRI venlafaxine. Further studies are warranted to clarify whether venlafaxine may exert similar effects in humans.

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Key words: Antidepressant, cytokines, EAE, multiple sclerosis, venlafaxine.

Introduction

Venlafaxine, a selective serotonin/norepinephrine reuptake inhibitor (SNRI), is a drug frequently used for the treatment of affective disorders. Besides its efficacy in the therapy of major depression a number of studies have suggested immunomodulatory effects of venlafaxine in vitro similar to those that have been demonstrated for other antidepressants such as fluoxetine, imipramine or amitryptiline (e.g. Maes, 2001; Obuchowicz et al., 2006). Venlafaxine has been shown to down-regulate interferon-γ (IFN-γ) production in whole-blood cells from patients with treatment-resistant depression while up-regulating anti-inflammatory cytokines such as interleukin-10 (IL-10) (Kubera et al., 2001). Furthermore, venlafaxine reduces the secretion of the pro-inflammatory cytokines interleukin-6 (IL-6) and IFN-γ from astrocytes and changes the phenotype of primary microglia from activated to resting morphology (Vollmar et al., 2008).

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of the central nervous system (CNS) of unknown aetiology. While a number of pro-inflammatory cytokines [e.g. IL-17, IFN-γ, tumour necrosis factor-α (TNF-α)] have been found in the cerebrospinal fluid (Ishizu et al., 2005) or in lesions during acute MS relapses (Lassmann et al., 2007), anti-inflammatory cytokines such as IL-10 and transforming growth factor-β (TGF-β) (Carrieri et al., 1998)

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have been detected during remission, suggesting an imbalance of pro- and anti-inflammatory cytokines in this disorder. Besides neurological deficits, fatigue and depressive episodes appear in the course of this disease in more than 50% of MS patients (Joffe, 2005).

Experimental autoimmune encephalomyelitis (EAE) is the well-known animal model for MS which allows the study of inflammation-related damage of CNS tissue. EAE can be induced in several animal strains by immunization with myelin components or by adoptive transfer of myelin-specific T cells (Gold et al., 2006; 't Hart and Amor, 2003; Zamvil and Steinman, 1990). The disease is clinically characterized by neurological deficits, mainly paresis, and histopathologically by perivascular infiltrates in the CNS. In most EAE models the disease is initiated by CD4+, MHC class II-restricted Th1 and Th17 cells (Stromnes et al., 2008). Activated CD4+ T cells can cross the blood–brain barrier (BBB), infiltrate the CNS and secrete chemokines and pro-inflammatory cytokines upon re-challenge by microglial cells and autoantigen. The secreted chemokines and cytokines will attract monocytes to the lesion and activate microglial cells which both significantly contribute to CNS tissue damage by secreting neurotoxic molecules. Furthermore, astroglial cells proliferate within demyelinating lesions of MS and EAE (Holley et al., 2003; Tani et al., 1996) and promote inflammation, oligodendrocyte damage and glial scarring (Ambrosini et al., 2005).

In-vivo findings on the clinical course of experimental autoimmune models demonstrate a significant impact of neurotransmitter reuptake modulation. 5-HT transporter-deficient mice develop less severe EAE with reduced infiltration of the CNS compared to wild-type animals (Hofstetter et al., 2005). Clinical signs of experimental allergic neuritis are less severe in 5-HT reuptake inhibitor-treated animals (Bengtsson et al., 1992). Further, the selective phosphodiesterase type-4 inhibitor rolipram which exerts antidepressant properties suppresses clinical and histological signs of EAE (Sommer et al., 1995). Recently, hyperforin was found to have suppressive effects on EAE by down-regulating effector functions of activated T cells (Cabrelle et al., 2008).

Thus far, modern antidepressants especially selective SNRIs have not been investigated for their impact on CNS autoimmunity in vivo.

In this study, we report that venlafaxine efficiently suppresses EAE clinically and histopathologically. In-vitro and in-vivo data suggest that this effect is mediated by the strong anti-inflammatory activity of venlafaxine.

Methods

Animals and induction of EAE

Female SJL/J mice were obtained from Harlan Winkelmann (Borchen, Germany). They were kept according to the regional animal guidelines and used from age 6–12 wk. All procedures were performed according to the local guidelines and the study was approved by the regional Animal Care Committee.

EAE was induced by the adoptive transfer of 5×10^6 to 2×10^7 cells i.p. into syngenic recipients according to previously published protocols (Nessler et al., 2006; Stromnes and Goverman, 2006). Briefly, SJL/J mice were subcutaneously immunized with 200 μg/animal proteolipid protein (PLP) 139–151 (HSLGKWLGHPDKF, single-letter code, Jerini, Berlin) emulsified in incomplete Freund's adjuvant (Sigma-Aldrich, Munich, Germany) supplemented with 5 mg/ml inactivated *M. tuberculosis* H37RA (Difco Laboratories, Detroit, MI, USA). The draining lymph nodes were removed 11 d later and single-cell suspensions were made. After in-vitro restimulation with 10 μg/ml PLP 139–151 for 4 d, 5×10^6 to 2×10^7 cells were injected intraperitoneally into syngenic recipients.

Clinical signs of EAE were ranked from 0 (normal), 1 (tail limpness), 2 (paraparesis with clumsy gait), 3 (hindlimb paralysis), 4 (hind- and forelimb paralysis), 5 (death) according to Kassiotis et al. (1999). All ratings were done by observers blinded to the treatment.

Venlafaxine treatment

Mice were treated daily with 6, 20 or 60 mg/kg venlafaxine p.o. (the substance was kindly provided by Wyeth Pharma, Münster, Germany) in 100 μl PBS starting at the day of induction or after the onset of clinical symptoms. Control mice received PBS alone. In another experiment, osmotic pumps (Alzet model 2002; Alzet, Palo Alto, CA, USA) were implanted subcutaneously 2 d prior to disease induction and vehicle or 60 mg/kg/d venlafaxine were continuously administered for 14 d.

Histology and immunohistochemistry

Mice were perfused with PBS and 4% paraformaldehyde intracardially. Brains and spinal cords were dissected and embedded in paraffin. Inflammation was assessed by haematoxylin staining (Nessler et al., 2007). The extent of inflammation is expressed as the mean number of inflammatory infiltrates per spinal cord cross-section (inflammatory index). A minimum of 10 spinal cord cross-sections per animal were examined.

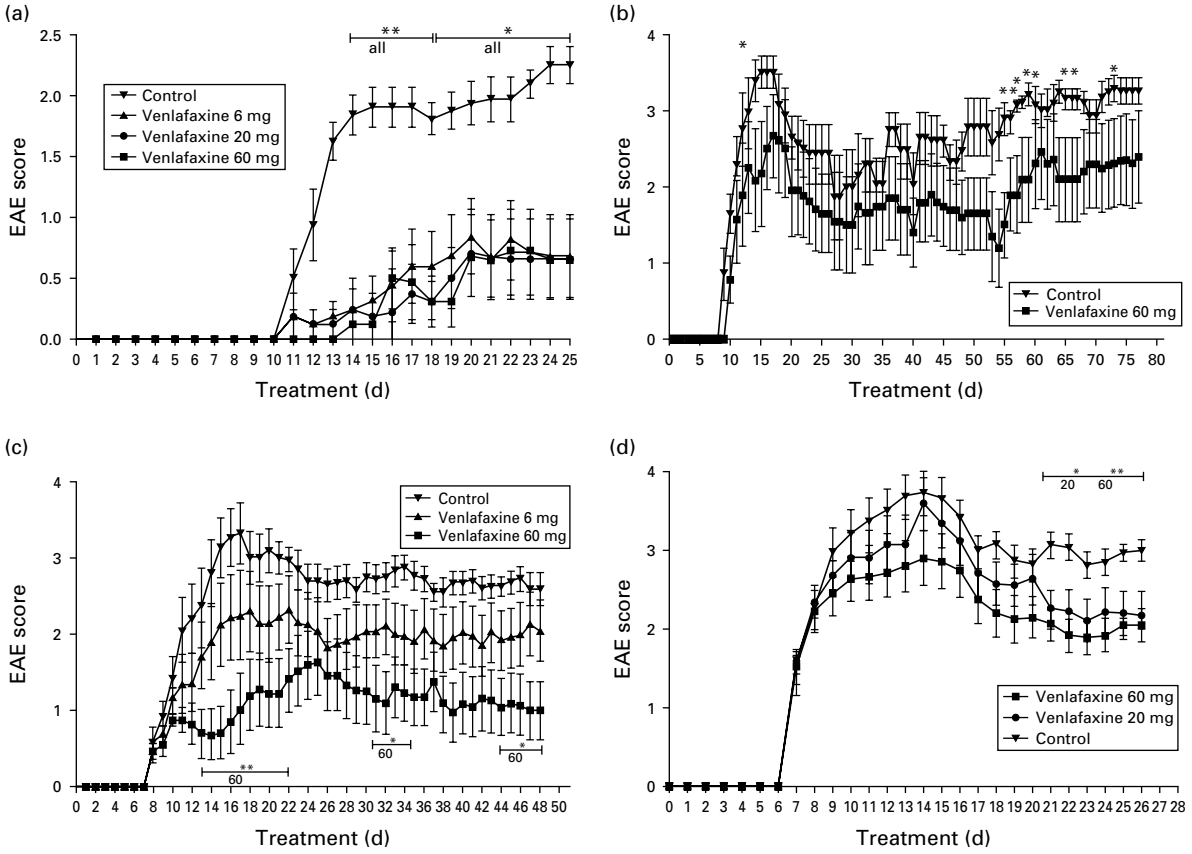

Figure 1. Venlafaxine treatment reduces EAE severity in SJL/J mice. Oral pretreatment of animals ($n=8$ animals/group) starting at the day of adoptive transfer (a) of 1×10^7 lymph node cells i.p. suppressed EAE in a dose dependent fashion. Non-sustained pretreatment with venlafaxine ($n=7$) animals/group for 14 days via s.c. implanted osmotic pumps (b) reduced the peak of disease and ameliorated relapses after transfer of 2×10^7 lymph node cells, but continuous oral therapy was superior in terms of EAE suppression. A significant and dose dependent reduction of adoptive transfer EAE was also observed when animals were treated at the beginning of first EAE symptoms (c, transfer of 5×10^6 cells, $n=10$ animals/group) or after manifestation of considerable EAE symptoms (d, transfer of 1×10^7 cells, $n=10$ animals/group). The therapeutic effect of venlafaxine is clearly dose dependent. However, even very low doses (6 mg/kg/d) were sufficient to ameliorate the clinical course of disease. Data are presented as mean clinical scores $\pm$ standard error, significant differences compared to control groups were determined by two-sided Kruskal-Wallis Test and are indicated. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Immunohistochemistry was performed with an antibody against the glial fibrillary acidic protein (GFAP, clone 6F2, Dako North America, Carpinteria, CA, USA) as described previously (Abdul-Majid et al., 2003). Tissues were pretreated with microwaving in 10 mM citrate buffer (pH 6.0) twice for 5 min. Bound antibody was visualized using an avidin-biotin technique (extravidin-peroxidase, Sigma-Aldrich). DAB chromogen (Dako) was applied for 2×5 min and the slides were counterstained with haematoxylin. Control sections were incubated in the absence of primary antibody or with non-immune sera. Slides were counterstained with haematoxylin and coverslipped.

Histological analyses were performed on spinal cord sections derived from the prevention experiment (Figure 1a).

Cell culture

Cell culture was performed in RPMI 1640 medium containing 10% fetal calf serum, non-essential amino acids, Hepes, L-glutamine, 2-mercaptoethanol and antibiotics (Sommer et al., 1997). The cells were cultivated at 37 °C in a humidified incubator at 5% CO_2

Cytokine production

In-vitro effects of venlafaxine were studied on the myelin–oligodendrocyte–glycoprotein (MOG) 35–55

specific encephalitogenic T-cell clone 5–8 (S. Nessler et al., unpublished observations), on PLP 139–51 specific splenocytes and on peritoneal macrophages activated with lipopolysaccharides (LPS).

T-cell clone 5–8 was restimulated with 10 μg/ml MOG 35–55 (MEVGWYRSPFSRVVHLYRNGK, single-letter code, Jerini, Berlin) and 4×10^6/ml irradiated antigen presenting cells (APC) for 48 h. Venlafaxine (titrated from 10^{-5} to 10^{-10} mol/l) was added at the time of restimulation. Supernatants were collected after 48 h and kept at -80 °C for further analysis by cytokine- and chemokine-specific ELISAs. Spleens from animals actively immunized with PLP 139–151 were removed at day 11 and single-cell suspensions were generated. These cells were restimulated with 10 μg/ml PLP in the presence of 10^{-4} to 10^{-10} mol/l venlafaxine and supernatants were removed after 48 h and processed as described above.

Finally, primary macrophages were isolated from the peritoneal cavity of mice 3 d after intraperitoneal injection with 3 ml of 3% (w/v) thioglycollate (BD Diagnostics Systems, Sparks, MD, USA) according to previously published protocols (Ousman et al., 2007). These cells were cultured with media alone for 48 h and then activated with 100 ng/ml LPS (Sigma-Aldrich) in the presence of 10^{-4} to 10^{-9} mol/l venlafaxine. Supernatants were harvested 24 h later and stored at -80 °C for further analysis.

The supernatants were analysed for IL-12 p40, IFN-γ, TNF-α, IL-6 and the chemokine RANTES (Ccl5) with commercially available ELISA kits (R & D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Cell viability

Viability of cells was analysed after exposure to venlafaxine by 7-amino-actinomycin D (7-AAD) staining (BD Pharmingen, San Diego, CA, USA) using flow cytometry. Briefly, naive spleen cells from SJL/J mice (activated with 0.25 μg/ml anti-CD3) cells were incubated for 48 h with different concentrations of venlafaxine (from 10^{-3} to 10^{-8} mol/l). Cells were stained with 7-AAD (0.25 μg/10^6 cells) and analysed on a Dako Cyan flow cytometer (see online Supplementary Figure).

RNA isolation and real-time PCR

Isolation of RNA from fresh spinal cord (Rneasy®, Qiagen, Hilden, Germany), its quantification, and the reverse transcription reactions (High-capacity RT kit®, Applied Biosystems, Foster City, CA, USA) were performed according to established protocols. Expression of messenger RNA (mRNA) for target genes and the endogenous control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assessed by real-time PCR (with TaqMan Gene Expression Assay products on a 7500 Fast real-time PCR system; Applied Biosystems). The probes of each gene contain a 6-carboxy-fluorescein phosphoramidite (FAM dye) label at the 5′ end of the gene and a minor groove binder and non-fluorescent quencher at the 3′ end and are designed to hybridize across exon junctions. The assays are supplied with primers and probe concentrations of 900 nM and 250 nM, respectively. PCR reactions were prepared in a final volume of 20 μl, with final concentrations of 1× TaqMan Universal Fast PCR Mastermix (Applied Biosystems) and cDNA derived from 20 ng of input RNA as determined by full-spectrum UV/Vis spectrophotometric measurements (Nanodrop, Peqlab; Erlangen, Germany). Thermal cycling conditions comprised DNA polymerase activation at 95 °C for 2 min, 40 cycles of denaturation at 95 °C for 3 s, and annealing and extension at 60 °C for 30 s. Each measurement was performed in duplicate and the threshold cycle was determined. The following gene expression assays have been used (Applied Biosystems): BDNF (Mm00432069_m1), Ccl2 (Mm99999056_m1), CD3 (Mm00599683_m1), CD8 (Mm01182107_g1), Granzyme B (Mm00442834_m1), IFN-γ (Mm00801778_m1), IL-6 (Mm00446190_m1), IL-12 p40 (Mm00434165_m1), TNF-α (Mm00443258_m1), RANTES (Mm01302428_m1).

Expression levels for each gene of interest were calculated by normalizing the quantified mRNA amount to GAPDH. Relative gene expression was determined and used to test significance between treatment and control groups.

Data analysis

Data are presented as mean ± standard error of the mean (S.E.M.). Statistical comparisons between EAE treatment groups were performed by the two-tailed Kruskal–Wallis test. Unpaired t tests were used for mRNA expression data and histopathological scores. Values of $p < 0.05$ were considered significant. Graphs were generated using GraphPad Prism software (San Diego, CA, USA).

Results

Venlafaxine treatment ameliorates clinical signs of EAE

Treatment with venlafaxine significantly ameliorated EAE when treatment was initiated at the day of disease

Table 1. Summary of the effects of venlafaxine pretreatment and active treatment on the cumulative score, maximum disease score and disease incidence

	Adoptive transfer experimental autoimmune encephalomyelitis in SJL/J mice			
			Active treatment	
	Pretreatment Mean (S.E.M.)	Pump treatment Mean (S.E.M.)	Mean (S.E.M.)	Mean (S.E.M.)
Cumulative score				
Vehicle	26.78 (1.82)	187.18 (16.75)	106.95 (7.21)	60.73 (3.70)
6 mg/kg	7.72 (3.19)*	n.a.	78.25 (14.75)	n.a.
20 mg/kg	6.31 (2.40)**	n.a.	n.a.	51.47 (4.83)
60 mg/kg	6.03 (2.02)*	96.26 (32.65)*	45.63 (13.23)**	46.43 (4.02)*
Maximum score				
Vehicle	2.4 (0.1)	3.75 (0.14)	3.88 (0.15)	3.95 (0.21)
6 mg/kg	1.19 (0.35)**	n.a.	3.15 (0.36)	n.a.
20 mg/kg	1.09 (0.34)**	n.a.	n.a.	3.6 (0.266)
60 mg/kg	1.31 (0.31)*	2.89 (0.48)	2.35 (0.33)*	3.3 (0.25)
Disease incidence				
Vehicle	8/8	7/7	10/10	10/10
6 mg/kg	4/8	n.a.	10/10	n.a.
20 mg/kg	4/8	n.a.	n.a.	10/10
60 mg/kg	4/8	6/7	10/10	10/10
Animals per group	$n=8$	$n=7$	$n=10$	$n=10$
Treatment period	25 d	14 d	40 d	20 d

Significant differences between venlafaxine-treated and vehicle-treated mice were assessed by two-tailed Kruskal–Wallis test. * $p<0.05$, ** $p<0.01$.

induction (see Figure 1a and Table 1 for means, standard errors and statistical comparisons with the Kruskal–Wallis test). Early oral venlafaxine treatment was effective at 6, 20 and 60 mg/kg. Whereas all animals in the PBS-treated control group developed signs of EAE the disease incidence in the treatment groups was only 50%. Moreover, disease severity was milder in the affected animals in the treatment groups compared to the control group.

When animals were treated using venlafaxine with osmotic pumps exclusively during the induction phase, EAE symptoms were delayed and less pronounced in the treatment than in the control group (Figure 1b). However, sustained administration of venlafaxine was clearly more efficient than the 2-wk pretreatment.

Therapeutic intervention with venlafaxine at the beginning of EAE symptoms (Figure 1c) showed a dose–response relationship with a significant reduction of EAE symptoms at 60 mg/kg venlafaxine compared to vehicle-treated animals. If venlafaxine treatment was started after manifestation of severe symptoms (Figure 1d) significant amelioration of EAE symptoms could be demonstrated for 20 mg/kg and 60 mg/kg venlafaxine after 2 wk therapy.

Venlafaxine treatment reduces the number of inflammatory infiltrates and prevents gliosis in EAE lesions

Histology of control mice with clinical signs of EAE after adoptive transfer of PLP-specific cells revealed dense subpial and perivascular infiltrates expanding to the parenchyma (Figure 2b). Venlafaxine-treated mice showed markedly reduced CNS inflammation and were largely devoid of inflammatory infiltrates in the brain and spinal cord (Figure 2a). The average number of inflammatory infiltrates per spinal cord cross-section (inflammatory index, Figure 2f) was 6.78 ± 0.73 in untreated animals compared to 2.03 ± 0.73 in the 6 mg/kg group ($t=4.56$, $p<0.0018$ revealed by unpaired t test) and 0.99 ± 0.42 in the 60 mg/kg group ($t=6.81$, $p=0.0001$).

In untreated mice (Figure 2d), severe astrogliosis in the parenchyma was observed in the chronic disease phase, whereas treated mice (Figure 2c) showed no

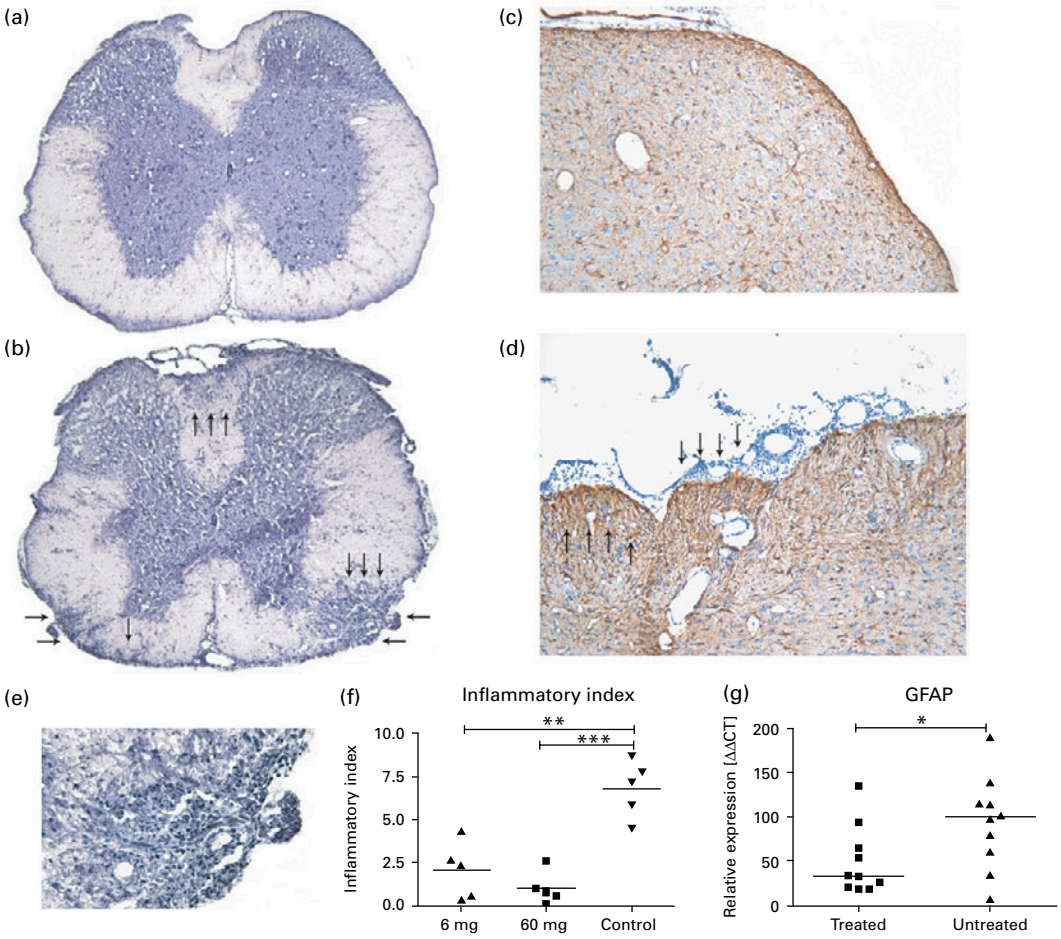

Figure 2. Venlafaxine reduces the histopathological manifestation of experimental autoimmune encephalomyelitis. (a) Shows a representative haematoxylin staining (40× original magnification) of the thoracic spinal cord from a venlafaxine-treated animal (20 mg/kg.d) without inflammatory foci after 3 wk of adoptive transfer. (b) Illustrates a spinal cord section of a vehicle-treated mouse with considerable amounts of inflammatory foci [(e) 200× magnification], with dense mononuclear cell infiltration around the meninges. Arrows indicate mononuclear cell infiltrates. (f) Shows the mean numbers of inflammatory infiltrates per spinal cord cross-section (inflammatory index). A minimum of 10 spinal cord cross-sections per animal were examined. Statistical difference was assessed by unpaired, two-tailed *t* test. Panels (c) and (d) illustrate reactive gliosis to inflammation in the brainstems of representative untreated [(d) 200× magnification] and treated (c) animals as revealed by GFAP immunostaining and haematoxylin counterstaining after 2 wk of disease onset. In untreated mice [(d) 200× magnification], inflammatory cell infiltration evokes severe astrogliosis in the parenchyma whereas treated mice are almost free of reactive gliosis. Data were confirmed (g) by quantitative GFAP gene expression analysis performed in mice receiving different doses of venlafaxine as preventive treatment. Statistical evaluation of relative GFAP gene expression was assessed by two-tailed Mann–Whitney *U* test (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

Figure 3. Venlafaxine reduces the mRNA expression in spinal cord tissue of EAE mice at day 48 after disease induction. Here, the quantitative mRNA expression of inflammation-related genes in the spinal cord tissue of venlafaxine- and vehicle-treated mice is illustrated. Gene expression analysis was performed on tissue from one therapeutic trial (Figure 1c). The GAPDH-normalized relative gene expression (ΔΔCT) is shown for single animals. Both doses of venlafaxine suppressed the in-vivo expression of the CD3 T cells, cytotoxic CD8 T cells and Granzyme B genes. However, the effect was more pronounced under high-dose treatment. Further, the antidepressant significantly reduced the gene expression of the pro-inflammatory cytokines, IL-12 p40, IFN-γ and TNF-α and the chemokines Ccl2 and Ccl5 whereas the expression of brain-derived neurotrophic factor (BDNF) was significantly increased. The *n*-fold change in mRNA expression compared to control group is summarized for each inflammation-related gene. Statistical difference was assessed by unpaired, two-tailed *t* test (* $p<0.05$, ** $p<0.01$).

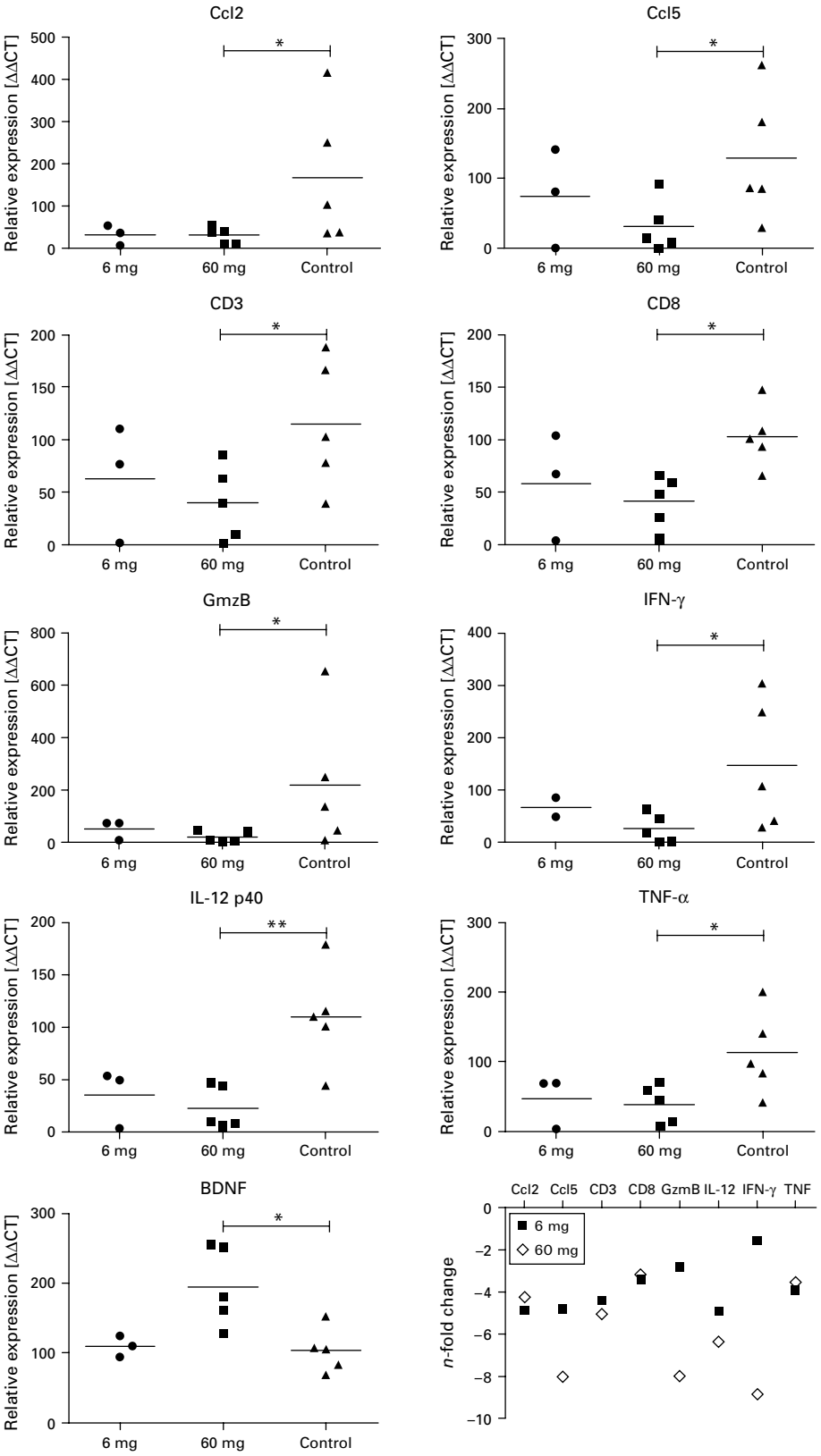

Figure 3. For legend see opposite page.

reactive gliosis as revealed by GFAP immunostaining. Data were confirmed by GFAP gene expression studies. GFAP gene expression was significantly higher in untreated mice compared to venlafaxine-treated mice.

Decreased expression of pro-inflammatory gene transcripts in EAE lesions during venlafaxine treatment

In line with the histopathological studies, treatment with venlafaxine reduced the expression of T-cell genes (CD3 and CD8) in inflamed spinal cord tissue (Figure 3). The effect was dose dependent and reached the highest suppressive effect at 60 mg/kg/d. Further, Granzyme B, a gene expressed by cytotoxic CD8 T cells and natural killer cells, was reduced in animals receiving high doses of venlafaxine. The drug also had a significant impact on IL-12 p40, TNF-α and IFN-γ, three pro-inflammatory cytokines produced by macrophages and T cells respectively. Moreover, gene transcripts of the chemokines Ccl2 and Ccl5 were strongly reduced in the lesions of treated animals. By contrast, brain-derived neurotrophic factor (BDNF) mRNA expression was significantly up-regulated in the inflamed spinal cord of EAE mice receiving a high dose venlafaxine.

Venlafaxine reduces the secretion of pro-inflammatory cytokines by T cells and macrophages in vitro

In vitro, venlafaxine reduced the release of pro-inflammatory cytokines in PLP-specific T cells and MOG 35–55 specific T-cell clones (Figure 4a, b). The effect was most pronounced for IFN-γ and IL-12 p40 with an overall reduction of cytokine secretion by 50%. Venlafaxine also reduced the expression levels of Ccl5, IL-6 and TNF-α in a dose-dependent manner. Peritoneal macrophages released less of the pro-inflammatory cytokines IL-6 and TNF-α upon challenge with venlafaxine (Figure 4c). The suppression of cytokine and chemokine secretions by venlafaxine was dose-dependent and observed at concentrations non-toxic to the cells as assessed by 7-AAD staining (data not shown). Toxicity was only observed when the concentration of venlafaxine exceeded 10^{-3} mol/l.

Discussion

In the present study, we have demonstrated that the selective SNRI venlafaxine can suppress the clinical and histopathological signs of EAE. Venlafaxine treatment ameliorates EAE even after disease has been established. Clinical and histopathological treatment effects of venlafaxine have been confirmed by significant and dose-dependent reductions of in-vivo mRNA expression levels of pro-inflammatory cytokines and immune cell markers in the inflamed CNS tissue.

First, gene expression data illustrate the anti-inflammatory properties of venlafaxine in autoimmune CNS diseases applying a method which is characterized by high sensitivity and objective quantifiability in the absence of any in-vitro manipulation. Second, gene expression data were confirmed by immune assays on the protein level. The in-vitro administration of venlafaxine suppressed cytokine production in (i) myelin-specific T cells, (ii) in T-cell clones with high encephalitogenicity and (iii) peritoneal macrophages at low doses, accounting for the immunosuppressive effects.

Venlafaxine strongly reduced the in-vivo gene expression and in-vitro secretion of IL-12 p40 which is considered essential in T-cell-mediated autoimmune diseases (Gran et al., 2004). This is based on the strong capacity of IL-12 to induce T-cell activation, Th1 cytokine differentiation and macrophage activation (Trinchieri and Scott, 1995).

The results of the present study are consistent with in-vitro findings on the anti-inflammatory effects of venlafaxine on primary astroglia and microglia (Kubera et al., 2001; Vollmar et al., 2008). Antidepressants impact on microglial cells by suppression of inflammation-induced cytokine production. Hashioka et al. (2007) showed for several substances (imipramine, fluvoxamine and reboxetine) reduced IL-6 and nitric oxide production after IFN-γ activation. Similar effects were found for amitryptiline in mixed glial and microglial cultures (Obuchowicz et al., 2006).

The mechanisms leading to the reduction of cytokine secretion are still unknown. One putative mechanism (Hindmarch, 2001) is the increase of transcription factors such as intracellular cyclic adenosyl monophosphate (cAMP) resulting in activation of neuroprotective proteins, such as BDNF (Xia et al., 1996), which was also up-regulated in the spinal cord of venlafaxine-treated animals in our study. Other in-vitro studies found antidepressant mediated reduction of cytokine-induced prostaglandin E2 and nitric oxide production by inflammatory cells from synovial tissue (Yaron et al., 1999).

Animals in the present study were treated by oral administration of venlafaxine and by the use of osmotic pumps. Mice treated with 5 mg/kg/d venlafaxine subcutaneously exhibited plasma concentrations of 178.4 ng/ml according to a study by Uhr et al. (2003). Plasma concentrations of venlafaxine up to 600–900 ng/ml have been reported in patients

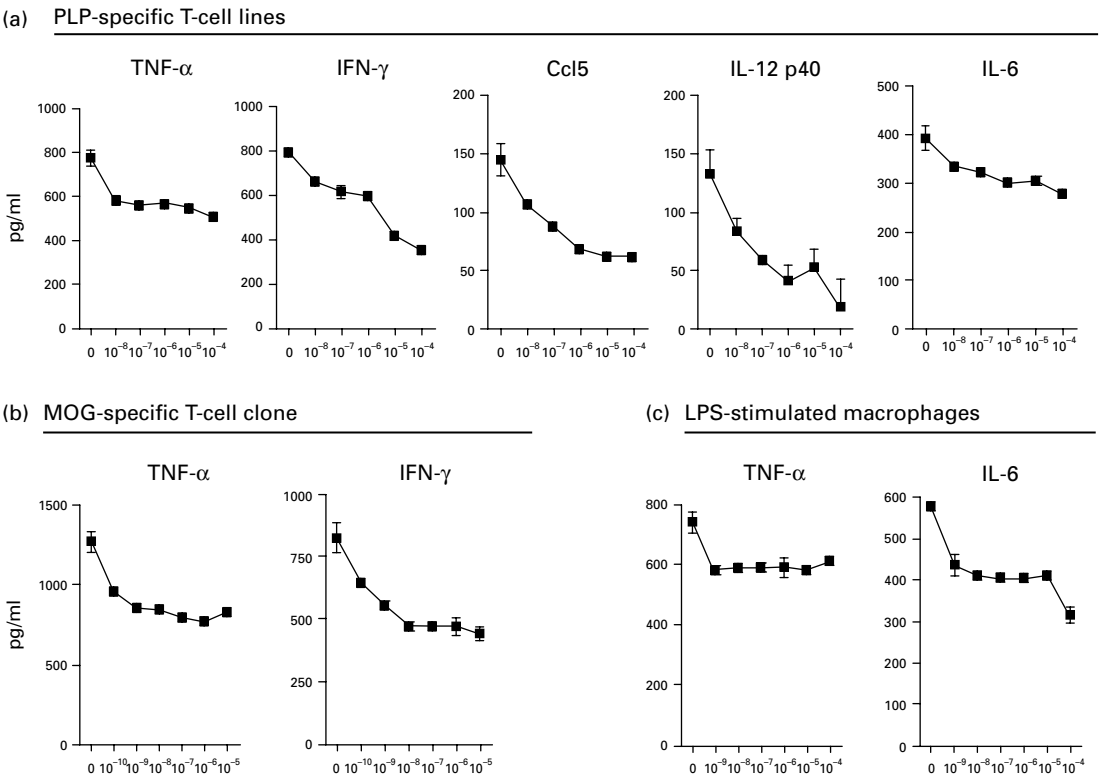

Figure 4. (a) Venlafaxine reduces the secretion of pro-inflammatory cytokines in encephalitogenic PLP-specific T cells. T cells (5×10^6/ml) were stimulated with PLP 139–51 (10 μg/ml) for 48 h. Venlafaxine or PBS was added at a concentration of 10^{-4} to 10^{-8} mol/l. The supernatant was analysed by ELISA for TNF-α, IFN-γ, IL-6, Ccl5 and IL-12 p40. Background cytokine production in the absence of the antigen was subtracted from PLP 139–51 stimulated production. (b) Venlafaxine decreases the secretion of TNF-α and IFN-γ in an encephalitogenic MOG-specific T-cell clone. Clone 5–8 (1×10^6 cells/ml) was restimulated with MOG 35–55 (10 μg/ml) and irradiated APC (4×10^6 cells/ml). Venlafaxine (10^{-5} to 10^{-10} mol/l) was added for 48 h and cytokine concentrations in the supernatants were determined with ELISA. (c) Venlafaxine also impacts on the cytokine production by lipopolysaccharide (LPS)-stimulated primary peritoneal macrophages. Adherent primary macrophages (1×10^6 cells/ml) were activated with 100 ng/ml LPS and incubated with PBS or venlafaxine (10^{-4} to 10^{-9} mol/l) for 24 h. In the supernatants, the cytokine concentration of TNF-α and IL-6 was determined by ELISA. The background cytokine production in the absence of LPS was subtracted from the stimulated production. All experiments were replicated at least three times.

chronically treated with 200–300 mg/d venlafaxine (Ilett et al., 2002). Assuming a linear dose–response relation, the applied 6 mg/kg/d and 20 mg/kg/d venlafaxine represent clinically relevant dosages. Oral administration of the drug was clearly superior to the continuous drug administration by osmotic pumps, suggesting that high plasma peaks are beneficial for the therapeutic efficacy of venlafaxine in our model.

At first view, affective disorders and autoimmune diseases such as MS or EAE are completely different diseases with distinct clinical phenotypes. However, the incidence of neuropsychiatric disorders among MS patients is remarkably high (Cetin et al., 2007; Ghaffar and Feinstein, 2007) and introduces a new perspective on the interaction of both diseases. Interestingly, a study by Mohr et al. (2001) found that MS patients with concomitant depression revealed a reduction of MOG-stimulated IFN-γ production under antidepressant therapy with sertraline and cognitive therapy. Even in EAE, Pollak et al. (2002) identified an 'EAE-associated behavioural syndrome' which is closely related to human major depression in terms of body weight reduction, changes in food and sucrose intake and a decrease in social exploration.

In the pathogenesis of mood disorders, the role of cytokines has attracted considerable interest during the last decade (e.g. Besedovsky and Rey, 2007). Studies found increased levels of IL-6, IL-1β and TNF-α in stimulated peripheral blood mononuclear cells of depressed patients (Cyranowski et al., 2007). There is growing evidence that the increase in cytokine concentrations accounts for 'sickness behaviour' and

depression (e.g. Irwin and Miller, 2007). Antidepressant treatment seems to have suppressing effects on cytokine secretion (Castanon et al., 2002) revealing a fundamental consequence of selective monoaminergic reuptake inhibition.

Interestingly, studies of antidepressant effects of a cyclooxygenase-2 (COX-2) inhibitor (Muller et al., 2006) which curtails prostaglandin E2 generation and the production of pro-inflammatory cytokines showed significant improvement in depressive patients under celecoxib add-on therapy. Further, the same COX-2 inhibitor has been found to have preventive effects in EAE through the suppression of pro-inflammatory cytokine secretion (Miyamoto et al., 2006). COX-2 inhibitors reduce the secretion of IL-12 (Muthian et al., 2006) revealing a mechanism of immunomodulation similar to the one we identified here for venlafaxine. These findings provide further evidence for a neuroimmune interaction and an inflammation-related pathogenesis of affective disorders.

In summary, the clinical effects of venlafaxine on CNS inflammation are marked and warrant replication in human trials to prove efficacy in MS patients.

Note

Supplementary material accompanies this paper on the Journal's website (http://journals.cambridge.org).

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Statement of Interest

None.

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Appendix II

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BRIEF REPORT

CINP

Venlafaxine exhibits an anti-inflammatory effect in an inflammatory co-culture model

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Abstract

Growing evidence indicates immunoregulatory effects of various antidepressants. Through the interaction of the nervous and immune systems, the norepinephrine–serotonin system was shown to modulate inflammatory CNS diseases. Thus, we examined the norepinephrine–serotonin reuptake inhibitor venlafaxine in an astroglia–microglia co-culture model which allows mimicking of an inflammatory milieu by increasing the cultured microglial fraction. Astrocytic membrane resting potential and intercellular coupling, two markers becoming severely impaired under inflammation, were assessed with the patch-clamp technique. We measured IL-6, IL-10, IFN-γ and TGF-β concentrations and analysed phenotypic changes of microglia. We found (i) a reversal of the inflammation-induced depolarization effect on the membrane resting potential, (ii) an augmentation of TGF-β release with a concomitant reduction in the secretion of pro-inflammatory IL-6 and IFN-γ, and (iii) a significant change of microglial phenotype from activated to resting morphology. Our data clearly indicate anti-inflammatory properties of venlafaxine which might be a result of monoamine-mediated immunomodulation.

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Key words: Antidepressants, glia, inflammation, norepinephrine, venlafaxine.

Introduction

In the past years, several studies uncovered immunoregulatory effects of antidepressant agents (e.g. Maes, 2001). Venlafaxine, fluoxetine and imipramine were found to have negative immunoregulatory effects by suppressing the interferon-γ–interleukin-10 (IFN-γ–IL-10) production ratio in whole-blood cells (Kubera et al., 2001). Further studies reported decreasing pro-inflammatory and increasing anti-inflammatory cytokine levels (Kenis and Maes, 2002; Xia et al., 1996) under antidepressant treatments. Recently, amitryptiline was shown to inhibit interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) production in rat mixed glial and microglial cultures (Obuchowicz et al., 2006).

Changes of the serotonin (5-HT) and norepinephrine (NE) transmitter systems have been reported in the pathogenesis of affective disorders which are efficiently treated with selective 5-HT and/or NE reuptake inhibitors. Both transmitter systems have been suggested to serve as mediators of bidirectional interactions between the nervous and the immune systems (Felten et al., 1992; Mossner and Lesch, 1998). For instance, 5-HT receptor-deficient transgenic mice when challenged with experimental allergic encephalomyelitis (EAE) revealed a reduction of inflammatory infiltrates in the CNS and of the neuroantigen-specific production of IFN-γ in splenocytes (Hofstetter et al., 2005). Further studies showed a suppression of clinical signs in experimental allergic neuritis when animals were treated with selective serotonin reuptake inhibitors (Bengtsson et al., 1992).

Earlier investigations on the effect of NE showed a suppression of clinical and histological signs of EAE after treatment with the β-adrenergic agonist isoproterenol (Chelmicka-Schorr et al., 1992).

Recently, de Keyser et al. (2004) discussed the role of astrocytic β_2-adrenoceptors in multiple sclerosis (MS) disease progression. Correspondingly, therapies

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designed to elevate cAMP levels in astrocytes may have potential effects to prevent both relapse and progression of MS.

To investigate the putative immunoregulatory effects in the CNS of both 5-HT and NE, we examined the antidepressant venlafaxine, a highly selective 5-HT and NE reuptake inhibitor (SNRI) which blocks both 5-HT and NE transporters (SERT and NET) respectively.

We employed an inflammatory astroglia–microglia co-culture model (Faustmann et al., 2003, Hinkerohe et al., 2005) which allows mimicking of inflammatory conditions in an in-vitro bioassay. Especially, the activation of microglia and response of astroglia to microglial activation can be monitored in this assay. Since astrocytes seem to play a crucial role in the pathogenesis of inflammatory diseases of the CNS and represent pharmacological targets of antidepressants (Hertz et al., 2004) we studied the influence of exogenously applied venlafaxine on inflammatory markers including the degree of the astroglial coupling, the interleukin-6 (IL-6), IL-10, transforming growth factor-β (TGF-β) and IFN-γ response and the morphological change of microglial phenotype.

Methods

Cell culture

Primary cell cultures of glial cells were prepared from hemispheres of postnatal (P0–P2) Wistar rats according to Dermietzel et al. (1991) and Faustmann et al. (2003). Depending on the extent of shaking, the fraction of microglial cells remaining in the co-cultures varies between 5% (M5), comparable to the concentration found in healthy adult brain tissue, and 30% (M30) as determined by counting after fixation and immunohistochemical staining with the microglia marker ED1 (see Hinkerohe et al., 2005 for detailed methodology). The study was approved by the Bioethical Committee of the Ruhr-University Bochum, and experiments were performed in accordance with accepted guidelines for care and use of animals in research.

Administration of the antidepressant substance venlafaxine

Based on findings by Uhr and co-workers (2003), we incubated cells with 300 ng/ml to mimic cerebrum concentrations which are found after 1-wk treatment with the optimal dose of venlafaxine. In a further condition, we reduced the concentration by a factor of 10 (30 ng/ml) to create suboptimal dosing conditions. Drugs were dissolved in 50 μl phosphate buffered saline (PBS) and were added to M5 and M30 co-cultures for 16 h.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels were quantified from cell culture supernatants by ELISA. Samples of supernatants were collected after incubation and stored at $-20\,^{\circ}$C until concentration determination. Quantikine-ELISA kits (R&D Sytstems, Minneapolis, MN, USA) were applied for quantification of rat IL-6, IL-10, IFN-γ and TGF-β according to the manufacturer's protocol. Cytokine concentrations of all control (incubation with PBS for 16 h) and venlafaxine-treated (incubation for 16 h) samples were determined in duplicate. All data represent means of four independent experiments.

Functional coupling and membrane resting potential (MRP)

Astroglial MRP was determined by employing the whole-cell patch-clamp mode (see Hinkerohe et al., 2005 for detailed procedure). The technique allows simultaneous intracellular injection of Lucifer Yellow and monitoring of dye transfer to neighbouring cells. Numbers of coupled astroglial cells were counted 10 min after dye injection.

Immunofluorescence

Immunofluorescence was applied to co-cultures in order to assess the fraction of microglia within the astrocytic cultures. Briefly, we determined the density of astrocytes by immunolabelling the glial fibrillary acidic protein (GFAP) with a polyclonal antibody (1:100; Sigma G9269, Taufkirchen, Germany). Microglia were labelled by using a monoclonal antibody directed to the ED1 epitope (1:250; Serotec MCA 341R, Eching, Germany), which allowed classification of microglia as resting ramified (RRT), intermediate (INT) and activated, rounded phagocytic (RPT) phenotypes (Faustmann et al., 2003). For quantification of cell numbers immunocytochemically labelled cells were counter-stained with DAPI (4,6-diamidino-2-phenyl-indol) (1:2500; Sigma D9542) to visualize the nuclei.

Statistical analysis

Significance of differences between mean cytokine concentrations (IL-10 and TGF-β) were tested using the t test. Significant differences for the mean IL-6 and IFN-γ concentrations were determined with the Mann–Whitney U test.

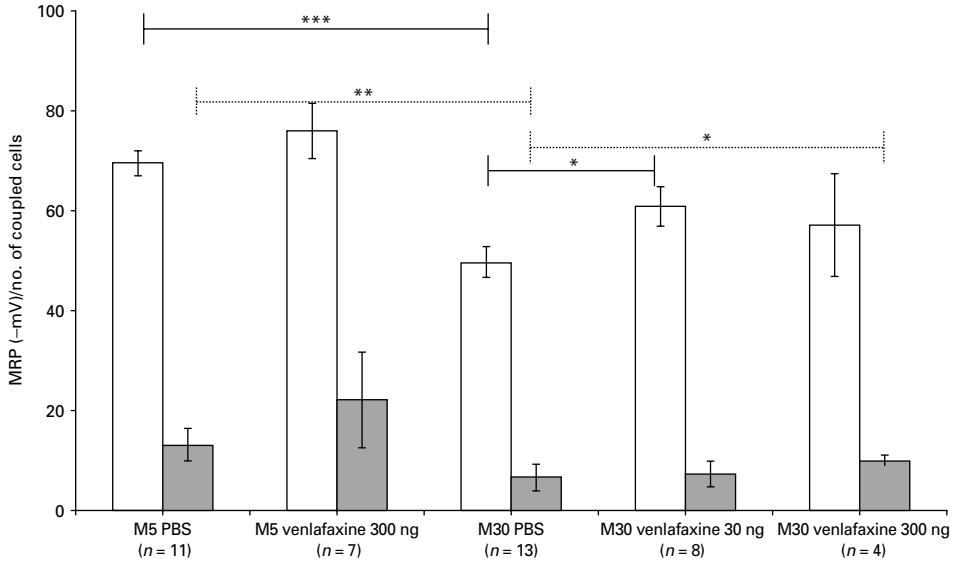

Figure 1. Reversal of membrane depolarization and augmentation of intercellular coupling by venlafaxine under inflammatory (M30) and non-inflammatory (M5) conditions. Cultures were incubated for 16 h with the indicated concentration of venlafaxine or vehicle (PBS). Each bar represents the mean number of coupled cells (■) or mean membrane resting potential (□) ± S.E.M. of single cells which have been investigated with patch-clamp technique and dye microinjection. Data are from at least four different experiments. Significant differences are indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Significant effects regarding the mean number of coupled astroglia were tested with the Mann–Whitney *U* test and differences between mean astroglial MRPs with the *t* test. Significant differences between mean fractions of active and resting microglia were tested with the *t* test. All statistical analyses were performed with SPSS, version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

MRP of astrocytes and intercellular coupling

In M5 (5%) cultures incubated with PBS mean MRPs of −69.56 mV (S.E.M. = 2.46) which is in the range of the normal astrocytic MRP were detected (Figure 1). Increase of the microglia fraction to 30% caused a significant depolarization ($t = 4.94$, $p < 0.001$) of the MRP (mean = –49.6 mV, S.E.M. = 3.07) compared to M5. Incubation of 30 ng/ml venlafaxine with M30 prevented depolarization ($t = -2.28$, $p < 0.05$) of the membrane potential of astrocytes (mean = −60.96 mV, S.E.M. = 3.94) significantly. Venlafaxine 300 ng/ml revealed slightly lower but non-significant MRPs (mean = −57.15, S.E.M. = 10.38).

For functional intercellular coupling (Figure 1), we found a significant reduction ($Z = -2.82$, $p < 0.01$) of the number of coupled cells comparing M5 PBS incubated cultures (mean = 13.18, S.E.M. = 3.36) with M30 cultures (mean = 6.69, S.E.M. = 2.72). The incubation of M30 with 300 ng/ml venlafaxine could almost restore ($Z = -2.05$, $p < 0.05$) the number of coupled cells (mean = 10, S.E.M. = 1.08).

Cytokine concentrations

M5 cultures incubated with PBS revealed a significantly lower concentration of pro-inflammatory (IL-6, IFN-γ) cytokines and a higher concentration of anti-inflammatory cytokines (IL-10, TGF-β) compared to the M30 cultures (Figure 2). Markedly, the incubation of M30 cultures with venlafaxine significantly altered cytokine concentrations dose-dependently through elevation of TGF-β and suppression of IL-6 and IFN-γ secretion. The incubation of 30 ng/ml venlafaxine significantly ($Z = -2.94$, $p < 0.01$) elicited a decrease of the mean IL-6 concentration from 185.71 pg/ml (S.E.M. = 31.56) to 116.6 pg/ml (S.E.M. = 11.58), whereas the effect was not significant for 300 ng/ml. By incubating M30 cultures with 30 ng/ml venlafaxine we found significantly higher ($t = -2.19$, $p < 0.05$) mean TGF-β concentrations under inflammatory conditions (M30 PBS = 14.46 pg/ml, S.E.M. = 3.18) compared to M30 venlafaxine-treated cultures (30 ng/ml = 23.1 pg/ml, S.E.M. = 1.87). For IFN-γ, the incubation with 300 ng/ml venlafaxine could reduce

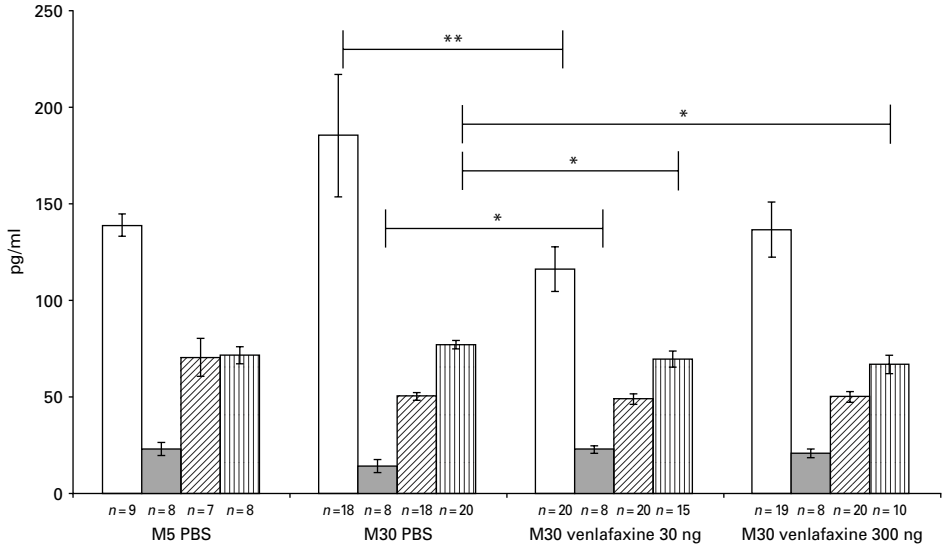

Figure 2. Reduction of pro-inflammatory and augmentation of anti-inflammatory cytokine concentrations by venlafaxine under M5 and M30 conditions. Each bar represents the mean ± S.E.M. concentration of the respective cytokine in separate cell supernatants after 16 h of incubation. Data are from at least four different experiments and were measured in duplicate. Significant differences are indicated (* $p<0.05$, ** $p<0.01$). □, IL-6; ■, TGF-β; ▨, IL-10; ▥, IFN-γ.

($t=-1.97$, $p<0.05$) mean concentration from 77.3 pg/ml (S.E.M. = 4.17) for M30 PBS to 67 pg/ml (S.E.M. = 4.5). For IL-10 concentrations, we did not find significant effects for venlafaxine; M5 PBS incubated cultures revealed significantly higher mean concentrations than all other conditions.

Change in microglial phenotype

While the RRT dominates in cultures with a low number of microglia (mean M5 = 37.6%, S.E.M. = 7.44; mean M30 = 11.73%, S.E.M. = 2.87; $t=3.166$, $p<0.01$) (Figure 3), the activated RPT dominates in cultures of M30. In M5 cultures, 29.07% (S.E.M. = 4.76) of the cells were activated whereas 61.58% (S.E.M. = 3.28) RPTs were found in M30 ($t=-5.551$, $p<0.001$). The incubation of venlafaxine with M30 co-cultures for 16 h resulted in a profound deactivation of microglia, comparable to the conditions observed in M5 conditions. When M30 co-cultures were treated with 30 ng/ml venlafaxine a ratio of 43.55% (S.E.M. = 8.07) RRT microglial cells and 34.80% (S.E.M. = 4.34) activated RPTs was determined. The differences reached high significance (RRT: $t=-4.59$, $p<0.001$; RPT: $t=4.80$, $p<0.001$). The addition of 300 ng/ml venlafaxine could also restore the activation pattern found in M5 cultures. Under incubation only 20.40% (S.E.M. = 2.23) of microglia were active ($t=9.65$, $p<0.001$) whereas 47.18% (S.E.M. = 4.16) were RRT ($t=-7.25$, $p<0.001$).

Discussion

Our results for M5 and M30 co-cultures are in good accord with previous findings described by Faustmann et al. (2003) and Hinkerohe et al. (2005). They recently provided evidence that an increase of microglia cells to about 30% elicits a significant activation compared to cultures obtained with 5% microglia. This phenotypic activation was accompanied by a decrease of astroglial MRP and reduced intercellular coupling indicating a clear-cut correlation between microglia fraction and basic astrocytic properties.

In this investigation, we yielded stringent evidence that the SNRI venlafaxine reversed the inflammatory conditions of M30 cultures in a dose-dependent fashion. Incubation of M30 cultures with venlafaxine was capable of preventing microglial activation, strengthening the astroglial coupling and minimizing pro-inflammatory cytokine secretion. Markedly, the lower dose (30 ng/ml) was more effective on changes of astrocytic properties compared to the higher dose application. Incubation with the higher dose (300 ng/ml) equals a treatment in humans of ~150 mg/d when considering plasma and cerebrum levels (Uhr et al., 2003) of venlafaxine-treated mice. When assuming a linear dose–serum concentration dependency (Reis et al., 2002), a human dose of 15 mg/d can be approximated for the lower concentration used in the present study.

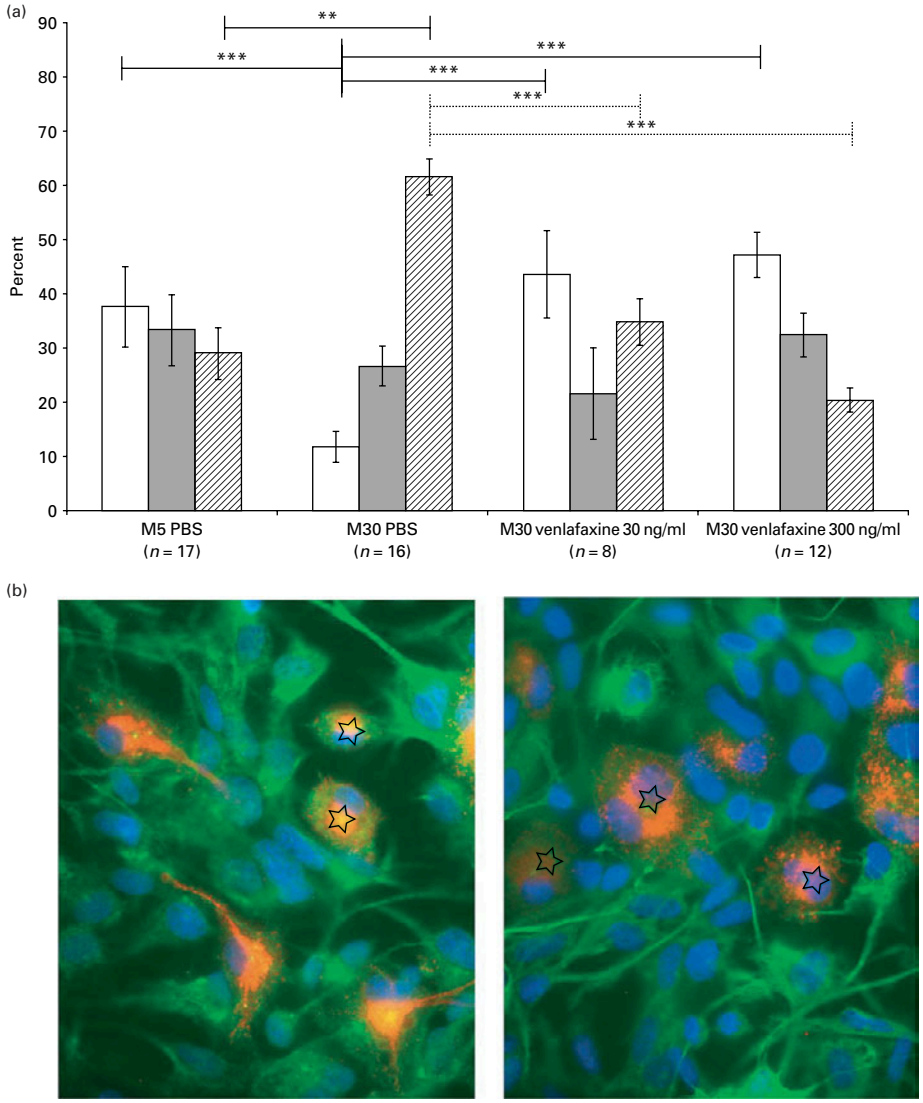

Figure 3. Change of microglial phenotype from activated to resting morphology by incubation with venlafaxine. Each bar (a) represents the mean percentage ± S.E.M. of resting (□), intermediate (■) or active (▨) microglial cells in the co-culture after 16 h of incubation with indicated substance concentration or vehicle. Data are from at least four different experiments. Significant differences are indicated (** $p < 0.01$, *** $p < 0.001$). The left image (b) displays astrocytes (green) and mainly resting ramified microglial cells (red, indicated by a star) whereas microglial cells in the right image largely constitute the round phagocytic phenotype (both ×63 magnification).

These results correlate with some previous findings on the neuroprotective effects of venlafaxine (Xu et al., 2003). Those authors described venlafaxine's effects on rats' hippocampal BDNF and reported an increase in BDNF-positive pyramidal neurons after chronic treatment with 5 mg/kg.d. The higher dose (10 mg/kg) decreased the intensity of BDNF immunostaining in all subareas.

Present results strongly promote the concept of anti-inflammatory properties of venlafaxine in vitro and may put a new complexion on the relationship between neuroinflammatory and other pathogenic CNS processes. Markedly, the substance exerts effects on astrocytes which recently emerged as potential targets for inflammatory CNS diseases (Ransom et al., 2003). Monoamine transporters (Inazu et al., 2003) as well as adrenergic receptors (Hertz et al., 2004) which have been identified on astrocytes might play a key role in mediating anti-inflammatory effects by antidepressants.

Cytokines which have been found to play a key role in the pathogenesis of inflammatory CNS diseases were greatly impacted by venlafaxine. For instance, IL-6 which is released in the CNS during various pathological conditions, including Alzheimer's disease, MS, CNS trauma, and viral and bacterial meningitis (Gruol and Nelson, 1997) was reduced by venlafaxine. Moreover, IFN-γ which was significantly lowered by venlafaxine is a suspected participant in the pathogenesis of MS (Becher et al., 1999). The augmentation of the anti-inflammatory cytokine TGF-β by venlafaxine strengthens the immunoregulatory effects since TGF-β was shown in rodents to prevent the development and/or exacerbation of disease symptoms in EAE (Johns et al., 1991). Besides the neuroimmunological involvement of cytokines, considerable clinical and experimental data support the existence of a relationship between cytokines and depression (Levine et al., 1999).

Interestingly, the well-established tricyclic antidepressant amitriptyline was recently found to inhibit the secretion of pro-inflammatory cytokines in rat mixed glial and microglial cell cultures (Obuchowitz et al., 2006). Similarly to venlafaxine, amitriptyline inhibits 5-HT and NE reuptake but acts in a more unspecific way, supporting the thesis of a noradrenergic- and serotonergic-mediated immunoregulation.

In conclusion, antidepressants targeting 5-HT and NE transporters seem to suppress inflammatory processes in vitro. Further studies in animal models (e.g. EAE) must be conducted to demonstrate antidepressants as a putative treatment option for inflammatory CNS pathologies.

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State of Interest

None.

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Appendix III

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Active Immunization with Amyloid-β 1–42 Impairs Memory Performance through TLR2/4-Dependent Activation of the Innate Immune System

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Active immunization with amyloid-β (Aβ) peptide 1–42 reverses amyloid plaque deposition in the CNS of patients with Alzheimer's disease and in amyloid precursor protein transgenic mice. However, this treatment may also cause severe, life-threatening meningoencephalitis. Physiological responses to immunization with $A\beta_{1-42}$ are poorly understood. In this study, we characterized cognitive and immunological consequences of $A\beta_{1-42}$/CFA immunization in C57BL/6 mice. In contrast to mice immunized with myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$/CFA or CFA alone, $A\beta_{1-42}$/CFA immunization resulted in impaired exploratory activity, habituation learning, and spatial-learning abilities in the open field. As morphological substrate of this neurocognitive phenotype, we identified a disseminated, nonfocal immune cell infiltrate in the CNS of $A\beta_{1-42}$/CFA-immunized animals. In contrast to MOG_{35-55}/CFA and PBS/CFA controls, the majority of infiltrating cells in $A\beta_{1-42}$/CFA-immunized mice were $CD11b^+CD14^+$ and $CD45^{high}$, indicating their blood-borne monocyte/macrophage origin. Immunization with $A\beta_{1-42}$/CFA was significantly more potent than immunization with MOG_{35-55}/CFA or CFA alone in activating macrophages in the secondary lymphoid compartment and peripheral tissues. Studies with TLR2/4-deficient mice revealed that the TLR2/4 pathway mediated the $A\beta_{1-42}$-dependent proinflammatory cytokine release from cells of the innate immune system. In line with this, TLR2/4 knockout mice were protected from cognitive impairment upon immunization with $A\beta_{1-42}$/CFA. Thus, this study identifies adjuvant effects of $A\beta_{1-42}$, which result in a clinically relevant neurocognitive phenotype highlighting potential risks of Aβ immunotherapy. ***The Journal of Immunology*****, 2010, 185: 000–000.**

Alzheimer's disease (AD) is the most common neurodegenerative disorder characterized by compact extracellular plaques, which are largely composed of amyloid-β (Aβ) peptide. Several therapeutic strategies have been developed to eliminate or reduce Aβ deposits within the CNS. Active immunization in which Aβ peptide is combined with an adjuvant to stimulate an Ab response against Aβ was shown to lower brain amyloid burden and partially restore behavioral deficits in animal models (1, 2). These promising observations led to a clinical trial with active immunization using synthetic $A\beta_{1-42}$. However, the trial was discontinued due to the occurrence of meningoencephalitis in 6% of cases without a clear correlation to the strength of the anti-Aβ Ab response in these patients (3–5). Furthermore, immunotherapeutic approaches in animal models of AD provided controversial results with respect to cognitive improvements. In amyloid precursor protein (APP) transgenic mice, behavioral tasks were not improved postimmunization (p.i.) with Aβ (6), whereas in another study, spatial memory was recovered in a small subset of immunized mice with very low hippocampal Aβ levels (7).

More recent data indicate that immunization with Aβ peptide provokes activation of microglia, which initiates inflammation within the CNS (8). Whereas microglia seems to play a key role in Aβ clearance, it remains to be determined whether microglial cells also play an active proinflammatory role when exposed to Aβ. Indeed, activated microglial cells located in close proximity to Aβ plaques are found in patients with AD (9) and transgenic mouse models of AD (10). Moreover, it has been shown that Aβ peptide itself provokes activation of microglia, stimulating critical signaling responses that lead to increased IL-6 production, inducing the death of cultured neurons (11). In addition, Aβ peptide was shown to enhance the action of TLR2 and 4 agonists in primary mouse microglial cells (12), suggesting a role of the TLRs in mediating $A\beta_{1-42}$-driven inflammatory responses.

Whereas the majority of studies on immune interventions targeting Aβ were conducted in APP transgenic mice with conditional overexpression of APP in the CNS, little is known about the systemic and organ specific inflammatory responses upon immunization with Aβ in nontransgenic animals. In this study, we

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The online version of this article contains supplemental material.

Abbreviations used in this paper: Aβ, amyloid β; AD, Alzheimer's disease; APP, amyloid precursor protein; BL, baseline; EAE, experimental autoimmune encephalomyelitis; GFAP, glial fibrillary acidic protein; KO, knockout; MOG, myelin oligodendrocyte glycoprotein; PAMP, pathogen-associated molecular pattern; p.i., postimmunization; PTX, pertussis toxin.

aimed at characterizing: 1) the cognitive profile and the histopathological manifestation of wild-type mice challenged with $A\beta_{1-42}$ immunization; 2) distinguishing the inflammatory response in $A\beta_{1-42}$-immunized animals from classical experimental autoimmune encephalomyelitis (EAE); and 3) unraveling the immunological mechanisms behind the inflammatory processes in $A\beta_{1-42}$-immunized mice in the systemic compartment and within the CNS.

Materials and Methods

Mice

Female C57BL/6 mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and were used in experimental paradigms at the age of 6–8 wk. TLR2/4-deficient mice on the C57BL/6 background were provided by C. Kirschning (Institute of Medical Microbiology, Technische Universität Munich, Munich, Germany). All procedures were conducted in compliance with the local guidelines for animal experimentation.

Immunization procedures

Animals were immunized s.c. with 100 μg/animal human $A\beta_{1-42}$ peptide (American Peptide Company, Sunnyvale, CA; EZBiolab, Carmel, CA) emulsified in CFA containing 5 mg/ml *Mycobacterium tuberculosis* extract (strain H37Ra, DIFCO Laboratories, Detroit, MI). EAE induction was performed by s.c. injection of 100 μg/animal of myelin oligodendrocyte glycoprotein (MOG) peptide 35–55 (Jerini, Berlin, Germany) emulsified in CFA. Control animals received CFA with PBS. On days 0 and 2, all animals were injected with 500 ng/animal pertussis toxin (PTX; Sigma-Aldrich, Munich, Germany) i.p.

Behavioral tests

Open field. For evaluation of habituation and visuospatial learning, the open field test was conducted as previously described (13, 14). Briefly, the open field was a square arena (30 × 30 × 40 cm) with clear Plexiglas walls and a grid square floor composed of nine equal quadrants. At the beginning of the test, mice were placed in the center of the open field and left to freely explore. The total number of quadrant borders the mice crossed and the number of rearings were counted by a blinded observer during a 10-min observation period. Baseline values were assessed prior to immunization.

According to O'Keefe and Nadel's cognitive map theory (15), exploration of a novel environment is used by the animal to construct a cognitive map, and activity wanes once such a map is established. Therefore, habituation to an open field is a measure of memory, and the faster a cognitive map is established, the sooner exploration activity will decrease. To asses a habituation learning measure (habituation learning index), the difference of crossed segments in the first and last 150 s of each 10-min observation period was determined (16). The open field test was repeated every 3 d.

Clinical signs of EAE were ranked with an established score from 0–5: 0 (normal); 1 (tail limpness), 2 (paraparesis with clumsy gait); 3 (hind limb paralysis); 4 (hind limb and forelimb paralysis); and 5 (death). All ratings were done by observers blinded to the treatment.

Visuospatial learning task. Visuospatial learning performance was tested in the open field paradigm with slight modifications from published protocols (17). For ethical reasons, the water maze paradigm was not applied, as some of the animals in the MOG_{35-55}/CFA-immunized control group developed severe pareses.

For 3 consecutive learning d, mice were placed into the open field in which two identical objects (bottles) in terms of height, color, shape, and surface texture were located. Spatial configuration did not change for three training sessions. On day 4, the bottle in the corner was moved to the opposite corner, leaving the configuration and distance of the objects undisturbed. The total exploration time for each object was determined during a 10-min observation period. Object exploration was defined as physical contact with the bottle by mouth, vibrissae, and forepaws. Compassing or sitting inactively next to the objects was not regarded as object exploration. For statistical evaluation, the initial exploration time for each stimulus in the first session was calculated, and the relative change in exploration time of the replaced stimulus in the fourth session was determined.

Macrophage depletion

For systemic depletion of macrophages, mice were given i.p. injections of clodronate liposomes according to established protocols (18). Briefly, mice received an initial dose of 100 mg/kg clodronate liposomes (kindly provided by R. Schwendener, Institute of Molecular Cancer Research, University of Zurich, Zurich, Switzerland) followed by subsequent injections of 50 mg/kg every fourth day. Control mice were injected with empty liposomes. Immunization with PBS/CFA or $A\beta_{1-42}$/CFA plus PTX was performed 3 d after the initial clodronate injection.

Serum Ab production

Serum Abs against human and murine $A\beta_{1-42}$ peptides were determined by ELISA according to established protocols (19). On day 28 p.i. with PBS/CFA or human $A\beta_{1-42}$/CFA, anti-$A\beta_{1-42}$ Abs in the sera of the animals were captured by solid-phase human or murine $A\beta_{1-42}$ followed by detection of mouse IgG with HRP-labeled goat anti-mouse IgG (AbD Serotec, Raleigh, NC).

Cell separation

Cells immunoreactive for CD11b and CD11b/CD11c were isolated from naive mouse spleen tissue by magnetic cell sorting with MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Purity of cells (>90%) was confirmed by FACS analysis.

Peritoneal macrophages

Primary macrophages were isolated from the peritoneal cavity of mice 12 d p.i. according to previously published protocols (20). For assessing cytokine production, these cells were cultured (2×10^6 cells/ml) in media (DMEM medium containing 10% FCS, nonessential amino acids, HEPES, L-glutamine, and antibiotics) for 48 h at 37°C in a humidified incubator at 5% CO_2. For gene expression studies, mRNA was isolated directly after harvesting the cells from the peritoneal cavity.

Isolation of mononuclear cells from the CNS

Mice were perfused with cold PBS through the left cardiac ventricle on day 10 p.i. The brain was dissected, and the spinal cord was flushed out by hydrostatic pressure. CNS tissue was cut into pieces and digested with 2.5 mg/ml Collagenase D (Roche Diagnostics, Indianapolis, IN) and 1 mg/ml DNAse I (Sigma-Aldrich) in DMEM medium at 37°C for 40 min. Single-cell suspensions were prepared using a 70-μm cell strainer followed by percoll gradient centrifugation (70/37%). Mononuclear cells were removed from the interphase, washed, and resuspended in culture medium.

Surface staining and flow cytometry

Mononuclear cells were stained for CD11b, CD14, and CD45 (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. Analyses were performed on a Dako CyAn flow cytometer system (DakoCytomation, Glostrup, Denmark). Flow cytometric data were analyzed with FlowJo (Tree Star, Ashland, OR).

Aβ peptide and cell stimulation

Lyophilized human $A\beta_{1-42}$ peptide (obtained from American Peptide Company or EZBiolab) was reconstituted with PBS at a concentration of 2 mg/ml. Dissolved peptide was stored at 4°C for up to 48 h. Where indicated, murine $A\beta_{1-42}$ (American Peptide Company) was used. In stimulation experiments, $CD11b^+$ and $CD11b^+CD11c^+$ cells (2×10^6 cells/ml) were stimulated with different concentrations of $A\beta_{1-42}$ peptide (0.1–50 μg/ml) or 100 ng/ml LPS (Sigma-Aldrich) for 48 h at 37°C in culture medium in a humidified incubator at 5% CO_2.

Cytokines

Cytokine levels were determined in sera and culture supernatants. Cell culture supernatants were collected after indicated incubation periods and stored at −80°C until analysis. Levels of IL-1β, IL-6, IFN-γ, and TNF were measured by commercial ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

RNA isolation and real-time PCR

Isolation of RNA (RNeasy, Quiagen, Hilden, Germany) from whole brain tissue and immune cells, its quantification, and the RT reactions (High-capacity RT Kit, Applied Biosystems, Foster City, CA) were performed according to established protocols. Expression of mRNA of target genes and the endogenous control gene GAPDH was assessed by real-time PCR (with TaqMan Gene Expression Assay products on StepOne Plus PCR System, Applied Biosystems) according to the manufacturer's recommendations. Expression levels for each gene of interest were calculated by normalizing the quantified mRNA amount to GAPDH. Relative gene expression was determined and used to test significance between different groups. The following gene expression assays (Applied Biosystems) were used: IL-1β (Mm00434228_m1),

IL-6 (Mm00446190_m1), CD14 (Mm00438094_g1), glial fibrillary acidic protein (GFAP; Mm01253033_m1), S100A8 (Mm00496696_g1), and TNF (Mm00443258_m1).

Histology

Mice were anesthesized with isoflurane and perfused with ice-cold PBS and 4% paraformaldehyde. Brains were dissected and embedded in paraffin. Immunohistochemistry was performed with a rat Ab against mouse Mac-3 (1:200; clone M3/84, BD Biosciences) as described previously (21, 22). Briefly, tissues were pretreated by microwaving in 10 mM citrate buffer (pH 6) for two cycles of 5 min each. Immunolabeling was detected by the avidin-peroxidase method and visualized with diaminobenzidine by incubation for 5 min. Control sections were incubated in the absence of primary Ab or with nonimmune sera. Slides were counterstained with hematoxylin and coverslipped.

Data analysis

For statistical comparisons, a one-way multiple-range ANOVA test for multiple comparisons was employed. Unpaired *t* tests were used for comparison of two groups. Values of $p < 0.05$ were considered significant. Graphs were generated using GraphPad Prism software (GraphPad, San Diego, CA).

Results

Immunization with $A\beta_{1-42}$ is associated with alterations of behavioral and cognitive performances

Because APP and its cleavage products, the Aβ peptides, are present in the normal CNS, we wished to investigate in more detail how immunotherapeutic approaches designed to remove Aβ deposits interfere with regular functions of the CNS. Active immunization with $A\beta_{1-42}$/CFA significantly altered the psychomotor and cognitive phenotype of mice in comparison with various control groups. Observations in the open field revealed pronounced deficits in three cognitive parameters: first, open field testing of $A\beta_{1-42}$/CFA-immunized mice showed a significant reduction of locomotion (Fig. 1*A*) as compared with MOG/CFA- or PBS/CFA-immunized animals. Changes in locomotion were detected as early as on day 10 p.i., and reduced locomotion in $A\beta_{1-42}$-immunized mice persisted over the entire observation period until day 28. Reduced rearing behavior was detected already on day 4 and persisted until day 18 (Fig. 1*B*). Second, we observed a significant decrease in habituation learning ability. Whereas control animals showed habituation to a persisting environment by reduction of exploration over time, $A\beta_{1-42}$/CFA-immunized mice had a significantly lower habituation learning index (Fig. 1*C*) from day 3 p.i. Even compared with MOG_{35-55}/CFA-immunized mice (EAE scores are shown in Fig. 1*D*), we found significant differences in $A\beta_{1-42}$- immunized mice on days 10, 17, and 28 p.i. Experiments in aged mice (12 mo old) revealed similar deficits in explorative behavior after $A\beta_{1-42}$/CFA immunization (Supplemental Fig. 1). Third, we found that mice immunized with $A\beta_{1-42}$/CFA developed profound deficits in visuospatial learning both in the acute (observation between days 9 and 14 p.i.) and chronic (observation between days 23 and 28 p.i.) phases of disease (Fig. 2*A*, 2*B*). As compared with controls, $A\beta_{1-42}$/CFA-immunized animals spent significantly less time to explore a novel stimulus in a known environment (reduced memory gain) both in the acute and chronic phases of disease. Together, these behavioral data indicate a profound and persistent decline in motivational and cognitive performance in $A\beta_{1-42}$/CFA-immunized animals.

$A\beta_{1-42}$ immunization results in macrophage infiltration and reactive astrogliosis in the CNS

In contrast to immunization with MOG_{35-55}/CFA or CFA only, immunization with $A\beta_{1-42}$ emulsified in CFA induced profound and persistent behavioral changes in wild-type animals. To investigate the potential immunological substrate of this behavioral phenotype, we performed immunohistochemical studies of CNS tissue specimens 18 d p.i. Immunohistochemistry revealed perivascular and subpial infiltrates of mononuclear cells in the brain and brainstem of $A\beta_{1-42}$/CFA-immunized mice (Fig. 3*B*), but not

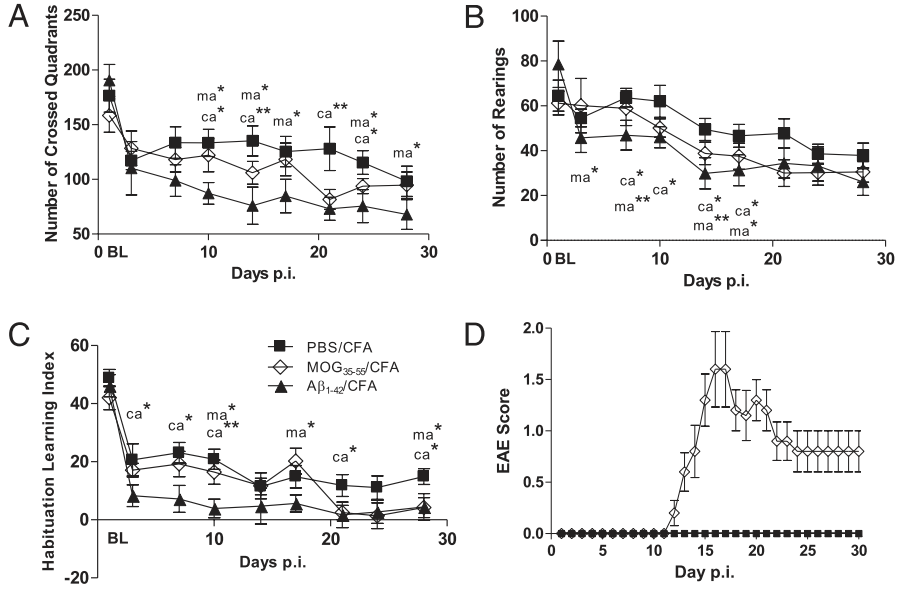

FIGURE 1. Active immunization with $A\beta_{1-42}$ impairs psychomotor functioning and habituation learning in the open field. Groups of female C57BL/6 mice ($n = 10$/group) were immunized with PBS/CFA, MOG_{35-55}/CFA, or $A\beta_{1-42}$/CFA plus PTX and evaluated for locomotion (*A*) and explorative behavior (*B*) as measured by the number of crossed quadrants and rearing events at different time points p.i. Habituation learning was assessed in a setting that tested the habituation to visuospatial cues (*C*; for habituation learning index, see *Materials and Methods*). Mean performances and SEM are illustrated for each group preimmunization and p.i. At least three independent experiments were performed. *D*, EAE scores illustrating that paralytic disease in the MOG_{35-55}/CFA group started around day 11, but did not mar the specific readout parameters of the open field tests. Statistical comparisons are based on the relative change to baseline performance. "ma" and "ca" denote significant differences between the MOG_{35-55}/CFA versus $A\beta_{1-42}$/CFA and PBS/CFA versus $A\beta_{1-42}$/CFA groups, respectively. $*p < 0.05$; $**p < 0.01$.

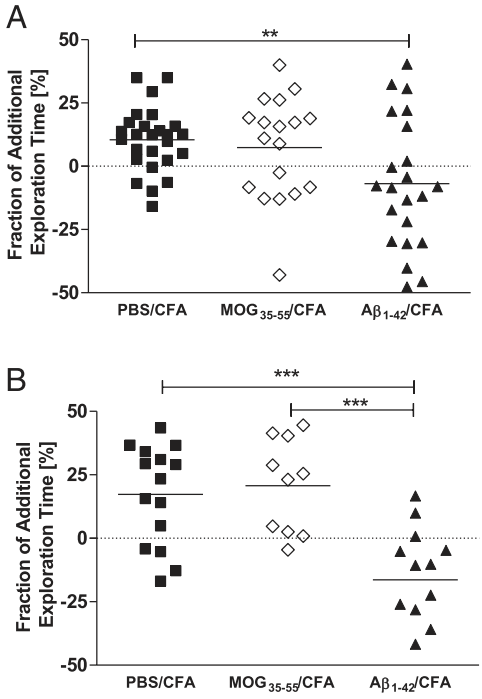

FIGURE 2. Active immunization with $A\beta_{1–42}$ impairs performance in a visuospatial object recognition task in the open field. Groups of female C57BL/6 mice were immunized with PBS/CFA, $MOG_{35–55}$/CFA, or $A\beta_{1–42}$/CFA plus PTX and evaluated in a visuospatial object recognition paradigm in the acute phase (*A*, acquisition period between days 9 and 14 p.i.) and the chronic phase of disease (*B*, acquisition period between days 23 and 28 p.i.). The fraction of additional exploration time of the novel stimulus in a habituated environment is illustrated for each individual mouse. Dashed lines indicate mean performances of individual groups. Results were replicated at least three times. Note that in contrast to $A\beta_{1–42}$/CFA immunization, immunization with $MOG_{35–55}$/CFA induced paralytic disease, but did not affect visuospatial memory performance. $^{**}p < 0.01$; $^{***}p < 0.001$.

in PBS/CFA controls (Fig. 3*A*). These infiltrates in the hippocampal region mainly consisted of macrophages as shown by MAC-3 staining with few $CD3^+$ T cells. Infiltrates in $A\beta_{1–42}$/CFA-immunized mice (Fig. 3*D*) were disseminated and nonfocal, whereas $MOG_{35–55}$/CFA controls showed focal meningeal and perivascular inflammatory infiltrates (Fig. 3*C*). We wondered whether the cellular infiltrate in $MOG_{35–55}$/CFA- versus $A\beta_{1–42}$/CFA-immunized mice was also quantitatively different. To quantify various immune cell populations in the CNS of immunized mice, we isolated mononuclear cells from the CNS and performed flow cytometric analysis. Because behavioral differences between the groups of $MOG_{35–55}$/CFA-versus $A\beta_{1–42}$/CFA-immunized animals were evident as early as 10 d p.i., whereas animals in the $MOG_{35–55}$/CFA group did not yet show signs of paralytic disease at this time point, we chose to perform quantitative analyses of CNS infiltrates on day 10 p.i. In PBS/CFA-immunized control animals, most of the CNS-derived $CD11b^+$ cells were $CD45^{low}$, indicating their microglial origin (Fig. 4*A*, 4*D*, 4*G*). Whereas in $MOG_{35–55}$/CFA-immunized mice, T cells (CD11b-$CD45^{high}$) were already starting to accumulate in the CNS on day 10 p.i., the majority of $CD11b^+$ cells were still $CD45^{low}$, again suggesting their microglial origin (Fig. 4*B*, 4*E*, 4*G*). In contrast, the majority of $CD11b^+$ cells isolated from the CNS of $A\beta_{1–42}$/CFA-immunized mice were $CD45^{high}$, indicating that these $CD11b^+$ cells were macrophages that had invaded the CNS as early as on day 10 p.i. (Fig. 4*C*, 4*F*, 4*G*). Moreover, the fraction of $CD14^+$ cells within the population of $CD11b^+CD45^{high}$ macrophages in the CNS was significantly higher in $A\beta_{1–42}$/CFA-immunized mice than in either control group (Fig. 4*H*, 4*I*). Together, these data indicate that $A\beta_{1–42}$/CFA immunization leads to early and massive recruitment of blood-borne macrophages into the CNS.

Consistent with the immunohistochemical and flow cytometric analyses, the expression of macrophage-associated genes, such as S100A8 and CD14 (Fig. 5*A*, 5*B*), was upregulated in whole brain tissue of $A\beta_{1–42}$/CFA-immunized animals compared with PBS/CFA and $MOG_{35–55}$/CFA controls. Quantitative RT-PCR from whole brain tissue isolated from $A\beta_{1–42}$/CFA-immunized mice demonstrated a 10-fold higher expression of S100A8 as compared PBS/CFA-immunized animals and a 3-fold higher expression as compared with $MOG_{35–55}$/CFA-immunized mice (Fig. 5*A*). CD14 expression was ~2-fold increased as compared with either control group (Fig. 5*B*). When comparing the CNS parenchyma between

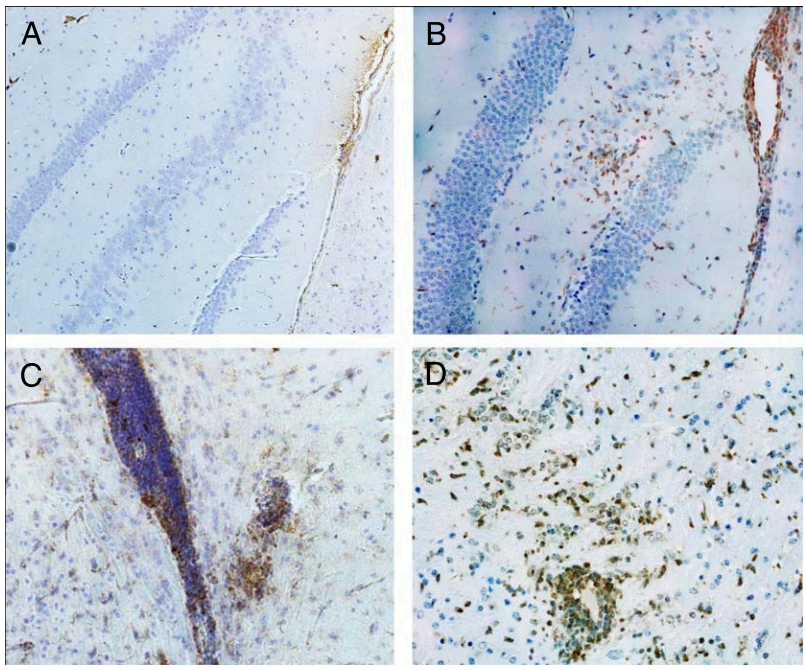

FIGURE 3. Disseminated, nonfocal immune cell infiltration in the CNS of $A\beta_{1–42}$/CFA-immunized mice. Mice were immunized with PBS/CFA, $MOG_{35–55}$/CFA, or $A\beta_{1–42}$/CFA plus PTX and prepared for histopathological analysis 18 d p.i. Representative MAC-3 immunostainings (original magnification ×20) of coronar sections from the hippocampus region prepared from PBS/CFA (*A*) and $A\beta_{1–42}$/CFA-immunized (*B*) mice are shown. Vessels with perivascular immune cell infiltrates (original magnification ×40) located in the cerebrum of $MOG_{35–55}$/CFA (*C*), and $A\beta_{1–42}$/CFA-immunized (*D*) mice are illustrated. Note that in $A\beta_{1–42}$/CFA-immunized mice, nonfocal infiltrates of macrophages are widely distributed within the hippocampus region and other regions of the cerebrum. PBS/CFA-immunized mice are largely devoid of infiltrating cells, whereas $MOG_{35–55}$/CFA-immunized animals showed EAE-typical focal meningeal and perivascular infiltrates.

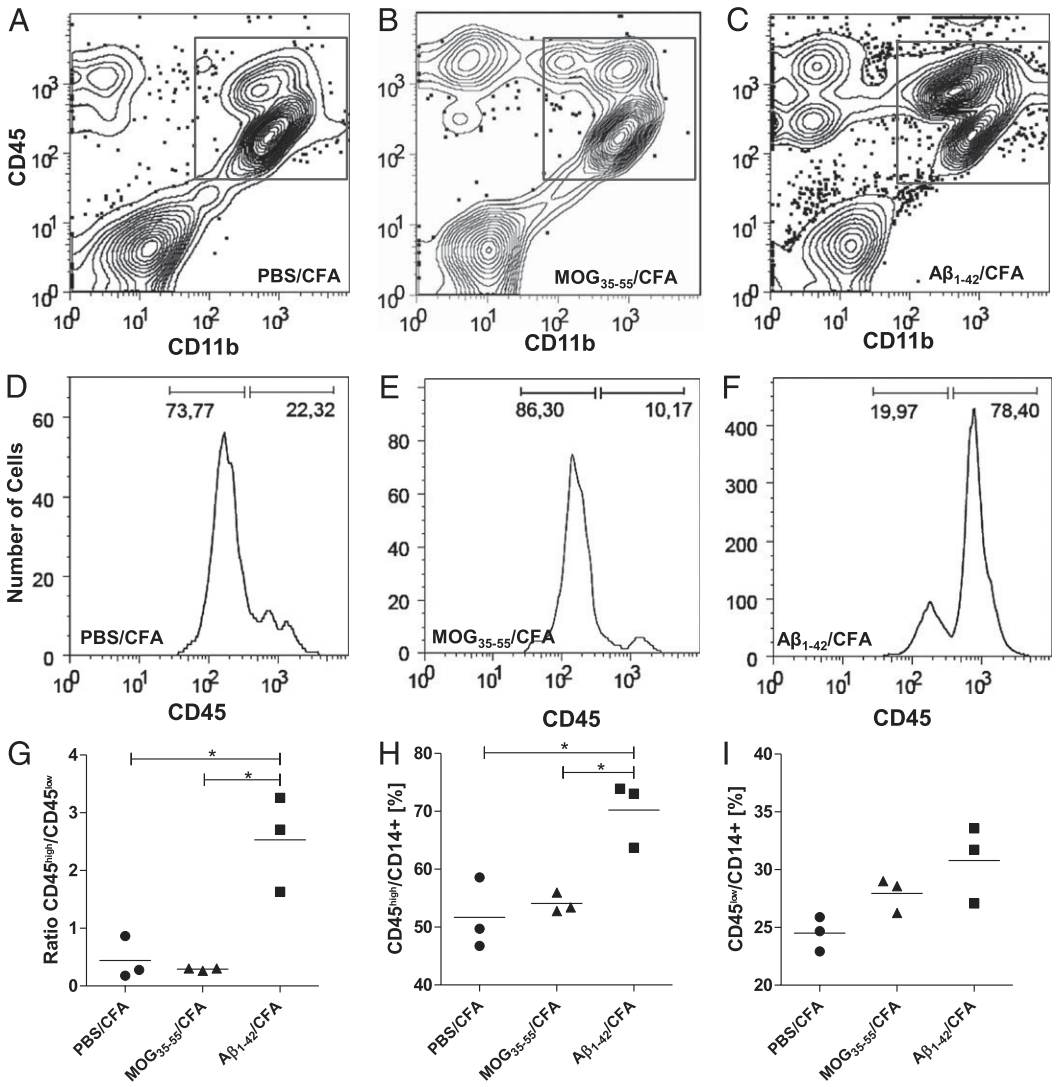

FIGURE 4. Infiltrating cells in $A\beta_{1-42}$/CFA-immunized animals are primarily of blood-borne macrophage origin. Mononuclear cells were isolated from the CNS of PBS/CFA, MOG_{35-55}/CFA, and $A\beta_{1-42}$/CFA-immunized animals on day 10 p.i. and analyzed by flow cytometry. Representative cytograms (*A–C*) and histograms (*D–F*) are shown to illustrate the fractions of $CD11b^+CD45^{low}$ cells (microglia) and $CD11b^+CD45^{high}$ cells (macrophages) in PBS/CFA, MOG_{35-55}/CFA, and $A\beta_{1-42}$/CFA-immunized animals, respectively. *G*, Ratio of macrophages versus microglial cells in the CNS of the three experimental groups. One symbol represents one animal. Note that in contrast to PBS/CFA and MOG_{35-55}/CFA-immunized controls, the majority of cells isolated from the CNS of $A\beta_{1-42}$/CFA-immunized mice on day 10 p.i. were $CD11b^+CD14^+CD45^{high}$ cells (*H*, *I*), indicating their macrophage origin. $*p < 0.05$ as determined by Mann-Whitney *U* test.

the groups at late stages of the disease (4 wk p.i.), we found prominent signs of astrogliosis in the $A\beta_{1-42}$/CFA-immunized mice as determined by a disproportionate upregulation of GFAP mRNA expression in $A\beta_{1-42}$/CFA-immunized animals (Fig. 5*C*).

Immunization with $A\beta_{1-42}$ induces systemic release of proinflammatory cytokines by activated macrophages

Cognitive changes in $A\beta_{1-42}$/CFA-immunized mice developed in the absence of focal neurologic symptoms. We wondered whether systemic and CNS specific release of inflammatory cytokines induced by immunization with $A\beta_{1-42}$/CFA was responsible for the neurocognitive phenotype. To test this hypothesis, we determined the level of TNF in the sera of mice immunized with $A\beta_{1-42}$/CFA. On day 15 p.i., serum TNF was significantly increased in $A\beta_{1-42}$/CFA-immunized mice as compared with control groups (Fig. 6*A*). To identify possible cellular sources of TNF, we measured both the expression and the production of TNF and IL-6 in peritoneal macrophages of the various experimental groups. Consistent with the elevated serum concentration of TNF, peritoneal macrophages isolated from $A\beta_{1-42}$/CFA-immunized mice showed a higher expression TNF, IL-6, CD14, and S100A8 on a per-cell basis and higher secretion of TNF and IL-6 into the culture supernatant than macrophages that were isolated from MOG/CFA- or CFA only-immunized mice (Fig. 6*B*, 6*C*). Taken together, these data suggest that immunization with $A\beta_{1-42}$/CFA induces an exaggerated systemic inflammatory response by activating cells of the innate immune system.

To assess the functional relevance of systemic activation of macrophages and their recruitment to the CNS for the neurocognitive phenotype of $A\beta_{1-42}$/CFA-immunized mice, we performed macrophage-depletion experiments. Clodronate liposomes were used to deplete macrophages in the secondary lymphoid tissues prior to immunization with $A\beta_{1-42}$/CFA or PBS/CFA. The depletion of macrophages by clodronate liposomes prevented $A\beta_{1-42}$-induced effects on psychomotor behavior (Fig. 7). In contrast to control mice receiving empty liposomes, macrophage-

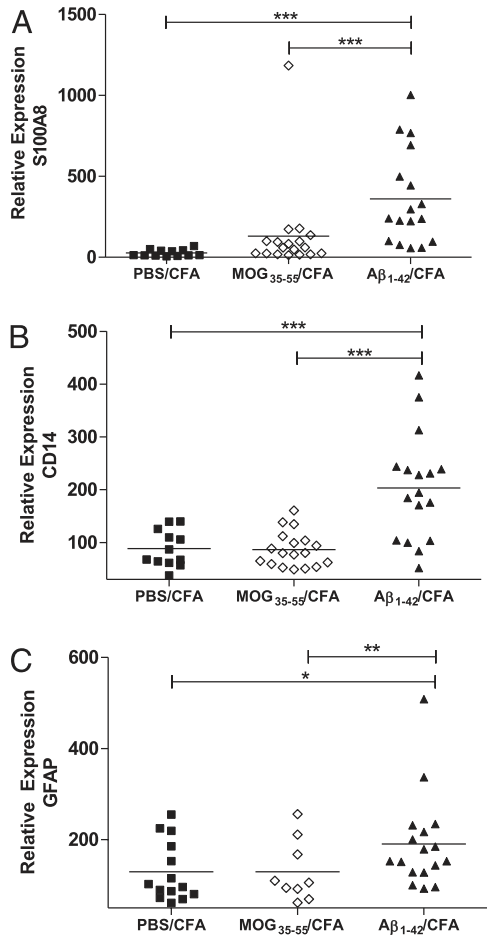

FIGURE 5. $A\beta_{1–42}$/CFA immunization induces mRNA expression of genes related to macrophage and glia cell activation. The expression of macrophage-associated genes was quantified by RT-PCR in whole CNS tissue. Expression of S100A8 (*A*) and CD14 (*B*) was significantly higher in $A\beta_{1–42}$/CFA-immunized mice compared with controls. Increased astrogliosis was confirmed in $A\beta_{1–42}$/CFA-immunized mice by quantitative GFAP expression analysis in whole brain tissue 4 wk p.i. (*C*). Numbers represent the relative gene expression for individual animals as determined by RT-PCR. Dashes indicate mean relative gene expression of individual groups. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

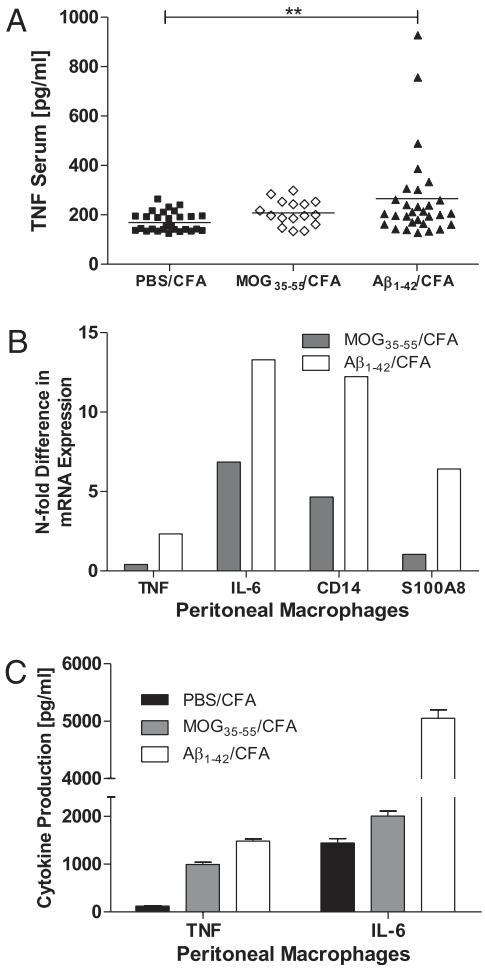

FIGURE 6. $A\beta_{1–42}$/CFA immunization induces systemic inflammation. *A*, Serum TNF levels were measured in groups of mice immunized with PBS/CFA, $MOG_{35–55}$/PBS, or $A\beta_{1–42}$/CFA 15 d p.i. Cytokine levels of single animals are depicted; dashes indicate mean concentrations. *B* and *C*, Peritoneal macrophages were isolated from PBS/CFA, $MOG_{35–55}$/CFA, or $A\beta_{1–42}$/CFA-immunized mice and tested for gene expression by quantitative RT-PCR directly ex vivo. The *n*-fold difference in gene expression of macrophages from $A\beta_{1–42}$/CFA- and $MOG_{35–55}$/CFA-immunized mice relative to the PBS/CFA group is shown (*B*). To confirm the mRNA data on the protein level, peritoneal macrophages were isolated from the individual groups of mice and cultured without further stimulation for 48 h. Secretion of IL-6 and TNF in the culture supernatant was measured by ELISA (*C*). Mean cytokine concentrations plus SD are shown. Similar results were obtained in three independent experiments. $**p < 0.01$.

depleted animals did not show impaired locomotion (Fig. 7*A*), rearing (Fig. 7*B*), and habituation performance (Fig. 7*C*) as a result of challenge with $A\beta_{1–42}$/CFA, suggesting that peripheral macrophages are crucial effector cells in inducing the clinical phenotype of $A\beta_{1–42}$/CFA-challenged animals. Accordingly, resident microglial cells ($CD11b^+CD45^{low}$) and not blood-borne macrophages ($CD11b^+CD45^{high}$) constituted the majority of $CD11b^+$ cells in the CNS of macrophage-depleted $A\beta_{1–42}$/CFA-immunized mice (Supplemental Fig. 3). Thus, clodronate administration was able to abrogate both the behavioral and the immunopathological phenotype in $A\beta_{1–42}$/CFA-immunized mice.

The stimulatory effects of $A\beta_{1–42}$ in macrophages and dendritic cells are TLR2/4 dependent

Because we observed a profound activation of the innate immune system p.i. with $A\beta_{1–42}$, we investigated the stimulatory properties of Aβ peptide in vitro and tested the relevance of specific TLR systems that have been implicated with immunostimulatory effects of Aβ peptide in previous studies. It has been reported that the activation of microglial cells by Aβ peptide requires both TLR2 and TLR4 pathways to activate intracellular signaling (23). In this study, stimulatory effects of $A\beta_{1–42}$ on $CD11b^+$ macrophages and $CD11b^+CD11c^+$ dendritic cells isolated from naive wild-type and TLR2/4-deficient mice were evaluated in vitro. $A\beta_{1–42}$ induced large amounts of IL-6 and TNF in macrophages (Fig. 8*A*, 8*B*) and IFN-γ in dendritic cells from wild-type mice in a dose-dependent manner (Fig. 8*C*). Similar effects were observed poststimulation with murine $A\beta_{1–42}$ peptide (Supplemental Fig. 2). In contrast, this effect was not detected in macrophages and dendritic cells derived from TLR2/4-deficient mice, suggesting that either TLR2 or TLR4 or the combined activation of these TLRs mediate the stimulatory effect of $A\beta_{1–42}$.

To corroborate whether activation of the TLR2/4 pathway by $A\beta_{1–42}$ was relevant in vivo, we immunized TLR2/4 knockout (KO) animals with $A\beta_{1–42}$/CFA. We consistently observed a differential disease-promoting effect of $A\beta_{1–42}$/CFA versus PBS/CFA treatment in wild-type animals. Indeed, we determined

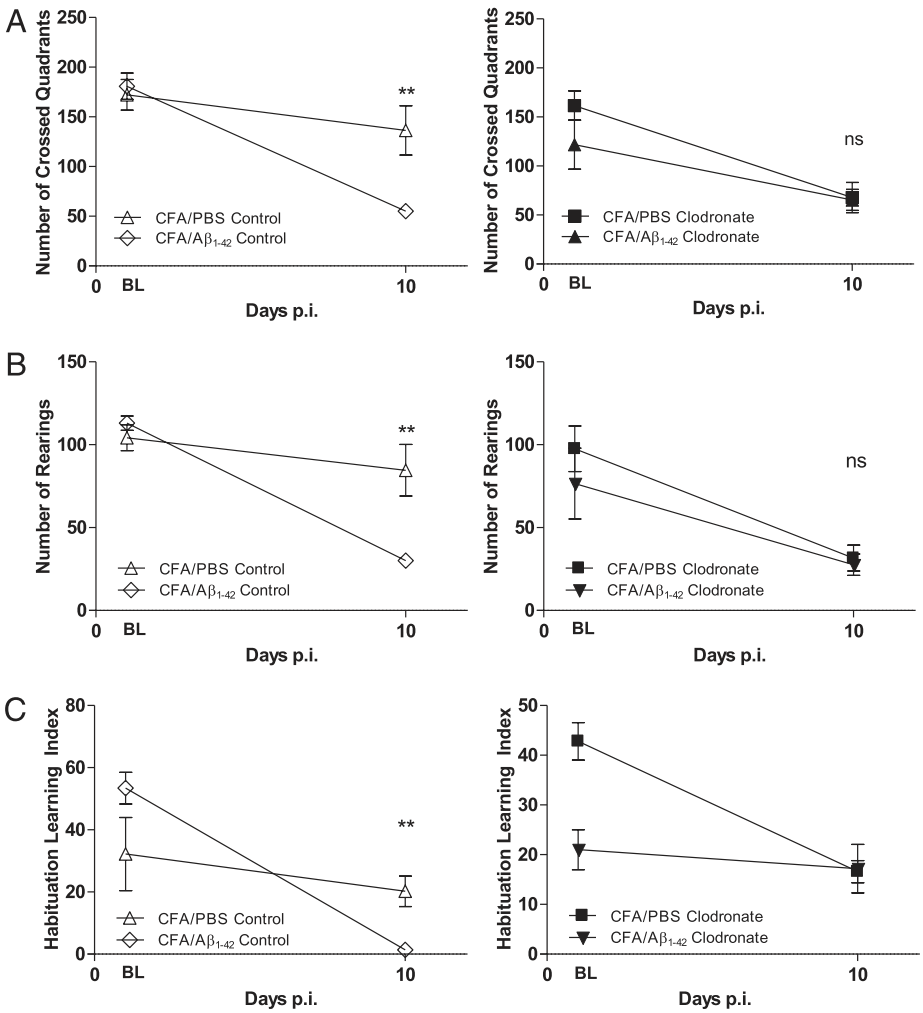

FIGURE 7. $A\beta_{1-42}$/CFA immunization does not affect the behavioral phenotype in macrophage-depleted mice. Groups of female wild-type mice ($n = 5$/group) were control treated or depleted of macrophages by clodronate as described in *Materials and Methods* followed by immunization with PBS/CFA or $A\beta_{1-42}$/CFA plus PTX. Mice were tested for locomotion (number of crossed quadrants in the open field) (*A*), rearing behavior (*B*), and habituation (*C*) preimmunization [2 d postdepletion, BL] and on day 10 p.i. The mean performances are summarized for both depletion and control groups after PBS/CFA or $A\beta_{1-42}$/CFA challenge. Statistical comparisons are based on the surplus effect of $A\beta_{1-42}$/CFA immunization as compared with PBS/CFA immunization within the respective treatment group. Note that the surplus effect of $A\beta_{1-42}$/CFA immunization was abrogated in the clodronate group. $^{**}p < 0.01$. BL, baseline.

a significant decrease in locomotion and rearing in wild-type C57BL/6 mice immunized with $A\beta_{1-42}$ as compared with immunization with CFA only (Fig. 9). In contrast, we did not find any additional neurocognitive phenotype (surplus effect) upon immunization with $A\beta_{1-42}$/CFA as compared with the CFA only condition in TLR2/4-deficient mice. When evaluating the surplus effect induced by $A\beta_{1-42}$/CFA immunization in wild-type animals versus TLR2/4 KO mice, the differences were significant as of day 4 p.i. regarding locomotion and as of day 8 with respect to the rearing behavior. Taken together, these data corroborate the critical involvement of the TLR2/4 pathways in the macrophage-induced behavioral changes following active immunization with $A\beta_{1-42}$ in vivo.

Discussion

In this study, we showed that active immunization with $A\beta_{1-42}$/CFA induced sustained cognitive and behavioral impairment in wild-type C57BL/6 mice. We identified a disseminated, nonfocal infiltrate of $CD11b^{+}CD14^{+}CD45^{high}$ cells in the CNS of $A\beta_{1-42}$/CFA-immunized mice. We propose that this infiltrate of activated macrophages represented the immunopathogenetic correlate of the neurocognitive phenotype in $A\beta_{1-42}$/CFA-immunized mice because challenge with $A\beta_{1-42}$/CFA failed to induce neurocognitive impairment in animals that had been depleted of macrophages. Furthermore, immunization with $A\beta_{1-42}$/CFA induced a systemic inflammatory response including the systemic release of cytokines. Peritoneal macrophages from $A\beta_{1-42}$/CFA-immunized animals were characterized by an increased activation state as compared with MOG_{35-55}/CFA-immunized mice, suggesting the capacity of Aβ peptide to activate the innate immune system in a manner reminiscent of pathogen-associated molecular patterns (PAMPs). Using TLR2/4-deficient mice, we showed that the TLR2/4 pathway mediated the $A\beta_{1-42}$-induced proinflammatory cytokine release from cells of the innate immune system. Accordingly, TLR2/4 KO mice were protected from cognitive impairment upon immunization with $A\beta_{1-42}$/CFA. We concluded that vaccination with $A\beta_{1-42}$/CFA lead to the activation of innate immune cells in the systemic and CNS compartments in a TLR-dependent manner. Thus, this study identified adjuvant effects of $A\beta_{1-42}$, which resulted in a clinically relevant and sustained neurocognitive phenotype.

Induction of EAE with myelin Ags emulsified in CFA is a widely used model to study autoimmune diseases of the CNS, such as multiple sclerosis. We used classical MOG_{35-55}/CFA-induced EAE as a control condition for our vaccination protocol with $A\beta_{1-42}$/CFA. MOG_{35-55}-induced EAE has been extensively investigated (24–27). In MOG_{35-55}/CFA-induced EAE, focal perivascular and parenchymal infiltrates of T cells and macrophages primarily in the spinal cord lead to demyelination and axonal damage resulting in paralytic disease. However, less is known about the neurocognitive status in MOG_{35-55}/CFA-immunized animals. A depression-like syndrome is reported, but cognitive alterations are rarely seen (28). In contrast, only a few days p.i. with $A\beta_{1-42}$/CFA, mice exhibited a significant decrease in their psychomotor

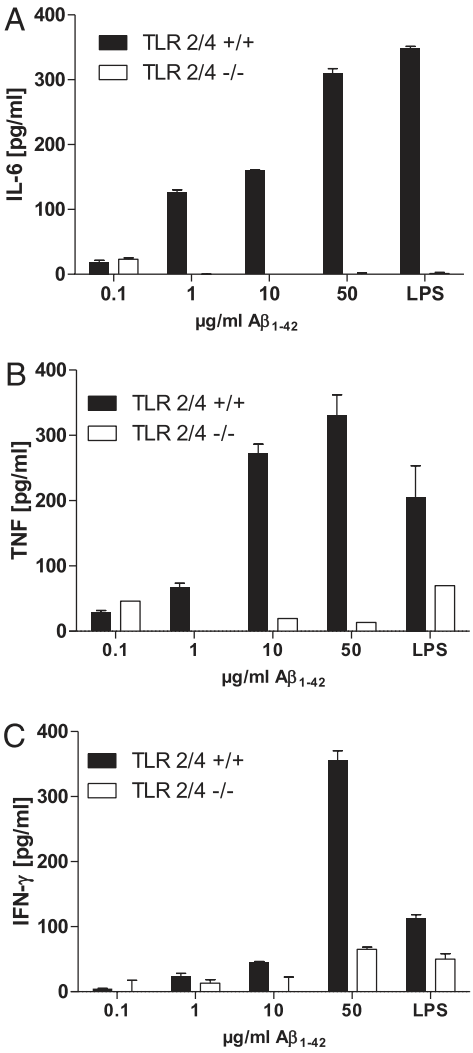

FIGURE 8. $A\beta_{1–42}$ induces IL-6 and TNF production in naive macrophages and IFN-γ in dendritic cells through activation of TLR2 and TLR4. MACS purified $CD11b^+$ cells (*A*, *B*, macrophages) and $CD11b^+CD11c^+$ cells (*C*, dendritic cells) from untreated wild-type or TLR2/4-deficient mice were stimulated with increasing concentrations of $A\beta_{1–42}$ for 48 h. Levels of IL-6, TNF, and IFN-γ were determined in the supernatants by ELISA. Data are representative of three independent experiments.

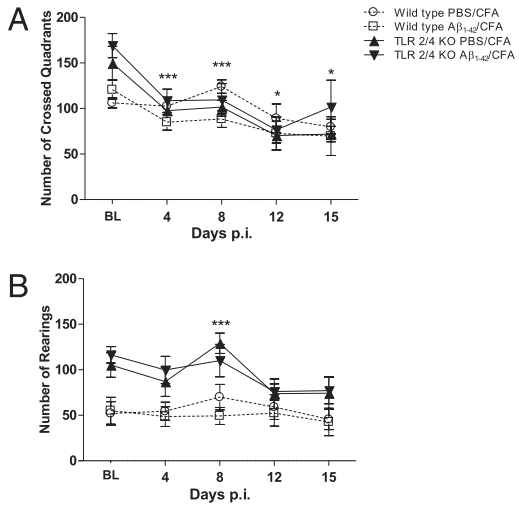

FIGURE 9. $A\beta_{1–42}$/CFA immunization does not affect the behavioral phenotype in TLR2/4-deficient mice. TLR2/4-deficient and wild-type mice (n = 8/group) were immunized with PBS/CFA or $A\beta_{1–42}$/CFA and evaluated for locomotion (*A*) and explorative behavior as measured by the number of crossed quadrants and rearing events (*B*) at different time points p.i. The mean performances pre- and p.i. are summarized for both wild-type and TLR2/4 KO mice upon PBS/CFA or $A\beta_{1–42}$/CFA challenge. Statistical comparisons are based on the surplus effect of $A\beta_{1–42}$/CFA as compared with PBS/CFA challenge within the respective mouse strain. Data were reproduced in three different experiments. Note that in contrast to wild-type mice, TLR2/4-deficient mice were resistant to $A\beta_{1–42}$/CFA induced neurocognitive impairment. Significant differences are indicated. $*p < 0.05$; $***p < 0.001$.

performance as well as deficient habitual learning abilities and impaired performance in complex visuospatial tasks in the absence of apparent focal neurologic deficits. Although neurocognitive phenotypes have been extensively characterized in previous reports and in our present study, memory performance in mice can only be captured in the context of locomotive behavior tasks that reflect both motivational and cognitive components. Dissection of these components is not possible in the absence of verbal communication. Thus, we cannot exclude that the extent of exploration in our setup was also influenced by the lack of motivation due to systemic release of proinflammatory cytokines.

The clinical syndrome exhibited by $A\beta_{1–42}$/CFA-immunized mice was reminiscent of the apathic condition that was the result of a cytokine release syndrome. In fact, deficits in visuospatial tasks are reported in mice injected with LPS. After LPS treatment, mice show impaired performance in tests of cognition that require effective integration of new information to complete a spatial task (29). A further study in mice provides evidence for hippocampus-dependent learning and memory impairment after LPS injection (30). Systemic administration of LPS is reported to induce TLR4-dependent secretion of proinflammatory cytokines such as IL-1β, IL-6, and TNF in the CNS (31–34). Furthermore, activation of TLR4 by LPS increases IFN-γ levels in mice and stimulates IDO in peripheral tissues and the brain (35). IDO activation results in decreased tryptophan levels and increased production of kynurenine promoting depression-like behavior in mice (36). LPS-induced sickness behavior is characterized both by systemic inflammation (35) and activation of local microglial cells (37) in the absence of cellular infiltrates in the CNS.

In contrast to detrimental effects of proinflammatory cytokines on cognitive functioning, other cytokines may have beneficial effects on cognitive processes in the normal brain (38, 39). For example, IL-4 in meningeal T cells is involved in maintaining cognitive abilities in spatial learning and memory tasks (40). In the absence of T cell-derived IL-4, mononuclear cells in the meningeal compartment become activated and contribute to impaired learning abilities. In line with this concept, we showed that extensive production of proinflammatory cytokines like TNF and IL-6 by $A\beta_{1–42}$-activated macrophages resulted in reduced cognitive performance.

A study by Furlan et al. (19) reports an $A\beta_{1–42}$-specific $CD4^+$ T cell response and mild neurologic signs p.i. of C57BL/6 mice with $A\beta_{1–42}$/CFA, but did not test for behavioral deficits. Mice develop an inflammatory disease of the CNS characterized by the presence of perivenular inflammatory foci containing macrophages, T and B cells, and Igs both in the brain and spinal cord. In our immunohistochemical analyses of $A\beta_{1–42}$/CFA-immunized mice, we consistently observed disseminated macrophage infiltrates without focal accumulation of immune cells. S100A8 and CD14 were more prominently expressed in the CNS of $A\beta_{1–42}$/CFA-immunized mice than in control animals. Calprotectin (S100A8) induces cytokine-like effects in the local environment

and is expressed in activated macrophages, endothelial cells, and epithelial cells (41). CD14 is reported to function as a coreceptor for $A\beta_{1-42}$ (42, 43). Both Ab blocking of CD14 and knockdown of the CD14 gene reduce the Aβ peptide-induced release of inflammatory cytokines and NO in microglial cells and peritoneal macrophages (44). We hypothesize that the upregulation of CD14 p.i. with $A\beta_{1-42}$ might be part of a positive feedback loop in that $A\beta_{1-42}$ induced components of its own receptor system both in the peripheral immune compartment and the CNS. Thus, induction and subsequent activation of the CD14 receptor would result in sustained secretion of proinflammatory cytokines in the presence of Aβ peptide.

$A\beta_{1-42}$/CFA immunization strongly stimulated the production of proinflammatory cytokines in the serum and in peritoneal macrophages. These data suggested that $A\beta_{1-42}$ acted in a PAMP-like manner on cells of the innate immune system. PAMPs (e.g., LPS) are recognized by pattern recognition receptors such as TLRs, triggering the expression of proinflammatory molecules (45). It has been demonstrated that $A\beta_{1-42}$ has the capability to engage TLR2 to transduce intracellular signaling into microglial cells (46). Mice transgenic for a chimeric mouse/human APP and the human presenilin-1 gene that are also deficient for TLR2 exhibit increased Aβ deposition in the CNS and accelerated cognitive decline (47) due to deficient microglia activation, indicating the possibility of a direct interaction of $A\beta_{1-42}$ with TLRs in the CNS. By activating TLR2, $A\beta_{1-42}$ induces the secretion of proinflammatory molecules like TNF, IL-6, and IL-1β in mouse primary microglia (23). Similarly, both TLR2 and -4 mediate $A\beta_{1-42}$-induced proinflammatory responses in human monocytic cell lines (48). In contrast, TLR2 and -4 are not required for the induction of EAE by active immunization with myelin Ags emulsified in CFA. In TLR2-deficient mice, the severity of MOG_{35-55}/CFA-induced EAE is similar to wild-type animals (49). TLR4 and TLR9 KO animals are even hypersusceptible to EAE (50). Thus, each of TLR2 and TLR4 are dispensable for inducing a paralytic syndrome upon immunization with MOG_{35-55}/CFA, suggesting that adjuvant effects of CFA are mediated by other pattern recognition receptors or a combination of these TLRs. However, the neurocognitive phenotype induced by immunization with $A\beta_{1-42}$/CFA was absolutely dependent on TLR2 and TLR4. Thus, we propose that unique effects of $A\beta_{1-42}$ were mediated by TLRs and were the molecular basis of the clinical neurocognitive phenotype induced by immunization with $A\beta_{1-42}$. Because there is also a weak Ag-specific T cell response to $A\beta_{1-42}$ promoting inflammation in tissues with relevant expression of Aβ (27), activated macrophages may subsequently be recruited to the CNS. In this study, macrophages were further activated and induced to release proinflammatory cytokines, resulting in clinically manifest psychomotor impairment.

In conclusion, $A\beta_{1-42}$ peptide had the capacity to stimulate cells of the innate immune system in a TLR2/4-dependent manner. We propose that $A\beta_{1-42}$ had adjuvant-like properties and by this mechanism induced a profound inflammatory response syndrome. $A\beta_{1-42}$, perhaps by activating TLR2, triggered the expression of CD14, which could act as a coreceptor for Aβ peptide. Therefore, this might represent a feed-forward loop enhancing $A\beta_{1-42}$-driven activation of macrophages, leading to sustained secretion of proinflammatory cytokines in situ. Thus, the current study highlights potential risks of Aβ immunotherapy and potential mechanisms involved in the induction of cognitive deficits.

Disclosures

The authors have no financial conflicts of interest.

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Appendix IV

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Implications of Antiinflammatory Properties of the Anticonvulsant Drug Levetiracetam in Astrocytes

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There is accumulating evidence that epileptic activity is accompanied by inflammatory processes. In the present study, we evaluated the effect of levetiracetam (Keppra), an anticonvulsant drug with decisive antiepileptic features, with regard to its putative antiinflammatory potential. We previously established an in vitro cell culture model to mimic inflammatory conditions: Primary astrocytic cultures of newborn rats were cocultured with 30% (M30) microglial cells. Alternatively, cocultures containing 5% microglia (M5) were incubated with the proinflammatory mediator, the cytokine interleukin-1β (IL-1β), and lipopolysaccharide (LPS), a potent bacterial activator of the immune system. For the M30 cocultures, we observed reduced expression of connexin 43 (Cx43), the predominant gap junction protein. Impaired functional dye coupling and depolarized membrane resting potential (MRP) were monitored in M30 cocultures as well as in M5 cocultures treated with IL-1β and LPS. We could show that the Cx43 expression, the coupling property, and the membrane resting potential on which we focused our inflammatory coculture model were normalized to noninflammatory level under treatment with levetiracetam (Keppra). Cumulatively, our results provide evidence for antiinflammatory properties of levetiracetam in seizure treatment.

Key words: levetiracetam; epilepsy; inflammation; astrocyte/microglial coculture; gap junction communication

Recent reports underscore the idea that inflammatory conditions are present during and after seizure activity (Vezzani, 2005). Previous investigations could detect an increase of proinflammatory cytokines, in particular interleukin-1 (IL-1), in experimental epilepsy models (De Simoni et al., 2000). A prolonged inflammatory condition was emphasized, demonstrating a role of inflammation in chronic epilepsy rather than in acute seizures. Moreover, inflammatory mediators were detected in the cerebrospinal fluid from patients with recent epileptic seizures (Peltola et al., 2000). Microglial cells among other cells, such as astrocytes, represent the main source of cytokines in the CNS (Hanisch, 2002), so we assume that the occurrence of inflammatory cytokines in epileptic seizures involves activation of microglia. The overexpression of cytokines triggers disturbance of the neuronal and glial enviroment.

More detailed knowledge about the link between inflammatory responses and the etiopathogenesis of epilepsy would help in developing novel and effective therapeutic modalities. Here we focused on the novel drug levetiracetam (LEV; Keppra; ucb L059; [S]-alpha-ethyl-2-oxo-1-pyrrolidine acetamide), which has been shown to possess antiepileptic efficacy and good tolerability in the treatment of refractory partial seizures in several clinial trials (Ben-Menachem and Falter, 2000; Cereghino et al., 2000). However, many studies devoted to the detailed molecular actions of LEV have suggested that LEV is devoid of impact on many targets accepted as accounting for classical antiepileptic drugs (AEDs; Klitgaard et al., 1998; Zona et al., 2001). Thus, the knowledge about the mechanisms responsible for its antiepileptic activity remains limited. The aim of the present study was to evaluate the ability of LEV to restrain the effects of inflammation on astrocytes, focusing on astrocytic connexin 43 (Cx43) expression, gap junction-mediated intercellular

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communication, and membrane resting potential within inflammation.

To mimic inflammatory conditions, we used a defined in vitro bioassay consisting of primary astrocytic cultures of newborn rats that were cocultured with 30% (M30) microglial cells. Astroglial cocultures containing 5% (M5) microglial cells served as controls, which corresponds to the physiological situation in healthy brain. Previously, we had demonstrated that M30 cocultures revealed a high fraction of activated round phagocytic-type (RPT) microglia, whereas the M5 cocultures contained mainly microglial cells of resting ramified type (RRT; Faustmann et al., 2003). The change of the morphological phenotype from RRT to RPT is considered to occur in line with enhanced phagocytic activity of the microglia and the capability to secrete inflammatory cytokines (Stoll et al., 1998; Ledeboer et al., 2000). In addition to the M30 coculture model, activation of microglia was also observed after exposure of M5 cocultures to diverse proinflammatory cytokines, including IL-1β (Hinkerohe et al., 2005).

Astrocytes have multiple functions, e.g., cell proliferation, metabolic and trophic support of neurons and the control of extracellular ion and neurotransmitter homoeostasis (Dermietzel and Spray, 1998; Reuss and Unsicker, 1998). These properties can be influenced by a well-functioning intercellular communication based on gap junction channels, which in the case of astrocytes are predominantly constituted by the gap junction protein Cx43 (Dermietzel et al., 1991; Giaume and Venance, 1998). Impairment of the astrocytic syncytium and subsequent disturbance of the dissipation and buffering capacity have been reported to occur in diverse pathophysiological processes, such as inflammatory conditions (John et al., 1999; Faustmann et al., 2003).

Here, we first evaluated the astroglial Cx43 epression in the M30 cocultures and the strength of gap junction-mediated intercellular communication (GJIC) within the astroglial syncytium with and without LEV. Furthermore, the astrocytic membrane resting potential (MRP) was measured by means of the patch-clamp technique. These parameters were studied in parallel using M5 cocultures pretreated with the proinflammatory cytokine IL-1β and lipopolysaccharide (LPS), which is a cell wall component of gram-negative bacteria and contributes to a distinct production of inflammatory cytokines by activation of immune component cells, such as astrocytes and microglia (Saukkonen et al., 1990; Lee et al., 1993).

MATERIALS AND METHODS

Cell Culture

Primary astroglial cell cultures were obtained from whole brain hemispheres of postnatal (P0–P2) Wistar rats based on a modified protocol reported by Dermietzel et al. (1991). Briefly, after removal of meninges and choroid plexuses, brains were treated with 0.1% trypsin/1% DNAse I (Invitrogen, Karlsruhe, Germany; Serva, Heidelberg, Germany) and passaged through a 60-μm nylon mesh. Cells (40,000 cells/coverslip) were then incubated in culture flasks (Becton Dickinson, Heidelberg, Germany) for 4–5 days in 87% Dulbecco's minimal essential medium (DMEM; Gibco, Karlsruhe, Germany), 10% fetal calf serum, 1% nonessential amino acids, 1% penicillin (50 μg/ml) streptomycin (50 μg/ml), and 1% glutamine (2 mM; Invitrogen) until confluence in humidified carbon gas (5% CO_2/95% a.a.) at 37°C. Non-adjacent microglia were removed by vigourously shaking the flask, followed by subsequent washing procedures. Depending on the extent of shaking, an astrocyte/microglia coculture containing about 5% microglia (M5) was acchieved, comparable to the concentration found in healthy brain tissue or left to obtain a coculture containing about 30% microglia cells (M30). To confirm whether the cocultures contatined a 5% or 30% fraction of migroglia, immunohistochemical staining and subsequent counting were performed as described below.

The study was approved by the Bioethical Committee of the Ruhr-University Bochum, and experiments were performed in accordance with accepted guidelines for care and use of animals in research. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Immunocytochemistry

Astrocytes/microglia cocultures (M5 and M30) on poly-L-lysine-coated glass coverslips (12 mm^2 diameter) were washed and fixed in 100% ethanol and treated with PBS blocking solution containing 10% horse serum and 1% bovine serum albumin (BSA) before incubating with the primary antibodies for 60 min at room temperature. Monoclonal mouse antiglial fibrillary acidic protein (GFAP; 1:100; G9269; Sigma; Taufkirchen, Germany) was used; microglia was labeled by using a monoclonal antibody to the ED1 marker (1:250; MCA 341R; Serotec, Eching, Germany), which allowed classification of microglia as resting ramified (RRT) or activated, rounded phagocytic (RPT) phenotypes (Faustmann et al., 2003); and Hoechst 33342 (1:2,500; B2261; Sigma, München, Germany) was used to counterstain the cell nuclei. Conjugated Alexa-Fluor 488 (green) or 568 (red) goat anti-rabbit and goat anti-mouse IgG were applied for 30 min (1:1,000; Molecular Probes, Leiden, The Netherlands) as secondary antibodies. Processed coverslips were observed via confocal scanning microspocy (Zeiss LSM 510 inverted confocal microscope; Zeiss, Göttingen, Germany) at ×630 magnification.

Enzyme-Linked Immunosorbent Assay

Cytokine levels in cell culture supernatants (M5, M30, M30 + LEV) were quantified by ELISA. The Quantikine-ELSIA-Kits were applied (R&D Systems, Minneapolis, MN) for quantification of IL-1β (RLB00), according to the R&D Systems protocol. Fifty microliters of dilutant and 50 μl of each supernatant sample were given to prepared wells and incubated for 2 hr at room temperature. After washing procedures, 100 μl of substrate solution was added to each well. This reaction was stopped after 30 min by adding 100 μl hydrochloric acid solution. Optical densities of each well were

determined by using a microplate reader (Bio-Rad 550) set to 450 nm. Wavelength correction was set to 570 nm.

Concentrations of cytokines were calculated by normalized standard twofold diluted series. All samples were determined in triplicate. All values are medians of three independent experiments. The same statistical analysis was performed as described for functional coupling and MRP (see below).

Administration of the Anticonvulsive Substance LEV, the Inflammatory Cytokine IL-1β, and LPS

Based on clinical findings of Grim and coworkers (2003), LEV at a concentration of 50 μg/ml was used to mimic serum concentrations that were found after 4 weeks of treatment with the sufficient dose of LEV. However, this concentration might be less than that found in the extracellular space in vivo, taking into account the findings of Rambeck et al. (2006), who showed that, similarly to the mechanisms in serum, protein binding play a crucial role in AED concentrations in affected brain sites. In the first set of experiments, the human recombined cytokine IL-1β (500 pg/ml; Pepro-Tech, Rocky Hill, NY) or LPS (100 ng/ml; 026:B6; L2654; Sigma) was added to the primary M5 cocultures for 2 hr. M30 cocultures that had been pretreated with LEV (50 μg/ml; Keppra; UCB Pharma) for 24 hr and M5 cocultures that had been pretreated the same way for 22 hr and additionally with 2 hr of IL-1β (500 pg/ml) or LPS (100 ng/ml) were assessed for the experiments.

Functional Coupling and MRP

The whole-cell patch clamp technique (Axon 200-B patch clamp amplifier; Axon Instruments, Burlingame, CA) was used for simultaneous measurement of MRP and functional coupling, which allows concurrent monitoring of GJIC. For this purpose, patch pipettes (GB 150-8P; Science Products, Hofheim, Germany) with 2–4 MOhm resistance were filled with intracellular solution (135 mM K-gluconate, 20 mM KCl, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM EGTA, pH 7.2) containing 1% (w/v) Lucifer yellow (LY). The number of coupled cells was counted 10 min after dye transfer using a Zeiss Axioskop with an FITC filter set. Significant differences between medians of coupled cells were analyzed by using the Mann-Whitney test (one-tailed; significance was determined at $^{\star}P = 0.05$ and high significance at $^{\star\star}P = 0.001$ or $^{\star\star\star}P = 0.0001$). GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA) was used for statistical analyses and graph design.

Protein Analysis

Astroglial M5 and M30 cocultures as well as M5 cocultures pretreated with IL-1β (500 pg/ml; Pepro-Tech) or LPS (100 ng/ml) treated with LEV 50 μg/ml (LEV 50) for 24 hr were subjected to Western blotting. Cells were lysed in Laemmli lysis buffer, and total protein content was determined by Bio-Rad's Bradford assay. Samples containing 5 μg of total protein were loaded on 10%-SDS gels and transferred onto nitrocellulose membranes by semidry blotting. The membranes were preincubated in 0.5% blocking reagent (catalog No. 1096176; Boehringer, Mannheim, Germany) in PBS for 1 hr and probed with a primary polyclonal affinity-purified antibody (diluted 1:1,000 in 0.2% blocking solution) from rabbit directed to the carboxy terminus of Cx43 (amino acids 360–382; Hofer et al., 1996) at 4°C overnight. The membranes were then washed twice in PBS + 0.05% Tween 20 (PBS-T) for 10 min and once in PBS for 5 min, followed by incubation with a horseradish peroxidase-coupled secondary anti-rabbit IgG antibody for 1 hr. After four washes in PBS-T, signals were visualized by using an ECL Kit (Amersham Pharmacia, Freiburg, Germany) according to the manufacturer's directions.

The membranes were rinsed with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, 2% (w/v) sodium dodecyl sulfate) and probed with anti-β-actin antibody (catalog No. A5441; Sigma, Taufenkirchen, Germany, 1:1,000 in 0.2% blocking solution) as described above to verify equal protein content in the blotted samples. The exposed X-ray films were scanned (Arcus II scanner, Agfa, Taiwan) and densitometrically evaluated with TINA software, version 2.09 (Raytest GmbH, Straubenhardt, Germany).

RESULTS

Definition of the Culture Conditions

As we described previously (Faustmann et al., 2003), the activation of microglia in a microglia/astrocyte coculture system can be achieved through variation of the amount of microglial cells. Whereas, in cultures with a low fraction of microglia (M5), the RRT dominates (Fig. 1A), M30 cocultures are characterized by an increase in the activated RPT (Fig. 1B).

ELISA

In M5 coculture supernatants, low concentrations of IL-1β were found (41.87 ± 6.3 pg/ml, n = 3). In M30 cocultures, IL-1β was almost six times higher than in M5 cocultures (242.4 ± 28.2 pg/ml, n = 3, $P < 0.05$), whereas M30 cocultures treated with levetiracetam at a concentration of 50 μg/ml for 24 hr revealed a decreased level of IL-1β (140.46 ± 15.2 pg/ml, n = 3, $P = 0.05$; Fig. 2). The increased release of IL-1β in M30 cocultures strengthens the assumption of inflammatory characterics of astroglial/microglial cocultures containing a high fraction of microglia.

GJIC

The number of coupled astrocytes in the cocultures was quantified at 10 min after LY application. M5 cocultures revealed a significant decrease of coupled cells when incubated with the proinflammatory cytokine IL-1β (M5: 36.9 ± 4.2, n = 30; M5 + IL-1β: 3.1 ± 0.7, n = 11, $P < 0.0001$) and LPS (2.25 ± 0.6, n = 12, $P < 0.0001$). Similar results were found for the M30 cocultures (13.6 ± 12.3, n = 19, $P < 0.0001$), demonstrating the effects of activated microglia on the astrocytic syncytium (Figs. 3, 4). The application of 50 μg/ml LEV for 24 hr to the M5 cocultures significantly

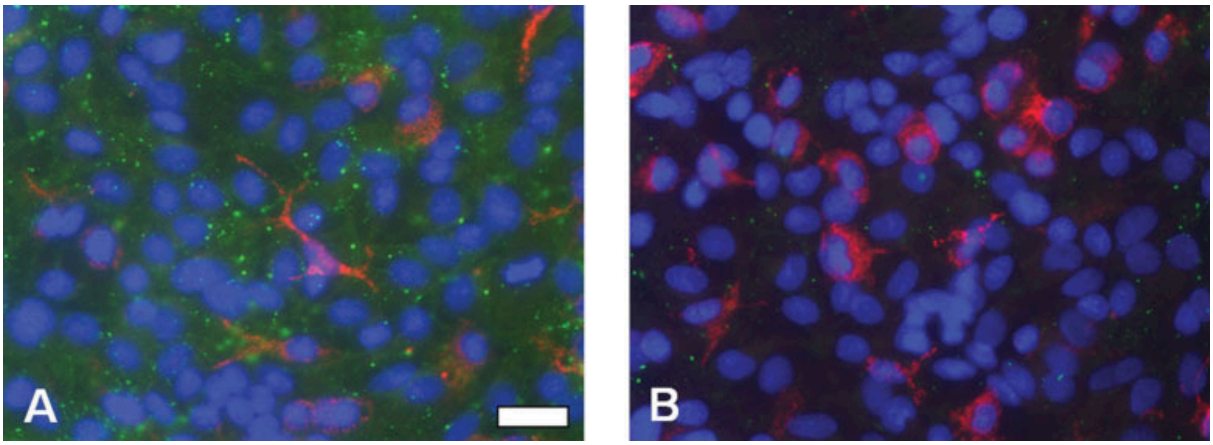

Fig. 1. Astroglial/microglial cocultures containing either 5% (M5) or 30% (M30) microglial cells. As shown in the ED1 immunostaining, the M5 cocultures contained mainly microglial cells of resting ramified type (RRT; **A**), whereas the M30 cocultures revealed a high fraction of activated round, phagocytic type (RPT) microglia (red; **B**). Scale bar = 40 μm.

restored the impaired coupling efficiency caused by incubation with IL-1β (14 ± 3.6, n = 10, $P = 0.0062$) and LPS (7.1 ± 2.6, n = 9, $P = 0.0345$). The incubation of the M30 cocultures with 50 μg/ml of LEV for 24 hr revealed an increase in coupled cells to 55.4 ± 12.3 (n = 16, $P = 0.0006$; Fig. 4). For M5, the incubation with 50 μg/ml LEV did not have any significant effects on the astrocytic coupling (39.6 ± 7.7, n = 12, $P = 0.3797$; Fig. 4).

MRP of Astrocytes

In M5 cocultures incubated with PBS, which served as controls, MRP was –74.9 ± 1.6 mV (n = 30), which is in the range of normal astrocytic MRP. A significant MRP depolarization to –48.4 ± 3.0 mV was induced by IL-1β (n = 11, $P < 0.0001$) and –62.7 ± 2.9 mV measured in M30 cocultures (n = 19, $P = 0.0001$; Fig. 5). M5 cocultures incubated with LEV at a concentration of 50 μg/ml showed no significant alteration vs. control samples (–74.7 ± 2.3 mV, n = 12, $P = 0.4889$; Fig. 5).

Prevention of the inflammatory effects on MRP of astrocytes could be shown under pretreatment with 50 μg/ml LEV for 24 hr: in M30 cocultures, an MRP of –76.2 ± 3.1 mV (n = 16, $P = 0.0023$) could be observed (Fig. 5). In M5 cocultures incubated with IL-1β, the MRP was –66.7 ± 2.7 mV (n = 10, $P = 0.0004$) when pretreated with LEV (50 μg/ml; Fig. 5).

Quantitative Analysis of Cx43 Protein Expression

Cx43 revealed a typical three-banded pattern on Western blots, which originates from its differently phosphorylated isoforms (Laird et al., 1991). To quantify the Cx43 concentration, density profiles of the three bands of treated cultures were obtained by assessing their densitometric values and set in ratio to the signals of the three bands from control cultures. PBS-treated cultures served as controls. Control medians were set to the hypothetical value of 100%, to which values for stimulated samples were normalized. The Cx43 concentration was found to decrease for M30 primary astrocyte/microglia cocultures to 49.2% ± 5.5% (n = 3). Cx43 concentration appeared at control level after pretreatment of the cocultures with LEV 50 (103.8% ± 9.0%; n = 3) for 24 hr (Fig. 6). No significant changes in Cx43 expression were observed after LEV treatment of M5 cocultures preexposed to IL-1β (500 pg/ml) or LPS (100 ng/ml; data not shown).

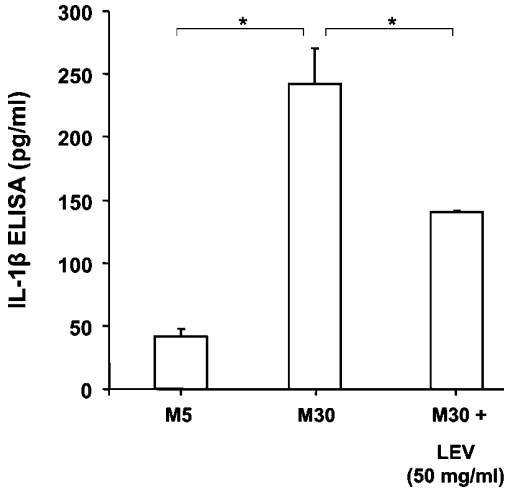

Fig. 2. ELISA IL-1β concentration in M5 vs. M30 cocultures. In comparison with M5 cocultures, supernatants of M30 cocultures revealed a six times higher concentration of IL-1β. The enhanced IL-1β level in M30 cocultures was decreased by LEV. $*P < 0.05$.

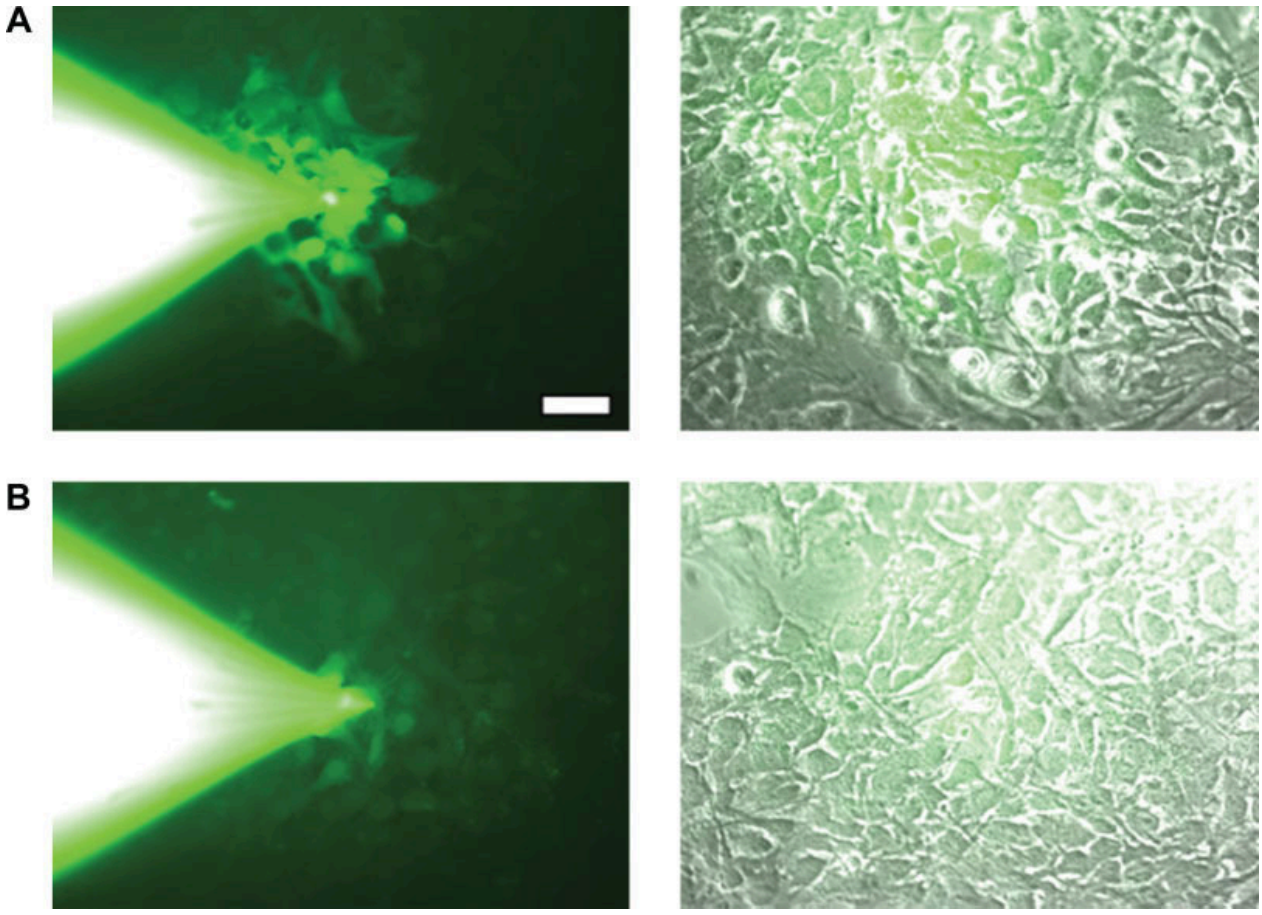

Fig. 3. Astroglial coupling in M5 and under inflammatory conditions in M30 cocultures (left, fluorescent; right, phase contrast). A high level of Lucifer yellow transfer was found under noninflammatory condotions in M5 (**A**) compared with low coupling under inflammatory conditions in M30 (**B**). Scale bar = 40 μm.

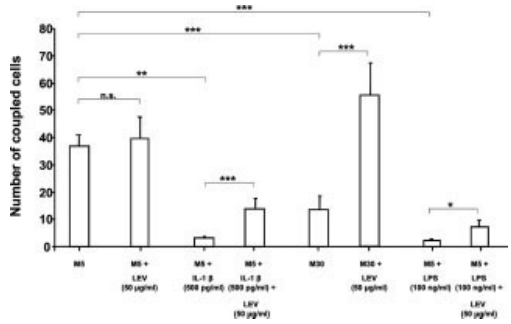

Fig. 4. Coupling efficiency after Lucifer yellow application using the patch-clamp technique in astroglia/microglia cocultures. The number of coupled cells was significantly reduced in the M5 cocultures treated with IL-1β (500 U/ml) and LPS (100 ng/ml) and in the M30 cocultures. Preincubation of the M5 cocultures with LEV (50 μg/ml) partially reversed the impaired coupling efficiency of astroglial cells elicited by IL-1β and LPS. The number of coupled cells in the M30 cocultured was also strongly increased after treatment with LEV (50 μg/ml). ⋆$P < 0.05$, ⋆⋆$P < 0.001$, ⋆⋆⋆$P < 0.0001$.

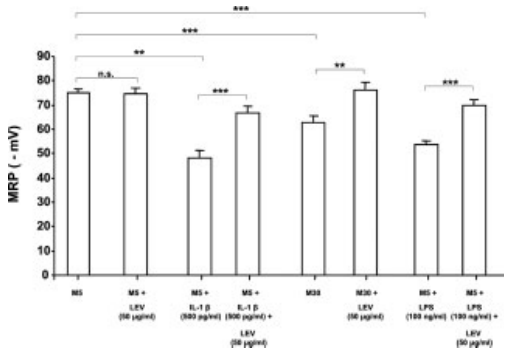

Fig. 5. Astroglial membrane resting potential under noninflammatory and inflammatory conditions. Addition of LEV at 50 μg/ml to the M5 cocultures did not significantly affect the astroglial MRP under noninflammatory conditions. The astroglial MRP was significantly depolarized in the IL-1β (500 U/ml)- and LPS (100 ng/ml)-preincubated M5 cocultures and in the M30 cocultures. Depolarization of MRP under the inflammatory conditions—IL-1β and LPS exposure and activated culture conditions (M30)—could be significantly reduced by application of LEV (50 μg/ml). ⋆⋆$P < 0.001$, ⋆⋆⋆$P < 0.0001$.

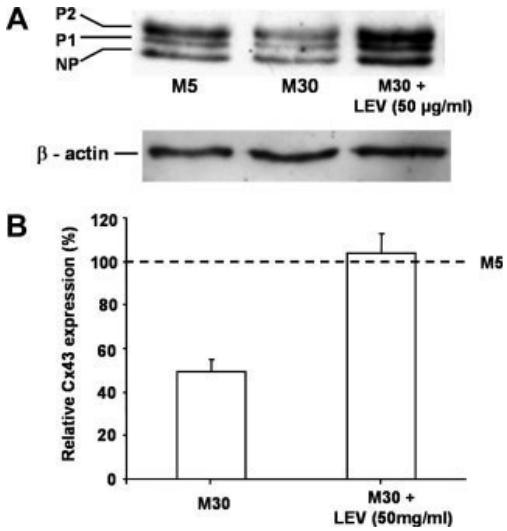

Fig. 6. The expression of Cx43 is reduced in the M30 coculture but can be restored by treatment with LEV. **A:** Western blot analysis of Cx43 expression was performed in the M5 cocultures and M30 cocultures with and without application of LEV (50 µg/ml). **B:** Densitometric evaluation of immunoblotting revealed a significant downregulation of the astroglial Cx43 expression under activated culture conditions (M30), which was raised to control level (M5) by application of LEV (50 µg/ml).

DISCUSSION

Several clinical trials of major study groups have demonstrated that adjunctive therapy with LEV was superior to placebo in suppressing seizures in patients with refractory partial epilepsy (Ben-Menachem and Falter, 2000; Cereghino et al., 2000). More recent studies even suggested that the use of LEV as monotherapy is safe and effective for partial seizures (Ben-Menachem, 2003). However, the anticonvulsive mechanisms of LEV are not completely understood, and many experimental studies performed so far have distinguished LEV from other AEDs in its structure, pharmakokinetics, and molecular effects (Patsalos, 2000; Klitgaard, 2001).

Some AEDs, such as phenytoin, carbamazepine, valproate, and Lamotrigine, are known to act on neuronal excitatory Na^+ channels (Macdonald and Kelly, 1995), whereas LEV failed to modify the profile of voltage-gated tetrodotoxin-sensitive inward Na^+ current in rat neocortical neurons. Likewise, a lack of effect on low-voltage-gated (T-type) Ca^{2+} current in hippocampal neurons has been reported (Zona et al., 2001), whereas an incomplete inhibition of high-voltage-activated Ca^{2+} current of N-type Ca^{2+} has been ascribed to LEV (Niespodziany et al., 2001; Lukyanetz et al., 2002).

As far as the GABAergic system is concerned, the existing results remain obscure. On the one hand, LEV appears to be devoid of impact on GABA metabolism and transport (Sills et al., 1997; Fraser et al., 1999) and fails to interact with the benzodiazepine site of the $GABA_A$ receptor (Klitgaard et al., 1998). On the other hand, it has also been reported that systemic administration of LEV induces alterations in GABA metabolism and turnover and that LEV reverses the action of negative allosteric modulators of neuronal GABA currents, such as zinc (Rigo et al., 2002).

With regard to the type of seizure activity, LEV lacks potent anticonvulsant activity in the acute maximal electroshock seizure test and in the maximal pentylenetetrazol seizure test in rodents (Loscher and Honack, 1993; Klitgaard et al., 1998) but shows potent protection against generalized epileptic seizures in electrically and pentylenetetrazol-kindled (Gower et al., 1995; Klitgaard et al., 1998).

Given these findings, LEV appears to have selective anticonvulsant activity in animal models of chronic epilepsy rather than in acute seizure models and exerts its effect through a distinctive profile of a mechanism that does not involve direct influence on synaptic neurotransmission via conventional ligand–receptor interaction with the classical receptors that are known to be targeted by other AEDs (Noyer et al., 1995; Klitgaard, 2001). Thus, recent studies were devoted to exploring alternative pathways that may account for the effects of LEV. For example, Lynch and coworkers (2004) identified the synaptic vesicle protein SV2A as the brain binding site of LEV. This membrane glycoprotein has been suggested to act as a modulator of vesicle fusion and thus to be of major importance for the neuronal release propabilitiy of synaptic vesicles (Xu and Bajjalieh, 2001). Another example of alternative effects of LEV is its impact on the synthesis of brain-derived neurotrophic factor (BDNF) and inducible nitric oxide synthase (iNOS) in astrocytes as possible candidates for targets of antiepileptic treatment, which has only rarely been considered so far (Cardile et al., 2003; Pavone and Cardile, 2003).

To explore the impact of LEV on glial cells, the focus of the present study was to investigate whether the antiepileptic profile of LEV involves modulation of impaired astroglial properties under inflammatory conditions. We had previously shown that primary astrocytic cultures of newborn rats that are cocultured with 30% (M30) microglial cells or incubated with IL-1β provide suitable environment for such investigations as demonstrated by the high fraction of activated microglia indicative for inflammatory responses (Faustmann et al., 2003; Hinkerohe et al., 2005). This model is characterized by a decrease in astroglial Cx43 expression, an impaired functional coupling within the astroglial syncytium, and a depolarized astrocytic MRP. The treatment of this inflammatory culture model with LEV displayed a modification of the functional properties of astrocytes.

LEV at therapeutic concentration (50 µg/ml) enhanced Cx43 expression in M30 cocultures and coupling strength of astrocytes and normalized the MRP to physiological levels. The ELISA results showed an increase of the inflammatory cytokine IL-1β in the supernatants of the M30 cocultures, which provides clear evidence that the morphological transformation of the microglia in these cultures is accompanied by functional changes (Fig. 2). Moreover, the ability of LEV to

decrease the enhanced IL-1β level in M30 cocultures shows an antiinflammatory mechanism. The finding that GJIC (Fig. 4) in IL-1β-treated M5 cocultures at the concentration of 500 pg/ml was only partially recovered after LEV treatment most likely is due to the twofold concentration of IL-1β compared with IL-1β measured in M30 cocultures (Fig. 2). Similarly, LEV had only limited effect on GJIC of LPS-treated M5 cocultures. This can be explained by a stronger and a broader range of LPS-induced cytokine sekretion (Saukkonen et al., 1990; Lee et al., 1993). The missing effect of IL-1β and LPS on Cx43 expression, which does not meet the results observed in M30 cocultures, suggests that mechanisms other than Cx43 expression are involved in altered GJIC. A feasible mechanism is the modification of Cx43 in astrocytic gap junctions, e.g., through phosphorylation, which results in impaired GJIC. It has been described that phosphorylation of Ser or Tyr residues in the C-terminal domain of Cx43 regulates the gating of gap junction channels and that this effect is achieved through activation of protein kinase C (PKC; Lampe et al., 2000). In this context, PKC has been shown to be among the distal effectors of IL-1β in modulating GJIC (Zvalova et al., 2004).

Epilepsy and Inflammation

The finding that LEV exhibits reversal of affected astroglial properties from inflammatory conditions supports the idea of a link between inflammatory processes and seizure activity. Indeed, in a number of recent studies, it has been suggested that activation of the innate immune system, such as the production of proinflammatory cytokines, accompanies the molecular and structural changes that take place during and after seizure activity (Vezzani, 2005). This is supported by clinical findings in which elevated levels of proinflammatory cytokines were observed in the cerebrospinal fluid from patients with recent epileptic seizures (Peltola et al., 2000) and in surgically resected human brain tissue from patients with intractable epilepsy (Sheng et al., 1994). Also, in various experimental seizure models, a rapid increase of proinflammatory cytokines on protein level as well as upregulated expression of messenger RNA has been observed after seizure induction (Gahring et al., 1997; Oprica et al., 2003). In particular, the accumulation of IL-1β persisting for 60 days (De Simoni et al., 2000) implies a prolonged activation of the immune system, which strengthens the idea that inflammation is linked to epileptic activity rather than being a mere event with minor implications. Furthermore, immunohistochemical studies demonstrated the enhancement of IL-1β accompanied by an increase of activated microglial cells after kainic acid-induced seizures (Vezzani et al., 1999).

Among the effector cells to respond to activated microglia, atrocytes are of major importance, insofar as they undergo loss of multiple regulatory properties, such as ion and neurotransmitter uptake and dissipation, which are crucial for the maintenance of efficient interneuronal signalling in normal brain. For example, glutamate uptake by the astrocytes is a well-known mechanism that provides low extracellular levels of glutamate, which are essential for proper neuronal activity (Oprica et al., 2003). There is clear evidence for a direct link between coupling capacity of astrocytes and uptake of glutamate. It was recently shown that decoupling of astrocytes with gap junction blockers resulted in decreased expression of the astrocytic glutamate transporter 1, which constitutes the major glutamate transporter subtype in the cortex (Figiel et al., 2007). Hence, impairment of GJIC, e.g., by IL-1β, possibly promotes proconvulsant effects through inhibition of glutamate uptake, leading to an increase in glutamate available for the activation of N-metyl-D-aspartate (NMDA) and non-NMDA receptors (Scimemi et al., 2005).

In conclusion, the results of our study suggest that the efficacy of LEV derives in part from its ability to prevent impairment of astroglial regulatory properties under inflammatory conditions. This observation contributes to a better understanding of how glial cells participate in seizure disorder. In this regard, astrocytes may serve as candidates for potential targets of future anticonvulsant therapeutics in consideration of antiinflammatory and neuroprotective aspects, an issue that has been largely ignored so far.

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Appendix V

IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3$^+$ regulatory T cells

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The conditions leading to the induction of adaptive Foxp3$^+$ regulatory T cells (T-regs) from peripheral T cells in vivo are incompletely understood. Here, we show that unresponsiveness of T cells to IL-6 by T cell-selective deletion of gp130 or immunization of wild-type mice with antigen in incomplete Freund's adjuvant (IFA), which fails to induce IL-6, promotes the conversion of peripheral CD4$^+$ T cells into adaptive Foxp3$^+$ T-regs. Thus, both T cell-conditional gp130 knockout (KO) mice immunized with MOG35-55 in complete Freund's adjuvant (CFA) and wild-type mice immunized with MOG35-55 in IFA develop overwhelming antigen-specific T-reg responses and are protected from experimental autoimmune encephalomyelitis (EAE). Depletion of T-regs restores T helper (Th)17 responses and clinical EAE in MOG/CFA-immunized T cell-conditional gp130 KO mice, but not in MOG/IFA-immunized wild-type mice. We conclude that in the absence of T-regs, IL-6 signaling is dispensable for the induction of Th17 cells, and alternative pathways exist to induce Th17 cells and EAE in the absence of IL-6 signaling. However, IL-6 signaling is dominant in inhibiting the conversion of conventional T cells into Foxp3$^+$ T-regs in vivo, and in the absence of IL-6 signaling, no other cytokine can substitute in inhibiting T-reg conversion. These data identify IL-6 as an important target to modulate autoimmune responses and chronic inflammation.

experimental autoimmune encephalomyelitis | multiple sclerosis | IL-21 | tolerance | incomplete Freund's adjuvant

Foxp3$^+$ regulatory T cells (T-regs) are critical for the maintenance of peripheral tolerance, and deletion of Foxp3$^+$ T-regs results in multiorgan autoimmunity (1). Naturally occurring Foxp3$^+$ T-regs are generated in the thymus (2) and are released into the peripheral immune compartment during early postnatal development. In the peripheral immune compartment, IL-2 is an essential growth factor for the proliferation of T-regs, whereas TGF-β is important for their maintenance (3). Apart from naturally occurring CD4$^+$CD25$^+$Foxp3$^+$ T-regs, several subsets of T-regs have been described that are induced from naïve conventional T cells in the peripheral immune compartment under specific circumstances (for review, see ref. 4). However, under physiological conditions, it is believed that induced Foxp3$^+$ T-regs are generated mainly in the gut and possibly in other immunological niches that contain high local concentrations of TGF-β and are colonized by specialized types of antigen-presenting cells (5, 6).

Recently, we have discovered a reciprocal developmental relationship between Foxp3$^+$ T-regs and T helper (Th)17 cells because TGF-β triggers the expression of Foxp3 in naïve T cells, whereas IL-6 inhibits the TGF-β-driven expression of Foxp3, and TGF-β plus IL-6 together induce retinoid-related orphan receptor (ROR)-γt triggering the developmental program of Th17 cells (7). In the absence of IL-6, IL-21, which is a member of the IL-2 family of cytokines, can substitute for IL-6, and activation with TGF-β plus IL-21 might constitute an alternative pathway to induce Th17 cells (8). Together, these findings suggested that IL-6 and possibly IL-21 are switch factors between the induction of T-regs and Th17 cells. IL-6 was initially described as B cell-stimulatory factor (9) and as an important trigger of acute-phase responses. IL-6 uses a receptor complex consisting of the ligand-binding subunit IL-6Rα (CD126) and the signaling subunit gp130 (10). Whereas gp130 is ubiquitously expressed, the expression of IL-6Rα is restricted to hepatocytes, intestinal epithelial cells, endocrine glands, and leukocytes with the exception of naïve B cells (for review see ref. 11). Mice deficient in gp130 have been generated. However, in contrast to *Il6*$^{-/-}$ mice, homozygous loss of gp130 is perinatally lethal (12). In fact, gp130 is the receptor signaling subunit for at least 6 additional members of the IL-6 family of cytokines, including IL-11, oncostatin M, leukemia inhibitory factor, cardiotrophin-like cytokine, ciliary neurotrophic factor, and cardiotrophin-1. Furthermore, gp130 is able to trigger 2 major signaling pathways, i.e., the SHP-2/ERK pathway and the STAT3 pathway (for review, see ref. 11). Interestingly, decreased gp130-triggered SHP/ERK signaling and increased gp130-triggered STAT3 signaling result in autoimmunity (13).

Here, we investigated the role of IL-6 in the generation of an immune response to MOG35-55, by using genetically modified mice in which unresponsiveness to IL-6 is restricted to T cells. We found that IL-6 critically prevented the conversion of naïve CD4$^+$ T cells into Foxp3$^+$ T-regs in vivo, and conversely, vaccination protocols that did not induce large amounts of IL-6 resulted in an immune response dominated by Foxp3$^+$ T-regs. Furthermore, we show that immunization with antigen emulsified in incomplete Freund's adjuvant promotes the de novo generation of Foxp3$^+$ T-regs to an extent that is sufficient to confer antigen-specific tolerance. Hence, we illustrate that "absence of inflammatory signals" is consistent with absence of IL-6-induction, which places this cytokine at a nodal point in the shaping of an adaptive immune response.

Results

Responsiveness of T Cells to IL-6 Determines Susceptibility to Experimental Autoimmune Encephalomyelitis (EAE). We and others have shown that IL-6-deficient mice are resistant to EAE (7, 8, 14). In the absence of IL-6, Th17 responses are impaired whereas T-reg

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responses are dominant, suggesting that IL-6 is a critical factor that shifts the immune response from a T-reg response toward a pathogenic Th17 response (8). However, IL-6 has also been shown to induce the expression of vascular cell adhesion molecule (VCAM) on endothelial cells (14). Because the interaction between the integrin very-late antigen 4 (VLA-4) on T cells and VCAM is crucial for the transmigration of encephalitogenic T cells across the blood brain barrier (15), the failure of IL-6-deficient mice to up-regulate VCAM has also been proposed to be responsible for their resistance to EAE.

Here, we sought to discriminate these 2 effects by investigating genetically modified mice (henceforth called *gp130*$^{-/-}$ mice) that were created by crossing CD4-Cre$^{+/+}$ mice with gp130$^{flox/flox}$ mice. As a consequence, responsiveness to IL-6 is selectively eliminated in T cells, whereas other cell types including endothelial cells are not affected. Additionally, we crossed *gp130*$^{-/-}$ mice with *Foxp3gfp*.KI mice (7, 16) so that we could track Foxp3^{+} T-regs based on the expression of green fluorescent protein (GFP). We immunized wild-type or *gp130*$^{-/-}$ mice with MOG35-55/complete Freund's adjuvant (CFA). Whereas wild-type animals developed EAE, *gp130*$^{-/-}$ mice were, like *Il6*$^{-/-}$ mice, completely resistant to EAE (Fig. 1*A*). This suggested that the absence of IL-6 signaling in T cells and not in other cellular targets of IL-6 was responsible for the resistance to the disease. We wondered whether *gp130*$^{-/-}$ mice, analogously to IL-6-deficient animals, had a reduced Th17 response. When we tested the recall response after immunization with MOG35-55/CFA, the antigen-specific production of IL-17 and IFN-γ was significantly reduced in *gp130*$^{-/-}$ mice (Fig. 1*B*). However, we detected an enhanced T-reg response in *gp130*$^{-/-}$ mice ex vivo upon immunization with MOG35-55/CFA (Fig. 1*C Upper*), and in contrast to wild-type mice, the fraction of Foxp3^{+} T-regs in *gp130*$^{-/-}$ CD4^{+} T cells increased even further after in vitro stimulation of MOG35-55-sensitized splenocytes (Fig. 1*C Lower*), suggesting that the lack of IL-6 signaling in T cells is sufficient to skew the immune response toward the expansion of Foxp3^{+} T-regs at the expense of Th17 cells. When tracking antigen-specific Foxp3^{+} T-regs by staining with MOG35-55/IAb tetramers, we found that the fraction of MOG35-55/IAb-reactive Foxp3^{+} T-regs in the compartment of activated CD4^{+} T cells was also increased in *gp130*$^{-/-}$ mice compared with their wild-type counterparts (Fig. 1*D*). Thus, lack of responsiveness to IL-6 seems to promote the generation (or expansion) of antigen-specific Foxp3^{+} T-regs despite the presence of an inflammatory milieu in vivo.

Both IL-6 and IL-21 are, together with TGF-β, capable of inducing Th17 cells. Therefore, we wanted to know whether the induction of Th17 cells would occur in the complete absence of IL-6 signaling in T cells. Naïve CD4^{+} T cells from wild-type and *gp130*$^{-/-}$ mice were purified by flow cytometry and differentiated in vitro in the presence of TGF-β plus IL-6 or TGF-β plus IL-21. Whereas wild-type cells responded to both cytokine mixtures by becoming Th17 cells, the induction of Th17 cells was abrogated in *gp130*$^{-/-}$ T cells in response to TGF-β plus IL-6 (Fig. 2*A*). However, TGF-β plus IL-21 induced Th17 cells as efficiently in *gp130*$^{-/-}$ T cells as in wild-type T cells (Fig. 2*A*), suggesting that the combination of TGF-β plus IL-21 is operational independently of IL-6-mediated signal transduction. To test this hypothesis in vivo, we treated *gp130*$^{-/-}$ mice with a control antibody or depleted them of CD4^{+}CD25^{+}Foxp3^{+} T-regs by means of a monoclonal antibody against CD25. This system allowed us to investigate the induction of pathogenic T cell populations in vivo in the absence of an exaggerated T-reg response that confounds the induction of effector T cell populations. Control antibody-treated *gp130*$^{-/-}$ mice were resistant to EAE; however, T-reg-depleted *gp130*$^{-/-}$ mice developed EAE with kinetics and severity similar to wild-type control animals (Fig. 2*B*). Despite the absence of IL-6 signaling, T-reg-depleted *gp130*$^{-/-}$ mice not only developed EAE, but also mounted a Th17 response both in the peripheral immune compartment and the CNS (Fig. 2*C*), suggesting that IL-6 signaling is dispensable for the induction of pathogenic Th17 responses in vivo, at least under conditions of reduced T-reg levels.

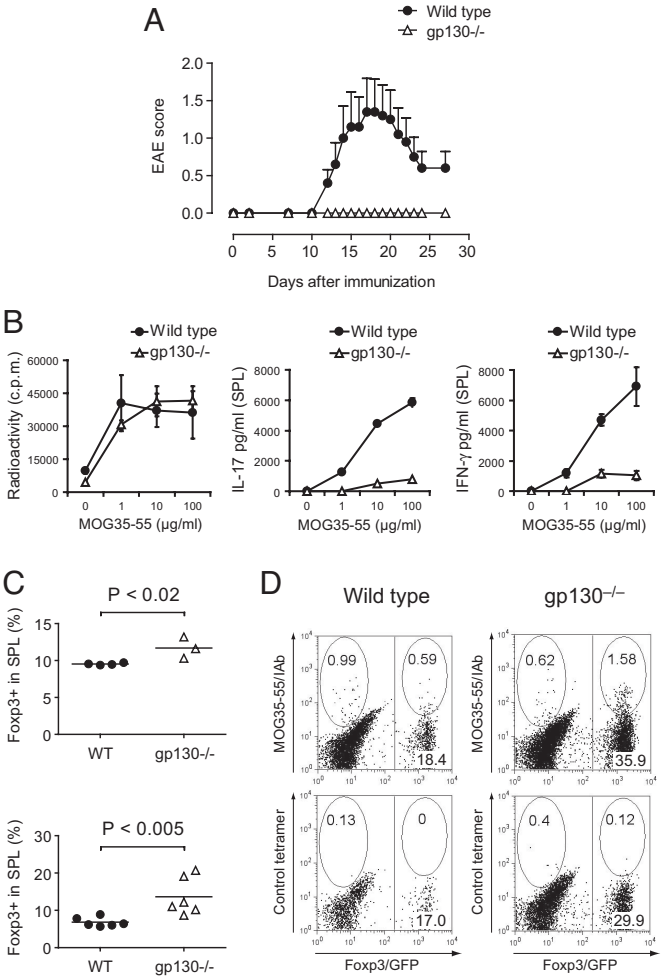

Fig. 1. Unresponsiveness of T cells to IL-6 confers resistance to EAE caused by lack of Th17 cells and an increased T-reg response. (*A*) Wild-type or T cell-conditional *gp130*$^{-/-}$ mice on the *Foxp3gfp*.KI background were immunized with MOG35-55/CFA plus pertussis toxin and followed for signs of EAE (mean clinical score ± SEM, $n = 10$). (*B*) On day 10 after immunization, splenocytes were isolated and restimulated with MOG35-55 in vitro. The proliferative response was measured by [^{3}H]thymidine incorporation, and the cytokine production in 48-h culture supernatants was determined by ELISA. Mean of triplicate cultures is shown. (*C*) Frequency of Foxp3^{+} T-regs in the splenic CD4^{+} T cell compartments of MOG35-55/CFA-immunized wild-type and *gp130*$^{-/-}$ mice as determined by the expression of Foxp3/GFP ex vivo (*Upper*) and after in vitro restimulation with MOG35-55 (*Lower*). (*D*) MOG35-55/IAb tetramer staining in MOG35-55-stimulated splenocytes from in vivo-sensitized wild-type and *gp130*$^{-/-}$ mice. The splenocytes were isolated on day 11 after immunization with MOG35-55/CFA followed by restimulation in vitro for 4 days. The gate was set on blasting CD4^{+} T cells. Representative cytograms are shown.

Immunization with Incomplete Freund's Adjuvant (IFA) Fails to Induce IL-6 and Th17 Cells but Induces Antigen-Specific T-Regs. Emulsion of protein and peptide antigens in IFA has commonly been described to result in a Th2 type of response (17). We postulated that exposure of wild-type mice to antigen emulsified in IFA would prevent IL-6 production and could therefore lead to the generation of Foxp3^{+} T-regs. Indeed, in contrast to immunization with MOG35-55/CFA, IL-6 was not induced when C57BL/6 mice were immunized with MOG35-55/IFA (Fig. 3*A*). Furthermore, immunization with MOG35-55/IFA, in contrast to immunization with MOG35-55/CFA, did not trigger antigen-specific production of

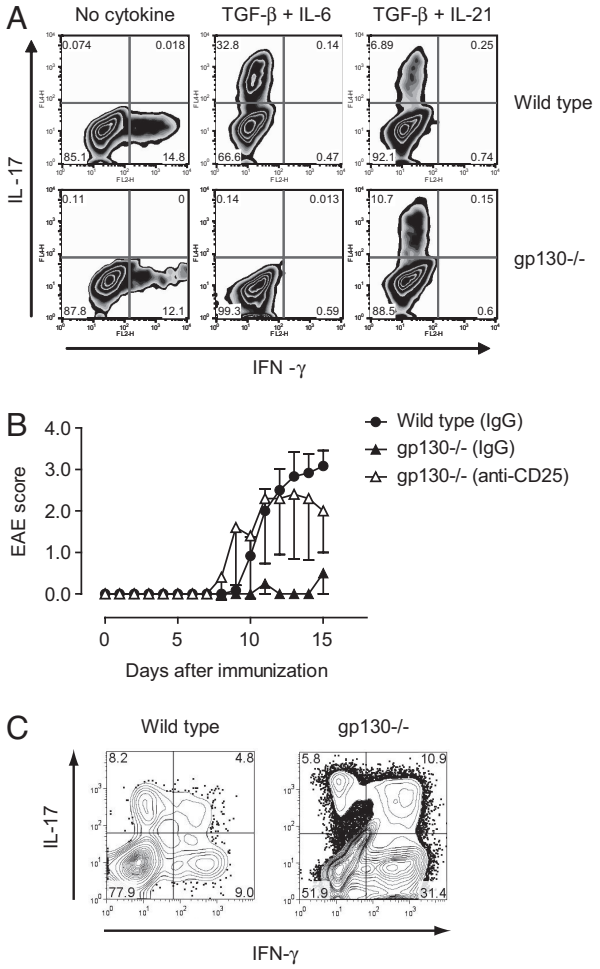

Fig. 2. The combination of TGF-β plus IL-21 induces Th17 cells in gp130$^{-/-}$ mice. (*A*) Naïve T cells were purified from wild-type or *gp130*$^{-/-}$ mice and differentiated in vitro with either TGF-β plus IL-6 or TGF-β plus IL-21. The frequency of IL-17- and IFN-γ-positive T cells was determined by intracellular cytokine staining. (*B*) *gp130*$^{-/-}$ mice were either treated with control IgG ($n = 3$) or depleted of T-regs by treatment with a monoclonal antibody to CD25 (PC61, 2 × 0.5 mg) (8) 5 and 3 days before immunization with MOG/CFA ($n = 5$). As further control group, T-reg-competent wild-type mice were included ($n = 6$). The mean EAE score of each group is shown. Data represent 1 of 3 independent experiments. (*C*) At the peak of disease, mononuclear cells were isolated from the CNS of wild-type animals and T-reg-depleted *gp130*$^{-/-}$ mice followed by stimulation with PMA/ionomycin and intracellular cytokine staining for IL-17 and IFN-γ. The numbers in the quadrants of the cytograms indicate percentages of cytokine-positive cells in the CNS-derived CD4$^+$ T cell compartment. One representative experiment is shown. Because *gp130*$^{-/-}$ mice that were not depleted of T-regs did not develop EAE, the T cellular infiltrate into the CNS of these mice was insufficient to perform intracellular cytokine staining.

IFN-γ and IL-17 (Fig. 3*A*). This finding confirmed and extended earlier results showing that in the absence of *Mycobacterium tuberculosis* extract as adjuvant, immunization of wild-type mice failed to induce productive Th1 responses (17). We did not observe a measurable induction of IL-4 or IL-10 (data not shown), suggesting that there was no strong skewing toward a Th2 type of response. We then tracked antigen-specific T cells in the Foxp3/GFP$^-$ (effector T cell, T-eff) and the Foxp3/GFP$^+$ (T-reg) compartment in *Foxp3gfp*.KI mice by using MOG35-55/IAb tetramer staining. Interestingly, we found that in contrast to immunization with MOG35-55/CFA (Fig. 3*B*), immunization with MOG35-55/IFA led to the preferential expansion of MOG-specific Foxp3$^+$ T-regs and only insufficiently supported the priming/expansion of antigen-specific T-eff cells (Fig. 3*B*).

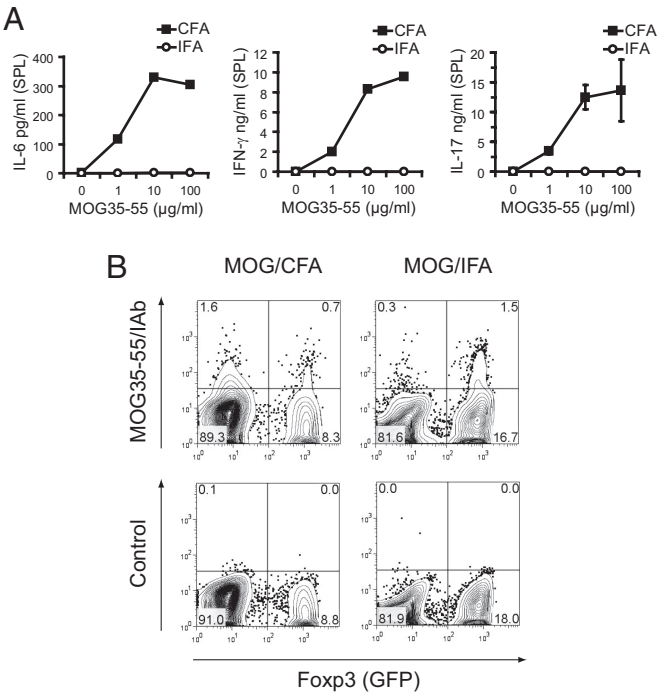

Fig. 3. Immunization with MOG35-55 in IFA results in the generation/expansion of MOG35-55 specific Foxp3$^+$ T-regs and does not support the induction of Th17 cells. Wild-type *Foxp3gfp*.KI mice were immunized with MOG35-55 emulsified in CFA vs. IFA. After 10 days, splenocytes were isolated and stimulated with MOG35-55. Supernatants were analyzed for the indicated cytokines by cytometric bead array (*A*), and the fractions of MOG35-55-specific CD4$^+$ T cells in the Foxp3$^-$ and Foxp3$^+$ compartments were determined by tetramer staining (*B*).

Next, we wanted to differentiate whether the lack of priming/expansion of IFN-γ and IL-17 producing CD4$^+$ T cells upon immunization with IFA was caused by an overwhelming T-reg response. Thus, similarly to the strategy in *gp130*$^{-/-}$ mice, naturally occurring CD4$^+$CD25$^+$ T-regs were depleted by using an anti-CD25 antibody, and the depleted mice were then immunized with IFA. Surprisingly, the number of IFN-γ-producing Th1 cells was greatly increased in T-reg-depleted compared with nondepleted mice immunized with MOG35-55/IFA (Fig. 4 *A* and *B*). However, antigen-specific Th17 cells were not generated in T-reg-depleted MOG/IFA-immunized animals (Fig. 4*C*), and MOG/IFA immunization failed to induce EAE despite the depletion of T-regs (Fig. 4*D*). It has recently been reported that adjuvant-free induction of IFN-γ in vivo appears to be innocuous and potentially even protective in autoimmune diseases (18). Taken together, in contrast to T-reg-depleted *gp130*$^{-/-}$ mice that developed a Th17 response and became susceptible to EAE upon immunization with MOG/CFA, immunization with MOG/IFA failed to induce Th17 cells and EAE in T-reg-depleted wild-type animals. Together, these data suggest that immunization with CFA leads to activation of alternative signaling pathways in T cells that allow for the generation of Th17 cells independently of IL-6/IL-6R signaling, whereas immunization with IFA fails to do so.

Lack of Responsiveness to IL-6 Promotes the Conversion of Conventional CD4$^+$ T Cells into Foxp3$^+$ T-Regs in Vivo. Alternative signaling pathways such as IL-21/IL-21R signaling can substitute for IL-6/IL-6R signaling in inducing Th17 cells. However, IL-6/IL-6R signaling appears to be dominant in inhibiting the de novo induction of Foxp3$^+$ T-regs in vivo. To confirm this hypothesis, CD4$^+$Foxp3$^-$ T cells derived from *Foxp3gfp*.KI mice were adoptively transferred into Rag1-deficient recipients followed by immunization with either MOG35-55/CFA or MOG35-55/IFA. After 20 days, CD3$^+$CD4$^+$ T

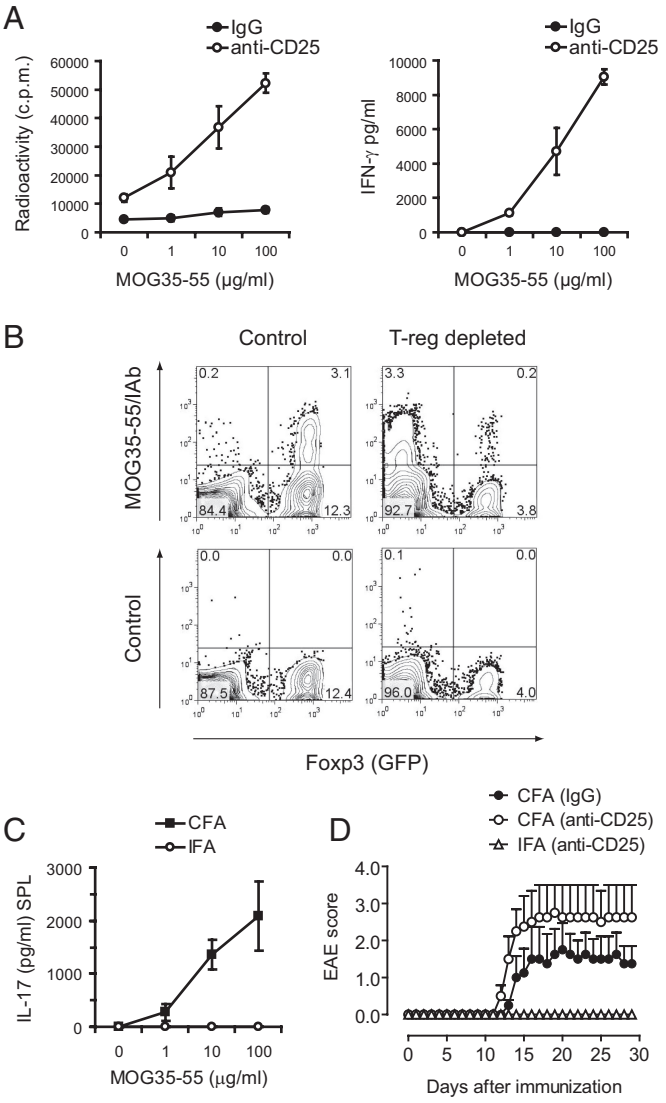

Fig. 4. Immunization with MOG/IFA fails to induce Th17 cells in vivo. (*A*) *Foxp3gfp*.KI mice were treated with control IgG or depleted of $CD4^+CD25^+$ T-regs by i.p. administration of a monoclonal antibody to CD25 followed by immunization with MOG/IFA. Splenic recall cultures were tested for antigen-specific proliferation and cytokine production by ELISA. Mean ± SD of triplicate cultures is shown. (*B*) The fraction of MOG35-55 specific T cells in the $Foxp3^-$ and $Foxp3^+$ compartments of MOG/IFA-immunized control and T-reg-depleted *Foxp3gfp*.KI mice was measured by tetramer staining. (*C*) Wild-type *Foxp3gfp*.KI mice were depleted of T-regs and immunized with MOG/CFA or MOG/IFA. After 10 days, the antigen-specific IL-17 response was tested in splenocytes by ELISA. Mean ± SD of triplicate cultures is shown. (*D*) In a parallel experiment, T-reg-depleted and MOG/CFA ($n = 4$, control) vs. MOG/IFA ($n = 7$) immunized mice were monitored for EAE. Mean clinical score ± SEM is shown.

cells were recovered from the spleens of host mice and tested for the expression of Foxp3. As reported before (16), immunization with CFA blocked the de novo generation of $Foxp3^+$ T-regs (Fig. 5*A*). In contrast, immunization with IFA consistently induced a small but significant fraction of $Foxp3^+$ T-regs (Fig. 5 *A* and *B*). Most importantly, when $CD4^+Foxp3^-$ T cells from *gp130*$^{-/-}$ mice (crossed to *Foxp3gfp*.KI) were transferred, significant conversion of $Foxp3^-$ T cells into $Foxp3^+$ T-regs was observed even after immunization with CFA (Fig. 5 *A* and *B*).

Collectively, these data illustrate that IL-6 signaling in $CD4^+$ T cells blocks the conversion of $Foxp3^-$ into $Foxp3^+$ T-regs. Furthermore, in the absence of IL-6 induction (immunization with

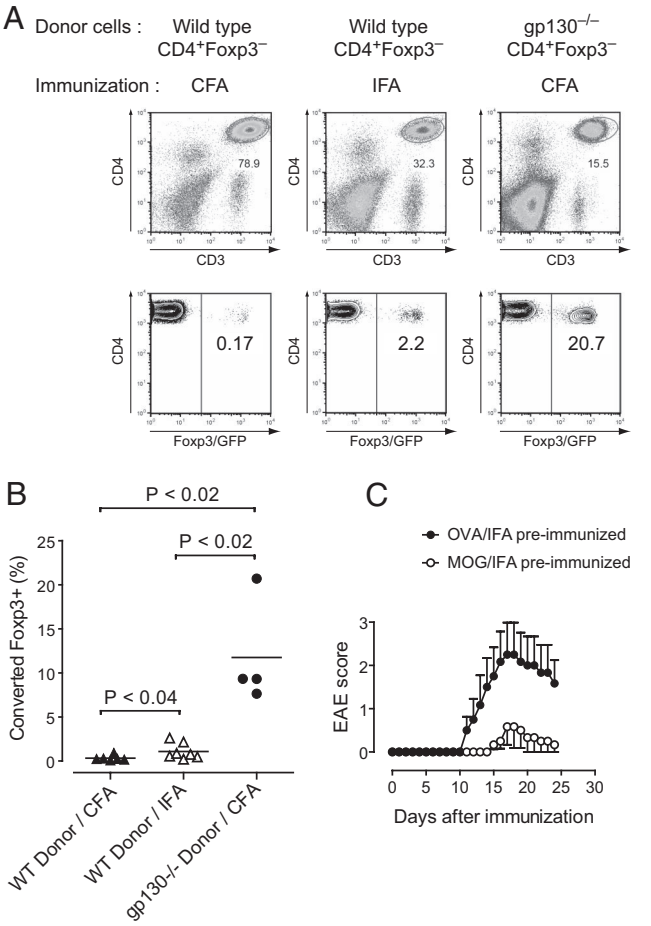

Fig. 5. IL-6 inhibits the conversion of conventional $Foxp3^-$ T cells into $Foxp3^+$ T-regs and blocks the generation of antigen-specific T-regs in vivo. (*A*) $CD4^+Foxp3^-$ T cells from naïve wild-type or *gp130*$^{-/-}$ mice on the *Foxp3gfp*.KI background were purified by flow cytometry and transferred into Rag1-deficient recipients followed by immunization with MOG/CFA or MOG/IFA as indicated. On day 20 after immunization, splenocytes were isolated, and the $CD4^+$ T cell compartment was assessed for expression of Foxp3. (*B*) Fraction of converted $Foxp3^+$ T cells in the transferred wild-type or gp130-deficient T cell populations after immunization with MOG35-55/CFA or MOG35-55/IFA as indicated. Statistical analysis was performed by using Student's *t* test. (*C*) Immunization with MOG35-55/IFA induces antigen-specific tolerance. Wild-type mice were either preimmunized with OVA323–339/IFA (control) or MOG35-55/IFA. After 7 days, both groups were rechallenged with MOG35-55/CFA plus pertussis toxin. Mean clinical score ± SEM ($n = 6$ in each group).

MOG35-55/IFA) or in the absence of IL-6 signaling in $CD4^+Foxp3^-$ T cells (transfer of *gp130*$^{-/-}$ T cells), de novo generation of T-regs does occur in vivo. In *gp130*$^{-/-}$ T cells, the conversion of $Foxp3^-$ T cells into $Foxp3^+$ T-regs cannot be suppressed even upon immunization with MOG/CFA, suggesting that no alternative signaling pathway can substitute for IL-6/IL-6R signaling in inhibiting T-reg conversion.

MOG35-55/IFA Induces Antigen-Specific Tolerance. In contrast to immunization with antigen/CFA where antigen-specific $Foxp3^+$ T-regs are exclusively recruited from preexisting (and expanding) naturally occurring T-regs, immunization with antigen/IFA leads to the establishment of a profound T-reg response that is fueled by conversion of $Foxp3^-$ into $Foxp3^+$ T-regs. To explore whether the induction of antigen-specific T-regs by immunization with MOG35-55/IFA could be exploited in a clinical setting, we compared disease development and clinical course of EAE in mice that were "tolerized" with an irrelevant peptide (OVA323-339) emulsified in IFA

or with MOG35-55/IFA followed by immunization with MOG35-55/CFA. Wild-type mice that did not receive a MOG35-55/IFA injection before immunization with MOG35-55/CFA developed regular EAE with paralysis of the hind limbs (OVA/IFA-preimmunized group, Fig. 5*C*). In contrast, most of the animals that were administered MOG35-55/IFA 1 week before rechallenge with MOG35-55/CFA were protected from EAE (Fig. 5*C*). In addition to the markedly reduced incidence, those animals in the MOG/IFA-tolerized group that nevertheless developed disease had a delayed onset of EAE and a milder disease course resulting in a significantly decreased disease burden. These results further support the idea that MOG35-55/IFA is a potent means to induce antigen-specific tolerance that relies on the de novo induction of antigen-specific Foxp3$^+$ T-regs.

Discussion

In this work, we investigated the role of IL-6 in the lineage decision of antigen-specific CD4$^+$ T cells during an autoimmune response in vivo. We found that unresponsiveness to IL-6 restricted to T cells is sufficient to mount a massive T-reg response in vivo that prevents the induction of Th1 and Th17 effector cells and results in complete resistance to EAE. However, the failure to induce Th17 cells in *gp130*$^{-/-}$ mice is not caused by an intrinsic inability of *gp130*$^{-/-}$ CD4$^+$ T cells to become Th17 cells because the combination of TGF-β plus IL-21 induced the expression of IL-17 in naive *gp130*$^{-/-}$ CD4$^+$ T cells. Also, T-reg-depleted *gp130*$^{-/-}$ mice were able to mount a pathogenic Th17 response upon immunization with MOG/CFA in vivo. Thus, alternative pathways exist to induce Th17 cells in the absence of IL-6 signaling. However, IL-6 has a dual role because it also suppresses the induction of Foxp3. Here, IL-6/IL-6R signaling in CD4$^+$ T cells constitutes a dominant pathway because in the absence of IL-6R signaling but in the presence of an intact IL-21/IL-21R system, the induction of Foxp3 was still not suppressed, and the mice developed an overwhelming T-reg response even if CFA was used as an adjuvant. Consistent with these findings, we show that immunization of wild-type mice with autoantigen in IFA fails to induce IL-6 and promotes the development of antigen-specific T-regs instead of antigen-specific effector T cells. This immunization protocol can be used to induce antigen-specific tolerance protecting from EAE.

IL-6 is a potent factor to switch immune responses from the induction of Foxp3$^+$ T-regs to pathogenic Th17 cells in vivo. There is accumulating molecular evidence that a single naïve T cell can develop into both a functional T-reg cell and an IL-17-producing T cell (19). TGF-β is necessary to induce the expression of both Foxp3, the master transcription factor of T-regs, and ROR-γt, the essential transcription factor of Th17 cells (20). Although necessary for the expression of both Foxp3 and ROR-γt, TGF-β enhances the function of Foxp3 but inhibits the function of ROR-γt (20). Only when additional signaling of "proinflammatory" cytokines such as IL-6 or IL-21 is operational, the TGF-β-mediated functional inhibition of ROR-γt is released, and Th17 cells are induced. Here, we show that after T-reg depletion, the development of Th17 cells is possible in the absence of IL-6 signaling, suggesting that other factors can compensate for IL-6 effects in inducing Th17 cells. It has recently been shown that STAT3, ROR-γt, and ROR-α are required to induce IL-17 in T cells (21–23). Although IL-6 and IL-21 use totally unrelated receptors, both recruit STAT3 as downstream signaling molecule (24). Thus, IL-21R signaling can bypass defects in IL-6R signaling and induce Th17 cells. STAT3 is also necessary and might even be sufficient to inhibit Foxp3 because STAT3-deficient T cells show excessive induction of Foxp3 when activated in the presence of TGF-β plus IL-6 (25 and data not shown). However, in the case of a deficient IL-6R system, the induction of Foxp3 cannot be suppressed either, and Foxp3$^+$ T-regs are massively induced, suggesting that activation of STAT3 by other factors such as IL-21 is qualitatively or quantitatively insufficient to compensate for IL-6 in the inhibition of Foxp3 induction and the generation of functional T-regs in vivo. We conclude that IL-6/IL-6R (gp130)/STAT3 signaling has a dominant function in the suppression of Foxp3 in vivo. This idea is supported by the fact that under conditions of high availability of IL-6, IL-21R KO mice do not exhibit enhanced induction of T-regs and are susceptible to EAE (26).

Collectively, these data illustrate why IL-6 is pivotal in dictating the balance between induced T-regs and Th17 cells in vivo and show that the de novo generation of Foxp3$^+$ T-regs actually occurs in the secondary lymphoid compartment in the absence of IL-6. Blockade of IL-6 signaling seems to be a promising strategy to control autoimmune responses, and a recent report confirmed that preventive administration of a monoclonal antibody to IL-6R that is already successfully used in juvenile idiopathic rheumatoid arthritis abrogates the buildup of inflammation in EAE caused by a decreased Th17 response (27). Interestingly, immunization with MOG/IFA provides an antigenic stimulus but fails to induce IL-6. IFA has long been known to induce "unresponsiveness" of T cells. However, the potential underlying mechanisms were poorly defined. On one hand, passive mechanisms such as anergy induction and deletion of autoreactive T cells were discussed (28). However, active mechanisms like immune deviation toward a Th2 type of response (17) and induction of regulatory T cells (29, 30) were reported. Active mechanisms of tolerance induction by immunization with antigen/IFA were supported by the possibility of transferring protection from the development of autoimmune disease to naïve host animals by adoptive transfer of T cells from IFA-immunized donor animals (30). We revisited this issue by using a unique combination of tools including *Foxp3gfp*.KI reporter mice and a MOG35-55/IAb tetramer to track well-defined Foxp3$^+$ regulatory T cells. Our data are consistent with early observations by Swanborg and colleagues (29, 30) who described the induction of "suppressor cells" in the peripheral immune compartment of MBP/IFA-immunized rats and in a later report suggested that these suppressor cells might use TGF-β to keep potentially autoreactive encephalitogenic T cells in check. It is likely that the suppressor cells described by Swanborg and colleagues are identical to antigen-specific Foxp3$^+$ T-regs that are overwhelmingly induced by immunization with antigen/IFA. In the present work, we also define that the mechanism by which IFA induces T-regs is conversion of Foxp3$^-$ into Foxp3$^+$ T cells. We demonstrate that lack of responsiveness to IL-6 in T cells or the failure to induce IL-6 is necessary and sufficient to promote this conversion. This sheds light on the mechanism of how conversion of Foxp3$^-$ T cells into Foxp3$^+$ T-regs might take place in vivo and explains why this phenomenon can be observed "under noninflammatory" conditions (31).

In conclusion, these findings have an important impact on the attempt to generate adaptive antigen-specific Foxp3$^+$ T-regs and skew immune responses for therapeutic applications in vivo. Indeed, as soon as IL-6 production or signaling is blocked, immunogenic vaccination protocols are likely to be converted into tolerizing regimens in that exposure to antigen in the absence of IL-6 promotes the induction of antigen-specific Foxp3$^+$ T-regs.

Materials and Methods

Animals. Foxp3gfp KI mice were generated as described (7, 16). CD4-Cre$^{+/+}$ mice and gp130flox/flox mice were provided by W. Müller (Faculty of Life Sciences, University of Manchester, UK) (32) and bred onto the *Foxp3gfp*.KI background. Because CD4-Cre deletes in all T cells when they are at the double-positive stage in thymic development, CD4-Cre$^{+/+}$ × gp130$^{flox/flox}$ mice lack gp130 in all T cells. All animals were on pure C57BL/6 background. Animals were kept in a conventional, pathogen-free facility at the Harvard Institutes of Medicine (Boston, MA), and all experiments were carried out in accordance with the guidelines prescribed by the standing committee of animals at Harvard Medical School, Boston.

Induction of EAE and Adoptive Transfer Experiments. EAE was induced by s.c. immunization of mice into the flanks with 100 μL of an emulsion of 100 μg of MOG35-55 peptide (MEVGWYRSPFSRVVHLYRNGK) and 250 μg of *M. tuberculo-*

sis extract H37 Ra (Difco) in adjuvant oil (CFA). In addition, the animals received 200 ng of pertussis toxin (List Biological Laboratories) i.p. on days 0 and 2. Where indicated, MOG35-55 was emulsified in IFA (without *M. tuberculosis* extract). Mice immunized with MOG35-55/IFA did not receive pertussis toxin. Clinical signs of EAE were assessed according to the following score: 0, no signs of disease; 1, loss of tone in the tail; 2, hind limb paresis; 3, hind limb paralysis; 4, tetraplegia; 5, moribund.

In the adoptive transfer experiments, recipient Rag1-deficient animals received 2×10^6 flow cytometrically sorted $CD4^+Foxp3^-$ T cells from naïve wild-type or *gp130*$^{-/-}$ donor mice i.p. in 0.5 mL of sterile PBS. The host mice were checked for proper reconstitution of CD4 T cells in the peripheral blood on day 10 after transfer and immunized s.c. with MOG/CFA plus pertussis toxin vs. MOG/IFA on day 20 after transfer. Three weeks later, splenocytes were isolated and tested for the expression of Foxp3/GFP by flow cytometry.

T Cell Proliferation and Differentiation. Cells were cultured in DMEM/10% FCS supplemented with 5×10^{-5} M 2-mercaptoethanol, 1 mM sodium pyruvate, nonessential amino acids, L-glutamine, and 100 units of penicillin and 100 μg of streptomycin per ml. In antigen-specific recall assays, 2.5×10^6/ml splenocytes or draining lymph node cells were cultured in round-bottom wells for 72 h with the indicated concentration of MOG35-55 without the addition of IL-2. During the last 16 h, cells were pulsed with 1 μCi of [3H]thymidine (PerkinElmer) followed by harvesting on glass fiber filters and analysis of [3H]thymidine incorporation in a β-counter (1450 Microbeta, Trilux, PerkinElmer).

For in vitro T cell differentiation, $CD4^+$ cells from naïve splenocytes and lymph node cells were isolated by using anti-$CD4^+$ beads (Miltenyi) and further purified by flow cytometry into $CD4^+CD62L^{high}Foxp3/GFP^-$ T cells. T cells were stimulated for 3 days with plate-bound antibody to CD3 (145-2C11, 4 μg/ml) plus soluble antibody to CD28 (PV-1, 2 μg/mL) or by soluble anti-CD3 (2 μg/mL) in the presence of irradiated syngeneic splenocytes as antigen-presenting cells. Where indicated, the medium was supplemented with recombinant cytokines (R&D Systems): human TGF-β1 (3 ng/mL), mouse IL-6 (30 ng/mL), and mouse IL-21 (100 ng/mL).

Cytokine Production. Culture supernatants were collected after 48 h, and cytokine concentrations were determined by ELISA or by cytometric bead array (BD Biosciences) according to the manufacturer's instructions.

MHC Class II IAb Construct and Generation of Soluble MHC Class II Molecules and IAb Multimeric Complexes. Generation of the cDNA constructs encoding the IAb α- and β-chains of the MOG35-55/IAb monomer and staining with MOG35-55/IAb tetramers have been described (16, 33). Briefly, MOG35-55-stimulated primary spleen or lymph node cells were incubated with IAb tetramers (30 μg/mL) in DMEM supplemented with 2% FCS (pH 8.0) at room temperature for 2.5 h. The percentage of tetramer$^+$ cells was determined in the CD4 gate of live (7-AAD$-$) cells. To control for unspecific binding, IAs control tetramers were used (33). Stained cells were analyzed on a FACSCalibur machine (BD Biosciences), and data analysis was performed by using FlowJo software (Tree Star, version 6.3.3).

Intracellular Cytokine Staining. For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma), ionomycin (1 μg/mL; Sigma), and monensin (GolgiStop 1 μL/mL; BD Biosciences) at 37 °C/10% CO_2 for 4 h. After staining of surface markers (CD4), cells were fixed, permeabilized, and stained for intracellular cytokines by using Cytofix/Cytoperm and Perm/Wash buffer and antibodies to mouse IL-17 and IFN-γ (BD Biosciences) according to the manufacturer's instructions.

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Appendix VI

REVIEW

CNS Neuroscience & Therapeutics

The Combination of Interferon-Beta and HMG-CoA Reductase Inhibition in Multiple Sclerosis: Enthusiasm Lost too Soon?

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Keywords
Immunomodulatory therapy; Interferon-beta; Multiple sclerosis; Statin.

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SUMMARY

Recent studies support the notion that statins, widely prescribed cholesterol-lowering agents, may target key elements in the immunological cascade leading to inflammation and tissue damage in the pathogenesis of multiple sclerosis (MS). Compelling experimental and observational clinical studies highlighted the possibility that statins may also exert immunomodulatory synergy with approved MS drugs, resulting in several randomized clinical trials testing statins in combination with interferon-beta (IFN-β). Some data, however, suggest that this particular combination may not be clinically beneficial, and might actually have a negative effect on the disease course in some patients with MS. In this regard, a small North American trial indicated that atorvastatin administered in combination with IFN-β may increase disease activity in relapsing-remitting MS. Although other trials did not confirm this finding, the enthusiasm for studies with statins dwindled. This review aims to provide a comprehensive overview of the completed clinical trials and reports of the interim analyses evaluating the combination of IFN-β and statins in MS. Moreover, we try to address the evident question whether usage of this combination routinely requires caution, since the number of IFN-β-treated MS patients receiving statins for lowering of cholesterol is expected to grow.

Introduction

Multiple sclerosis (MS) is considered a chronic autoimmune disease with complex genetic background in which autoreactive T cells infiltrate the central nervous system (CNS) and initiate inflammatory and destructive processes leading to permanent neurological disability [1]. In light of the initial clinical manifestation in early adulthood, uncertainty of prognosis, and limited impact of disease-modifying drugs (DMD) on disability progression, this, often devastating, disease poses a significant burden on patients, families, and caregivers. Over the years, several DMDs have been approved for the treatment of MS, including interferon-beta-(IFN-β)-1a (Avonex®, Rebif®), IFN-β-1b (Betaseron/Betaferon®), glatiramer-acetate (GA; Copaxone®), mitoxantrone (Novantrone®), and natalizumab (Tysabri®). Although the arsenal of treatment options is constantly growing, insufficient response in a subgroup of patients, considerable side effects, and tedious regular and parenteral application have been challenging for some patients [2]. Thus, one strategy to increase efficacy is the combination of partially effective agents [3].

Statins are orally administered inhibitors of the 3-hydroxy-3-methyl-glutaryl (HMG)-CoA reductase, an enzyme that catalyzes the rate-limiting step of cholesterol biosynthesis (Figure 1). These substances are well established in the treatment of cardiovascular disease and have attracted significant interest in autoimmune disorders due to an expanding knowledge of additional immunomodulatory, antiinflammatory, and neuroprotective effects. Indeed, both *in vivo* and *in vitro* experiments demonstrated pleiotropic effects on the immune system

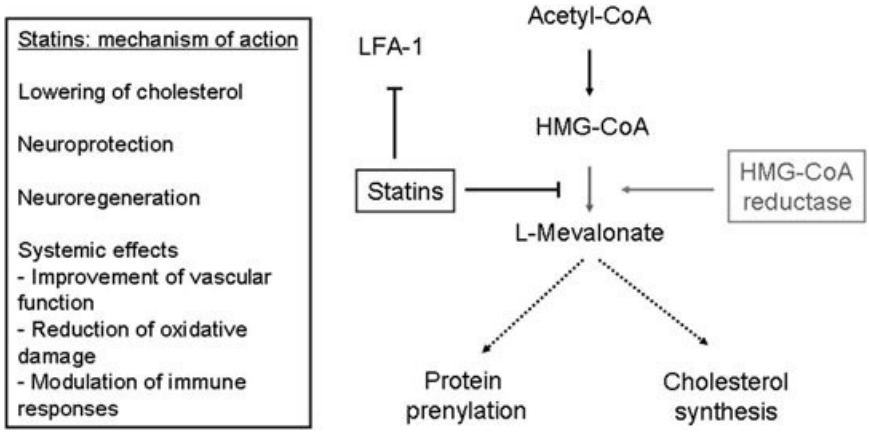

Figure 1 Statins exhibit different mechanisms of action by interfering with cholesterol synthesis and protein prenylation. Statins inhibit the conversion of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to L-mevalonate through competitive inhibition of the rate-limiting enzyme HMG-CoA reductase. This inhibition results in a decrease in the downstream biosynthesis of cholesterol and other intermediate metabolites. The latter are involved in the isoprenylation of proteins which serve as essential adjuncts in the posttranslational modification of numerous key proteins including Ras, Rac, Rab, cdc42, RhoB, and Rho. Less cholesterol also impairs the lipid raft formation and thus has impact on expression of molecules on the cell surface and cell proliferation.

that might be beneficial in the treatment of MS [4–6]. In this regard, two small open-label trials involving a total of 35 patients with relapsing-remitting MS (RRMS) confirmed that a statin monotherapy is safe and indicated potential efficacy on short-term clinical and magnetic resonance imaging (MRI) measures. The first study (2003), in which 7 RRMS patients were treated with 40 mg lovastatin for 12 months noticed a decrease of the mean annual relapse rate and no adverse events [7]. In the second study (2004) the 80 mg simvastatin treatment for 6 months was associated with a lowering of mean number (−44%) and volume of contrast-enhancing lesions (CELs) by (−41%) compared to pretreatment scans [8]. A very recent double-blind, placebo-controlled trial evaluated atorvastatin in prevention of progression from clinically isolated syndrome (CIS) to MS [9]. While the primary endpoint with development of >3 new T2 lesions or one clinical exacerbation by 12 months was not met, patients in the atorvastatin group were more likely to remain T2 lesion-free compared with placebo (odds ratio 3.93; $P = 0.012$). Based on these findings, considerable enthusiasm developed to also investigate the effect of statins in combination with IFN-β. Unexpectedly, combination trials to date generated preliminary data indicating that the concomitant administration of statins and IFN-β may not provide a superior efficacy over IFN monotherapy. In this regard, a placebo-controlled randomized study in which 28 RRMS patients were treated with 40 or 80 mg atorvastatin for 6 months in combination with high-dose IFN-β-1a even suggested a potential increase of clinical and MRI activity [10]. Evidence for a divergent action of the two substances on immune mechanism had already been shown *in vitro* [5,11]. The investigators presented further data, which suggested that statins interfere with the phosphorylation of the transcription factor STAT1, which also medicates the transcription of interferon beta response genes [12,13]. Based on these observations, a lively discussion emerged whether further combination studies of IFN-β and statins should be halted. Remarkably, interim and final reports of additional combination studies were presented since then and could not confirm potential adverse effects on clinical and MRI measures (Table 1).

This review provides a comprehensive overview on the current knowledge of statin-IFN-β combination therapy in patients with MS. Specifically, clinical trials and potential obstacles of this combination therapy will be discussed.

Statins Are Well-Tolerated Oral Agents with Immunomodulatory and Neuro-Protective Properties

The most common side effects of statins are gastrointestinal symptoms and muscle ache. Hepatotoxicity, indicated by increases in serum amino transaminase levels, occurs in less than 1% of patients even at high dosages, but the risk of liver toxicity and rhabdomyolysis increases under combination therapies [20]. Other side effects include myopathy, rash, peripheral neuropathy, insomnia, and cognitive problems. Systemic HMG-CoA inhibition was

Table 1 Lineup of clinical studies evaluating effects interferon-β (IFN-β) in combination with statins in patients with clinically isolated syndrome (CIS) and relapsing-remitting multiple sclerosis (RRMS)

	First author, year of publication	Study type	Patients	Allocation	Interferon-β (IFN-β)	Statin and dosage per day	Primary endpoint	Secondary endpoints
Original articles								
1	Paul F et al. [14], 2008	Phase II	RRMS	IFN-β + statin (n = 16), statin (n = 25)	IFN-β-1a 22 μg s.c. thrice weekly or IFN-β-1b s.c. every other day	Atorvastatin 80 mg	CEL at months 6–9: decrease/trend for combitherapy in number and volume of CEL	Changes in EDSS and MSFC: not stated
2	Birnbaum G et al. [10], 2008	Safety study	RRMS	IFN-β (n = 9), IFN-β + statin (n = 17)	IFN-β-1a 44 μg s.c. thrice weekly	Atorvastatin 40 mg (n = 7) and 80 mg (n = 10)	EDSS change, CEL or new lesion: greater clinical and MRI disease activity for patients on combitherapy	
3	Rudick RA et al. [15], 2009	Post-hoc analysis of other trial (SENTINEL)	RRMS	IFN-β (n = 542), IFN-β + statin (n = 40)	IFN-β-1a 30 μg i.m. once weekly	Most frequently atorvastatin (65%) and simvastatin (32.5%)	Annualized relapse rate, disability progression, number CEL, number of new/enlarging T2-lesions after 2 years: no differences	
4	Lanzillo R et al. [16] 2010	Open-label randomized study	RRMS	IFN-β (n = 24), IFN-β + statin (n = 21)	IFN-β-1a 44 μg s.c. thrice weekly	Atorvastatin 20 mg	Number of CEL after 24 months: reduction comparable between the groups. Combitherapy; significantly reduced when compared to baseline	Relapse rate: significantly lower for combitherapy. EDSS and laboratory data: no difference
Communications								
1	Sörensen PS et al. [17] 2007	Safety study, interim analysis	RRMS	Total (n = 8), IFN-β, IFN-β + statin	IFN-β-1a 30 μg i.m. once weekly	Simvastatin 80 mg	First time to documented relapse after a mean of 6.9 months: no differences	Relapses, new/enlarging T2-lesions: n.c.
2	Markovic-Plese et al. [18] 2007	Safety study	CIS	IFN-β (n = 9), IFN-β + statin (n = 10)	IFN-β-1a 30 μg i.m. once weekly	Simvastatin 80 mg	Clinical and MRI activity: no differences	
3	Oztekin NS et al. 2009 [19]	Preliminary data at 18 months (of 24)	RRMS	IFN-β (n = 11), IFN-β + statin (n = 7)	IFN-β-1a 44 μg s.c. thrice weekly	Atorvastatin 20 mg	MRI activity: comparable between the groups	Relapses, EDSS, safety laboratory data: n.c.

RRMS, relapsing-remitting MS; CEL, contrast-(Gadolinium) enhancing lesions; EDSS, expanded disability status scale; MSFC, multiple sclerosis functional composite score; s.c., subcutaneous application; i.m., intramuscular application; n.c., not communicated.

shown to affect brain cholesterol production but not brain cholesterol content [21]. Moreover, due to the long half-life of brain cholesterol, only extended usage of statins was able to reduce cholesterol levels in the cerebrospinal fluid (CSF) [22]. It should also be noted that cholesterol is an indispensable component of myelin membranes and cholesterol availability in oligodendrocytes is a rate-limiting factor for brain maturation [23].

The source of effector mechanisms on the immune system can be generally divided into HMG-CoA reductase

dependent and independent pathways [24–26]. To this end statins were shown to exert neuroprotection by activating neuroprotective-signaling pathways and as a consequence of different systemic effects [27].

HMG-CoA Reductase-Dependent Effects

The majority of statin-mediated effects on the immune system appear to be related to the competitive inhibition of HMG-CoA reductase (Figure 1). The subsequent decrease in the production of its substrate l-mevalonate and its metabolites interferes with gene regulation and posttranslational modification of proteins that are associated with proliferation and differentiation of various cells and tissues. In this regard, the synthesis of isoprenoid metabolites is downregulated, which serve as lipid attachements for a number of intracellular signaling molecules including the GTP-binding proteins Ras, Rac, and Rho [28]. Apart from influencing GTP-binding proteins, isoprenyelation of these molecules also interferes with transcription factors such as nuclear factor (NF); statins have shown to limit TNF-related NF-kB accumulation and the increase of inhibitor IkB [29]. The effects of HMG-CoA reductase inhibition on the immune system were recently summarized in a review by Greenwood and colleagues as follows: there is a decrease of (1) leukocyte motility, (2) antigen uptake, processing, and presentation, (3) leukocyte activation, proliferation, and function, (4) phagocytosis, (5) leukocyte transvascular migration, and (6) endothelial-cell immune function [30] under statin therapy. The effects evident from various experimental studies potentially beneficial in MS include the inhibition of expression and secretion of proinflammatory cytokines, inhibition of major histocompatibility complex (MHC) class II expression on antigen-presenting cells (APCs), and costimulatory molecules, and the suppression of Th1 differentiation. Indeed, simvastatin intake had an inhibitory effect on the differentiation and maturation of dendritic cells from patients with optic neuritis (ON), and selectively reduced T-cell proliferation [31]. Likewise, simvastatin treatment in RRMS was associated with inhibition of peripheral-blood mononuclear cell (PBMC) proliferation, antigen presentation by blocking expression of MHC class II DR molecules in CD14+ monocytes, activation and differentiation of T cells, and attenuation of gene expression of early proinflammatory cytokines via inhibition of T-bet, a master controller of the Th1 cytokine pathway [32]. The effect of statins on Th1 differentiation has been consistent throughout many published experimental autoimmune encephalomyelitis (EAE) studies [4,33–36]. There is also a reduced activation of the transcription factor STAT (signal transducer and activator of transcription)-4, which is required for IL12-dependent Th1-differentiation [4,25]. The induction of an antiinflammatory Th2 phenotype, which is associated with induction and secretion of antiinflammatory Th2 cytokines (IL4, IL5, and IL10) is less consistent and was related to the enhanced activation of STAT6, which is involved in IL4-dependent Th2 differentiation [4]. Most interestingly, atorvastatin was shown to enhance the Th2-promoting effects of glatiramer-acetate in EAE, indicating that a combination of statins with an established immunomodulator may be an exciting concept for future clinical trials [36]. Further studies in MS and healthy controls revealed that simvastatin inhibits Th17 cell differentiation, a recently identified CD4+ T-cell subset supposed to play a critical role in autoimmunity [37]. Immunomodulatory effects of statins on T-cell activation and differentiation were indeed related to inhibition of prenylation of regulatory proteins [28]. Simvastatin however interferes with remyelination by directly impacting oligodendrocyte progenitor cell function and affecting mature oligodendrocyte numbers at immunomodulatory concentration by interference with Ras and Rho signaling [38,39]. Another *in vitro* study revealed that the inhibition of the mevalonate pathway by atorvastatin was associated with reduced length of neurites and ultimatively cell death of primary cortical neurons [40]. In contrast, Paintilla reported that lovastatin promoted myelin repair by inhibition of Rho and augments survival and differentiation of oligodendrocyte progenitors [41,42]. To this end, the combination of IFN-β and atorvastatin lowered serum levels of high-sensitivity C-reactive protein (CRP) in RRMS, pointing at the potential additional antiinflammatory effect of statins [43].

HMG-CoA Reductase-Independent Effects

Among the HMG-CoA reductase-independent effects of statins is the binding and inhibition of β2-integrin leukocyte function antigen 1 (LFA-1), which is also known as αL-β2 or CD11a/CD18. LFA-1 is constitutively expressed on the surface of leukocytes and binds to intercellular adhesion molecule (ICAM-1 or CD54) with subsequent leukocyte recirculation and infiltration of inflamed tissue [44]. Likewise, in EAE, an animal model of MS, treatment with lovastatin lead to reduced immune activation, leukocyte infiltration in the brain and subsequent paralysis [33,45]. *In vitro*, simvastatin was shown to inhibit the expression of ICAM-1 on PBMCs, whereas VLA-4 and LFA-1 were unaltered [5]. Eventually, *ex vivo* treatment with statins impede the migration of monocytes and lymphocytes taken from MS patients across a blood-brain barrier model due to reduced secretion of chemokines CCL2 and CXCL10 by endothelial cells [46].

Neuroprotective Action of Statins

Several systemic effects of statins have been described which are likely to contribute to neuroprotection. These effects include (1) reduction of oxidative damage, (2) improvement of vascular function by regulation of nitric oxide production, inhibition of coagulation, and effects on angiogenesis, and (3) modulation of the peripheral inflammatory response [27,47]. Further observations suggest that statins provide neuroprotection by attenuation of inflammation-induced glutamate/calcium excitotoxicity, an important component of axonal injury in MS [48,49]. In addition, treatment of rodents with statins following brain injury increased neurogenesis and synaptogenesis, most likely via the release of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) [50–52]. Neuroprotective pathways directly involved in statin-mediated neuroprotection are protein kinase B (PKB/Akt) and the Ras-(extracellular-signal-regulated cascade) ERK signaling cascade [35,53,54]. However, several *in vitro* studies indicate that particularly lipophilic statins exert neurotoxic action and induce cell death in neurons and glial cells. Yet, the concentrations required for these effects are not expected in the CNS and were achieved under cholesterol- or LDL-depleted medium, which do not mirror physiological conditions [27].

Clinical Trials Evaluating the Combination of IFN-β and Statins in MS

Previously, various placebo-controlled, randomized clinical trials in CIS and RRMS had shown the positive impact of IFN-β on modifying the disease course, with short-term trials altering relapse rate, disability progression, and MRI measures, and long-term treatment delaying secondary progression [2]. Thus, the interest in the combination of statins with IFN-β was reflected by the aim to improve the efficacy of IFN-β on the one hand and the difficulties to perform treatment trials versus IFN-β in MS on the other. Indeed, placebo-controlled trials in MS have been becoming increasingly difficult to perform since the establishment of immunomodulatory treatment, both for ethical and practical reasons [55]. The precise IFN-β mechanisms of action, however, remain unclear. Several biological effects have been described such as attenuation of proliferation of leukocytes and APCs, the modulation of cytokine and chemokine production toward an antiinflammatory phenotype, and the potential to inhibit T-cell migration across the blood-brain barrier [56].

Numerous MS trials that tested different IFN-βs and statins were presented at European and North American conferences between 2005 and 2009 and reflected the lively interest in evaluating this drug combination in MS. F. Paul and colleagues had published the encouraging results of their phase II trial evaluating 80 mg atorvastatin (40 mg twice daily) with or without additional subcutaneous (s.c.) IFN-β in 41 RRMS patients (n = 16 with comedication). A peculiarity of this study was the inclusion criteria of at least one CEL and the baseline-to-treatment concept, which involved a baseline period of 3 months prior to start of HMG-CoA reductase inhibition, followed by a 9-month treatment duration. A nonsignificant reduction in CEL number and volume in the group receiving the combination was observed in a multivariate analysis, providing further evidence for a potential immunomodulatory synergy. The authors reported that the combined treatment of IFN-β with high-dose atorvastatin was safe and well tolerated in the majority of the patients. A temporary mild elevation of liver enzymes with no consistent timeframe of occurrence after initiation of statin treatment was reported in 16 out of 41 patients. In 5 patients treatment with statins had to be discontinued temporarily, and was resumed after liver enzymes returned to normal. The most frequent side effects, however, were respiratory tract infections including rhinitis, sinusitis, and bronchitis.

The report by Birnbaum and colleagues in 2008 on IFN-β—statin combination therapy, however, lead to a critical rethinking of this therapeutic approach. This double-blind, placebo-controlled trial evaluated 26 RRMS patients who had been clinically stable on IFN-β-1a; the treatment groups consisted of placebo (n = 9) or 40 (n = 7) or 80 mg (n = 10) atorvastatin daily (Table 1). Perhaps unexpectedly, atorvastatin-treated subjects were at greater risk for experiencing either clinical and MRI disease activity relative to controls ($P = 0.019$). Of the 17 patients treated with atorvastatin, 10 developed either new lesions on MRI or had clinical relapses, contrasting 1 in 9 placebo-treated patients. Noteworthy, some relapses occurred after years of stable disease and a cox-proportional hazard model analysis rebutted that group differences in baseline demographics influenced the risk of disease activity. Certainly, the study participants were relatively old (group mean age 38.4, 40.1, and 45.1 years) with a mean disease duration of around 7 years and relatively short time on IFN-β (mean 1.8, 2.0, and 2.2 years, respectively). In this study, no significant changes of liver enzymes and creatine kinase (CK) was found between the three treatment groups, whereas total cholesterol levels were reduced in subjects receiving atorvastatin.

The third study was published in 2009 and represented a post-hoc analysis of the SENTINEL trial, a prospective study which determined the effects of natalizumab plus intramuscular (i.m.) IFN-β 1a in RRMS. The IFN-β-1a arm included 40 patients who received statins to treat

hyperlipidemia; clinical and MRI outcomes of 542 patients who were not treated with statins served as reference. No significant differences were observed between the groups with regard to adjusted annualized relapse rate, disability progression, number of CEL, or number of new or enlarging T2-hyperintense lesions after 2 years. The authors concluded that statin therapy did not affect clinical effects of i.m. IFN-β-1a in RRMS patients. The incidence of muscle-related pain was higher in patients of the statin group. Other commonly reported adverse events of the statin group were fatigue, headache, back or extremity pain, arthralgia, depression, and asthenia.

In the most recent study, the ACTIVE trial by Lanzillo and colleagues, patients with RRMS who continued to have CEL or relapses while on therapy with IFN-β-1a for 12 months were randomized to a combination therapy with 20 mg atorvastatin (n = 21) or remained on IFN-β-1a (n = 24) [16]. The analysis of the primary endpoint, the number of CEL at 24 months, revealed that both groups had a decrease in the number of CEL. The difference between baseline and 24-month follow-up was significant for the combination therapy ($P = 0.007$) but not in the monotherapy group. However, a statistical analysis between the groups did not show differences. Secondary outcome measures were number of relapses, expanded disability status scale (EDSS) variation, and laboratory safety data. Patients treated with the combination therapy had a significantly lower relapse rate ($P < 0.005$), while comparison of the EDSS after 24 months did not show differences between the groups. In either groups laboratory parameters such as CK and liver enzymes remained unchanged, and no muscle pain or cramps were reported. The authors concluded that "low-dose atorvastatin may be beneficial as add-on therapy in poor responders to IFN-β-1a alone."

Information on further combination trials is available but is restricted to interim analyses in abstract form (n = 2) and a letter to the editor (n = 1). Among these three mostly safety trials (Table 1), no major concerns of the IFN-β and statin combination were noted in general and with regard to clinical or MRI outcomes. Taken together, seven trial reports evaluating a combination therapy of IFN-β and statins are available for analysis, even though three need to be regarded as too preliminary being interim study reports and only being published as conference proceedings. Yet, both the study by Paul et al. and Lanzillo et al. suggested a trend for an additive effect on MRI measures, whereas the Rudick et al. trial did not find differences with regard to their outcome parameters. Lanzillo even reported a significantly lower relapse rate with the combination therapy compared to the two prerandomization years. Most importantly, among the trials no further study indicated a potential detrimental effect of this combination. Indeed, in an accompanying editorial, Goldman and Cohen raise the possibility that the results of Birnbaum et al. may be an artifact [57]. This argumentation is based on the small sample size, which could magnify potential group imbalances, differences in compliance with assigned treatment, unblinding, differences in event ascertainment, or outliers in on-study disease activity. Further issues that make comparisons between the trials difficult include the usage of different IFN-β and statins, as well as different statin dosages.

Potential Pharmacological Interference of a Combination Therapy

An immunoregulatory effect by IFN-β in the context of recently described Th17-cell-mediated autoimmune response has been attributed to the STAT1-induced decrease in the frequency of IL17-producing CD4+ cells [58,59]. Accordingly, *in vitro* studies suggested that the increase of clinical and MRI disease activity in the Birnbaum study may have been related to abrogation of IFN-β signaling by statins (Figure 2). This potential loss of therapeutic efficacy was shown in cell culture experiments to be induced by blocking tyrosine phosphorylation of the STAT1 transcription factor (P-Tyr STAT1) by statins, which is essential for type I IFN-(α/β) signaling [13,61].

A study by Zhang et al. evaluating the effects of simvastatin on monocytes derived from MS patients reported the inhibition of IL6 and IL23 and induction of IFN-γ, IL4, and IL27 resulting from increased SOCS3 protein expression and inhibition of STAT1 and STAT3 phosphorylation [62]. Of note, simvastatin inhibited the expansion of Th17 cells *in vitro* but enhanced the differentiation of Foxp3(+) CD4(+) T cells [63]. Additional experiments revealed that in Jurkat cells stimulated with different IFN-β preparations, atorvastatin starts to inhibit P-Tyr STAT1 activation and subsequent IFN-α/β responses after 3 h for a duration of 24 h [12]. The inhibitory effect was more pronounced in monocytes (25–100%) than in T cells (15–40%). Subsequently, amelioration of IFN-β effects by statins was also determined *in vivo* in a subgroup of RRMS patients receiving the combination therapy [12]. Moreover, whether the increase of infectious complications in the German trial are related to an attenuation of antiviral immune responses by statins via the STAT1 signaling pathway remains speculative. To this end, studies evaluating interference of IFN-β signaling by statins are currently only published in abstract form.

In contrast, four independent studies evaluated markers of IFN-β activity including IFN-induced genes MxA and TRAIL and could not confirm a loss of IFN-β signaling in patients cotreated with statins *in vivo* (Table 2)

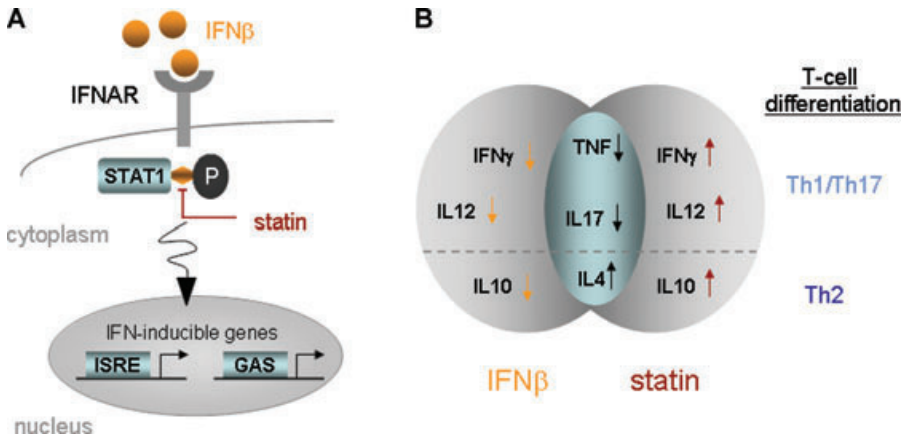

Figure 2 Two potential grounds for attenuation of IFN-β bioactivity by statins. (**A**) Blocking of IFN-β induced phosphorylation of STAT1 and (**B**) differential impact on cytokine secretion and subsequent T-cell differentiation. (**A**) Simplified scheme of the IFN-α/β signaling pathway: binding of IFN-α/β with the receptor complex IFNAR leads to activation of the receptor associated Tyk2 and Janus kinase (Jak1) [60]. This is followed by the tyrosine phosphorylation (P) of STAT1 and STAT2, which can be blocked by statins *in vitro* [12,13]. Activation of the STATs leads to formation of two transcriptional-activator complexes which subsequently activate ISRE and GAS, respectively, in the nucleus. (**B**) IFN-β and statins exert differential impact on modulation of cytokine responses and subsequently T-cell differentiation. A diverse action of IFN-β and statins was shown *in vitro* for Th1 cytokines IFN-γ and IL12 and Th2 cytokine IL10 [5]. IFN, interferon; IFNAR, IFN-α/β receptor; STAT1, member of the Signal transducer and activators of transcription family of transcription factors; ISRE, IFN-stimulated response element; GAS, IFN-γ activated site; IL, interleukin; TNF, tumor-necrosis factor.

[14,15]. Paul et al. even determined supraadditive effects on inhibition of MBP-specific T-cell proliferation *in vitro* but could not confirm these data *in vivo* [14]. Unfortunately, the Birnbaum study did not include an evaluation of IFN-β bioactivity. The other four studies did not show a substantial inhibition of IFN-β signaling by statins *in vivo*.

The immunomodulatory action of statins is reflected by modification of the expression of several molecules crucially implicated in the pathogenesis of MS. Both, similarities and differences of statins and IFN-β with regard to their immunomodulatory actions and potency were observed *in vitro* [5]. These differences particularly refer to an increase of proinflammatory cytokines such as IFN-γ and IL12 and decrease of the antiinflammatory IL10 (Figure 2). While IFN-β-1b reduces and simvastatin increases the expression IFN-γ and IL12 *in vitro* [5], patients on a combination therapy of IFN-β-1b and atorvastatin had significantly increased IL12p70 levels [11]. Likewise, *in vitro* IL10 expression is raised by IFN-β-1b and decreased by simvastatin [5], and a trend for an increase of IL10 serum levels was found *in vivo* by the combination treatment [11]. The role of Th1/Th2/Th17 immunity in EAE has become more apparent during recent years. However, the situation in MS is more complex and the exact role of immunomodulatory treatments such as IFN-β and statins are yet to be determined. The evaluation of soluble CD95 and CD95L confirmed previously described effects by IFN-β and no further alteration by additional treatment with atorvastatin [67]. To this end, it was reported that simvastatin may increase the proteolytic activity MMP9, a protease essential for degradation of the extracellular matrix and subsequent migration of leukocyte to the brain [69,70]. Indeed, treatment with statins increased influx of leukocytes to the inflamed peritoneum [71]. *In vivo* we could confirm that MMP9 activity is attenuated by IFN-β but the net effect is not altered after joint treatment with atorvastatin [66]. Treatment with IFN-β was shown to enhance gene expression of certain chemokines in peripheral blood including CCL1, CCL2, CCL7, CXCL10, CXCL11, and this peripheral upregulation was suggested to reduce chemoattraction of leukocytes to the CNS [72]. Statins, however, were reported to restrict leukocyte migration by attenuation of chemokine secretion (CCL2, CXCL10) by endothelial cells [46].

Hence, many of the *in vitro* findings pointing at a potential interference of IFN-β and statins or a differential action are only partially confirmed *in vivo*. Additional mechanisms may be involved in supraadditive or antagonizing effects and further evaluations are required. Particularly whether certain statins are more likely to affect IFN-β bioactivity due to different pharmacodynamic characteristics and immunomodulatory potency and interfere with physiological and regenerative pathways within the CNS due to lowering of cholesterol are important questions that need to be addressed.

Table 2 *In vivo* evaluation of IFN-β bioactivity and potential alterations in combination with statins

Reference	Measures	Clinical Trial	Specimen	Method	Findings: IFN-β versus IFNβ + statin
Marker of IFN-β activity					
[15]	IFN-stimulated gene	[15]	PBMC	cDNA macroarray	No differences
[14]	TRAIL	[14]	PBMC	rtPCR	No alteration by atorvastatin
[17]	MxA, TRAIL	[17]	PBMC	Affymetrix gene chip	No differences
[64]	IFN-β induced genes	[18]	PBMC	Affymetrix gene chip	No differences
Modulation of immune system					
[14]	TNF, IFN-γ, IL4, IL10	[14]	Supernatant of ConA-stimulated PBMC	Multiplex bead array	Atorvastatin: increase of IL10
[11]	IL1β, IL2, IL6, IL12p70, TNF, IFN-γ, IL4, IL5, IL10	[65]	Serum	Multiplex bead array	combination: increase of IL12p70
[66]	MMP9, TIMP1	[65]	Serum	ELISA	No differences
[67]	soluble Fas (CD95), soluble FasL (CD95L)	[65]	Serum	Multiplex bead array	No differences
[14]	T-cell proliferation	[14]	PBMC	^{3}H thymidine incorporation assay	Atorvastatin: no anti-proliferative effect
Leukocyte migration					
[68]	Transendothelial migration	[68]	T cells	*In vitro* BBB model	Combination therapy: migrational capacity decreases
[46]	Transendothelial migration	treatment *ex vivo*	Monocytes/ lymphocytes	*In vitro* BBB model	Statin treatment *ex vivo*: restricts migration
Antiinflammatory effects					
[43]	High sensitivity CRP	[65]	Serum	ELISA	Combination therapy: reduces hs-CRP

BBB, blood-brain barrier**;** ConA, concanavalin A; CRP, C-reactive protein; TRAIL, TNF-related apoptosis-inducing ligand; TNF, tumor necrosis factor; hs, high sensitivity; IFN, interferon; IL, interleukin; MMP, matrix-metalloproteinase; PBMC, peripheral-blood mononuclear cells; TIMP, tissue inhibitor of MMP.

Treatment of Hyperlipidemia in Patients with MS

At this time, no clear statement can be made on the value of statins as potential DMDs in MS. However another important issue is certain to emerge in clinical practice. Treatment of hyperlipidemia is an essential component of primary and secondary prevention of cardiovascular events and can be achieved through HMG-CoA reductase inhibition. Hypercholesterolemia is among the most frequent comorbidities in MS (37%) [73] and a substantial proportion of these MS patients will require pharmacological treatment for lowering cholesterol with statins. In many patients, lowering of cholesterol will likely be in concert with IFN-β. Based on all available data there is no rationale to stop IFN-β in these patients but a higher rate of adverse events including elevation of liver enzymes, CK, and muscle pain can be expected and a close clinical follow-up including laboratory examinations is indicated. Yet, whether a certain statin is better tolerated when used together with IFN-β and whether lower statin dosages should be preferred in this case still need to be elucidated.

Conclusions

The approval of immunomodulatory drugs in the early 1990s was a major therapeutic advance and while it is accepted that IFN-β modifies the inflammatory disease phase of MS, little is known about their exact mechanisms of action. Statins, the well-established therapeutic agents in cardiovascular medicine have been considered a potentially interesting add-on agent for many years. Compelling experimental and preliminary clinical background provided the rationale for several small Phase II trials evaluating different combinations of IFN-β preparations and statins in CIS and RRMS. The combined treatments were generally well tolerated; the side effects

with most adverse events related to hepatic and muscle problems were in the expected range and need to be kept in mind for both further clinical trials and patients on IFN-β with the need of treating hyperlipidemia by HMG-CoA reductase inhibition. A single trial, however, raised concerns toward this combination by reporting a possible abrogation of IFN-β effects by statins. These findings illustrate the problematic issue of translating *in vitro* and animal studies into clinical practice and more importantly how to draw conclusions from small trials evaluating of short-term effects. While additional trials, admittedly mostly interim and safety studies, did not confirm these findings and particularly did not detect a loss of IFN-β bioactivity *in vivo*, further clinical and experimental interest in this direction have almost certainly been significantly diminished. Partly, the approval of natalizumab and the introduction of other oral DMDs including cladibrine (Leustatin®) and FTY720 (fingolimod) as well as highly specific and effective biologics such as alemtuzumab (Campath®) or rituximab (MabThera®) may have been involved in this development. The use of statins as DMDs outside of controlled MS trials or beyond the treatment of hyperlipidemia in MS patients, regardless if mono- or combination therapy cannot be advised until further study evidence is available. A large, prospective, randomized, double-blind, placebo-controlled trial will be required to make a definite statement with regard to the value of this potential treatment strategy of IFN-β and statins and may subsequently rehabilitate this drug combination. However, such a trial is currently not scheduled and it can be hoped that a critical analysis of the shortly finished trials will shed light on the potential impact of combining IFN-β and statins on the course of RRMS.

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Conflict of Interest

The authors declare no conflict of interests.

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