Streptococcus pneumoniae Infection Aggravates Experimental Autoimmune Encephalomyelitis via Toll-Like Receptor 2

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The course of autoimmune inflammatory diseases of the central nervous system (CNS) can be influenced by infections. Here we assessed the disease-modulating effects of the most frequent respiratory pathogen Streptococcus pneumoniae on the course of experimental autoimmune encephalomyelitis (EAE). Mice were immunized with myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) peptide, challenged intraperitoneally with live S. pneumoniae type 3, and then treated with ceftriaxone. EAE was monitored by a clinical score for 35 days after immunization. EAE was unaltered in mice infected with S. pneumoniae 2 days before and 21 days after the first MOG35-55 injection but was more severe in animals infected 7 days after the first MOG35-55 injection. The antigen-driven systemic T-cell response was unaltered, and the intraspinal Th1 cytokin mRNA concentrations at the peak of disease were unchanged. The composition of CNS-infiltrating cells and subsequent tissue destruction were only slightly increased after S. pneumoniae infection. In contrast, the serum levels of tumor necrosis factor alpha and interleukin-6 and spinal interleukin-6 levels were elevated, and the expression of major histocompatibility complex class II molecules, CD80, and CD86 on splenic dendritic cells were enhanced early after infection. Serum cytokine concentrations were not elevated, and EAE was not aggravated by S. pneumoniae infection in Toll-like receptor 2 (TLR2)-deficient mice. In conclusion, infection with S. pneumoniae worsens EAE probably by elevation of proinflammatory cytokines and activation of dendritic cells in the systemic circulation via TLR2 and cross talk through the blood-brain barrier.

Multiple sclerosis (MS) is a frequent inflammatory demyelinating disease of the central nervous system (CNS). Cell-mediated autoimmunity underlies its pathogenesis (21). Autoimmune diseases such as thyroiditis, diabetes type I (9, 10), and MS (1, 4) can be evoked or aggravated by infectious agents (11, 23, 26, 27). In MS patients in particular viral or virus-like infections (e.g., Mycoplasma pneumoniae and Chlamydia pneumoniae) of the respiratory tract can induce aggravation or cause relapse (4, 14, 42). Similar to the findings in humans, viral infections are important triggering factors for the animal model of MS, experimental autoimmune encephalomyelitis (EAE). Examples are Semliki Forest virus or gammaherpesvirus infections, which can strongly evoke relapses (25, 48).

Thus far, only a few studies have addressed the question of whether bacterial infections can initiate relapses of MS or EAE. Enterotoxins from Staphylococcus aureus, which act as T-cell activating superantigens, can exacerbate or induce EAE (17, 30, 37, 38). Chlamydia pneumoniae injected intraperitoneally is able to induce or exacerbate EAE (7). The mode of action, how these pathogens probably influence the course of autoimmune diseases, became apparent after the discovery of Toll-like receptors (TLRs). TLRs recognize specific patterns of microbial components and regulate the activation of both innate and adaptive immunity (28, 40). The quantity of TLR agonists released by bacteria is influenced by the mode of antibiotic treatment (3, 39, 45).

Here we report that EAE is aggravated by a mild infection with live Streptococcus pneumoniae, the most common pathogen of bacterial respiratory tract infections (41). Clinical aggravation of autoimmune encephalomyelitis was not accompanied by an augmentation of myelin oligodendrocyte glycoprotein (MOG)-specific autoreactive lymphocytes but by an increase of proinflammatory cytokines and the activation of dendritic cells in the systemic circulation. In TLR2−/− mice the levels of tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6) were not increased after infection, and EAE was not worsened. Therefore, we postulate that exacerbation of EAE by S. pneumoniae infection is TLR2 dependent but T cell independent.

MATERIALS AND METHODS

Animals. Female C57BL/6 mice (age 6 to 8 weeks) were purchased from Charles River (Sulzfeld, Germany). TLR2−/− mice (47) were bred in the Central Animal Care Facility of the University Hospital Göttingen, Germany. All animal experiments were approved by the District Government of Braunschweig, Lower Saxony. Mice were housed in pathogen-free conditions and received water and food ad libitum.

Induction of EAE and infection with S. pneumoniae. C57BL/6 mice were immunized subcutaneously (s.c.) in both flanks with 200 μg of myelin oligodendrocyte glycoprotein 35-55 (MOG35-55) in 50 μl of phosphate-buffered saline (PBS) and 100 μl of incomplete Freund adjuvant (IFA; Sigma-Aldrich, Deisenhofen, Germany) containing 1 mg of desiccated M. tuberculosis (H37Ra; Difco Laboratories, Detroit, MI) on day 0 and 2 after the first immunization. Mice were injected intraperitoneally with 100 ng of Bordetella pertussis toxin (Sigma-Aldrich, Deisenhofen, Germany) in 300 μl of PBS on day 0 and 2 after the first immunization.

EAE mice were infected intraperitoneally with 2.5 × 10⁶ CFU of S. pneu-
 activists were chosen to elucidate whether infection aggravated the course of EAE when it occurred within the early asymptomatic phase. By use of an infection time 21 days after the first immunization, we clarified whether infection could cause relapse beyond the peak of disease, when the clinical symptoms of EAE slowly resolved. Starting 12 h after infection, all mice received antibiotic treatment with ceftriaxone (Roche; Hoffmann-La Roche, Grenzach-Wyhlen, Germany) at 100 mg/kg twice daily for 3 days. This short interval between S. pneumoniae application and antibiotic therapy was necessary to ensure a mild infection. At 12 h after blood was drawn from 11 mice and plated on blood agar plates.

**Clinical evaluation.** Mice were weighed and starting from day 7 scored daily for clinical EAE signs according to the following scoring system: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis and discrete hind limb weakness; 2, complete tail paralysis and strong hind limb weakness; 2.5, unilateral hind limb paralysis; 3, complete hind limb paralysis; 3.5, complete hind limb paralysis and forelimb weakness; 4, tetraplegia; and 5, death of EAE (2). Mice were killed by cervical dislocation when the EAE score was >3.0 or the loss of weight was >20% of the maximum weight. One of thirty-four mice died of infection, and three of the thirty-three mice in the S. pneumoniae-infected group (see results in Fig. 1B) had to be killed because of an EAE score of 3.5.

**Histology.** Mice were perfused with 4% paraformaldehyde in PBS at the peak of disease. Spinal cords were fixed in 4% buffered formalin and embedded in paraffin. Luxol fast blue (LFB) was used to assess the degree of demyelination. The following antibodies were used for immunohistochemical analysis of the spinal cord tissue: monoclonal rat anti-mouse MAC3 (clone M3/84, dilution 1:200; BD Biosciences, Heidelberg, Germany) for macrophage staining, monoclonal rat anti-human/mouse CD3 (clone CD3-D12, dilution 1:200; Serotec, Düsseldorf, Germany) for T cells, monoclonal rat anti-mouse B220 (clone RA3-6B2, dilution 1:200; BD Biosciences, Heidelberg, Germany) for B cells, and a mouse anti-amyloid precursor protein (APP; clone 22C11, dilution 1:100; Chemicon, Temecula, CA) for axonal degeneration (secondary antibody for CD3, MAC3, and B220: biotinylated goat anti-rat immunoglobulin [RPN1001, dilution 1:200]; Amersham Biosciences, Freiburg, Germany), secondary antibody for APP: sheep anti-mouse immunoglobulin [RPN1001, dilution 1:200; Amersham Biosciences, Freiburg, Germany], using avidin-biotin amplification bridge method with peroxidase as a substrate). The three cross sections with the strongest infiltrate of each animal were taken for further analysis of immunohistochemical and LFB stainings: the number of positive cells per square millimeter was calculated, and the total white matter and demyelinated areas were quantified on a computerized imaging system (BX51 microscope [Olympus, Hamburg, Germany], AnalySIS software [Special SIS Docu; Soft Imaging System] by planimetry. The demyelination was expressed as a percentage of the total area of the white matter.

**Real-time PCR.** Total RNA was extracted from spinal cords of EAE mice at the peak of disease using RNeasy Minikit (QIAGEN, Hilden, Germany). The samples were treated with DNase I (Roche, Mannheim, Germany), and 1 μg of RNA was transcribed into cDNA using oligo(dT) primers and the SuperScript II RT kit (Invitrogen, Carlsbad, CA). A total of 2.5 μl of cDNA was transferred into a 96-well Multiplex PCR plate (Sarstedt, Germany), and 12.5 μl of Abololute QPCR SYBER Green master mix (ABgene, Surrey, United Kingdom) plus 19.6 μl of double-distilled H2O was added. The following primer probe pairs were used: IL-10, sense (5'-GGTTGCCCAAAGCTCTCGAG-3') and antisense (5'-ACCTGCCATGCTTGTTCT-3'); TNF-α, sense (5'-CTGCTCCAAAATGCTGATGAA-3') and antisense (5'-GAGTAGACAGGATACACCC-3'); and GAPDH, sense (5'-CTCTGCCACACCACCTGGTATGACACC-3') and antisense (5'-GTTCTGCACCTGGGATGACCTGGC-3'). The primer pairs for IFN-γ and IL-2 were purchased from R&D Systems (Minneapolis, MN). After an initial Taq activation step at 95°C and 45 s at 63°C, amplification was measured during the annealing phase. A total of 45 cycles were performed (iCyler, analysis data with iCyler analysis software version 2.3; Bio-Rad, Hercules, CA). The reaction efficiency for each primer was always at least 90%, based on the amplification efficiency in serial dilutions.

**Fluorescence-activated cell sorting (FACS).** Monocytes were killed at the peak of disease, and spinal cord tissue was homogenized and passed through a 70-μm-apore-size nylon filter (Fisher Scientific, Schwerte, Germany). The homogenate was centrifuged, resuspended in 70% isotonic Percoll separation solution (Biochrom, Berlin, Germany), and overlaid with 37% isotonic Percoll. The gradient was centrifuged at 600 × g for 25 min at room temperature. Flow cytometry (FACSCalibur flow cytometer [CellQuest software], postacquisition analysis with WinMDI 2.8 software [Scrivens Research Institute, La Jolla, CA]) was performed with the following antibodies: CD45-PerCP, CD11b-fluorescein isothiocyanate, and GR-1-phycocyanin (Becton & Dickinson Biosciences, Heidelberg, Germany).

**Fluorescence-activated cell sorting (FACS).** Analysis of CD11c+ cells was performed with samples from the spleens of immunized mice 12 h after infection with S. pneumoniae or intraperitoneal saline injection. Samples were prepared at 4°C in buffer solution (PBS containing 2% fetal calf serum and 0.2% NaN3) and stained with fluorescein isothiocyanate-labeled anti-CD86 or anti-MHC class II (all BD Pharmingen). After lysis of erythrocytes with FACS lysing solution (Becton Dickinson, San Jose, CA) and washing, cell suspensions were analyzed on a FACSCalibur flow cytometer with CellQuest software. Viable cells were gated by the forward and side scatter of light.

**Lymphocyte proliferation assay.** Seven days after the initial MOG35–55 immunization (13 h after bacterial infection and 1 h after the first antibiotic dose) and 15 days after the first immunization, respectively, animals were killed, and draining axillary and inguinal lymph nodes were removed and homogenized (2). T cells were placed in a 96-well plate (5 × 105/well) and cultured in triplicate in the presence of 50 μg of MOG35–55 peptide/ml for 48 h in RPMI complete medium ( Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal calf serum (PAA Lab GmbH, Pasching, Austria), 100 U of penicillin/ml and 100 μg of streptomycin/ml (Biochrom, Berlin, Germany). The cells were harvested by using a Harvest II-110-96 (Inotec AG, Dottikon, Switzerland) after incubation for 15 h with 0.5 μg of 1Hlumidine (Amersham Biosciences, Freiburg, Germany) was performed. Lymphoid incorporation was assessed by using an MTS beta-450 BetaCounter (Perkin-Elmer, Boston, MA), and IFN-γ was measured in the supernatants by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Bergisch-Gladbach, Germany).

**Cytokine ELISA.** At 1 week after the initial MOG35–55 immunization (13 h after bacterial infection, 1 h after the first antibiotic dose), the animals were decapitated and exsanguinated. Blood was allowed to clot and then centrifuged at 6,000 × g for 10 min. For the measurement of cytokine concentrations in the spinal cord, mice were transcardially perfused with PBS. Then the spinal cords were homogenized and centrifuged. The concentrations of TNF-α and IL-6 in the serum and tissue supernatants were measured by commercially available ELISAs (Quantikine ELISA Systems; R&D Systems, Bergisch-Gladbach, Germany). The color read was read in a microplate reader (SLT; Spectra Lab Instruments, Crailsheim, Germany) at an absorbance of 450 nm, with values obtained at 540 nm as a reference. Release was calculated as picograms of cytokine per milliliter.

**Statistical analysis.** Statistical evaluation and graphical presentation was performed by using GraphPad Prism 4.0 for the unpaired Student t test and GraphPad Instat 3.05 (GraphPad Software, San Diego, CA) for the Fisher exact test. Moreover, nonparametric repeated measures analysis of variance (ANOVA) was performed on the disease severity-versus-time curves of mice infected 7 days after the first immunization and the respective control animals. The data are expressed as means ± the standard error of the mean (SEM). Differences were considered statistically significant when the P value was <0.05.

**RESULTS**

Infection with S. pneumoniae increased the severity of autoimmune encephalomyelitis. S. pneumoniae infection was either performed 2 days before the first MOG immunization (Fig. 1A) or 7 (Fig. 1B) or 21 (Fig. 1C) days after the initial MOG35–55 immunization. At 12 h after infection, animals were clinically asymptomatic. The blood contained (5.0 ± 2.4) × 104 CFU of S. pneumoniae/ml. S. pneumoniae was not able to substantially modulate the course of autoimmune encephalomyelitis when mice were infected 2 days before or 21 days after the first immunization. In contrast, S. pneumoniae aggravated the disease course, when infection was performed 7 days after the initial MOG35–55 immunization. The increased severity of EAE after S. pneumoniae infection was reflected by significant differences in the maximum disease score (infected versus uninfected mice, mean ± SEM: 2.7 ± 0.2 versus 2.0 ± 0.2; P = 0.035). Accordingly, the cumulative scores also differed among...
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was significantly aggravated (maximum disease score, \( V.11000 \)) in the course of autoimmune encephalomyelitis. MOG35-55 immunized mice received intraperitoneal injections of 2.5 \( \times 10^5 \) CFU \( S. pneumoniae \) (\( \Delta \); day \(-2, n = 12 \); day \(+7, n = 33 \); day \(+21, n = 12 \)) or 0.9% NaCl (\( \Delta \); day \(-2, n = 12 \); day \(+7, n = 32 \); day \(+21, n = 12 \)) either prior (day \(-2, A \)) or after immunization (day \( 7, B \); day \( 21, C \)). After 12 h, treatment with ceftriaxone was started twice daily for 3 days. Mice were weighed and scored for signs of EAE every day. Graphs show the mean clinical scores of the animals \( \pm \) the SEM. When mice were infected 7 days after the first MOG35-55 immunization.

The cytokine mRNA profile in spinal cords of both groups (40.5 \( \pm 3.5 \) in \( S. pneumoniae \)-infected mice and 29.5 \( \pm 4.1 \) in saline-treated mice; \( P = 0.046 \)). The disease severity-versus-time curves of mice infected 7 days after the first immunization and the respective control animals were significantly different (\( P = 0.048 \)). Disease incidence was higher in the \( S. pneumoniae \) group (94%) compared to control animals (75%) (\( P = 0.044 \)). All subsequent EAE experiments were then performed with \( S. pneumoniae \) infection day 7 after the first MOG35-55 immunization.

No substantial changes of the histopathology in the CNS after infection. In spinal cords examined histologically at the peak of disease (14 infected and 13 uninfected mice) (Fig. 2), the number of CD3-positive T cells was mildly elevated in \( S. pneumoniae \)-preinfected animals (160 \( \pm 41 \) cells/mm\(^2\) versus 96 \( \pm 30 \) cells/mm\(^2\) in controls) (\( P = 0.23 \), Fig. 2B). T-cell influx was accompanied by slightly more MAC3\(^+\) cells in mice after \( S. pneumoniae \) infection (101 \( \pm 27 \) cells/mm\(^2\)) than in the uninfected mice (82 \( \pm 27 \) cells/mm\(^2\)) (\( P = 0.63 \)). The number of B220\(^+\) B cells was also elevated in \( S. pneumoniae \)-infected mice (36 \( \pm 9 \) cells/mm\(^2\) versus 16 \( \pm 5 \) cells/mm\(^2\)) (\( P = 0.08 \)). In the inflamed regions, APP-positive axonal structures indicating acute axonal damage were slightly enhanced in infected mice (57 \( \pm 18 \) deposits/mm\(^2\) compared to 37 \( \pm 14 \) deposits/mm\(^2\); Fig. 2C) (\( P = 0.39 \)), and the demyelinated area was slightly increased in mice challenged with \( S. pneumoniae \) (14\% \( \pm 4\% \) compared to 10\% \( \pm 4\% \); Fig. 2D) (\( P = 0.44 \)).

Flow cytometry was subsequently used to further differentiate MAC3\(^+\) cells into either CD11b\(^+\) CD45\(^hi\)-infiltrating macrophages or CD11b\(^-\) CD45\(^lo\) endogenous microglia, respectively (Fig. 3). Mice at the peak of EAE exhibited a similar ratio of both myeloid cell populations (14.5% CD11b\(^+\) CD45\(^hi\) cells versus 12.2% CD11b\(^+\) CD45\(^lo\) cells in \( S. pneumoniae \)-challenged mice compared to 18.6% CD11b\(^+\) CD45\(^hi\) cells versus 18.5% CD11b\(^-\) CD45\(^lo\) cells in control mice), indicating that the slightly elevated number of MAC3\(^+\) cells after \( S. pneumoniae \) preinfection was due to enhanced numbers of both macrophages and microglia. The frequencies of CD11b\(^+\) Gr1\(^+\) granulocytes were almost equal in the infected and uninfected groups (54.4% compared to 68.5%).

Unaltered Th1 cytokine mRNA profile in spinal cord after \( S. pneumoniae \) infection at peak of disease. The cytokine mRNA profile in spinal cords of \( S. pneumoniae \)-infected and control mice taken at peak of disease (10 days after disease onset) (Fig. 4) revealed no shift toward a Th1 response. The amounts of IL-2 and IFN-\( \gamma \) mRNA did not differ significantly in infected and infected animals (\( P > 0.05 \)). Proinflammatory TNF-\( \alpha \) mRNA and IL-10 mRNA levels also were not increased in the spinal cord after infection with \( S. pneumoniae \).

\( S. pneumoniae \) did not enhance T-cell activation. Lymphocytes derived from \( S. pneumoniae \)-challenged and -unchallenged mice killed on day 8 after the first MOG35-55 injection developed comparable proliferative activity (Fig. 5A) and similar levels of IFN-\( \gamma \) (Fig. 5B) in response to their cognate antigen MOG35-55 by the recall assay (2). MOG35-55-specific T-cell proliferation was not altered at a later time point of disease (day 15 after immunization; data not shown).

Strong induction of proinflammatory cytokines and activation of dendritic cells in the systemic circulation of infected mice early after infection. Serum and spinal cord levels of proinflammatory cytokines IL-6 and TNF-\( \alpha \) were determined
13 h after bacterial infection, i.e., 1 h after the first antibiotic dose (Fig. 6). IL-6 serum levels were 3.4-fold higher in the infected group (448 ± 86 pg/ml) than in uninfected mice (131 ± 24 pg/ml; \( P = 0.002 \)). TNF-\( \alpha \) serum levels rose from undetectable levels to a mean of 106.5 ± 23 pg/ml (\( P = 0.003 \)). TNF-\( \alpha \) was detectable in six of nine mice with \textit{S. pneumoniae} infection, whereas none of nine uninfected mice exhibited measurable serum levels of TNF-\( \alpha \). At the same time, in the spinal cords, IL-6 concentrations were almost twice as high in infected (693 ± 92 pg/ml) than in uninfected mice (395 ± 14 pg/ml) (\( n = 6 \) each, \( P = 0.01 \)), whereas spinal TNF-\( \alpha \) concentrations were only slightly increased in infected mice (412 ± 14 pg/ml versus 353 ± 33 pg/ml; difference not significant). A rapid and strong upregulation of MHC class II and costimulatory molecules CD80 and CD86 was observed in splenic CD11\(^d\) dendritic cells prepared from mice infected with \textit{S. pneumoniae} and sacrificed 13 h after infection (Fig. 7).

Disease aggravation was mediated by engagement of TLR2 in vivo. Cell wall components of gram-positive bacteria are recognized by TLR2 on antigen-presenting cells such as macrophages and dendritic cells (32, 40, 43, 49). In TLR2\(^{-/-}\) mice antigen-specific proliferation of T cells, histopathological changes, as well as the clinical course of EAE, was comparable to wild-type mice (27a). We therefore addressed the question

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**FIG. 2.** Histopathological profile in EAE mice after \textit{S. pneumoniae} infection. (A) Animals were killed at the peak of disease (10 days after the first clinical symptoms), and immunohistochemistry/histology was performed for demyelination (LFB), macrophages/microglia (MAC3), T cells (CD3), B cells (B220), and APP representing axonal damage (the scale bar in the top row is 500 \( \mu \)m; for all other rows it is 200 \( \mu \)m). In all cases, animals with high clinical scores are shown. (B to D) Quantification of mononuclear infiltrates (B), axonal damage (C) or demyelination (D) in \textit{S. pneumoniae}-infected (■) and uninfected (□) mice. The data represent means ± the SEM from three spinal cord sections of 13 mice in the uninfected and 14 mice in the \textit{S. pneumoniae}-treated group.
of whether disease aggravation by \textit{S. pneumoniae} infection depended on the presence of TLR2 in vivo in TLR2-deficient mice (Fig. 8). The absence of TLR2 prevented disease aggravation by \textit{S. pneumoniae} infection, indicating the critical involvement of TLR2 (mean maximum disease score in infected versus uninfected TLR2 \textsuperscript{-/-} mice of 2.6 \pm 0.2 versus 2.9 \pm 0.1 \[P = 0.2\]; mean cumulative score of 42.7 \pm 4.0 versus 49.2 \pm 2.6 \[P = 0.2\]). At 1 h after the first ceftriaxone dose (13 h after infection with \textit{S. pneumoniae} or saline administration), the serum concentrations of IL-6 in TLR2 \textsuperscript{-/-} mice immunized with MOG \textsubscript{35-55} were 78.1 \pm 25.9 pg/ml versus 62.6 \pm 16.0 pg/ml (infected versus uninfected mice; \(P = 0.6\)). The TNF-\(\alpha\) serum levels were below the quantification limit in both infected and uninfected TLR2 \textsuperscript{-/-} mice (\(n = 7\) each group).

**DISCUSSION**

Bacterial and viral components can be costimulatory factors for disease development in EAE. Lipopolysaccharides of gram-negative bacteria, as well as DNA rich in unmethylated cytosine-guanosine motifs (CpG), are potential modifiers of autoimmune inflammation (12, 33, 34). These components are able to activate the innate immune system via specified receptors such as TLRs situated on antigen-presenting cells (40). Despite some reports on the effect of gram-negative bacteria and their components on the course of EAE (12, 35), very few data are available on the EAE-modulating effects of \textit{S. pneumoniae}, the most frequent pathogen of bacterial respiratory tract infections (41). Exacerbation of MS after active immunization with a pneumococcal vaccine has been reported (5).

In the present study, a mild \textit{S. pneumoniae} infection during the preclinical phase of EAE led to a clear aggravation of clinical symptoms. In contrast, \textit{S. pneumoniae} infection at earlier (2 days before the first immunization) or later (21 days after the first immunization) time points did not influence the
course of EAE. It is tempting to speculate that humans with MS probably are not equally vulnerable to *S. pneumoniae* infections during the course of their disease, the susceptibility being greatest in the subclinical phase prior to the manifestation of an MS event. This finding speaks in favor of a dependence of the susceptibility to bacterial challenge on the time of infection. The present data clearly indicate that *S. pneumoniae* infections are capable of boosting the immune process of EAE. We therefore studied several mechanisms of potential relevance for this phenomenon.

Planimetry of the demyelinated areas, as well as of axonal damage, showed a tendency toward more pronounced tissue damage in infected animals; however, as a consequence of the high interindividual variation of demyelination and axonal injury within both groups, the differences failed to reach statistical significance. Accordingly, the intraspinal production of the key Th1 cytokines TNF-α, IL-2, and IFN-γ was not substantially influenced by *S. pneumoniae* preincubation. These results make it unlikely that MOG-specific, encephalitogenic T lymphocytes and macrophages invading the brain after infection were responsible for disease aggravation. Indeed, when we examined the ability of *S. pneumoniae* to influence MOG-specific T-cell proliferation and IFN-γ production in vitro, no modulation was detectable. These data are in contrast to work by others who showed that systemic T-cell activation occurred after bacterial infection or administration of bacterial components in vivo (7, 20, 33, 46).

Teichoic and lipoteichoic acids and peptidoglycans are major constituents of the cell wall of *S. pneumoniae*. They are stimulants of the innate immune system and are ligands of TLR2 and Nod1/Nod2 (8, 40, 43, 49). Macrophages lack N-acetyl-muramyl l-alanine amidase required for the complete degradation of peptidoglycans (13). The persistence of peptidoglycan may be an important factor in various chronic autoimmune diseases, including MS (31). Cell wall components from gram-positive bacteria can substitute antigens from *M. tuberculosis* in EAE induction (46). TLR2 mRNA is upregulated during the course of EAE. Expression of the mRNA encoding TLR2 starts to increase 4 to 8 days after immunization with MOG and reaches its maximum at 3 weeks (50).

The action of pneumococcal cell wall products probably depends on a dendritic cell-mediated expression of costimulatory molecules and immunomodulatory cytokines. In the

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**FIG. 6.** Induction of proinflammatory cytokines in the systemic circulation of mice after *S. pneumoniae* challenge. Sera of 10 mice per group for the IL-6 ELISA and 9 mice per group for the TNF-α ELISA were collected 1 h after the start of antibiotic therapy (13 h after *S. pneumoniae* infection). TNF-α and IL-6 were measured by ELISA. The graphs show the mean serum levels ± the SEM. Please note the strong differences of the cytokine concentrations in the sera of infected and uninfected animals. n.d., not detectable.

**FIG. 7.** Costimulatory molecules on CD11c<sup>+</sup> dendritic cells were upregulated in mice infected during the subclinical phase of EAE. In three mice infected with *S. pneumoniae* (EAE SP3) and three control animals 7 days after the first MOG<sub>35-55</sub> immunization (EAE NaCl), and in one mouse which did not receive MOG<sub>35-55</sub> (isotype) the expression of MHC class II molecules and the costimulatory molecules CD80 and CD86 were studied in CD11c<sup>+</sup> splenic dendritic cells 1 h after start of antibiotic therapy (13 h after *S. pneumoniae* infection) by flow cytometry. The mean fluorescence intensity (MFI) was determined (means ± the SEM). The expression of MHC class II, CD80, and CD86 were strongly increased in infected EAE mice.
present study, dendritic cells from the systemic circulation were activated early after infection. Since in mice lacking TLR2 S. pneumoniae infection failed to increase the severity of EAE (Fig. 8), stimulation of TLR2 by pneumococcal components plays a central role in the aggravation of EAE by this pathogen. TLR4, stimulated at sublytic concentrations by pneumolysin (8), does not appear as important as TLR2 for the pathogen. TLR4, stimulated at sublytic concentrations by pneumolysin (8), does not appear as important as TLR2 for the pathogen. TLR4 did not influence the course of MS in 890 patients. The most likely mechanism in our model appears to be a sepsis-like symptom induced by the release of proinflammatory modulators by circulating immune cells and activation of dendritic cells in the presence of S. pneumoniae. In turn, this probably leads to a cross talk of activated leukocytes and microglial cells through the blood-brain barrier in the preclinical phase. The systemic inflammation was milder in infected TLR2−/− mice, and there was no difference in the clinical course of EAE in these mice.

Our data stress the importance of early treatment of respiratory bacterial infections in MS patients. They encourage epidemiological studies on a possible link between MS onset or exacerbation and infections with S. pneumoniae. Whether the detrimental effect of bacterial infections on the course of EAE can be attenuated by antibiotic therapy tailored to minimize the release of proinflammatory bacterial products (3, 39, 45) remains to be studied.

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