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*J Immunol* 2012; 188:5723-5733; Prepublished online 27 April 2012; doi: 10.4049/jimmunol.1103109 http://www.jimmunol.org/content/188/11/5723

**Supplementary Material**

Supplementary Material http://www.jimmunol.org/content/suppl/2012/04/27/jimmunol.1103109.DC1

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Ceramide Synthase 6 Plays a Critical Role in the Development of Experimental Autoimmune Encephalomyelitis

Susanne Schiffmann,* Nerea Ferreiros,* Kerstin Birod,* Max Eberle,* Yannick Schreiber,* Waltraud Pfeilschifter,† Ulf Ziemann,† Sandra Pierre,* Klaus Scholich,* Sabine Grösch,* and Gerd Geisslinger*

Ceramides are mediators of apoptosis and inflammatory processes. In an animal model of multiple sclerosis (MS), the experimental autoimmune encephalomyelitis (EAE) model, we observed a significant elevation of C16:0-Cer in the lumbar spinal cord of EAE mice. This was caused by a transiently increased expression of ceramide synthase (CerS) 6 in monocytes/macrophages and astroglia. Notably, this corresponds to the clinical finding that C16:0-Cer levels were increased 1.9-fold in cerebrospinal fluid of MS patients. NO and TNF-α secreted by IFN-γ–activated macrophages play an essential role in the development of MS. In murine peritoneal and mouse-derived RAW 264.7 macrophages, IFN-γ–mediated expression of inducible NO synthase (iNOS)/TNF-α and NO/TNF-α release depends on upregulation of CerS6/C16:0-Cer. Downregulation of CerS6 by RNA interference or endogenous upregulation of C16:0-Cer mediated by palmitic acid in RAW 264.7 macrophages led to a significant reduction or increase in NO/TNF-α release, respectively. EAE/IFN-γ knockout mice showed a significant delay in disease onset accompanied by a significantly less pronounced increase in CerS6/C16:0-Cer, iNOS, and TNF-α compared with EAE/wild-type mice. Treatment of EAE mice with l-cycloserine prevented the increase in C16:0-Cer and iNOS/TNF-α expression and caused a remission of the disease. In conclusion, CerS6 plays a critical role in the onset of MS, most likely by regulating NO and TNF-α synthesis. CerS6 may represent a new target for the inhibition of inflammatory processes promoting MS development. The Journal of Immunology, 2012, 188: 5723–5733.

Multiple sclerosis (MS) and its prototype animal model, experimental autoimmune encephalomyelitis (EAE), are induced by autoimmune responses against myelin components in the CNS. Activated autoreactive T cells proliferate and secrete proinflammatory cytokines, which in turn stimulate microglia, macrophages, and astrocytes, and recruit B cells, ultimately resulting in damage to myelin and the myelin-forming oligodendrocytes and axons (1). One hypothesis concerning the development of MS is that IFN-γ secreted from activated Th1 cells activates macrophages and initiates an inflammatory reaction in the CNS. The inflammatory phase includes the synthesis of NO and TNF-α by activated macrophages. In the CNS, NO can act during the onset of the disease as a tissue-damaging free radical (2), and TNF-α can initiate a myelin-directed immune response (3). The partial reduction of NO (4) and TNF-α (5) synthesis prevents the development of EAE. Furthermore, NO metabolites detected in the cerebrospinal fluid of MS patients are associated with the progression of the disease (6). These data indicate an important role of NO and TNF-α in the onset of MS.

The sphingolipid transduction pathway induces apoptosis, proliferation, growth arrest, and inflammation depending upon cell and receptor types and downstream targets (7) indicating that sphingolipids may play a role in the disease process of MS (8). Ceramides are the backbone of several complex sphingolipids (sphingomyelins, glycosylceramides). Ceramides can be generated by de novo synthesis or by degradation of complex sphingolipids. A rate-limiting step in the biosynthesis of ceramides is the attachment by the ceramide synthases (CerS) of various acyl-CoA side chains to a sphingoid base. CerS1–CerS6 act chain length specifically and introduce side chains to form C14–C26 ceramides. Thus, CerS1 synthesizes mainly C18-Cer, CerS4 synthesizes C18-/C20-Cer, CerS5 and CerS6 synthesize mostly C16-Cer, CerS2 synthesizes mainly C22-/C24-Cer, and CerS3 synthesizes very long chain ceramides (9). In addition to de novo synthesis, the salvage pathway also supplies ceramides mainly via the activation of sphingomyelinases (10).

Recent findings indicate that ceramides play an important role in inflammatory processes. In the inflammatory disease cystic fibrosis, ceramides induce the upregulation of proinflammatory mediators by a yet unknown mechanism (11). In the current study, we demonstrate that C16:0-Cer is involved in the induction of EAE through activation of NO/TNF-α synthesis. C16:0-Cer and CerS6 are upregulated in macrophages and astroglia in EAE mice. The inhibition of C16:0-Cer synthesis by l-cycloserine caused reduced inducible NO synthase (iNOS)/TNF-α expression and remission of...
the clinical symptoms in EAE mice. Furthermore, C16:0-Cer levels were increased 1.9-fold in the cerebrospinal fluid of MS patients. In cell culture experiments, we demonstrate that C16:0-Cer plays a critical role in IFN-γ-induced iNOS/TNF-α expression and NO/TNF-α release. Accordingly, EAE/IFN-γ knockout (KO) mice showed a significant delay in disease onset accompanied by a less pronounced increase in CerS6/C16:0-Cer, iNOS, and TNF-α compared with EAE/wild-type (WT) mice. Overall, this study reveals an important role for C16:0-Cer in the inflammatory process and subsequently in the induction of EAE.

Materials and Methods

Cells and reagents

RAW 264.7 mouse macrophages (courtesy of Prof. J. Pfeilschifter, University of Frankfurt, Frankfurt, Germany) and peritoneal macrophages were cultured and incubated in RPMI 1640 medium containing 10% FCS and 1% penicillin/streptomycin. Cells were cultured at 37°C in an atmosphere containing 5% CO₂, t-cycloserine, fusuminon B1, palmitic acid, and 60μM methylprednisolone were purchased from Sigma-Aldrich (Hamburg, Germany). IFN-γ, TNF-α, and IL-β were purchased from PeproTech (Hamburg, Germany). Small interfering RNAs (siRNAs) for CerS6 (s190529) were purchased from Ambion (Darmstadt, Germany). Sphingolipids were purchased either from Avanti Polar Lipids (Alabaster, AL) or Matreya LLC (Pleasant Gap, PA). EAE (EK-0115) and control (CK-0115) kits were purchased from Hooke Laboratories (Lawrence, MA).

Induction and evaluation of EAE

In all experiments, ethics guidelines for investigations in conscious animals were obeyed, and the experiments were approved by the local ethics committee for animal research. Eight- to ten-week-old female C57BL/6 or IFN-γ KO (C57BL6 background) mice weighing 18–20 g were obtained from Harlan Laboratories (Horst, The Netherlands) or The Jackson Laboratory (Bar Harbor, ME). The procedure used for the induction of EAE was conducted as recommended by Hooke Laboratories. Briefly, EAE mice received a s.c. injection in the upper and lower back of encephalitogenic myelin oligodendrocyte protein (MOG)35–55 (200 μg) emulsified in CFA containing 400 μg Mycobacterium tuberculosis. Two hours thereafter, and again 24 h later, the mice received an i.p. injection of pertussis toxin (PTX). Because CFA alone also exerts inflammatory properties (12), two control groups were used. In one group (control group), mice received neither CFA/PTX nor MOG, and in the other group, CFA-treated mice were treated with CFA containing M. tuberculosis and twofold higher PTX dose. Two days after the injection, the mice were examined daily for development of disabilities. Most (90%) of the EAE mice developed clinical symptoms after 13 ± 2 d. Mice that developed no clinical symptoms were excluded. Clinical scores were defined as follows: 0, no signs; 0.5, limp tail; 1, limp tail and weakness of hind legs; 2, limp tail and paraesthesia of hind legs; 3, limp tail and paralysis of hind legs.

Treatment study

Both EAE mice and CFA-treated mice received treatment with t-cycloserine, which has been shown to inhibit sphingolipid synthesis in the CNS (12). Two control groups were used. In one group (control group), mice received neither CFA/PTX nor MOG, and in the other group, CFA-treated mice were treated with CFA containing M. tuberculosis and twofold higher PTX dose. Two days after the injection, the mice were examined daily for development of disabilities. Most (90%) of the EAE mice developed clinical symptoms after 13 ± 2 d. Mice that developed no clinical symptoms were excluded. Clinical scores were defined as follows: 0, no signs; 0.5, limp tail; 1, limp tail and weakness of hind legs; 2, limp tail and paraesthesia of hind legs; 3, limp tail and paralysis of hind legs.

Cerebrospinal fluid from patients

Cerebrospinal fluid samples were collected from 16 MS patients and 10 control patients who suffered from other neurologic diseases (Tables I, II). Cerebrospinal fluid from patients was collected at the Department of Neurology of Goethe University (Frankfurt/Main, Germany). All patients gave written informed consent prior to participation, and the protocol was approved by the local ethics committee. All samples were stored at −80°C. Cerebrospinal fluid samples were collected from 16 MS patients and 10 control patients who suffered from other neurologic diseases (Tables I, II).

Preparation of tissue for histology studies

Animals were killed with carbon dioxide inhalation and perfused transcardially with PBS (for mRNA, protein, and sphingolipid analysis) followed by 4% paraformaldehyde (for immunohistochemistry). Spinal cord (lumbar segments) was extracted and stored at −80°C for mRNA, protein, and sphingolipid analysis. The tissue for immunohistochemistry (spinal cord) was kept in 4% paraformaldehyde for 1 h, placed overnight in 30% sucrose, embedded in tissue freezing medium (Jung: Leica Microsystems GmbH, Nussloch, Germany), then quickly frozen on dry ice and stored at −80°C.

Immunohistochemistry

Fourteen-micrometer sections were permeabilized in PBS containing 0.1% Triton X-100 for 10 min, then blocked in PBS containing 3% BSA and 0.1% Triton X-100 for 30 min at room temperature. The sections were incubated with the primary Ab at 4°C overnight, followed by fluorescence-labeled Abs diluted 1:800 for 2 h in PBS containing 0.1% Triton X-100. The following Abs were used in the dilution indicated: anti-CerS6 (1:100), anti-ionized calcium-binding adapter molecule 1 (1:200), anti-glial fibrillary acidic protein (1:1000), anti-CD11b (1:100), anti-F4/80 (1:100), anti-iNOS (1:100), and anti-death receptor 5 (1:50). The CerS6 (goat polyclonal) and death receptor 5 (DR5) (rat polyclonal) Abs were purchased from Santa Cruz Biotechnology (Heidelberg, Germany), and glial fibrillary acidic protein (GFAP) (rabbit polyclonal) Ab was purchased from Sigma-Aldrich (Schnelldorf, Germany). The ionized calcium-binding adapter molecule 1 (Iba1) (rabbit polyclonal) Ab was purchased from Wako Chemicals GmbH (Neuss, Germany). The Abs against CD11b (rat polyclonal) and F4/80 (rat polyclonal) were from Serotec (Düsseldorf, Germany). The Ab against iNOS (rabbit polyclonal) was purchased from Becton Dickinson (Heidelberg, Germany).

Preparation of crude protein extracts

RAW 264.7 macrophages were seeded in 5-cm dishes at a density of 5 × 10⁶ cells/dish. Cells were treated with 10 ng/ml or 0.5 ng/ml IFN-γ, 5 ng/ml TNF-α, 1 ng/ml IL-β, and 25 μM palmitic acid for the indicated time points. IFN-γ (10 ng/ml) treated RAW 264.7 macrophages were cotreated with 70 μM fusuminon B1, 500 μM t-cycloserine, or 1 μM methylprednisolone for 16 h. Cells were preincubated with methylprednisolone and t-cycloserine for 90 min or for 30 min with fusuminon B1. IFN-γ (0.5 ng/ml) treated RAW 264.7 macrophages were cotreated with 25 μM palmitic acid for 20 h. Vehicle-treated cells were used as controls. At the end of the incubation period, the crude extracts were prepared and used as described recently (14). Tissue samples from spinal cord were homogenized in Tris–CHAPS buffer (10 mM Tris–HCl–20 mM CHAPS, pH 7.4) supplemented with protease inhibitors. The homogenate was centrifuged, and the pellet (CerS6) (resuspended in Tris–CHAPS buffer) and the supernatants (iNOS) were collected and stored at −80°C. Protein concentrations were assessed using the Bradford method.

Western blot analysis

Immunoblotting was performed as described previously (14). In brief, 30 μg cell lysate proteins and 30 μg tissue homogenates were used. Membranes were analyzed on the Odyssey infrared scanner from LI-COR (Bad Homburg, Germany). The Abs used were diluted as follows: primary Ab against iNOS (1:200), CerS6 (1:100), and GAPDH (1:1000). The GAPDH Ab was purchased from Ambion (Darmstadt, Germany).

Real-time quantitative PCR

RAW 264.7 macrophages and lumbar spinal cord were analyzed for mRNA levels by quantitative PCR as previously described (15). The expression levels of CerS1–CerS6, TNF-α, IFN-γ, and iNOS were determined by TaqMan analysis using the SYBR Green kit (ABgene Limited, Epsom, UK) with an ABI Prism 7500 Sequence Detection System (Applied Biosystems, Austin, TX). Relative expression of ceramide synthase family genes, GAPDH, TNF-α, IFN-γ, and iNOS was determined using the comparative Ct method normalizing relative values to the expression level of 18S RNA (spinal cord samples) or GAPDH (macrophages) as housekeeping gene. The designed primer sets for ceramide synthases, iNOS, and IFN-γ were adopted from Lavial et al. (16), from Chiang et al. (17), and from Nath et al. (18), respectively. The sequence for the TNF-α primer set was designed as follows: forward 5’-TGACAGGCC-TGTAGCCCAAGC-3’, reverse 5’-GCTTTGTCCTTGAAGAGAAC-3’. Linearity of the assays was determined by serial dilutions of the templates for each primer set separately.

Screemining of ceramide synthase with siRNA

RAW 264.7 macrophages were transfected with 150 pmol CerS6 siRNA or 100 pmol scrambled siRNA as control. siPort Amine (Ambion, Darmstadt, Germany) was used for transfection according to the manufacturer’s protocol and as described previously (15). Briefly, Opti-MEM medium was incubated with transfection reagent for 10 min at room temperature, then
added to the siRNA solution consisting of Opti-MEM medium and siRNA, followed by an incubation for 10 min at room temperature. RAW 264.7 macrophages (3.5 × 10^5) were incubated with siRNA transfection solution, and the process was repeated after 24 h. After 41 h, the transfected RAW 264.7 macrophages were either harvested for mRNA isolation (RNA Isolation Kit; Qiagen, Hilden, Germany) or treated with IFN-γ for 16 h and subjected to sphingolipid analysis and NO determination. The effectiveness of the siRNA knockdown was verified using quantitative PCR.

**Determination of sphingolipid concentrations**

The quantification of sphingolipid in macrophages was achieved as previously published (19). Briefly, cells were seeded at a density of 0.5 × 10^5/cm^2 dish and incubated for 24 h. Subsequently, cells were treated with various substances. Cells were counted in a Neubauer chamber and stored at −80 °C. The counted cell number was used to normalize the measured sphingolipid concentrations to cell number. The lipids were extracted with methanol after addition of internal standards (C17:0-Cer). For the quantification of sphingolipid concentrations in tissue samples, ~20–100 mg of tissue was homogenized in PBS on ice. Aliquots (20 μl) of tissue suspension (0.02 mg/ml) were extracted in 600 μl chloroform/methanol (7:1) after the addition of the internal standard (C17:0-Cer) and 80 μl water. The suspension was vigorously vortexed at 25 °C for 1 min and centrifuged for 5 min at 25°C and 14,000 rpm. The supernatants were collected, and the extraction step was repeated. The combined organic phases were dried under a stream of nitrogen at 45°C and redissolved in 50 μl methanol for quantification. C14:0-Cer, C16:0-Cer, C18:0-Cer, C20:0-Cer, C24:1-Cer, and C24:0-Cer and the internal standards were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (19, 20). For the quantification of sphingolipid concentrations in cerebrospinal fluid, a 50-μl sample was extracted in 600 μl chloroform/methanol (7:1) after the addition of the internal standard (C17:0-Cer). Concentrations of the calibration standards, quality controls, and unknowns were evaluated using the Analyst software version 1.5 (Applied Biosystems). Linearity of the calibration curve was confirmed for C14:0-Cer from 0.3 to 500 ng/ml, for C16:0-Cer/C24:0-Cer from 3 to 5000 ng/ml, for C18:0-Cer from 0.9 to 1500 ng/ml, and for C20:0-Cer/C24:1-Cer and C24:0-Cer and the internal standards were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) as described previously (19, 20).

**Preparation of peritoneal macrophages**

RAW 264.7 macrophages and primary peritoneal macrophages were treated with various substances for the time points indicated. The cells were harvested and counted using the Neubauer chamber. The counted cell number was used for normalization of the measured products (TNF-α/NO). The supernatant was harvested, centrifuged (1200 rpm, 3 min, 4°C) and stored at −20 °C. The release of NO was assessed by measuring concentrations of nitrate and nitrite in the supernatant (1 ml) using the Griess method (21). The amount of TNF-α was determined using the mouse TNF-α ELISA detection kit from BioLegend (Uithoorn, The Netherlands). The amount of IFN-γ in the lumbar spinal cord of EAE, CFA-treated, and control mice was determined using the mouse IFN-γ ELISA detection kit from BioLegend. The concentrations of IFN-γ and TNF-α were determined according to the manufacturer’s protocol.

**Statistics**

Results are presented as means ± SEM. Cell culture data and animal data were analyzed using one-way ANOVA. Significant differences were analyzed by the Bonferroni post hoc test (PASW Statistics 18 software). The clinical score data from t-cycloserine and saline-treated EAE mice were analyzed by calculating the area under the curve (AUC). The AUC values were analyzed using the t test. The level of significance was set at p < 0.05.

**Results**

C16:0-Cer levels are increased at the onset of EAE

Because ceramides play a support role in inflammatory processes, we hypothesized that ceramides are modified during the development of MS. To assess this hypothesis, we used the chronic EAE model in mice as an animal model for MS. After 13 ± 2 d, the mice started to develop the first signs of reduced motor skills, which deteriorated within 4 ± 1 d to complete paralysis of the hind paws (Fig. 1A). The ceramide levels in the lumbar spinal cord were determined in CFA-treated (only treated with CFA-emulsion and PTX) and EAE mice using LC-MS/MS. The EAE mice were divided into two groups based on scores: sc0.5–sc1.5 (onset of the disease) and sc2–sc3 (acute phase of the disease). The concentrations of C14:0-Cer, C16:0-Cer, C18:0-Cer, C20:0-Cer, C18:1-Cer, C24:1-Cer, and C24:0-Cer were determined. The concentration of C16:0-Cer from 0.13 μmol/mg tissue C16:0-Cer, 10.05 ± 3.76 μmol/mg tissue C18:0-Cer, 14.41 ± 6.44 μmol/mg tissue C24:0-Cer, and 19.06 ± 8.91 μmol/mg tissue C24:0-Cer were below the detection limit of 0.096 ng/ml (data not shown). The ceramide concentrations in untreated mice were 0.47 ± 0.13 μmol/mg tissue C16:0-Cer, 10.05 ± 3.76 μmol/mg tissue C18:0-Cer, 14.41 ± 6.44 μmol/mg tissue C24:0-Cer, and 19.06 ± 8.91 μmol/mg tissue C24:0-Cer. The lumbar spinal cord revealed a significant increase in C16:0-Cer at the onset (sc0.5–sc1.5) of the disease, which persisted through the acute phase (sc2–sc3) of the disease (Fig. 1B). All other ceramide levels were unaltered in these mice. CFA-treated mice showed no changes in ceramide levels (Fig. 1B).

C16:0-Cer levels are increased in the cerebrospinal fluid of MS patients

Given these results in the EAE model of MS, we were interested whether C16:0-Cer levels are also upregulated in MS patients. Therefore, ceramide levels were measured by LC-MS/MS in the cerebrospinal fluid of MS patients and control patients not suffering from MS (Tables I, II). In the cerebrospinal fluid of MS patients (n = 16), the C16:0-Cer levels were significantly upregulated (1.9-fold) compared with those of control patients (n = 10) (Fig. 1C), strongly suggesting that C16:0-Cer may play a role in the development of MS.

C16:0-Cer increase in EAE mice is linked to raised CerS6 expression

Next, we studied the extent to which the raised C16:0-Cer levels in EAE mice are due to increased expression of a specific ceramide synthase. For this purpose, mRNA levels of all ceramide synthases—apart from CerS3, which was not detectable—were determined in the lumbar spinal cord of CFA-treated and EAE mice (sc0.5, sc1.5, sc3). Quantitative PCR revealed that the mRNA expression of CerS6 was significantly increased in mice at the onset of EAE (Fig. 1D). The mRNA data were confirmed at the protein level by Western blot analysis. The expression of CerS6 protein in homogenates of the lumbar spinal cord of untreated, CFA-treated, and EAE mice (sc0.5, sc1.5, sc3) was determined. Sc1.5 mice showed a significant increase in CerS6 compared with that of CFA-treated mice, whereas in sc3 mice, CerS6 expression was reduced to the level of that of CFA-treated mice (Fig. 1E). Thus, the mRNA and protein expression profiles suggested a transient increase in CerS6 and C16:0-Cer during development of EAE.

CerS6 is expressed in inflammatory cells and in astroglia in EAE mice

Next, we assessed which cells are responsible for the increase in CerS6 and C16:0-Cer levels. Immunohistochemical analysis
of the lumbar spinal cord of untreated and EAE mice showed that CerS6 was expressed in oligodendrocytes (Supplemental Fig. 1A, data shown for untreated mice). In EAE mice (sc1.5), CerS6 was additionally expressed in the lesion site in the white matter of the lumbar spinal cord (Fig. 2A). CerS6 was expressed in macrophages as shown by colocalization of CerS6 and F4/80 (macrophage marker) (Fig. 2B). Furthermore, Ajami et al. (22) have shown recently that the lesion site of EAE mice con-

Table I. Demographic data of MS patients

<table>
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<th>Duration (mo)</th>
<th>Treatment</th>
<th>Number of T2 Lesions</th>
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<td>&gt;9</td>
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CIS, Clinically isolated syndrome; EDSS, Expanded Disability Status Scale; RRMS, relapsing-remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis.
sists of infiltrating monocytes (CD11b positive), which differentiate to macrophages (CD11b positive, Iba1 positive) during the progress of the disease. Therefore, we investigated whether CerS6 is localized in these cell types. CerS6 was expressed in CD11b-positive and Iba1-positive cells (Iba1, CD11b, CerS6 triple staining) (Fig. 2C). These results pointed strongly to expression of CerS6 in macrophages, but it could not be excluded that microglia cells also express CerS6 because CD11b and Iba1 also stain microglia. CerS6 is also expressed in astroglia (GFAP staining) (Supplemental Fig. 1B). Because C16:0-Cer is considered to be involved in apoptotic processes, we investigated whether CerS6 is detectable in apoptotic cells. CerS6 was not expressed in DR5-positive cells (Supplemental Fig. 1C).

IFN-γ induces an increase in ceramides in macrophages

Next, we sought to investigate whether C16:0-Cer plays a role in the induction of EAE. Recent studies have revealed an important role of the Th1-mediated response in the development of EAE (23). Th1 cells secrete predominantly IFN-γ, IL-1β, and TNF-α, which in turn activate macrophages. Because CerS6 was expressed in macrophages, we used RAW 266.7 macrophages as a cell culture system. We studied the influence of IFN-γ, IL-1β, and TNF-α on

Table II. Demographic data of control patients

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<th>Patient No.</th>
<th>Sex</th>
<th>Age (y)</th>
<th>Specification of Other Neurologic Disease</th>
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FIGURE 2. CerS6 is selectively elevated at the onset of the disease in inflammatory cells. (A) Expression of CerS6 (red channel) in the lesion site in the white matter of the ventral horn (lumbar spinal cord) of sc1.5 EAE mice at two magnifications as indicated. (B and C) Expression of CerS6 (red channel) in macrophages in the lumbar spinal cord of sc1.5 EAE mice. (B) Staining with F4/80 (green) illustrates macrophages, and staining with DAPI illustrates the cell nucleus. Merge = overlay of CerS6 (red channel), F4/80 (green channel), and DAPI (blue channel). (C) Costaining of CerS6 (red), CD11b (green), and Iba1 (blue) in the lumbar spinal cord of sc1.5 EAE mice. Merge = overlay of CerS6 (red channel) and cell type-specific proteins (CD11b, green channel; Iba1, blue channel). All data are representative images of three independent experiments. Scale bar, 10 μm (A, original magnification ×5); scale bar, 25 μM (A, original magnification ×20). (D–H) The IFN-γ, iNOS, and TNF-α expression in EAE and CFA-treated mice. The relative mRNA levels (D) and protein levels of IFN-γ (E), mRNA expression levels of TNF-α (F), and mRNA levels (G) and protein levels (H) of iNOS from EAE and CFA-treated mice. The relative mRNA expression levels of IFN-γ, iNOS, and TNF-α were normalized to 18S RNA and were calculated using the mRNA levels of untreated mice of the same age as those at basal level. Data are means ± SEM of number of mice as indicated, and each measurement was made in triplicate.*p < 0.05, **p < 0.01, ***p < 0.001 (significant differences between EAE and untreated mice).
the ceramide levels in RAW 264.7 macrophages. Only IFN-γ induced in RAW 264.7 macrophages a significant elevation of C16:0-Cer after 16-h treatment (Supplemental Fig. 1D). IFN-γ led to a time-dependent marked increase in C14:0-Cer and C16:0-Cer and to a slight increase in C24:0-Cer (Fig. 3A). The fact that dihydroceramides were elevated (data not shown) indicated an activation of sphingolipid de novo synthesis. The mRNA levels of the various ceramide synthases in IFN-γ-treated RAW 264.7 macrophages supported the LC-MS/MS data and revealed a significant increase in the mRNA levels of CerS6 (Supplemental Fig. 1E). The mRNA expression of CerS6 in RAW 264.7 macrophages was transiently increased as in the lumbar spinal cord of EAE mice (Fig. 1D). The protein expression of CerS6 was also time-dependently elevated in IFN-γ-treated RAW 264.7 macrophages with a significant increase already after 6 h (Fig. 3B). The transient increase in CerS6 in the lumbar spinal cord, as well as in IFN-γ-treated RAW 264.7 macrophages, and the subsequent marked increase in C16:0-Cer in both experimental setups indicated that the in vitro experiment simulates very closely the in vivo situation.

C16:0-Cer mediates IFN-γ–induced NO release in RAW macrophages

By releasing NO, macrophages contribute to oligodendrocyte degeneration in demyelinating diseases (24, 25). One of the key events in macrophage responses to IFN-γ is the expression of iNOS and the subsequent formation of NO. IFN-γ has been identified in MS lesions and has been implicated as a disease-promoting factor (26). In EAE mice, the mRNA and protein expression of IFN-γ was already increased at the onset of the disease (Fig. 2D, 2E). Moreover, in EAE mice we also observed an increase in mRNA and protein expression of iNOS, which correlated with the progress of the disease (Fig. 2G, 2H). RAW 264.7 macrophages treated with IFN-γ (10 ng/ml) exhibited increased iNOS mRNA and protein expression and subsequently released NO (Fig. 3C).

The upregulation of CerS6 in RAW 264.7 macrophages started at 6 h (Fig. 3B), which was much earlier than the upregulation of the iNOS expression after 16 h (Fig. 3C), indicating that CerS6 acts upstream of iNOS. If NO synthesis is regulated by C16:0-Cer, IFN-γ induced NO should be reduced by treatment with specific

**FIGURE 3.** C16:0-Cer mediates IFN-γ–induced NO release in RAW 264.7 macrophages. (A and B) The time-dependent effect of IFN-γ-treated RAW 264.7 macrophages on ceramide levels (A) and on the expression of CerS6 (B). RAW 264.7 macrophages were treated at the time points indicated with IFN-γ (10 ng/ml) or were left untreated. (A) The ceramide levels were normalized to the number of treated cells. The relative increase in specific ceramides was calculated taking the ceramide levels of untreated cells as 100%. (B) Densitometric and Western blot analysis of CerS6 expression. CerS6 expression was related to the expression level of GAPDH, which was used as loading control. One representative blot of three is shown. Data are means ± SEM of three independent experiments. (C) Protein expression, expression of iNOS mRNA, and NO release were increased in a time-dependent manner in IFN-γ (10 ng/ml) treated RAW 264.7 macrophages. For Western blot analysis, GAPDH was used as the loading control (one of two independent experiments is shown). The amount of NO was determined in the supernatant and normalized to the number of cells. The relative expression of iNOS mRNA was normalized to GAPDH and was calculated using the mRNA level of untreated cells as the basal level. (D and E) Fumonisin B1 (FB1; 70 μM), L-cycloserine (Cyclo; 500 μM), and methylprednisolone (MP; 1 μM) prevent the IFN-γ (10 ng/ml) induced increase in NO synthesis in RAW 264.7 macrophages. After IFN-γ incubation (16 h), NO release (D) and the level of iNOS mRNA (E) were measured in RAW 264.7 macrophages. For normalization, the amount of NO was related to the number of treated cells. The relative expression of iNOS mRNA was normalized to GAPDH and was calculated using untreated cells at the same time point as the basal level. Data are means ± SEM of three independent experiments, each carried out in duplicate. *p < 0.05, **p < 0.01, ***p < 0.001 (significant differences between IFN-γ–treated and untreated RAW 264.7 macrophages).
inhibitors of sphingolipid synthesis. l-Cycloserine (an inhibitor of serine palmitoyltransferase) and fumonisin B1 (a nonselective inhibitor of ceramide synthases) prevented the increase in ceramides induced by IFN-γ in RAW 264.7 macrophages (Supplemental Fig. 2A). The inhibition by l-cycloserine of IFN-γ-induced ceramide synthesis indicates that IFN-γ induces de novo synthesis rather than the salvage pathway of ceramide synthesis. Importantly, l-cycloserine and fumonisin B1 were as effective in inhibiting IFN-γ-induced NO release as the anti-inflammatory glucocorticoid methylprednisolone (Fig. 3D). Additionally, l-cycloserine and FB1 prevented the IFN-γ-induced upregulation of the mRNA expression of iNOS (Fig. 3E), thus ruling out nonselective inhibition of the activity of iNOS. Neither fumonisin B1 nor l-cycloserine reduced cell viability in IFN-γ-pretreated macrophages (Supplemental Fig. 2B). The temporal relationship between CerS6 and iNOS expression and the fact that inhibition of ceramide synthesis prevented IFN-γ-induced NO release suggest that CerS6 acts upstream of iNOS.

To verify that predominantly C16:0-Cer and CerS6 mediate the NO increase in IFN-γ-treated macrophages, CerS6 was downregulated with RNA interference technology. siCerS6-pretreated RAW 264.7 macrophages were stimulated with 10 ng/ml IFN-γ for 16 h. CerS5, which has a similar substrate specificity to that of CerS6, was not regulated by siCerS6 (Supplemental Fig. 2C). siCerS6 prevented IFN-γ-induced CerS6 upregulation, whereas scrambled siRNA had no effect (Supplemental Fig. 2C). The downregulation of CerS6 selectively prevented the increase in C16:0-Cer (data not shown) and C16:0-Cer (Supplemental Fig. 2D), but not that in C24:0-Cer (data not shown), which was also slightly upregulated in IFN-γ-treated cells (Fig. 3A). As expected, siCerS6 prevented IFN-γ-induced iNOS mRNA expression and synthesis of TNF-α (Fig. 3A) and TNF-α release (Fig. 4A) in RAW 264.7 macrophages when compared with scrambled siRNA-treated RAW 264.7 macrophages. These findings indicate that CerS6 and C16:0-Cer mediated the activation of iNOS expression in IFN-γ-stimulated RAW 264.7 macrophages. Because the downregulation of C16:0-Cer prevented IFN-γ-induced NO synthesis, specific endogenous upregulation of C16:0-Cer would be expected to increase NO release. Importantly, exogenously added palmitic acid (25 μM), which led to a specific increase in C16:0-Cer (Fig. 4C, Supplemental Fig. 2E), significantly amplified the IFN-γ (0.5 ng/ml) induced NO synthesis in RAW 264.7 macrophages (Fig. 4D).

C16:0-Cer mediate IFN-γ-induced TNF-α release in RAW macrophages

Because TNF-α, like NO, is a mediator of inflammatory processes, we investigated whether TNF-α is also regulated by C16:0-Cer. As postulated, TNF-α was upregulated in EAE mice in a disease-dependent manner (Fig. 2F). IFN-γ induced the expression of TNF-α mRNA in macrophages, which could be inhibited by methylprednisolone (1 μM) (Fig. 4E). IFN-γ-induced TNF-α mRNA expression and TNF-α release were also inhibited by fumonisin B1 and l-cycloserine (Fig. 4E, 4F). As expected, siCerS6 prevented IFN-γ-induced TNF-α mRNA expression (Fig. 4G) and TNF-α release (Fig. 4H) in RAW 264.7 macrophages compared with scrambled siRNA-treated RAW 264.7 macrophages. Moreover, exogenously added palmitic acid (25 μM) amplified the IFN-γ (0.5 ng/ml) induced TNF-α synthesis in RAW 264.7 macrophages (Fig. 4F).

**FIGURE 4.** Downregulation of CerS6 prevents, at least partially, the IFN-γ–induced release of NO/TNF-α, which is amplified by exogenously added palmitic acid. (A, B, G, and H) Levels of iNOS mRNA (A) and TNF-α mRNA (G) as well as NO release (B) and TNF-α release (H) in scrambled (scr.) siRNA and siCerS6-treated RAW 264.7 macrophages that were coincubated with 10 ng/ml IFN-γ for 16 h. C16:0-Cer (C) and NO (D) levels are upregulated in RAW 264.7 macrophages stimulated simultaneously with 25 μM palmitic acid and 0.5 ng/ml IFN-γ for 20 h. (E and F) Fumonisin B1 (FB1), l-cycloserine (Cyclo), and methylprednisolone (MP) prevent the IFN-γ–induced increase in mRNA expression and synthesis of TNF-α. Exogenously added palmitic acid and IFN-γ led to an increase in TNF-α synthesis. Fumonisin B1 (70 μM), l-cycloserine (500 μM), or methylprednisolone (1 μM) were coincubated with 10 ng/ml IFN-γ for 16 h. RAW 264.7 macrophages were stimulated with 25 μM palmitic acid and 0.5 ng/ml IFN-γ for 20 h. The level of TNF-α mRNA in the cell pellet (E) and of TNF-α release (F) in the supernatant were determined. C16:0-Cer/NO/TNF-α amounts were related to the number of treated cells. The relative mRNA levels were calculated using untreated cells at the same time point as those at basal level. Data are means ± SEM of one of three comparable independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (significant differences between IFN-γ stimulated and IFN-γ/palmitic acid or IFN-γ-specific inhibitor costimulated RAW 264.7 macrophages).
C_{16:0}-Cer mediates IFN-γ–induced TNF-α and NO release in peritoneal macrophages

In peritoneal macrophages, we investigated whether NO and TNF-α release are dependent on C_{16:0}-Cer. In peritoneal macrophages, IFN-γ induced a time-dependent, transient increase in CerS6, which resulted in a significant increase in C_{16:0}-Cer after 16 h (Fig. 5A, 5B). Notably, the IFN-γ–induced release of NO and TNF-α could be inhibited by fumonisin B1 as well as by l-cyclosorine (Fig. 5C, 5D). These data indicate that in primary macrophages, the synthesis of NO and TNF-α is also C_{16:0}-Cer dependent.

C_{16:0}-Cer concentrations and iNOS, TNF-α, and CerS6 expression are reduced during the initial phase of disease in IFN-γ KO EAE mice

Next, we were interested in whether IFN-γ is relevant for the elevated C_{16:0}-Cer/CerS6 levels in the EAE model. For this purpose, we induced EAE in IFN-γ KO mice. We observed a significant delay in disease onset, as described by Pil et al. (27). EAE/IFN-γ KO mice developed initial signs of clinical symptoms at day 12 ± 1 and EAE/WT mice at day 10 ± 1. Ceramide levels and mRNA expression were determined in EAE/WT and EAE/IFN-γ KO mice, when sc0.5 was reached. Notably, the expression of mRNAs for CerS6, iNOS, and TNF-α were significantly reduced in EAE/IFN-γ KO mice when compared with EAE/WT mice (Fig. 5E). Furthermore, in EAE/IFN-γ KO mice, the C_{16:0}-Cer level was significantly lower compared with that of EAE/WT mice (Fig. 5F). Most importantly, CerS6 and iNOS protein was also clearly lower in EAE/IFN-γ KO mice than in EAE/WT mice (Fig. 5G). The reduced CerS6 and iNOS expression in EAE/IFN-γ KO mice confirms our in vitro results showing that IFN-γ induces the synthesis of CerS6/C_{16:0}-Cer, which subsequently is relevant for the induction of iNOS.

Inhibition of C_{16:0}-Cer prevents worsening of the clinical symptoms in the EAE model

NO and TNF-α production were related to tissue-damaging effects and the initiation of a myelin-directed immune response, respectively, during the onset and in the acute phase of the EAE model (2, 3). Because C_{16:0}-Cer was linked to NO and TNF-α release in peritoneal macrophages, we studied the effects of inhibition of ceramide synthesis in EAE mice by l-cyclosorine, given at the onset of the disease. Mice were treated with 75 mg/kg l-cyclosorine (i.p.) once daily starting when the mice showed the first signs of clinical symptoms (sc0.5). The control EAE mice were treated with vehicle. The AUC for clinical score in l-cyclosorine–treated EAE mice was 10.51 ± 1.8 score units/day and that in vehicle-treated EAE mice was 17.69 ± 2.3 score units/day. Treatment with l-cyclosorine significantly prevented (p = 0.041) the development of clinical symptoms (Fig. 6A). Fig. 6B reveals...
that in l-cycloserine–treated EAE mice, C16:0-Cer levels were significantly lower compared with vehicle-treated EAE mice. Moreover, l-cycloserine prevented the increase in mRNA and protein levels of iNOS and of TNF-α in the lumbar spinal cord (Fig. 6C–F). These data indicate that C16:0-Cer mediates iNOS and TNF-α generation during the development of EAE (Fig. 7).

Discussion
It is known that sphingolipids play essential roles in the induction and progress of inflammation (28), but only few publications deal with the role of specific ceramides in the inflammatory process (29). For the first time, to our knowledge, we show that CerS6 and its product, C16:0-Cer, specifically contribute to the inflammatory process by inducing iNOS and TNF-α expression in the initial phase of EAE. This was implied by the following results: 1) In EAE mice in vivo, C16:0-Cer and CerS6 expression are increased in inflammatory cells such as migrated monocytes/macrophages and astroglia. Furthermore, l-cycloserine—an inhibitor of the de novo synthesis of ceramides—attenuated the clinical symptoms of EAE mice and prevented increases in C16:0-Cer as well as in iNOS and TNF-α. 2) In the cerebrospinal fluid of MS patients, C16:0-Cer was elevated 1.9-fold. 3) In IFN-γ–induced RAW 264.7 macrophages, IFN-γ, predominantly derived from activated T cells, induces C16:0-Cer-dependent TNF-α/NO release. NO and TNF-α are prominent mediators of oligodendroglial death, demyelination, and axonal degradation [Steinman 2001 (43), Hendriks et al. 2005 (44)].

FIGURE 6. The serine palmitoyltransferase inhibitor l-cycloserine prevents, at least partially, the development of clinical symptoms in EAE mice and the increase in C16:0-Cer, iNOS, and TNF-α expression. (A) Clinical score of EAE mice that were treated daily with 75 mg/kg l-cycloserine or vehicle (saline) by i.p. injection. The EAE and CFA-treated mice were treated with l-cycloserine or vehicle (saline) when the EAE mice reached sc0.5. Data are means ± SEM of number of mice as indicated. The results were obtained from two different studies. (B) The ceramide levels of the l-cycloserine–treated or vehicle-treated EAE mice and CFA-treated mice were related to the ceramide levels of age-matched control mice. (C–F) The relative expression of iNOS mRNA (C), iNOS protein (D), TNF-α mRNA (E), and TNF-α protein (F) from l-cycloserine–treated and vehicle-treated EAE mice is shown. The mRNA expression levels were calculated using data of untreated age-matched control mice as baseline values. The Western blot analysis shows a representative blot of two determinations. Data are means ± SEM of number of mice as indicated. *p < 0.05 (significant difference between l-cycloserine–treated and saline-treated EAE mice). Cyclo, l-cycloserine.

FIGURE 7. The putative role of C16:0-Cer in IFN-γ–induced NO and TNF-α release from macrophages. IFN-γ, predominantly derived from activated T cells, induces C16:0-Cer-dependent TNF-α/NO release. NO and TNF-α are prominent mediators of oligodendroglial death, demyelination, and axonal degradation [Steinman 2001 (43), Hendriks et al. 2005 (44)].
Glatiramer acetate, both licensed for the treatment of relapsing–remitting multiple sclerosis (MS), suppresses inflammation (disease-inhibiting effect) at the onset of the disease, and 2) later in the course of the disease, IFN-γ (disease-stimulating effect) increases NO level before disease onset reduces T cell proliferation (disease-inhibiting effect) 2) while elevated NO level in the CNS at the onset of the disease acts as a tissue-damaging free radical (disease-stimulating effect). We showed that inhibition of C16:0-Cer synthesis at the onset of the disease reduces TNF-α and NO synthesis and abates disease progression. Unfortunately, specific inhibitors of C16:0-Cer synthesis or CerS6 KO mice are not available, as these would be helpful in confirming the relevance of C16:0-Cer in the development of EAE.

We postulate that C16:0-Cer acts as an inflammatory mediator. A prerequisite for mediators is sufficient mobility to reach potential target proteins. But ceramides are not water-soluble and therefore less mobile. This raises the question how ceramides could be transferred to their potential target proteins? It is known that ceramides can be transported by a specific transport protein CERT (37), by vesicles (38), and in neuronal cells by binding to cytoskeleton proteins (38). Furthermore, the occurrence of several ceramide synthases at different subcellular sites and therefore the formation of ceramides in different subcellular organelles might also overcome the immobility of ceramides (39). In the cerebrospinal fluid, C16:0-Cer is probably also bound to proteins. Albumin could be a carrier protein for ceramides as it binds hydrophobic substances, and ceramides have been reported to interact with the hydrophobic domains of proteins (40).

In conclusion, our data reveal that C16:0-Cer/CerS6 play a crucial role in the inflammatory process during the initial phase of EAE by regulating NO and TNF-α synthesis. Importantly, we also demonstrated that in the cerebrospinal fluid of MS patients, C16:0-Cer levels are upregulated, and Wheeler et al. (41) observed a slight increase in C16:0-Cer in the white matter of MS samples. Recently, Nikić et al. (42) showed that axonal damage induced by NO released from macrophages occurs in axons with intact myelin sheaths and that this process is reversible. These findings indicate that the early reduction in NO through inhibition of CerS6 may prevent axonal damage and subsequently prevent the development of MS (42). Therefore, CerS6 could be a promising target for the development of novel drugs to treat MS, and C16:0-Cer may be a potential novel biomarker in MS for diagnosis and treatment monitoring.

Acknowledgments
We thank Prof. Dr. Michael Parnham for the linguistic revision of the manuscript.

Disclosures
G.G., K.S., U.Z., and S.S. have a patent pending concerning the use of CerS6 as a target for the development of new MS drugs. The other authors have no financial conflicts of interest.

References

C16:0-Cer/CerS6 ARE UPREGULATED IN THE EAE MODEL

The transient increase in C16:0-Cer/CerS6 at the beginning of the inflammatory process points toward a significant role of C16:0-Cer in the initial phase of MS. Furthermore, our results demonstrate for the first time, to our knowledge, a direct correlation between de novo synthesized C16:0-Cer and IFN-γ–induced NO and TNF-α synthesis (Fig. 7). Up to now, only ceramides synthesized by the salvage pathway or glycosylated ceramides have been reported to induce NO and TNF-α synthesis (30, 31). Innate or classical activation of macrophages by LPS or IFN-γ (32), respectively, induces sphingomyelinases (31) or de novo synthesis (as indicated by our results), respectively. Notably, in the initial phase of EAE, mainly Th1 cells that secrete IFN-γ are involved in the inflammatory process (33), suggesting an important role of de novo-synthesized ceramides in the initial phase of EAE.

Indeed, the induction of CerS6/C16:0-Cer was significantly reduced in EAE/IFN-γ KO mice compared with that in EAE/WT mice and was accompanied by a significant delay in disease onset in EAE/IFN-γ KO mice in comparison with EAE/WT mice. These results strengthen our hypothesis that IFN-γ–induced CerS6/C16:0-Cer play a critical role in the initial phase of the disease. However, EAE/IFN-γ KO mice, in comparison with EAE/WT mice treated with l-cycloserine, exhibited no disease amelioration; this might have been due to the dual anti-inflammatory and proinflammatory properties of the IFN-γ/IFN-α/NO/TNF-α pathway, as discussed below. The increase in C16:0-Cer in EAE/IFN-γ KO mice indicates that not only IFN-γ but also other stimuli regulate the C16:0-Cer level. We observed an increase in CerS6 in both macrophages and in astroglia. Kim et al. (34) showed that an increase in C16:0-Cer in astroglia correlates with oligodendrocyte apoptosis, which is a pathological hallmark of EAE contributing to disease worsening. The role of C16:0-Cer in other cell types will be the topic of further studies.

TNF-α and NO exert contradictory properties during the development of EAE/MS. A dual role for TNF-α was proposed: 1) TNF-α initiates a myelin-directed immune response (disease-stimulating effect) at the onset of the disease, and 2) later in the chronic phase it causes depletion of autoreactive lymphocytes and suppresses inflammation (disease-inhibiting effect). IFN-β and glatiramer acetate, both licensed for the treatment of relapsing–remitting MS, also reduce the TNF-α level in MS patients (35, 36). For NO, a similar dual role was suggested: 1) A high blood NO level before disease onset reduces T cell proliferation (disease-inhibiting effect) 2) while elevated NO level in the CNS at the onset of the disease acts as a tissue-damaging free radical (disease-stimulating effect). We showed that inhibition of C16:0-Cer synthesis at the onset of the disease reduces TNF-α and NO synthesis and abates disease progression. Unfortunately, specific inhibitors of C16:0-Cer synthesis or CerS6 KO mice are not available, as these would be helpful in confirming the relevance of C16:0-Cer in the development of EAE.


Supplement 1: The lumbar spinal cord was incubated with CerS6 antibody (red channel) and with cell marker antibodies (green channel) OSP (oligodendrocytes) (A), GFAP (astroglia) (B) or DR5 (apoptotic cells) (C). All data are representative images of three independent experiments. The magnification is 60x. Scale bar, 10 μm. D) RAW 264.7 macrophages were treated for 16h with 10 ng/ml IFN-γ, 5 ng/ml TNF-α or 1 ng/ml IL-1β. The ceramide levels were normalized to the number of treated cells. The relative increase of the specific ceramides was calculated using the ceramide levels of untreated cells as 100%. Data are mean ± s. e. m. of three independent experiments, each made in duplicate. * (p < 0.05), ** (p <0.01) indicate significant differences between cytokine-treated and untreated RAW 264.7 macrophages. E) RAW 264.7 macrophages were treated or not for the time points indicated with 10 ng/ml IFN-γ. The relative mRNA expression of CerSs was calculated using the mRNA level of untreated cells at the same time point as basal level. Data are means ± s. e. m. of three independent experiments, each carried out in triplicate. * (p < 0.05), ** (p < 0.01), *** (p < 0.001) indicate significant differences between IFN-γ treated and untreated cells.
Supplement 2: A) RAW 264.7 were preincubated with 500 μM L-cycloserine (Cyclo) or 70 μM Fumonisin B1 (FB1) for 90 min or 30 min, respectively, and subsequently 10 ng/ml IFN-γ were added for 16h. The ceramide levels were related to the number of treated cells. The amount of ceramides in the control cells were set as 100%. Data are means ± s. e. m. of three comparable independent experiments. * p<0.05, ** p<0.01, *** p<0.001 indicate significant differences between IFN-γ treated cells and cells co-treated with IFN-γ and an inhibitor of the sphingolipid synthesis. B) The cell viability was determined with the WST proliferation assay (Roche Diagnostics). RAW 264.7 macrophages were co-treated with 10 ng/ml IFN-γ and Fumonisin B1 (70 μM) or L-cycloserine (500 μM) for 16h. The cell viability was determined by using IFN-γ treated cells as 100 %. Data are means ± s. e. m. of two comparable independent experiments. C) CerS5/CerS6 mRNA and ceramide levels (D) in scrambled (scr.) siRNA and siCerS6 treated RAW 264.7 macrophages, which were co-incubated with 10 ng/ml IFN-γ for 16 h. * (p < 0.05), *** (p < 0.001) indicate significant differences between siCerS6 treated and scrambled siRNA treated RAW 264.7 macrophages. E) RAW 264.7 macrophages were incubated with DMSO (control), 25 μM palmitic acid, 0.5 ng/ml IFN-γ or 25 μM palmitic acid and 0.5 ng/ml IFN-γ for 20 h. The ceramide levels were related to the number of treated cells. The relative mRNA levels were calculated using untreated cells at the same time point as the basal level. Data are means ± s. e. m. of two comparable independent experiments each done in duplicate. (ns; not significant)