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Original Paper

Pharmacological Inhibition of Acid Sphingomyelinase Ameliorates Experimental Autoimmune Encephalomyelitis

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Key Words
Multiple sclerosis • Acid sphingomyelinase • Amitriptyline • Ceramide antibodies • Experimental autoimmune encephalomyelitis

Abstract
Background/Aims: Multiple sclerosis (MS) is one of the most common autoimmune disorders of the central nervous system (CNS) and the leading cause of neurological disability among young adults in the Western world. We have previously shown that the acid sphingomyelinase plays an important role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis. ***Methods:*** We induced adoptively transferred EAE in wildtype and acid sphingomyelinase-deficient mice. In addition, we immunized mice with $MOG_{aa35-55}$ to induce active EAE and treated the mice with amitriptyline, a functional inhibitor of the acid sphingomyelinase. We investigated symptoms of EAE, blood-brain barrier integrity and neuroinflammation. ***Results:*** In the model of adoptively transferred EAE we demonstrate that expression of acid sphingomyelinase in the recipients rather than on transferred encephalitogenic T cells contributes to the clinical development of EAE symptoms. To test if pharmacological targeting of acid sphingomyelinase can be explored for the development of novel therapies for MS, we inhibited acid sphingomyelinase with amitriptyline in mice in which EAE was induced by active immunization. We demonstrate that pharmacological inhibition of acid sphingomyelinase using amitriptyline protects against the development of EAE and

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markedly attenuates the characteristic detrimental neuroinflammatory response. ***Conclusion:*** The studies identify the acid sphingomyelinase as a novel therapeutic target for treating MS patients.

Introduction

Multiple sclerosis (MS) is the most important inflammatory disorder of the central nervous system (CNS) and the leading cause of neurological disability among young adults in the Western world [1, 2]. MS is characterized by focal blood-brain barrier (BBB) breakdown and inflammation in the CNS eventually leading to demyelination, loss of neurons, glia scars and neurological symptoms, such as motor and sensory deficits or impairment of vision. The inflammation is orchestrated by an initial infiltration of $CD4^+$ and $CD8^+$ T cells followed by macrophages [1, 3-5], and recent studies have also shown an important role of B lymphocytes in the pathogenesis of MS [6]. Immune cells determine the inflammatory effector phase in the CNS that ultimately leads to myelin sheath damage.

Although we know some of the pathophysiological events leading to MS, the etiology of the disease is presently still unknown. Thus, no causative treatment is available.

There are many therapeutic options available and several new drugs modifying the disease and mainly influencing lymphocyte migration, production of inflammatory mediators or lymphocyte proliferation or survival, have been introduced into the clinic [7, 8]. These immunomodulatory drugs clearly reduce MS relapses, but disease progression cannot completely be prevented nor can these drugs control progressive disease courses. Furthermore, many of these drugs can have severe adverse effects, such as detrimental cerebral infections, induction of tumors, influenza-like symptoms, vascular dysfunctions and macula damage [9, 10]. Therefore, safer novel therapies for the treatment of MS patients need to be identified and established.

We have previously shown that genetic deficiency of acid sphingomyelinase prevented the characteristic detrimental neuroinflammatory response typical for MS, blocked BBB disruption and subsequent intracerebral infiltration of inflammatory cells into the CNS in mice [11]. Therefore, acid sphingomyelinase-deficient mice were protected against clinical symptoms in experimental autoimmune encephalomyelitis (EAE). Acid sphingomyelinase is an ubiquitously expressed enzyme that has been previously shown to be critically involved in cellular stress responses [12-14]. The acid sphingomyelinase is primarily expressed in lysosomes and serves the consumption of sphingomyelin to ceramide. However, upon fusion of secretory lysosomes with the plasma membrane the enzyme can be transported to the plasma membrane to generate ceramide at the plasma membrane [15]. It is this ceramide that aggregates spontaneously to form ceramide-enriched membrane platforms which serve as signalling platforms, for instance by clustering stress or death receptors [15]. These platforms often mediate the role of the acid sphingomyelinase/ceramide system in physiological and pathological conditions [15-17]. In addition, the acid sphingomyelinase can be secreted and this secretory acid sphingomyelinase might be also able to release surface ceramide on cells [18]. The acid sphingomyelinase can be functionally inhibited by antidepressants such as e.g. desipramine, amitriptyline or imipramine [19-23]. These inhibitors are weak bases that diffuse into lysosomes, are protonated and thereby trapped in lysosomes and competitively displace the acid sphingomyelinase from the inner leaflet of the lysosomal membrane [20]. The release of the acid sphingomyelinase from the lysosomal membrane then results in proteolytic degradation of the enzyme in the lysosome [19-21, 24]. Similar mechanisms may apply to the inhibition of surface acid sphingomyelinase by these drugs.

Here, we investigated the cellular source of acid sphingomyelinase for EAE induction and tested whether targeting the acid sphingomyelinase with functional inhibitors of the acid sphingomyelinase is able to prevent or reduce symptoms of EAE.

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Materials and Methods

Mice

All animal experiments were performed in compliance with the German Guide for the Care and Use of Laboratory Animals and the Swiss legislation on the protection of animals. All studies were approved by the Landesamt für Gesundheit und Verbraucherschutz, Saarbruecken (approval numbers: 34/2008; 41/2009; 33/2009; 19/2012; 06/2013) and the Veterinary Office of the Canton of Bern (permission numbers: BE 42/14, BE 72/15, and BE 31/17). Female SJL/J mice aged 6 to 8 weeks were obtained from Charles River Laboratories (Sulzfeld, Germany). Female 6 to 8 week-old wildtype C57BL/6 littermates and acid sphingomyelinase-deficient mice (protein, Asm; gene symbol, *Smpd1*) on a C57BL/6 genetic background were bred in house. Mice were kept in a pathogen-free environment according to the Federation of Laboratory Animal Science Associations (FELASA) criteria. The genotype of acid sphingomyelinase-deficient mice was confirmed by polymerase chain reaction (PCR) before experimentation.

Induction of transfer EAE and FACS analysis of Th polarization

Transfer EAE in C57BL/6 wildtype and acid sphingomyelinase-deficient mice were induced as previously described [25]. Briefly, 6-8 week-old female donor acid sphingomyelinase-deficient mice and wildtype littermates were immunized with an emulsion of 1 mg myelin-oligodendrocyte glycoprotein peptide amino acids 35-55 ($MOG_{aa35-55}$; Genscript, NJ, USA) per mL complete Freund´s adjuvant (CFA). An aliquot of 200 μL of the suspension was subcutaneously injected into the backside of the mice in close proximity to the axillary and inguinal lymph nodes. Ten days later, mice were sacrificed and swollen axillary and inguinal draining lymph nodes and spleens were collected. Single cell suspension was prepared and erythrocytes were lysed with a mixture of 9 volumes of 155 mM NH_4Cl and 1 volume of 170 mM Tris-HCL, pH 7.6 for 5 min at 37°C. Cells were adjusted to $4x10^6$ cells/mL in re-stimulation medium [RPMI-1640 supplemented with 10% FBS (Thermo Fisher Scientific), 10 U/ml penicillin-streptomycin, 2 mM L-glutamine, 1% (v/v) non-essential amino acids, 1 mM sodium pyruvate, and 0.05 mM β-mercaptoethanol] and stimulated with 20 μg/mL MOG, 15 ng/ml mIL-12 (Fitzgerald Industries, MA, USA), 5 ng/mL mIL-23 (R&D, Wiesbaden, Germany), 10 μg/mL anti-IFNγ (clone XMG1.2; BD Biosciences) and plated in 12-well plates. Antigen-specificity of the collected cells was checked by additional proliferation assays of isolated donor lymphocytes. To this end, 2 days later cells were fed with 1 mL restimulation medium per well. Cells were collected 3 days later and viable cells were isolated by Ficoll gradient 1:1 (Sigma- Aldrich, St. Louis, USA).

CD4 positive cells were collected by magnetic bead negative selection (Invitrogen, Darmstadt, Germany) according to the manufacturer's instruction and $3x10^6$ cells were injected intraperitoneally per mouse into syngeneic recipient wildtype or acid sphingomyelinase-deficient mice. Assessment of clinical disease activity was performed twice daily as described before [26, 27] and scored as follows: 0, healthy; 0.5, limp tail; 1, hind leg paraparesis; 2, hind leg paraplegia; 3, hind leg paraplegia with incontinence.

Cytokine profile of activated $CD4^+$ T cells was analyzed by flow cytometry. Therefore, $5x10^6$ cells in re-stimulation medium were plated in a 60 mm dish and stimulated with 50 ng/mL phorbol myrisate acetate (PMA) + 1 μg/mL ionomycin (BioVision, CA, USA) and 3.3 μl/5 mL Golgi Stop (BD Bioscience, Th1/Th2/Th17 phenotying kit) for 5 hrs at 37°C. Thereafter, cells were collected, washed in FACS buffer (phosphate buffered saline (PBS; 137 mM NaCl, 10.2 mM Na_2HPO_4, 2.7 mM KCl, and 1.7 mM KH_2PO_4, pH 7.3) + 0.1% NaN_3+2.5%FCS), and distributed into 10 wells of a 96 well plate. Cells were re-suspended in 200 μL/well Cytofix/Cytoperm buffer (BD Bioscience, Th1/Th2/Th17 phenotyping kit) and incubated for 20 min on ice. Cells were washed with FACS buffer at room temperature and incubated with antibodies diluted in perm/wash buffer (BD Bioscience, Th1/Th2/Th17 phenotying kit) for 30 min on ice. The following antibodies and respective isotype controls were used: CD45-PE, -PerCP, -APC, -FITC (30-F11, Biolegend), CD4-PerCP (GK1.5, BD Pharmingen), IFNγ-FITC (XMG1.2, BD Bioscience), GM-CSF-FITC (MP1-22E9, Biolegend), IL-17-PE (TC11-18H10.1, BD Bioscience), IFNγ-PE (XMG1.2, BD Bioscience), IL-13-PE (eBio13A, eBioscience), IL-5-PE (TRFK5, eBioscience), IL-4-APC (11B11, Biolegend), IL-2-APC (JES6-5H4, BD Pharmingen), IL-10-APC (JES5-16E3, eBioscience). Cells were washed twice with perm/wash buffer and re-suspended in 200 μl 1% paraformaldehyde (PFA), kept at 4°C until measurement with FACS Calibur.

Induction and scoring of active EAE

C57BL/6 mice were immunized with 300 μg $MOG_{aa35-55}$ (Charité Medical University, Berlin, Germany). SJL/J mice received 50 μg proteolipid protein ($PLP_{aa139-151}$; AnaSpec, Fremont, CA). Both antigens were carefully emulsified 1:1 (v/v) in incomplete Freund's adjuvant (IFA; DIFCO, BD Bioscience, Heidelberg, Germany) supplemented with 4 mg/mL non-viable, desiccated *Mycobacterium tuberculosis* (H37RA; DIFCO, BD Bioscience). The suspension was then subcutaneously injected into hint leg flanks in close proximity to the inguinal lymph nodes and at the tail root. On days 0 and 2 after injection, 300 ng pertussis toxin (Axxora, Lörrach, Germany) was injected intraperitoneally (i.p.). To determine the clinical disease of EAE, we used the same score as described above. Mice were scored daily.

EAE treatments

To inhibit acid sphingomyelinase pharmacologically, we injected 25 mg/kg amitriptyline (Sigma-Aldrich, Deisenhofen, Germany) in 0,9% NaCl i.p. every 12 hrs starting on day 1 after immunization. We chose the twice-daily dosing schedule to maintain plasma concentrations of amitriptyline within the therapeutic range [21]. Control mice were injected with 0.9% NaCl. Mice were sacrificed as indicated, and brain and spinal cord samples were subjected to histological examination.

Histopathological studies

Mice were sacrificed and perfused with 0.9% NaCl for 2 min followed by 4% PFA for 15 min through the left ventricle of the heart. Brain and spinal cord were removed and snap-frozen in Tissue Tek (Sakura Finetek; Hartenstein, Würzburg, Germany) in melting isopentane with liquid nitrogen. The frozen tissue was cut serially into 6-μm sections with a cryostat microtome (Leica Microsystems, Wetzlar, Germany). The sections were air-dried overnight, and fixed with acetone at -20°C and subjected to immunohistochemical analysis with a three-step immunoperoxidase technique in a humidified chamber. The following monoclonal antibody was used: anti-CD45 (BD Bioscience). The isotype control IgG2aκ was obtained from IQ Products (Houston, TX). Sections were incubated with the primary or secondary antibodies for each 30 min and washed between the steps with phosphate-buffered saline (PBS). Primary antibodies were labelled with biotinylated secondary goat-anti-rat IgG (Vectastain; Linearis, Wertheim-Bettingen, Germany) and finally by a horseradish peroxidase–conjugated streptavidin (Vectastain; Linearis). Sections were developed with 0.07% aminoethylcarbazole (AEC; Sigma-Aldrich) and 0.009% hydrogen peroxide in 0.01 mol/L acetate buffer (pH 5.2) for 10 min and then counterstained with hematoxylin-eosin and cover-slipped with Aquatex (Merck, Darmstadt, Germany).

Lymphocyte adhesion assay

To measure adherence of lymphocytes to an endothelial monolayer, 16-well chamber slides (VWR International, Darmstadt, Germany) were coated with 50 μg/mL fibronectin (Roche, Mannheim, Germany). The wells were plated with 2×10^4 bEnd.3 cells (ATCC; LGC Standards, Wesel, Germany) each for 2 days prior to the experiment. The bEnd.3 cells are a mouse brain endothelial cell line derived from BALB/c mice. Lymphocytes were isolated from EAE-immunized C57BL/6 or SJL/J mice on day 14 after immunization. To this end, axillary and inguinal lymph nodes were homogenized in Hank's Balanced Salt Solution (HBSS)/10% FCS/25 mM HEPES solution, strained through a 70-μm cell filter, cells were centrifuged and resuspended in Dulbecco`s Modified Eagle Medium (DMEM)/2% GlutaMAX/1% penicillin-streptomycin/25 mM HEPES/5% FCS. 3×10^5 lymphocytes were added per well to the endothelial cells for 40 min at 4°C on a rocking shaker. Co-incubation was terminated by washing off non-adherent lymphocytes with PBS and fixation of the slides with 2.5% glutaraldehyde for 2 hrs. After the fixation period, slides were removed and adherent lymphocytes were counted with a light microscope. All experiments were performed in duplicate and repeated as indicated. Either lymphocytes or bEnd.3 cells were pre-incubated with amitriptyline or anti-ceramide antibodies.

Ceramide clustering

To determine clustering of ceramide and the formation of ceramide-enriched domains on the cell surface of T cells, we performed confocal microscopy of lymphocytes co-incubated with a bEnd.3 monolayer. Two days before the assay, 1×10^5 bEnd.3 cells were plated on glass coverslips in a 24-well plate. On day 14 after immunization, lymphocytes were harvested from wildtype mice as described above, and 5×10^5 T cells

were co-incubated with the bEnd.3 monolayer for 5 min. The samples were washed in ice-cold PBS and fixed in 4% PFA for 15 min. After fixation, the slides were washed in PBS and blocked for 10 min in H/S supplemented with 5% FCS and 0.05% Tween 20. Anti-ceramide antibodies, clone S58-9 (Glycobiotech), were diluted 1:100 in H/S + 1% FCS, and cells were incubated for 45 min with anti-ceramide antibodies. Samples were then washed 3-times in PBS, incubated for 45 min with Cy3-coupled anti-mouse IgM $F(ab)_2$ fragments (Jackson ImmunoResearch, Dianova, Hamburg, Germany), washed an additional 3-times in PBS and embedded in Mowiol. Immunofluorescence was measured with the Leica TCS SL software program, version 2.61.

Acid sphingomyelinase activity

On day 14 after immunization, lymphocytes were obtained from EAE-induced C57BL/6 mice as described above. Two days before the assay, 5×10^4 bEnd.3 cells were plated in 24-well plates. At the time of the assay, 1×10^5 lymphocytes were co-incubated with the bEnd.3 monolayer for 0 or 5 min. Although only a small fraction of T cells is activated upon immunization, only these cells with interact with the endothelial cells and activate the acid sphingomyelinase allowing us to determine activity of the acid sphingomyelinase on a low background. Cells were washed, lysed in 250 mM sodium acetate (pH 5.0), 1% NP40, and 10 μM $ZnCl_2$ for 5 min. Lysates were diluted 1:10 with 250 mM sodium acetate (pH 5.0) and 10 μM $ZnCl_2$ and were then incubated with 50 nCi [^{14}C]sphingomyelin per sample for 30 min at 37°C. Sphingomyelin was dried before the assay; resuspended in 250 mM sodium acetate (pH 5.0), 0.1% NP40, and 10 μM $ZnCl_2$; and bath-sonicated for 10 min. The reaction was stopped by the addition of 800 μL chloroform/methanol (2:1, v/v). Phases were separated by centrifugation, and radioactivity of the aqueous phase was measured with liquid scintillation counting to determine the release of [^{14}C]phosphorylcholine from [^{14}C]sphingomyelin as a measure of acid sphingomyelinase activity.

Ceramide measurement

Lymphocytes and bEnd.3 cells were co-incubated as above. Cells were lysed in 200 μL H_2O and extracted in 600 μL $CHCl_3$:CH_3OH:1N HCl (100:100:1, v/v/v). The lower phase was collected, dried, resuspended in 20 μL of a detergent solution (7.5% [w/v] n-octylglucopyranoside, 5 mM cardiolipin in 1 mM diethylenetriamine-pentaacetic acid [DTPA]), and sonicated for 10 min. The kinase reaction was initiated by the addition of 70 μL of a reaction mixture containing 10 μL diacylglycerol (DAG) kinase (GE Healthcare Europe, Munich, Germany), 0.1 M imidazole/HCl (pH 6.6), 0.2 mM DTPA (pH 6.6), 70 mM NaCl, 17 mM $MgCl_2$, 1.4 mM ethylene glycol tetraacetic acid (EGTA), 2 mM dithiothreitol, 1 mM adenosine triphosphate (ATP), and 5 μCi [^{32}P]γATP. Samples were gently shaken and incubated for 30 min at room temperature. The kinase reaction was terminated by the addition of 1 mL $CHCl_3$:CH_3OH:1N HCl (100:100:1, v/v/v), 170 μL buffered salt solution (135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES; pH 7.2), and 30 μL of a 100-mM ethylenediaminetetraacetic acid (EDTA) solution. Samples were separated, and the lower phase was collected, dried, and separated on Silica G60 thin-layer chromatography (TLC) plates with chloroform / acetone / methanol / acetic acid / H_2O (50:20:15:10:5, v/v/v/v/v). The TLC plates were analysed with autoradiography films, the spots were removed from the plates, and incorporation of [^{32}P] γATP into ceramide was measured by liquid scintillation counting. Ceramide amounts were determined by comparison with a standard curve using C_{16}-ceramide to C_{24}-ceramide as substrates.

Statistical analysis

All data are displayed as mean ± SD. Statistical analysis was performed with Student *t* test or ANOVA, as appropriate. For analysis of disease frequency the chi-square test was applied. Statistical significance was defined as $p < 0.05$.

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Results

Acid sphingomyelinase deficiency does not affect encephalitogenic potential of MOG-specific T cells

We have previously shown that deficiency of the acid sphingomyelinase protected mice from development of an active EAE [11]. Here, we aimed to identify mechanisms and the relevant cellular source of acid sphingomyelinase involved in EAE pathogenesis. We first investigated whether a lack of acid sphingomyelinase expression in MOG-specific T cells affects their encephalitogenic potential. To this end, we performed adoptive transfer EAE (tEAE) studies and found that acid sphingomyelinase-deficient MOG-specific T cells transfer EAE to wildtype recipients indistinguishable from wildtype MOG-specific T cells (Fig. 1).

Thus, acid sphingomyelinase activity is not required in auto-aggressive T cells for transferring EAE.

Adoptive transfer of EAE with wildtype encephalitogenic T cells into acid sphingomyelinase-deficient mice ameliorated EAE when compared to wildtype mice (Fig. 1). This reduced disease severity correlated to a reduced number of $CD45^{high}$ inflammatory cells, which could be isolated of the CNS of these mice (wildtype recipients: brain 157 cells, spinal cord: 56 cells; acid sphingomyelinase-deficient recipients: brain 22 cells, spinal cord 12 cells; data not shown). Most importantly the acid sphingomyelinase-deficient recipient mice almost completely recovered from clinical EAE by day 28 post disease induction (Fig. 1). To determine significant differences of the curves in Fig. 1a, we determined the area under the curve ± SD and performed a t-test. The calculations show a significant difference ($p<0.05$) between the group "Wt in Wt" and "Wt in $Asm^{-/-}$" and "$Asm^{-/-}$ in Wt" and "Wt in $Asm^{-/-}$". These data underscore that acid sphingomyelinase is relevant for disease development.

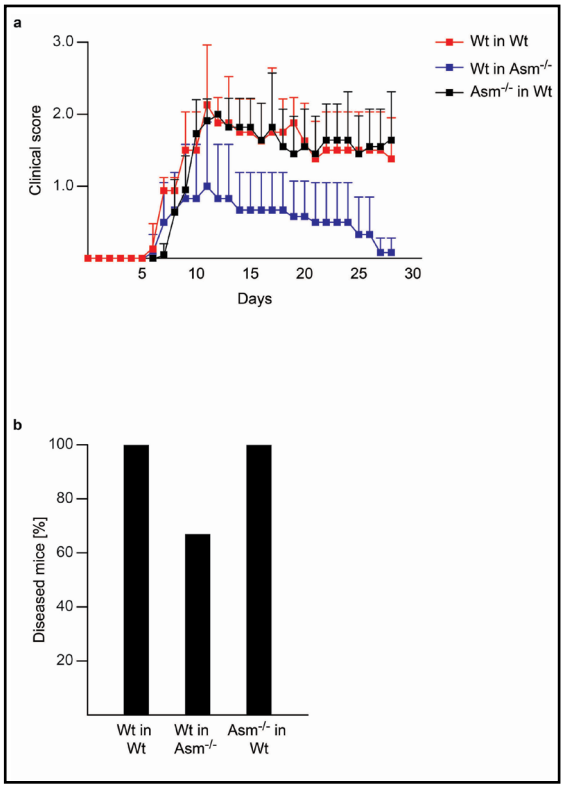

Fig. 1. Acid sphingomyelinase-deficient C57BL/6 develop ameliorated adoptively transferred EAE. Acid sphingomyelinase-deficient mice and C57BL/6 wildtype littermates received $3x10^6$ cells encephalitogenic T cells intraperitoneally. Mice were weighed and scored twice daily. Shown is the clinical course of a transfer EAE experiment (a) and the number of diseased mice in all groups (b). Shown are means ± SD, n= 8 for wildtype T cells in wildtype recipients (red line), n= 11 for acid sphingomyelinase deficient T cells in wildtype recipients (black line) and n= 6 for wildtype T cells in acid sphingomyelinase deficient recipients (blue line).

Pharmacological inhibition of acid sphingomyelinase activity prevents EAE

Next, we investigated whether blocking the acid sphingomyelinase/ceramide system can be developed into a clinically relevant therapeutic option for MS. To this end, we tested whether a pharmacological intervention with the functional acid sphingomyelinase inhibitor amitriptyline [19, 20] ameliorates or blocks the development of actively induced EAE. We treated wildtype mice after $MOG_{aa35-55}$-induced EAE twice daily with amitriptyline that was administered i.p. at a dose of 25 mg/kg body weight starting on day 1 after immunization. Control mice were injected with the same volume of 0.9% NaCl. We used two mouse models in these experiments, C57BL/6 mice and SJL/J mice, representing chronic and

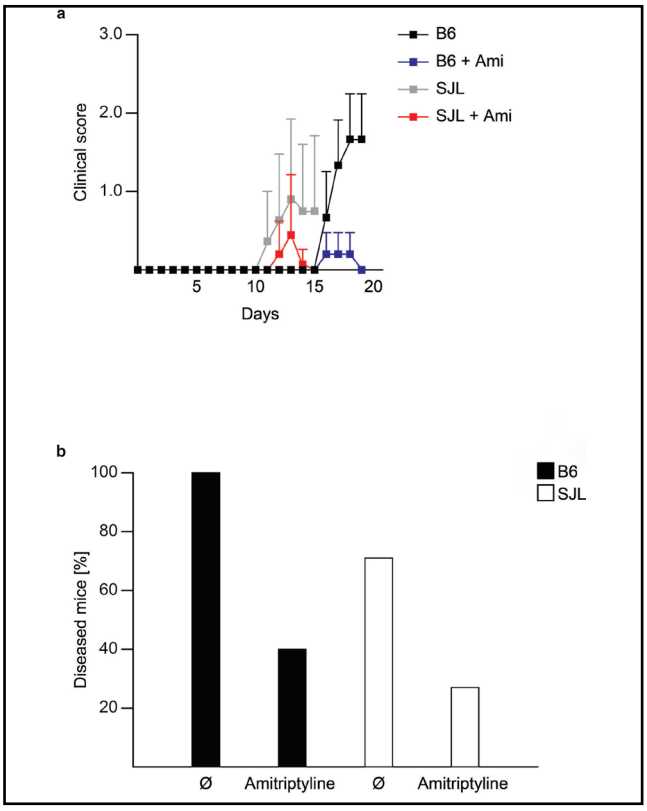

Fig. 2. Pharmacological inhibition of acid sphingomyelinase prevents EAE. SJL/J and C57BL/6 mice were immunized for experimental autoimmune encephalomyelitis (EAE) and treated with amitriptyline starting on day 1 after immunization. The disease was scored daily. Shown is a clinical course of one representative EAE experiment of each mouse strain (a) and the incidence of active EAE clinical symptom development (b). Shown are means ± SD, n = 5 for C57BL/6 and n= 11 for SJL/J mice.

relapsing-remitting EAE disease course, respectively. The results show that inhibition of acid sphingomyelinase activity by amitriptyline significantly reduced the number of mice developing chronic and relapsing remitting EAE symptoms (Fig. 2). To determine significant differences of the curves in Fig. 2a we determined again the area under the curve ± SD and performed a t-test. The calculations show a significant difference ($p<0.05$) between untreated and amitriptyline treated groups in Fig. 2a.

Consistent with the clinical findings, treatment of SJL and C57BL/6 mice after induction of EAE with amitriptyline also prevented the development of histological features of EAE (Fig. 3).

Activated lymphocytes interact with endothelial cells via the acid sphingomyelinase/ ceramide system

A key step of the development of EAE is the adhesion of lymphocytes to brain endothelial cells and we therefore tested if acid sphingomyelinase regulates adhesion of lymphocytes to brain endothelial cells. The studies demonstrate binding of lymphocytes from immunized SJL/J mice to endothelial cells *in vitro*, which was markedly reduced

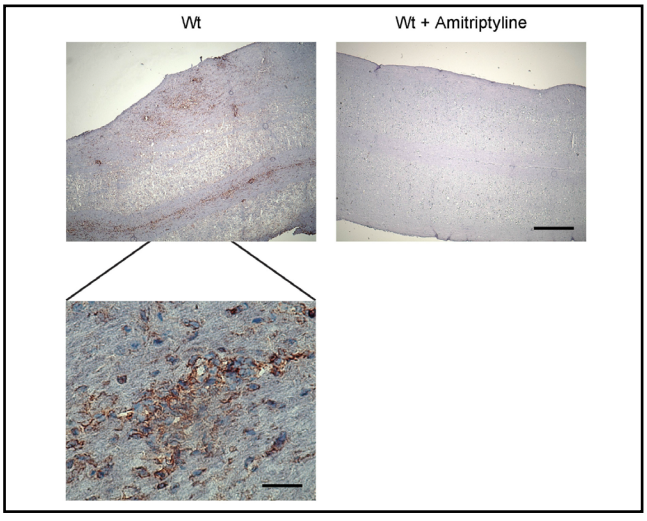

Fig. 3. Pharmacological inhibition of acid sphingomyelinase prevents histopathological changes of EAE. Amitriptyline treatment reduces inflammatory infiltration of the central nervous system. Experimental autoimmune encephalomyelitis (EAE) was induced by immunization with MOG_{35-55} in CFA on day 0 and mice were treated with amitriptyline starting on day 1 after immunization. Mice were anesthetized, perfused, the brain and the spinal cord were removed, fixed, embedded, sectioned and immunostainings with CD45 antibody were performed. Shown are representative histological pictures of 5 untreated or amitriptyline treated C57BL/6 mice. Scale bar is 250 µm and 100µm, respectively.

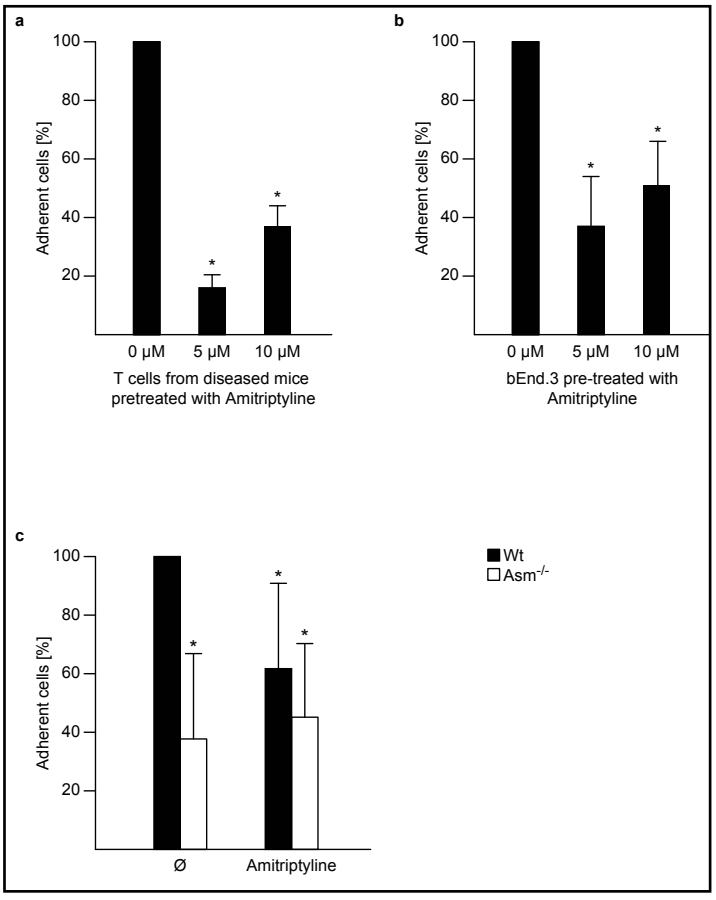

Fig. 4. Pharmacological inhibition of acid sphingomyelinase prevents adhesion of activated lymphoytes to brain endothelial cells. Adhesion of lymphocytes isolated from SJL/J mice immunized with $PLP_{139-151}$/CFA to mouse brain endothelial bEnd.3 cells is reduced by pre-incubation of lymphocytes or endothelial cells with amitriptyline (a, b; n=3). The same result was observed, when lymphocytes from C57Bl/6 mice immunized with $MOG_{aa35-55}$/CFA were treated with amitriptyline (c, wt: n = 21). Lymphocytes from immunized acid sphingomyelinase-deficient mice (n= 12) served as control and underlined that the pharmacological approaches are able to reduce adhesion to their same extend. Shown are means ± SD; *p ≤ 0.05.

by pre-incubation of lymphocytes (Fig. 4a) or endothelial cells (Fig 4b) with amitriptyline to the co-cultures. The same result could be observed for adhesion of lymphocytes derived from immunized C57/Bl6 mice (Fig. 4c).

We have previously shown that the activity of the acid sphingomyelinase often results in the formation of ceramide-enriched membrane platforms that are also involved in adhesion of cells to endothelial cells [28]. Thus, we tested whether ceramide-enriched membrane platforms are generated upon contact of lymphocytes isolated from C57BL/6 EAE-immunized mice with cultured brain endothelial cells. To this end, we prepared lymphocytes from draining lymph nodes of EAE-immunized wildtype mice and incubated them with bEnd.3 cells for 5 min. We observed rapid formation of ceramide-enriched membrane platforms when lymphocytes of mice suffering from EAE interacted with bEnd.3 cells (Fig. 5a), but we did not observe such clustering when lymphocytes, isolated from mice that were not immunized, were co-incubated with endothelial cells (Fig. 5a). The formation of ceramide-enriched membrane platforms was associated with rapid activation of the acid sphingomyelinase (Fig. 5b) and release of ceramide (Fig. 5c) when lymphocytes isolated from immunized mice were co-incubated with endothelial cells.

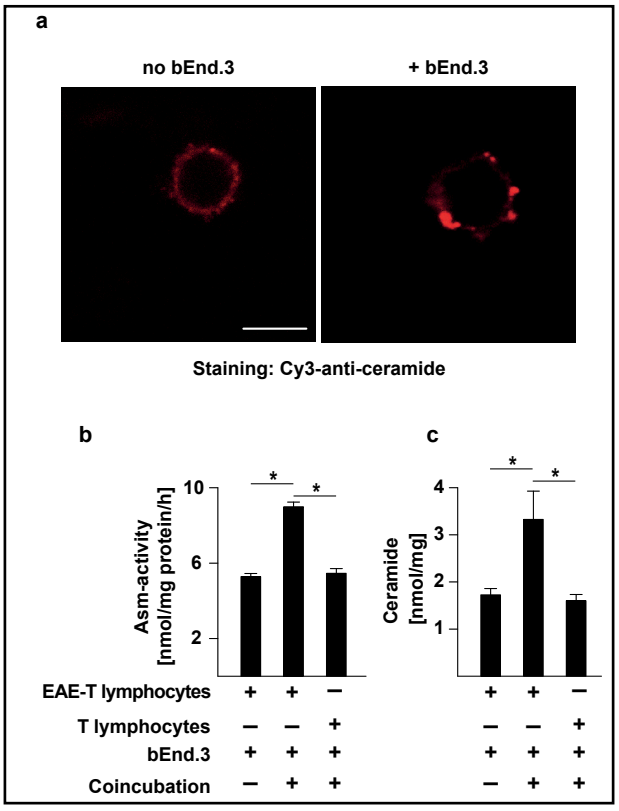

Fig. 5. Interaction of activated lymphocytes with brain endothelial cells induces formation of ceramide-enriched membrane platforms, activation of the acid sphingomyelinase and release of ceramide. (a) Confocal microscopy images of lymphocytes from immunized wildtype mice after 5 min incubation with mouse brain endothelial bEnd.3 cells show formation of ceramide-enriched membrane platforms. Acid sphingomyelinase activity (b) and ceramide concentrations (c) increase only upon co-incubation of lymphocytes from immunized mice with bEnd.3 cells, but not if wt lymphocytes from non-immunized mice were co-incubated with bEnd.3 cells. The confocal microscopy studies indicated that only a small fraction of immunized T lymphocytes interacted with endothelial cells and showed clustering of ceramide upon contact with the endothelial cells. Shown are representative confocal microscopy images or means ± SD of 5 independent experiments; *p, 0.05 Scale bar, 5 μm in a.

Discussion

Here, we provide evidence for the notion that acid sphingomyelinase is a pharmacological target to treat MS. We show that pharmacological inhibition of the acid sphingomyelinase system strongly protects against experimental MS. Endothelial adhesion of leukocytes and subsequent transmigration through the BBB into the CNS are hallmarks in MS pathophysiology. Pharmacological inhibition of acid sphingomyelinase by amitriptyline prevents the disease. Thereby, the detrimental pathophysiological cascade initiated by adhesion of lymphocytes to brain endothelial cells and followed by disruption of BBB tight junctions, migration of T lymphocytes through the BBB and finally intracerebral inflammatory cell recruitment, causing EAE symptoms is blocked. This finding is of clinical importance as it provides a potential novel therapeutic strategy.

Amitriptyline is a well-known antidepressant that has been widely used in clinical practice for more than 50 years [29]. The cationic amphiphilic drug amitriptyline competitively inhibits acid sphingomyelinase activity by displacing the enzyme from lysosomal membranes, in particular intralysosomal vesicles, thereby causing its degradation [19-24]. Because of this mechanism, therapeutic doses of amitriptyline never inhibit more than 60% to 80% of acid sphingomyelinase activity [20] and, thus, do not cause the severe clinical picture of Niemann-Pick disease type A, which is characterized by a reduction in enzyme activity of at least 90%. This finding is consistent with the clinical observation that even long-term amitriptyline treatment of patients with major depression is not associated with any severe adverse effects with regard to immune function. Therefore, given this long-term clinical experience with amitriptyline and its observed therapeutic effect in EAE, amitriptyline or its analogues are very good candidates for an immediate clinical study with MS patients. In fact, a study with a limited number of patients (n=19) found that treating MS patients with fluoxetine, a structurally related inhibitor of the acid sphingomyelinase and antidepressive drug, has a beneficial effect, although the published findings did not address molecular mechanisms [30].

Ceramide has been previously shown to form ceramide-enriched membrane platforms that are essential for the clustering of activated receptors [15]. Receptor clustering results in a very high local density of cognate receptor molecules, thereby permitting and greatly amplifying signal transduction by this particular receptor. The molecular details of ceramide-mediated receptor clustering of these molecules are unknown, although it has been suggested that the conformation and length of the transmembrane domain of a receptor molecule determine its partitioning into ceramide membrane domains [31, 32]. The results of the present study suggest that ceramide-enriched membrane platforms are important in the pathogenesis of EAE, but further studies are required to identify receptors and signalling pathways that are involved in this process.

The results of the transfer studies demonstrate that acid sphingomyelinase in cells of the recipient rather than in encephalitogenic T cells from the donor are important for EAE pathogenesis. At least in the transfer EAE model, the acid sphingomyelinase activity is not required in auto-aggressive T cells for development of an EAE. It should be indicated that these data do not exclude a role of the acid sphingomyelinase in T lymphocytes *in vivo*, since the *in vitro* treatment that is employed in the tEAE model may bypass events that require the acid sphingomyelinase in T lymphocytes *in vivo*.

Thus, acid sphingomyelinase-deficient recipients developed ameliorated EAE when compared to wildtype recipients and more importantly almost entirely recovered from disease pathogenesis on day 28 after tEAE induction. The tEAE data further encourage the notion that blocking acid sphingomyelinase bears great hope for the treatment of multiple sclerosis especially in the chronic phase of the disease, where there is no adequate treatment available so far.

Our studies investigating the adhesion of lymphocytes to endothelial cells indicate the formation of large ceramide-enriched membrane platforms on the surface of T lymphocytes that might be important for development of the disease. However, this seems to be inconsistent with the finding that expression of the acid sphingomyelinase in encephalitogenic T cells is not required for induction of the disease, at least in the transfer EAE model. This finding could be explained by the release of acid sphingomyelinase from activated endothelial cells upon contact with lymphocytes isolated from immunized mice, similar to the previously shown release of the acid sphingomyelinase from platelets upon contact with tumor cells [28]. The released acid sphingomyelinase then induces ceramide on the surface of these lymphocytes and mediates adhesion, which is a prerequisite for the subsequent extravasation of the encephalitogenic T cells and the development of an EAE. Our previous studies have shown that expression of the acid sphingomyelinase is required for induction of an EAE. Here, we show that lack of expression of the acid sphingomyelinase in other cells than immune cells reduces the severity of a transfer EAE, although it does not completely abrogate it. Thus, expression of the acid sphingomyelinase in the host is important for the development of an EAE. We suggest that expression of the acid sphingomyelinase in endothelial cells might contribute to the development of an EAE, but this hypothesis certainly does not exclude that expression of the acid sphingomyelinase in other cells, for instance neurons or glia cells is also required for development of an EAE. It might be possible that activation of the acid sphingomyelinase in these cells promotes attraction of immune cells and/or induces cell death [33, 34].

It needs to be tested whether the acid sphingomyelinase has additional functions in the development of an EAE or whether expression of the acid sphingomyelinase in endothelial cells and the mediation of lymphocyte adhesion is the primary and sole function of the acid sphingomyelinase in the development of an EAE *in vivo*.

In summary, the results of the present study demonstrate a therapeutic effect of acid sphingomyelinase in EAE pathophysiology. Pharmacologic inhibition of acid sphingomyelinase with amitriptyline blocks the detrimental neuroinflammatory effector phase in EAE. Amitriptyline is a safe drug that has been used in clinical practice for decades and, thus, would immediately be ready for clinical development as novel MS therapy.

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Statement of Ethics

Animal experiments conform to internationally accepted standards and have been approved by the appropriate institutional review body.

Author Contributions

SW, KF, TB, BE and EG planned the studies. SW, RH, LD, LS, AS and KAB performed the EAE and amitriptyline experiments, EG did the acid sphingomyelinase, ceramide and clustering measurements, NHJ, CB, SW and BE performed the tEAE investigations.

All authors read the manuscript and commented on it.

Disclosure Statement

The authors declare to have no conflict of interests.

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