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Selective CB2 receptor activation ameliorates EAE by reducing Th17 differentiation and immune cell accumulation in the CNS

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Abstract

CB2, the cannabinoid receptor expressed primarily on hematopoietic cells and activated microglia, mediates the immunoregulatory functions of cannabinoids. The involvement of CB2 in EAE has been demonstrated by using both endogenous and exogenous ligands. We showed previously that CB2 selective agonists inhibit leukocyte rolling and adhesion to CNS microvasculature and ameliorate clinical symptom in both chronic and relapsing EAE models. Here we showed that Gp1a, a highly selective CB2 agonist, with a four log higher affinity for CB2 than CB1, reduced clinical scores and facilitated recovery in EAE in conjunction with long term reduction in demyelination and axonal loss. We also established that Gp1a affected EAE through at least two different mechanisms, *i.e.* an early effect on Th1/Th17 differentiation in peripheral immune organs, and a later effect on the accumulation of pathogenic immune cells in the CNS, associated with reductions in the expression of CNS and T cell chemokine receptors, chemokines and adhesion molecules. This is the first report on the *in vivo* CB2-mediated Gp1a inhibition of Th17/Th1 differentiation. We also confirmed the Gp1a-induced inhibition of Th17/Th1 differentiation *in vitro*, both in non-polarizing and polarizing conditions. The CB2-induced inhibition of Th17 differentiation is highly relevant in view of recent studies emphasizing the importance of pathogenic self-reactive Th17 cells in EAE/MS. In addition, the combined effect on Th17 differentiation and immune cell accumulation into the CNS, emphasize the relevance of CB2 selective ligands as potential therapeutic agents in neuroinflammation.

Keywords

cannabinoid receptor; Gp1a; EAE; Th17 differentiation; chemokines

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INTRODUCTION

The cannabinoid system consists of cannabinoid receptors and both exogenous and endogenous receptor ligands. Most cannabinoid actions are mediated through the classical CB1 and CB2 receptors, with a preferential but not exclusive distribution in the CNS and periphery, respectively [reviewed in [1–4]]. The exogenous ligands include natural (plant derived) ligands and synthetic CB1/CB2 agonists and inverse agonist/antagonists [2, 4]. The best characterized endocannabinoids include anandamide (AEA) and 2-arachydonoylglycerol (2-AG), both partial CB1/CB2 agonists [4, 5].

The CB1 receptors are located primarily in the CNS at neuronal terminals and regulate neurotransmitter release and psychoactivity. In contrast, the CB2 receptors are expressed primarily on immune cells in the periphery, and during neuroinflammation on activated microglia in the CNS [2, 4]. Changes in cannabinoid receptor expression and in endocannabinoid levels have been reported in several pathological conditions, leading to proposed roles of CB1/CB2 receptors in various diseases [reviewed in [1]]. The therapeutic potential of CB2 receptor signaling in a wide array of diseases has been reviewed recently [2]. Important considerations for developing highly selective CB2 receptor agonists include absence of psychoactive effects, sustained anti-inflammatory activity, tissue/cell protection, lack of cardiovascular adverse effects, and efficacy in several disease models, including models of neuroinflammation [1, 2].

Multiple sclerosis (MS) is a neuroinflammatory disease characterized in terms of pathology by the presence of CNS inflammatory infiltrates, demyelination and axonal damage, and in terms of symptoms by sensory and motor impairment, ataxia, spasticity, and cognitive impairment [6]. In MS patients, the majority of lesions (plaques) are present in the brain periventricular white matter, cerebellum, brainstem and optic nerves, and in many cases also in the spinal cord. Studies using the experimental autoimmune encephalomyelitis (EAE) animal model established a sequence of events initiated by activation of myelin-specific CD4 T cells in the periphery, followed by extravasation and reactivation within the CNS, and ultimately leading to potent inflammatory events which result in neuronal and oligodendrocyte cell death [reviewed in [7,8]]. Both myelin-specific Th1 and pathogenic Th17 cells were shown to induce EAE upon adoptive transfer, with the Th17/Th1 ratio being a determining factor for lesion localization [reviewed in [8]].

CB1 and CB2 receptors were shown to play a role in EAE [reviewed in [5, 9]]. Both CB1-deficient and CB2-deficient mice developed more severe EAE [10–13], and cell specific receptor knock-outs established the protective effect of neuronal CB1 and of T cell CB2 receptors [10]. The major effect of neuronal CB1 appears to be the dampening of TNF α potentiation of glutamate induced excitatory postsynaptic currents [13], whereas CB2 signaling has a general anti-inflammatory effect, affecting immune cell activation and traffic to the CNS [4, 10, 11, 14].

We reported previously that systemic administration of the selective CB2 receptor ligand O-1966 attenuated both chronic and relapsing EAE and reduced rolling and adhesion of endogenous leukocytes to pial microvasculature [15]. However, the mechanisms involved in the protective effect of CB2 signaling in EAE, especially in terms of pathogenic Th1/Th17 differentiation and function, have not been elucidated. Here we used the recently developed, highly selective, CB2 agonist Gp1a (N-(piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-c]pyrazole-3-carboxamide) to investigate the protective mechanisms involved in early and delayed Gp1a administration in EAE, and its effects on the in vivo and in vitro Th1/Th17 differentiation.

MATERIALS AND METHODS

Mice

Six-8 weeks old C57BL/6 mice (H-2^b) and C57BL/6-Tg (Tcra2D2, Tcrb2D2)1Kuch/J (MOG₃₅₋₅₅ specific TCR) were purchased from Jackson Laboratory (Bar Harbor, ME). Transgenic mice were bred and maintained in the Temple University School of Medicine (TUSM) animal facility (Philadelphia, PA) under pathogen-free conditions. CB2R (*Cnr2*)^{+/-} mice on C57BL/6 background were originally obtained from the NIH and bred in our TUSM facility to generate *Cnr2*^{-/-} and *Cnr2*^{+/+} littermates. All mice used in experiments were between 6 and 10 wks of age. Mice were handled and housed in accordance with the guidelines of the Temple University Animal Care and Use Committee.

Reagents

Recombinant murine IL-12, IL-6, IL-1 β were purchased from Peprotech Inc (Rocky Hill, NJ). Gp1a was purchased from Abcam (Cambridge, MA). Lipopolysaccharide (LPS) (*Escherichia coli* O55:B5), pertussis toxin (PT), streptavidin-peroxidase, phorbol myristate acetate (PMA) and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO). Neutralizing anti-mouse IFN γ and APC-conjugated anti-mouse IFN γ were purchased from eBioscience (San Diego, CA). FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse IL17, APC-conjugated anti-mouse CD45, PE-conjugated anti-mouse CD11b, FITC-conjugated anti-mouse CD3, recombinant mouse IFN γ , capture and biotinylated anti-mouse IFN γ , GolgiPlug, Cytofix/Cytoperm, Perm/Wash buffer and TMB Substrate Reagent Set were purchased from BD PharMingen (San Diego, CA). 7-AAD Viability Staining Solution was purchased from Biolegend (San Diego, CA). CellTrace™ CFSE Cell Proliferation Kit, MOG₃₅₋₅₅, 1xHBSS, 10xHBSS and Trizol reagent were purchased from Invitrogen Corporation (Carlsbad, CA). Capture and biotinylated anti-mouse IL17 and recombinant mouse IL17, recombinant TGF β were purchased from R&D Systems (Minneapolis, MN). DNase I grade II and Liberase TL were purchased from Roche (Indianapolis, IN). Ketamine HCl was purchased from Fort Dodge Animal Health (Fort Dodge, IA). Xylazine was purchased from Butler Animal Health Supply (Dublin, OH). 0.5 M EDTA was purchased from Promega Corporation (Madison, WI). Percoll was purchased from GE Healthcare (Piscataway, NJ). *Mycobacterium tuberculosis* H37 RA was purchased from Difco (Detroit, MI). CD4+ CD62L+ T cell isolation kit II was purchased from Miltenyi Biotec (Auburn, CA). The BrdU flow kit was purchased from BD PharMingen.

EAE induction and treatment

C57BL/6 mice were immunized with 100 μ g MOG₃₃₋₅₅ peptide emulsified in complete Freund's adjuvant containing 2 mg/ml of *Mycobacterium tuberculosis* H37 RA, s.c. on day 0 and 100 ng pertussis toxin (PT) was administered i.p. on day 0 and day 2. Mice were treated with Gp1a (5mg/kg in PBS) or vehicle (PBS) via tail vein injection, twice per week. The treatment started from day 0 or day 7 depending on the experiments. Clinical scores were as follows: 0, no overt signs of disease; 1, limp tail or hind limb weakness but not both; 2, limp tail and hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund state, euthanized.

Isolation of mononuclear cells from central nervous system (CNS)

C57BL/6 mice were immunized as described before. Mice were anesthetized with 20 μ l of mix of ketamine HCl and xylazine and perfused through the left cardiac ventricle with 30 ml of HBSS containing 2mM EDTA. The brain was dissected and spinal cord was flushed out with HBSS. CNS tissue was digested with 10 ml HBSS containing DNase I (0.1 mg/ml for brain and 0.05 mg/ml for spinal cord) and Liberase (0.05 mg/ml for brain and 0.025 mg/ml

for spinal cord) for 45 min at 37°C with shaking, followed by blocking solution (10% FCS, 10 mM EDTA in HBSS). The tissue was pelleted and resuspended in 10 ml of 30% isotonic Percoll (diluted with 10x HBSS and distilled water), underlaid with 5 ml of 70% isotonic Percoll. Mononuclear cells were isolated from the 30/70 interphase after gradient centrifugation. Cells were washed with RPMI 1640 medium. FACS analysis was undertaken to characterize mononuclear cells and detect IFN γ and IL17 producing CD4 T cells.

In vivo CD4 T cell differentiation

C57BL/6 mice were immunized as described before. On day 11 the spleens were harvested. Splenocyte single cell suspensions from individual mice were prepared after erythrocyte lysis. Splenocytes (5×10^6 cells/ml) were restimulated with 50 μ g MOG_{35–55} in RPMI 1640 medium supplemented with 10% FBS and L-glutamine. Cells were collected and subjected to qRT-PCR after 48h of culture to detect transcription factor (*Tbx21*, *Rorc*, *Foxp3*, and *GATA3*) expression. After 72h of MOG_{35–55} activation, the cells were restimulated with PMA and ionomycin in the presence of GolgiPlug in order to measure intracellular cytokine production by FACS.

In vivo T cell proliferation

C57BL/6 mice were immunized with MOG_{35–55} and treated with Gp1a starting on day 7. In vivo T cell proliferation was assessed according to the BrdU labeling and the BrdU flow kit staining protocol (BD PharMingen). Briefly, on day 29 the mice received 100 μ l of 10 mg/ml BrdU solution i.p. 24h later mononuclear cells were isolated from spinal cord, stained with anti-CD3, anti-CD45, anti-CD11b followed by intracellular anti-BrdU antibody according to the manufacturer's protocol. BrdU⁺ cells were identified by flow cytometry in the CD45^{hi}CD11b⁺, CD45^{lo}CD11b⁺ and CD45⁺CD3⁺ populations.

In vitro CD4 T effector cell differentiation

Naïve CD4 T cells were prepared from spleens of 6–8 wks old 2D2 TCR-transgenic mice. Erythrocytes were eliminated from splenocytes with red blood cell lysis buffer according to manufacturer's protocol (eBioscience). Naïve CD4 T cells were isolated using the CD4+CD62L+ T cell isolation kit II (Miltenyi Biotec). Naïve CD4 T cells (1×10^6 cells/ml) were cultured with CD4 T cell-depleted splenocytes (4×10^6 cells/ml) in RPMI 1640 medium supplemented with 10% FBS, penicillin, streptomycin, amphotericin B, L-glutamine and 2ME. The cells were stimulated with 20 μ g/ml of MOG_{35–55}. Polarizing conditions: CD4 T cells were differentiated in the presence of 20 ng/ml IL-12 for Th1 cells, and of 5 ng/ml of TGF β 1, 20ng/ml of IL-1 β , 20ng/ml of IL-6 and 10 μ g/ml of anti-IFN γ for Th17 cells. To test the effects of CB2 agonist (Gp1a) on CD4 T cell differentiation, cells were also treated with Gp1a (5 and 10 μ M). After 72h of culture, supernatants and cells were collected and subjected to ELISA and FACS analysis, respectively, to detect cytokine production.

Viability of CD4 T cells

7-AAD staining was used to determine the viability of CD4 T cells. After 72h of activation with MOG_{35–55}, 1×10^6 splenocytes were washed with cold PBS 3 times, followed by CD4 staining for 30 min at 4°C. Cells were washed with cold PBS 2 more times and resuspended in 0.5 ml cell staining buffer. 5 μ l 7-AAD was added and the mixture was incubated in the dark for 5–10 min. Flow cytometry was used to detect 7-AAD positive cells.

In vitro CD4 T cell proliferation assay

Naïve 2D2 Tg CD4 T cells were purified and labeled with 5 μ M of CFSE according to the manufacturer's (InVitrogen) protocol, and cultured (1×10^6 cells/ml) with 4×10^6 /ml splenocytes in 200 μ l medium in 96-well plates. Cells were treated with 20 μ g/ml MOG_{35–55}

and in the absence and presence of Gp1a (5 and 10 μ M). After 72h of culture, T cell proliferation was assessed by CFSE dilution in the CD4 gated cells using FACS analysis.

FACS Analysis

Cells were analyzed by FACS in a 3-color FACS Calibur (BD Biosciences, Mountain View, CA). Data were collected for 10,000 cells and analyzed using Cellquest software from BD Biosciences (San Jose, CA). In order to determine IFN γ and IL17 producing CD4 T cells, mononuclear cells or activated splenocytes were stimulated with PMA (50 ng/ml) and ionomycin (500ng/ml) in the presence of GolgiPlug (1 μ l/ml) for 4 h. After extensive wash with cold PBS, the cells were stained with FITC anti-CD4 for 30 min, fixed and permeabilized using Cytotfix/Cytoperm and Perm/Wash buffer according to the manufacturer's instructions. Then cells were stained with APC anti-IFN γ and PE anti-IL17 for an additional 30 min. FACS analysis was performed and T cells were identified through gating on CD4 cells. The numbers of cytokine producing cells were calculated from the percentage of cytokine-positive CD4 T cells. We determined the number of CD4 T cells in the CNS through multiplication of the percentage of positive cells by total number of mononuclear cells isolated from the CNS. To characterize subsets of mononuclear cells, cells were stained with APC-conjugated anti-mouse CD45, PE-conjugated anti-mouse CD11b and FITC-conjugated anti-mouse CD3 and analyzed by flow cytometry. The number of each type of cells was calculated based on percentages of each type of cells and total number of mononuclear cells.

RT² Profiler PCR array

Spinal cord tissue pooled from 3 mice in each group was obtained at peak of disease, homogenized, and the expression of proinflammatory cytokines, chemokines, chemokine receptors and mediators of autoimmune disease and inflammatory response was evaluated using the RT² Profiler PCR array (Qiagen, Valencia, CA) based on the SYBR green PCR technique. First, 20 μ L of cDNA was diluted with 91 μ L of RNase free H₂O. Then 102 μ L of diluted cDNA was mixed with 1350 μ L of 2 $\times$ SYBR green-containing PCR master mix and 1248 μ L of RNase free H₂O to prepare 2700 μ L of experimental mixture. 25 μ L of the mixture was loaded in each well of a 96-well plate pre-coated with primers for different genes. The PCR array was performed using the StepOnePlus Real-Time PCR System (AB Applied Biosystems). The cycling conditions were 15 sec at 95°C, and 1 min at 60°C for 40 cycles, followed by a melting point determination of dissociation curves. Cycle threshold values were determined by automated threshold analysis, and the results were standardized based on 5 housekeeping genes, including glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*), β_2 microglobulin (*B2m*), glucuronidase beta (*Gus* β), heat-shock protein 90 alpha (*Hsp90sb1*), and β -actin.

qRT-PCR

The expression of *Tbx21*, *Rorc*, *Foxp3*, *GATA-3*, *P19*, *P35*, *P40*, *Il10*, *Il1 β* , *Tnfa*, *Il17*, *Ifn γ* , *Nos2*, *Ccl2*, *Ccl5*, *Cxcl10*, *Ccr5*, *Ccr2*, *Cxcr3*, *VLA4*(α 4), *VLA4*(β 1) and *Vcam1* was detected by SYBR Green-based qRT-PCR. RNA was prepared from CD4 T cells, splenocytes or homogenized spinal cord tissue using Trizol reagent according to the manufacturer's instructions. RNA (1 μ g) was reversed transcribed to cDNA and subjected to qPCR. The PCR mixture (20 μ l), consists of 4 μ l diluted cDNA, 16 μ l of SYBR Green containing the PCR master mix and 150 nM of each primer. Real-time PCR was performed using StepOnePlus Real-Time PCR System (AB Applied Biosystems). The following primers were used: *Tbx21* sense, 5'-CGGTACCAGAGCGGCAAGT-3', and antisense, 5'-CATGCTGCCTTCTGCCTTTC-3'; *Rorc* sense, 5'-GCGGAGCAGACACACTTACA-3', and antisense, 5'-TCCACCACCACAGCTGAGAGG-3'; *GATA-3* sense, 5'-

TACTTGC GTTTTTC GCAGGA-3', and antisense 5'-GATCTGTCGCTTTCGGGCT-3'; *Foxp3* sense, 5'-CAGCTGCCTACAGTGCCCCTA-3' and antisense 5'-CATTTGCCAGCAGTGGGTAG-3'; *P19* sense, 5'-TGCTGGATTGCAGAGCAGTAA-3' and antisense, 5'-ATGCAGAGATTCCG AGAGA-3'; *P35* sense, 5'-GAGGACTTGAAGATGTACAG-3' and antisense, 5'-TTCTATCTGTGTGAGGAGGGC-3'; *P40* sense, 5'-GACCCTGCCGATTGAACTGGC-3' and antisense, 5'-CAACGTTGCATCCTAGGATCG-3'; *Il10* sense, 5'-CCTGGTAGAAGTGATGC CCC-3' and antisense, 5'-TCCTTGATTCTGGGCCATG-3'; *Il1β* sense, 5'-CCCTGCAGCTG GAGAGTGTGGA-3' and antisense, 5'-TGTGCTCTGCTTGTGAGGTGC TG-3'; *Tnfa* sense, 5'-GACCCTCACACTCAGATCATCTTCTTCT-3', and antisense, 5'-CCTCCACTTGGTGGT TTGCT; *Il17* sense, 5'-CCTGGACTCTCCACCGCAAT-3' and antisense, 5'-ATGTGGT GGTCCAGCTTTCC-3'; *Ifnγ* sense, 5'-AGTCATCCGAGTGGTC CAC-3' and antisense, 5'-GCTTCCTGAGGCTGGATACC-3'; *Nos2* sense, 5'-CGCAGCTGGGCTGTACAA-3' and antisense, 5'-TGATGTTTGCTTCGGACATCA-3'; *Ccl2* sense, 5'-CACAGTTGCCGGCTGG AGCA-3' and antisense, 5'-CAGCAGGTGAGTGGGGCG TT-3'; *Ccl5* sense, 5'-GCTGCTTTG CCTACCTCTCC-3' and antisense, 5'-TCGAGTGACAAAGACTGC-3'; *Cxcl10* sense, 5'-ATTCTTTAAGGGCTGGTCTGA-3' and antisense, 5'-CACCTCCACATAGCTTACAGT-3'; *Vcam1* sense, 5'-TGTGCGCTGTGACCTGTCTGC-3', antisense 5'-TCTCCCATGCACAAG TGGCCC-3'; *VLA4(α4)* sense, 5'- TTTGGCTACTCGGTGGTGCT-3', and antisense, 5'-TGTCTTCCCACAAGGCTCTC-3'; *VLA4(β1)* sense, 5'-ATCCCAATTGTAGCAGGCGT-3', and antisense, 5'-AGTTGTCACGGCACTCTTGT-3'; *Ccr2* sense, 5'-GGTGTGGTGGCCCCCTTCATCA-3', and antisense, 5'-GCCTGCAAAGACCAGGAAGGC-3'; *Ccr5* sense, 5'-CAGAATGGTAGTGTGAGCAGGAA-3' and antisense, 5'-CATCGATTATGGTATGTCAGC-3'; *Cxcr3* sense, 5'-CCAGCCAAGCCATGTACCTT-3', and antisense, 5'-TCGTAGGGAGAGGTGCTGTT-3'; *GAPDH* sense, 5'-GGAGCGAGACCCCACTAA-3', and antisense, 5'-ACATACTCAGCACCGGCCCTC-3'. The cycling conditions were 95°C for 15 s, 75°C for 1 min, 57°C for 30 sec, for 40 cycles, followed by a melting point determination or dissociation curves. The expression level of each gene is indicated by the number of cycles needed for the cDNA amplification to reach a threshold. The amount of DNA is calculated from the number of cycles by using standard curves and the results are normalized to GAPDH.

Cytokine ELISA

Supernatants from splenocyte cultures were harvested and subjected to sandwich ELISA for IFN γ and IL17. The detection limits were: 15 pg/ml for IL17, 30 pg/ml for IFN γ .

Histology studies

A comparison was made between Gpl α - and vehicle-treated mice. The EAE mice in which treatment was started on day 0 were euthanatized at the peak of disease; the EAE mice in which ν treatment was started on day 7 were euthanatized on day 30 (end of observation period). The mice were perfused with 1xPBS and fixed with 4% paraformaldehyde in PBS. A 5-mm segment of the spinal cord in lumbar was extracted and stored in 4% paraformaldehyde in 1xPBS solution for less than 24 h, then transferred to a solution containing 30% sucrose in 1xPBS. After dehydration the tissue was embedded in optimal cutting temperature compound from Tissue-Tek (Sakura Finetek), and frozen at -80°C until sectioning. The tissue was sliced into 16- μ m serial sections for hematoxylin and eosin (HE) stain, luxol fast blue and periodic acid Schiff reagent stain (Sigma Aldrich) and various immunohistochemical stains. The tissue was also sliced into 30- μ m serial sections for Bielschowsky's silver stain (Hitobiotec Inc.). Macrophages/microglia were stained with

ionized calcium-binding adapter molecule 1 (Iba-1, Wako, Richmond, VA) (Iba-1, rabbit anti-mouse, 1:500) overnight, followed by the secondary antibody (FITC-goat antirabbit IgG, 1:500; Invitrogen) for 1 h. T cells were stained with CD3 antibody (1:50, BD). The stained tissue sections were visualized with an Olympus BX 51 microscope and photographed using a Nikon DP70 camera.

Statistical analysis

Results are described as mean $\pm$ SD for in vitro experiments or mean $\pm$ SEM for in vivo experiments. Comparisons between two groups were done using Student *t* test, whereas comparisons among multiple groups were done by one way ANOVA. Bonferroni test was used for post hoc comparisons among multiple groups where appropriate. Linear regression with 95% confidence interval was used for analysis of clinical scores between vehicle and Gp1a groups. Statistical significance was determined as *p* values less than 0.05. All statistical analyses were performed using GraphPad Prism 5.

RESULTS

Gp1a attenuates EAE development

EAE was induced in C57BL/6 mice through active immunization with MOG_{35–55} as described in Methods. Gp1a was administered i.v. twice weekly starting on day 0. Mice were evaluated daily for clinical scores. The Gp1a treated group had a lower disease incidence (60 versus 100% in vehicle-treated controls), a delay in disease onset, and displayed lower clinical scores, with statistically significant differences at both early and late disease stage (Fig. 1A and B). The maximum and cumulative scores in the Gp1a-treated group were approximately half of the control values (maximum score: 1.80 vs. 3.25, cumulative score: 18.05 vs. 31.75). These results suggest that early treatment with the CB2 selective agonist Gp1a attenuates EAE development.

Gp1a decreases CNS accumulation of CD4 T cells

To assess the presence of CD4 T cells in the CNS, we isolated mononuclear cells from brain and spinal cord of individual mice at peak of disease. The cells were restimulated with PMA and ionomycin, and Th1 and Th17 cells were identified by flow cytometry using surface (CD4) and intracellular (IFN γ and IL17) staining. The Gp1a treatment significantly decreased the number of total mononuclear cells. Moreover, the numbers of CD4 T cells, Th1 cells and Th17 cells were also dramatically reduced in the Gp1a group (spinal cord and brain) (Fig. 2A).

Histology studies were also performed at peak of disease. The HE stain (Fig. 2B 4x and 40x) showed that infiltrating immune cells in control EAE mice were mainly distributed in the spinal cord subpial white matter around the anterior funiculus and the anterior median fissure (Fig 2B, middle panel). T cells and activated microglia/macrophages were identified through CD3 and Iba-1 staining, respectively (Fig. 2C and D, middle panels). Spinal cord sections from Gp1a-treated mice had fewer infiltrating immune cells, fewer CD3 T cells, and fewer Iba-1+ microglia/macrophages (Fig 2B, C and D, right panels). Since lower numbers of microglia/macrophages could be due to Gp1a-induced apoptosis, we purified CNS mononuclear cells at peak of disease and analyzed apoptosis through annexin V staining of the CD11b+ population. We observed no changes in the percentage of annexin V positive cells from vehicle-or Gp1a-treated mice (supplemental figure 1). Collectively, these results suggest that EAE attenuation following early treatment with Gp1a could be attributed to decreased immune cell infiltration, and reduced microglia activation.

Gp1a inhibits both in vivo and in vitro CD4 T cell differentiation

To investigate the mechanisms underlying the reduced numbers of CNS Th1/Th17 cells following early Gp1a treatment, we tested the in vivo and in vitro effects of Gp1a on CD4 T cell differentiation. Splenocytes harvested on day 11 from EAE animals treated with vehicle or Gp1a were restimulated with MOG₃₅₋₅₅, and the expression of signature Th1/Th17/Treg/Th2 transcriptional factors was evaluated. Expression of Tbet (Th1), ROR γ t (Th17), Foxp3 (Treg), and GATA-3 (Th2), was quantified by qRT-PCR. Cells isolated from Gp1a-treated mice showed significant lower levels of Tbet and especially ROR γ t, and increased Foxp3 and GATA-3 expression (Fig. 3A). In agreement with the reduction in Tbet and ROR γ t, we observed decreased IFN γ and IL17 production (intracellular cytokine staining and ELISA) for splenocytes harvested from Gp1a-treated EAE mice (Fig. 3B, C).

To confirm that Gp1a acted through the CB2 receptor, we treated MOG₃₅₋₅₅-immunized CB2-deficient and wildtype mice with Gp1a, and measured the amounts of IFN γ and IL-17 secreted from splenocytes harvested on day 11 and restimulated ex vivo with MOG₃₅₋₅₅. As expected, splenocytes from Gp1a-treated wildtype mice produced less IL-17/IFN γ compared to vehicle-treated mice, whereas IL-17/IFN γ production from spleen cells of CB2-deficient mice was not reduced by the in vivo Gp1a treatment. Actually the cells from CB2 knockouts produced slightly higher, although not statistically significant, levels of IL-17/IFN γ suggesting a possible antiinflammatory role of endocannabinoids.

To directly assess the effect of Gp1a on T cell differentiation, we performed in vitro experiments. Purified naïve 2D2 CD4 T cells were cultured with splenic APCs and MOG₃₅₋₅₅ in the presence or absence of Gp1a for 72h. Gp1a did not affect MHCII and costimulatory molecule (CD40, CD80, CD86) levels on DCs (results not shown), nor T cell viability (Fig. 4A left panels). We also assessed the effects of Gp1a on T cell differentiation by using CFSE-labeled 2D2 T cells stimulated with MOG₃₅₋₅₅ and APCs in the presence and absence of Gp1a. Although 5 μ M Gp1a did not affect antigen-induced T cell proliferation, we observed a decrease in proliferation for 10 μ M Gp1a (Fig. 4A, right panels).

IFN γ and IL17 production was detected by intracellular staining and ELISA. The frequency of IFN γ producing Th1 cells was reduced by Gp1a in a dose dependent manner (Fig. 4B). Dramatic reduction of IFN γ secretion by Gp1a was detected by ELISA (Fig. 4C – lower panel). Although the numbers of IL17+ T cells were too low for comparison between control and Gp1a-treated cultures (Fig. 4B), the amount of secreted IL17 measured by ELISA was significantly decreased by Gp1a (Fig. 4C, upper panel). In agreement with the ELISA results, we found that *Ifn γ* and *Il17* mRNA expression was reduced by Gp1a (Fig. 4D). In contrast, high Gp1a concentrations (10 μ M) increased the expression of the anti-inflammatory cytokine IL-10 (Fig. 4D). IL-4 production in the presence or absence of Gp1a was undetectable. In terms of signature transcription factors, we observed Gp1a-induced decreases in Tbet and ROR γ t, increases in Foxp3, and no effect on GATA-3 expression (Fig. 4E).

T cell differentiation into Th1/Th17 subsets requires specific cytokines produced by DCs, *i.e.* IL-12 for Th1, and TGF β 1, IL-6 and IL-1 β for Th17. To rule out the possible impact of Gp1a on cytokine production from splenic DCs, we assessed the effect of Gp1a on Th1/Th17 differentiation in the presence of exogenous cytokines (polarizing conditions). Naïve 2D2 CD4 T cells were co-cultured with splenic APCs and MOG₃₅₋₅₅ in the presence of IL-12 (for Th1) or TGF β 1+ IL-6+IL-1 β (for Th17). Gp1a reduced the percentage of IFN γ + and IL17+ CD4 T cells, as well as IFN γ and IL17 secretion in a dose dependent manner (Fig. 4F, G). Similar to the results obtained for the in vivo Gp1a treatment, the inhibition was more pronounced for Th17 cells. Taken together, the in vivo and in vitro data suggest

that Gp1a inhibits Th1 and Th17 differentiation most probably through direct effects on CD4 T cells.

Delayed Gp1a treatment does not affect EAE initiation but promotes recovery

Next, we investigated whether Gp1a has a therapeutic effect when administered at a later time point. The Gp1a treatment was started on day 7 post-immunization, when CD4 T cells were already activated and differentiated, and continued twice a week. In contrast to early Gp1a administration (Fig. 1), the delayed Gp1a treatment group had similar incidence and maximum clinical score with the control group (Fig. 5A and B). However, the Gp1a-treated mice recovered faster, with a statistically significant difference in cumulative scores (Fig. 5A and B).

To assess whether Gp1a had any effect on the *in vivo* Th1/Th17 differentiation we restimulated spleen cells harvested on day 11 and evaluated the expression of signature transcription factors (at 48h) and cytokine production (at 72h). Although we observed a reduction in *Rorc* expression for the Gp1a-treated group, there were no differences in percentage of splenic IFN γ ⁺ and IL17⁺CD4 T cells, or in the levels of secreted IFN γ and IL17 (Fig. 6). Overall, these results suggest that the therapeutic effect of Gp1a in terms of accelerated EAE recovery is not due to a significant reduction in peripheral Th1/Th17 differentiation.

Delayed Gp1a treatment inhibits the expression of proinflammatory cytokines and chemokines in the spinal cord of EAE mice

Since the delayed Gp1a treatment did not affect Th1/Th17 cell differentiation in spleen, we focused on CNS pathophysiology. The active EAE model used in our experiments affects primarily the spinal cord. Therefore, we used microarrays to evaluate the expression profile of more than 80 inflammatory genes in spinal cord at peak of disease. Fold changes were normalized based on values obtained for spinal cord of naïve (non-EAE) mice. Compared with vehicle treatment, Gp1a showed a significant decrease in the expression of several proinflammatory molecules (supplemental Table 1). Major differences expressed as fold change for Gp1a/control including the cytokines *Il1 β* and *Tnf α* , the chemokines *Ccl2*, *Ccl5*, *Ccl7*, *Ccl12* and *Cxcl10*, the chemokine receptors *Ccr1*, *Ccr2* and *Ccr7*, and the Toll-like receptors *Tlr2* and *Tlr4* are summarized in Table 1. Using qRT-PCR, we confirmed that treatment with Gp1a resulted in significant decreases in the expression of the proinflammatory cytokines IL-12p40, IL23p19, TNF α , IL1 β , IFN γ and IL17, the chemokines CCL2, CCL5 and CXCL10, the adhesion molecule VCAM-1, and iNOS (Fig. 7). Decreases in VLA4 (α 4 and β 1) as well as the chemokine receptors CCR2, CCR5 and CXCR3 were also observed in CD4 T cells and T cell-depleted populations isolated from the spleen of Gp1a treated EAE mice at peak of disease (Fig. 8). Gp1a reduction in CNS expression of p40, TNF α , CXCL10, VCAM-1, and especially IFN γ and IL17 were apparent on day 30 as well (Fig. 9). Gp1a-induced reduction in the expression of T cell adhesion molecules and chemokine receptors, and in CNS chemokines and adhesion molecules, suggests that CNS migration of immune cells could be impaired by Gp1a treatment.

Delayed Gp1a treatment reduces the accumulation of infiltrating immune cells in the CNS

To determine the presence of immune cells in the CNS following delayed Gp1a treatment, mononuclear cells were isolated from brain and spinal cord and subjected to flow cytometry analysis. Staining for CD3, CD45 and CD11b was used to identify T cells (CD45^{hi}CD3⁺), macrophages (CD45^{hi}CD11b⁺) and microglia (CD45^{lo}CD11b⁺). Delayed Gp1a treatment reduced the total number of mononuclear cells and dramatically decreased the number of macrophages (CD45^{hi}CD11b⁺) in both spinal cord and brain (Fig. 10A and B). CD3 T cells showed a trend towards lower number in the brain of Gp1a treated mice and statistically

significant reduction in spinal cord. Lower numbers of microglia (CD45^{lo}CD11b⁺) were also observed in the spinal cord of Gp1a-treated EAE mice (Fig. 10A and B). Since lower numbers of T cells, macrophages and microglia could be due to Gp1a-induced inhibition of cell proliferation, we measured the proliferation of CNS T cells, macrophages and microglia in vehicle- and Gp1a-treated mice by administering BrdU *in vivo* and determining the percentages of BrdU positive cells in the CD45^{hi}CD3⁺, CD45^{hi}CD11b⁺ and CD45^{lo}CD11b⁺ MNC populations. We observed a reduction (from 9% to 2% BrdU⁺ cells) in the CD45^{hi}CD11b⁺ macrophage population, whereas T cell proliferation was unaffected. The results regarding CD45^{lo}CD11b⁺ microglia were inconsistent (results not shown).

Histology analysis of the spinal cord of vehicle-treated EAE mice identified CD3 T cells in both white and adjacent gray matter. Presence of Iba-1⁺ microglia/macrophages, both close to the sites of T cells infiltration (data not shown) and in the gray matter around the central canal, was much more pronounced in vehicle-treated compared to Gp1a-treated EAE mice (Fig. 10C).

Gp1a reduces demyelination and axonal damage in spinal cord of EAE mice

To evaluate the effect of Gp1a on demyelination and axonal damage, spinal cord tissue collected on day 30 from EAE mice following delayed Gp1a or vehicle treatment was subjected to staining with luxol blue/periodic acid Schiff reagent (LFB-PAS) for myelin and Bielschowsky's silver stain for axons. Spinal cord sections from Gp1a-treated EAE mice showed significantly less demyelination in the white matter of anterior funiculus (Fig. 11A – right panel). Vehicle-treated EAE mice exhibited decreased axonal density and thickness compared to the Gp1a-treated group, indicative of more severe axonal damage. Collectively, the histology results indicate that the Gp1a treatment resulted in less demyelination and axonal damage, in agreement with improved clinical recovery.

DISCUSSION

Recent evidence indicates that regulation of neuroinflammation represents one of the major physiological functions of the endocannabinoid system [reviewed in [3, 4, 16]]. The beneficial effect of cannabinoids in MS, an autoimmune neuroinflammatory disease, has received special attention. EAE, a well described animal model, characterized by demyelination, and neurological dysfunction, has similarities to human MS and is widely used for mechanistic studies [17]. CB2, the major cannabinoid receptor expressed in hematopoietic cells and activated microglia, is the main mediator of the immunoregulatory effect of cannabinoids. The involvement of CB2 as a protective agent in EAE is supported by experiments targeting endogenous as well as exogenous ligands. The beneficial role of endocannabinoids is supported by the fact that treatment of EAE mice with CB2 antagonists leads to an early increase in CB2 receptor expression in spleen, followed by increased expression in spinal cord [18], and by the fact that CB2-deficient mice develop exacerbated EAE symptoms associated with reduced clinical recovery and increased axonal loss [10, 11, 18]. In addition, treatment with the endocannabinoid 2-AG resulted in milder EAE clinical scores, and reduced axonal pathology [19]. In terms of exogenous cannabinoids, we reported previously that treatment with the CB2 selective agonist O-1966 resulted in milder clinical scores in active chronic and relapsing-remitting EAE models, as well as in the adoptive transfer model [15].

Here we confirm that Gp1a, a highly selective CB2 agonist, with a four log higher affinity for CB2 than for CB1, reduces clinical scores and facilitates recovery in EAE in conjunction with long term reduction in demyelination and axonal loss. We also establish that Gp1a affects EAE through at least two different mechanisms, *i.e.* an early effect on Th1/Th17 differentiation in peripheral immune organs, and a later effect on pathogenic T cell

accumulation in the CNS, associated with a reduction in CNS chemokine and adhesion molecule expression. It remains to be established whether early or delayed Gp1a treatment has a long term beneficial effect, suppressing chronic EAE.

The fact that CB2 receptors mediate cannabinoid effects on a variety of cell types such as T cells, microglia/macrophages, BBB endothelial cells, is in agreement with their wide distribution on hematopoietic cells [20, 21]. CB2 receptors were reported to be involved in the inhibition of proinflammatory cytokine production, antigen processing and induction of apoptosis in DCs, macrophages and microglia [15, 22–25]. Various CB2 ligands were shown to induce T cell apoptosis and reduce proliferation [10, 26–28]. In addition, Δ -9-tetrahydrocannabinol (THC), a CB1/CB2 ligand was reported to inhibit Th1 differentiation through CB1 and to promote Th2 differentiation through CB2 [29–31]. Nagarkatti and colleagues [32] reported that the beneficial effect of THC in experimental autoimmune hepatitis, mediated through both CB1 and CB2, was partially due to the increase in Foxp3+CD25+Treg. More recently, the highly selective CB2 ligand Gp1a was reported to reduce inflammation in several disease models, including sepsis, neuroAIDS, and experimental cystitis, through mechanisms involving both inhibition of proinflammatory cytokines and migration of immune cells [33–35].

Here, we report for the first time that the in vivo administration of Gp1a inhibits the differentiation of Th17, and to a lesser degree of Th1 cells, in a CB2-dependent manner. We also show in vitro that Gp1a inhibits Th17 and Th1 differentiation of naïve CD4 T cells in both non-polarizing and polarizing conditions. This is in agreement with two recent publications which reported in vitro inhibition of Th17 differentiation, using purified human T cells and anandamide [14], and a murine MOG-specific T cell line treated with THC or cannabidiol (CBD) [36]. The in vivo CB2-mediated effect of Gp1a on the differentiation of encephalitogenic Th17 cells is particularly important in view of recent studies emphasizing the importance of pathogenic self-reactive Th17 cells in EAE [37, 38]. In addition to proinflammatory cytokine release and neutrophil recruitment, Th17 cells have been reported to promote CNS formation of ectopic lymphoid tissue containing and supporting pathogenic B cells, and to induce neuronal cell death directly, irrespective of antigen specificity [39, 40]. Th1 cells also play a role in EAE, since the Th17/Th1 ratio acts as the determining factor for whether inflammation occurs in brain (high ratio) or is restricted to the spinal cord (low ratio) [8, 41].

Early Gp1a treatment (day 0) resulted in a beneficial effect in terms of clinical symptoms throughout the entire observation period (up to day 36). This was accompanied by a lower number of CNS infiltrating Th1 and Th17 cells, which could result from reduced Th1/Th17 differentiation in the peripheral immune organs. Indeed, we observed significant reductions in the generation of splenic Th1, and especially Th17 cells, in the Gp1a-treated EAE mice, and corresponding reductions in the expression of splenic Tbet and ROR γ t, the signature transcription factors for Th1 and Th17, respectively. In vitro experiments using naïve MOG-specific CD4 T cells treated with Gp1a also indicated a significant inhibition of Th1 and Th17 differentiation. This could result from effects on cytokine release by APCs as well as direct effects on T cells. Indeed, inhibition of the essential Th17 polarizing cytokine IL-6 by CB2 agonists such as JWH-133 and Gp1a, has been reported in models of stroke and sepsis [35, 42]. We also reported inhibition of IL-6, IFN γ and IL17 in T cell/DC co-cultures treated with O-1966 [15]. However, in the experiments reported here, we observed Gp1a-induced inhibition of Th17/Th1 differentiation even in the presence of exogenous polarizing cytokines (IL-12 for Th1 and TGF β +IL-6+IL-1 β for Th17). This suggests that Gp1a might affect T cells directly, in addition to the possible inhibitory effect on APC cytokine release. Cencioni and colleagues reported a similar direct effect of the endocannabinoid anandamide on human T lymphocytes, resulting in reduction of in vitro Th17 differentiation [14].

In contrast to the effects of Gp1a on Th1/Th17 differentiation, we observed an increase in Foxp3 and GATA-3 expression in spleen cells from Gp1a-treated EAE mice. This is in agreement with previous reports on CB2 signaling promoting *in vivo* Th2/Treg differentiation [30, 32]. Our *in vitro* data indicate significant increases in Foxp3, but not in GATA-3 expression. It remains to be established whether the increase in Foxp3 observed both *in vivo* and *in vitro* is associated with increased regulatory activity.

In contrast to the effect of the early treatment, delayed Gp1a administration (day 7) promoted clinical recovery without significantly affecting the EAE initiation phase. As expected, delayed treatment did not affect Th1/Th17 differentiation in spleen. However, effects on cytokine/chemokine expression in spinal cord were apparent at later time points. Significant inhibitory effects were observed for the Th17 polarizing cytokines IL-1 β and IL-23 (*p40* and *p19* expression), the cytokines IFN γ (Th1) and IL17 (Th17), as well as the proinflammatory genes *Tnf α* and *Nos2*. The reduction in IL-23 expression is particularly relevant, since IL-23 is an essential cytokine for the proliferation of Th17 and maintenance of the Th17 functional phenotype [43]. These results suggest that although the delayed Gp1a treatment does not affect Th17 differentiation in the periphery, it might still affect the migration, proliferation and reactivation of CNS infiltrating Th17 cells. The Gp1a treatment had a profound inhibitory effect on spinal cord expression of monocyte- and T cell- chemoattractant chemokines (CCL2, CCL5 and CXCL10) and of the chemokine receptors CCR1, 2 and 7. We also observed a reduction in the expression of VLA-4 and chemokine receptors CCR2, CCR5 and CXCR3 in splenic T cells from EAE mice treated with Gp1a. Together with the reduction in VCAM-1 which is involved in the BBB transmigration of activated T cells, these results suggest that Gp1a could affect T cell migration. The involvement of CB2 receptors in immune cell migration has been reported previously. Various CB2 agonists were reported to reduce transendothelial migration of CD4 and CD8 T cells [44]. By using intravital microscopy we showed previously that CB2 agonists reduced rolling and adhesion of endogenous leukocytes to the cerebral microvasculature in EAE mice [15, 45], and a similar effect was reported for JWH-133 and inflamed retina in experimental autoimmune uveitis [46]. Various CB2 agonists were shown to reduce MMP9 enzymatic activity in activated Jurkat T cells [44] and we reported recently that Gp1a inhibits MMP9 expression in DC, macrophages and microglia, reducing subsequent DC migration *in vitro* and *in vivo* [47].

The reduction in the numbers of T cells in the CNS could also result from apoptosis or reduced proliferation as reported for other CB2 selective ligands [10, 26–28, 48]. However, although 10 μ M Gp1a induced *in vitro* apoptosis of T cells activated with anti-CD3 and anti-CD28, we did not observe apoptosis in MOG-specific CD4 T cells activated and differentiated with MOG-APCs. It is possible that CB2-induced apoptosis depends on the strength of T cell activation, *i.e.* occurring only in the presence of non-physiological potent CD3/CD28 signals. In terms of CB2 effects on T cell proliferation, our *in vitro* studies showed a reduction only at the highest Gp1a concentration (10 μ M), and we did not detect any reduction in the *in vivo* proliferation of CD3 T cells isolated from the CNS of Gp1a-treated EAE mice as compared to vehicle controls. Therefore, taken together, the reduction in total numbers of CNS T cells in Gp1a-treated EAE mice, the previously described inhibition of leukocyte rolling and adhesion, the reduction in the expression of T cell VLA-4 and chemokine receptors, and the reduction in CNS expression of VCAM-1 and chemokines, suggest that CB2 signaling could reduce immune cell migration into the CNS. However, at the present time we cannot discard possible effects on T cell survival, reactivation, and function within the CNS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APC	antigen-presenting cell
BBB	blood brain barrier
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CNS	central nervous system
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
FACS	fluorescence activated cell sorter
LPS	lipopolysaccharide
MNCs	mononuclear cells
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
PBS	phosphate buffered saline
PT	pertussis toxin
qRT-PCR	quantitative RT-PCR
TCR	T cell receptor

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Highlights

The selective cannabinoid receptor 2 ligand Gp1a ameliorates clinical EAE

Gp1a inhibits encephalitogenic Th17/Th1 differentiation in vivo and in vitro

Gp1a decreases immune cell accumulation in the CNS during EAE

Delayed Gp1a treatment inhibits proinflammatory CNS chemokines and adhesion molecules

Gp1a reduces demyelination and axonal damage in EAE

B

Clinical features of EAE in animals treated with Gp1a from day 0

	Vehicle	Gp1a
Incidence	100%	60%
Death of severe disease	0%	0%
Disease onset (days)	13.13±1.36	14.17±1.72
Maximum score	3.25±0.31	1.80±0.64 *
Cumulative score	31.75±5.62	18.05±5.01*

Data represent the mean $\pm$ SEM. Statistically significant at * $p < 0.05$, compared with vehicle group.

Figure 1. Gp1a attenuates EAE development

C57BL/6 mice (n=10/group) were immunized with MOG₃₅₋₅₅ (100 μ g/mouse) as described in Methods. Gp1a treatment (5mg/kg) started on day 0 and continued twice weekly until the end of the observation period. The control group received the same treatment regimen with vehicle (PBS). Clinical scores were followed daily for 36 days. Clinical scores are presented as mean $\pm$ SEM. Comparison between Gp1a-treated and vehicle-treated mice for each time point* $p < 0.05$.

A**Spinal cord** **Brain**

Figure 2. Gp1a decreases CNS infiltration of immune cells

C57BL/6 mice were immunized and treated with Gp1a as described in Figure 1. CNS mononuclear cell analysis and histology studies were performed at peak of disease. (A) Mononuclear cells (MNCs) were isolated from brain and spinal cord as described in Methods and restimulated with ionomycin and PMA. Gated CD4 T cells were analyzed for intracellular IFN γ and IL17 by flow cytometry. Data are representative of two independent experiments. Data are presented as mean $\pm$ SEM, n=3. (B) Hematoxylin and eosin staining (HE) of spinal cord in normal and EAE mice treated with vehicle or Gp1a (3 mice/group). Upper panels show the whole spinal cord (4 $\times$ 10 magnification), lower panels show a 40 $\times$ 10 magnification of the anterior median region. (C) CD3 staining (T cells) (20 $\times$ 10 magnification); (D) Iba-1 staining (microglia activation) (20 $\times$ 10 magnification).

Figure 3. Gp1a inhibits in vivo Th1/Th17 differentiation

C57BL/6 mice were immunized and treated with Gp1a as described in Figure 1. On day 11, splenocytes were harvested and restimulated with MOG₃₅₋₅₅ (50 μ g/ml). (A) 48h later, cells were collected and subjected to qRT-PCR for *Rorc*, *Tbx2*, *Foxp3*, *GATA-3* expression. (B) 72h later, gated CD4 T cells were analyzed for intracellular IFN γ and IL17 expression. (C) Supernatants were collected and secreted IFN γ and IL17 were detected by ELISA. (D) CB2 KO and control WT mice (groups of 3) were immunized and treated with Gp1a as described in Figure 1. On day 11, splenocytes were harvested and restimulated with MOG₃₅₋₅₅ (50 μ g/ml). 72h later supernatants were collected and secreted IFN γ and IL17 were detected by ELISA. Levels in cells from vehicle-treated mice were considered as

100%. Data are representative of two independent experiments and presented as mean $\pm$ SEM, n=3.

Figure 4. Gp1a suppresses *in vitro* CD4⁺ T cell differentiation

Naïve splenic 2D2 CD4⁺ T cells were co-cultured with T-depleted spleen cells in the presence of MOG₃₅₋₅₅ (20 μ g/ml) and Gp1a (5 and 10 μ M). (A) Left panels: Cell viability was determined by 7 AAD staining and flow cytometry (72h). Right panels: 2D2 naïve T cells were labeled with CFSE and cultured as above for 72h. Flow cytometry analysis was performed. (B) Intracellular staining was performed to detect IFN γ and IL17 producing cells (72h). Analysis was based on gated CD4⁺ cells. (C) Supernatants were collected and subjected to ELISA to measure IFN γ and IL17 production (72h). (D and E) Cells were collected to detect *Ifn* γ , *Il17* and *Il10* (D) and *Foxp3*, *Tbx2*, *Rorc*, and *GATA-3* (E) expression using qRT-PCR (48h). (F and G) Splenic 2D2 naïve T cells were co-cultured as above and polarized under Th1 conditions (F) or Th17 conditions (G) as described in Methods. IFN- γ and IL-17 producing CD4⁺ T cells were determined by flow cytometry as described in B (left panels). Supernatants were collected and subjected to ELISA to measure

IFN- γ and IL-17 secretion (right panels). * $p < 0.05$ compared with control, ** $p < 0.01$, compared with control (vehicle). One of three independent experiments is shown. Data are presented as mean $\pm$ SD.

A

B

Clinical features of EAE in animals treated with Gp1a from day 7

	Vehicle	Gp1a
Incidence	10/10 (100%)	10/10 (100%)
Death of severe disease	1/10 (10%)	0/10(0%)
Disease onset (days)	13.20 ± 0.44	14.44 ± 0.87
Maximum score	3.98 ± 0.22	3.33± 0.18
Cumulative score	36.65 ± 3.12	25.44 ± 2.40*

Data represent the mean ± SEM. Statistically significant at * $p < 0.05$, compared with Vehicle group.

Figure 5. Therapeutic effects of Gp1a

C57BL/6 mice (n=10/group) were immunized with MOG₃₅₋₅₅ (100µg/mouse) as described in Methods. Gp1a treatment (5mg/kg) started on day 7 and continued twice weekly until the end of the observation period. The control group received the same treatment regimen with vehicle (PBS). Left panel: clinical scores followed for 30 days. Right panel: linear regression curve; dashed lines showed 95% confidence interval. Clinical scores are presented as mean ± SEM. Comparison between Gp1a-treated and vehicle-treated mice for each time point* $p < 0.05$; ** $p < 0.01$.

Figure 6. Delayed Gp1a treatment does not affect in vivo splenic Th1/Th17 differentiation

C57BL/6 mice were immunized and treated with Gp1a as described in Figure 5. On day 11, splenocytes were harvested and restimulated with MOG₃₅₋₅₅ (50 μ g/ml). (A) 48h later, cells were collected and subjected to qRT-PCR for *Rorc* and *Tbx21* expression. (B) 72h later, gated CD4 T cells were analyzed for intracellular IFN γ and IL17 expression. Supernatants were collected and secreted IFN γ and IL17 were detected by ELISA. Data are representative of two independent experiments and presented as mean $\pm$ SEM, n=3.

Figure 7. Delayed Gp1a treatment inhibits proinflammatory gene expression in spinal cord
C57BL/6 mice were immunized and treated with Gp1a as in Figure 5. At peak of disease spinal cord tissue was harvested and homogenized. Gene expression of cytokines, chemokines, and adhesion molecules was analyzed by qRT-PCR. One of two independent experiments is shown. Data are presented as mean ± SEM, n=3.

Figure 8. Gp1a treatment inhibits VLA-4 and chemokine receptor expression in splenic T cells CD4 T cells were isolated from the spleen of EAE mice treated with vehicle or Gp1a. As control, CD4 T cells were also isolated from spleens of naïve mice. CD4 T cells and CD4 T cell-depleted splenocytes were subjected to qRT-PCR to detect the expression of *Ccr5*, *Ccr2*, *Cxcr3*, *VLA4($\alpha 4$)*, and *VLA4($\beta 1$)*. Data are presented as mean $\pm$ SD.

Figure 9. Sustained inhibition of proinflammatory gene expression following delayed Gp1a treatment

C57BL/6 mice were immunized and treated with Gp1a as in Figure 5. On day 30 spinal cord tissue was harvested and homogenized. Gene expression of cytokines, chemokines, and adhesion molecules was analyzed by qRT-PCR. One of two independent experiments is shown. Data are presented as mean $\pm$ SEM, n=3.

Figure 10. Sustained reduction in immune cell infiltration following delayed Gp1a treatment
 C57BL/6 mice were immunized and treated with Gp1a as described in Figure 5. CNS mononuclear cell analysis and histology studies were performed on day 30. **(A, B)** Mononuclear cells (MNCs) were isolated from spinal cord and brain as described in Methods. Cells were stained with anti-CD45, anti-CD3 and anti-CD11b. CD45⁺CD3⁺, CD45^{hi}CD11b⁺, and CD45^{lo}CD11b⁺ cells were identified as T cells, macrophages and microglia, respectively. Data are representative of two independent experiments. Data are presented as mean $\pm$ SEM, n=3. **(C)** Immunohistochemistry (4 mice/group) using CD3 and Iba-1 staining.

Figure 11. Gp1a reduces demyelination and axonal damage

C57BL/6 mice were immunized and treated with Gp1a as in Figure 5. Histology staining of spinal cord in normal and EAE mice on day 30 (4 mice/group). **(A)** Spinal cord myelin was stained with luxol fast blue and periodic acid Schiff reagent in the white matter adjacent to the left ventral horn (20×10 magnification). **(B)** Bielschowsky's silver staining of spinal cord axons; upper panels show whole spinal cord, lower panels: the anterior median region (40×10 magnification).

Table 1

Gene expression fold changes for Gp1a-treated mice were based on values obtained from vehicle-treated mice.

Gene symbols	Gp1a/ Vehicle
C3ar1	0.54
Ccl12	0.51
Ccl2	0.12
Ccl5	0.54
Ccl7	0.16
Ccr1	0.24
Ccr2	0.23
Ccr7	0.28
Cxcl10	0.15
Il1b	0.20
Il1rn	0.10
Itgb2	0.47
Tlr4	0.58
Tlr2	0.47
Tnf	0.34