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New strategy for MS treatment with autoantigen-modified liposomes and their therapeutic effect

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ABSTRACT

As current treatments for multiple sclerosis (MS) remain chemotherapeutic ones directed toward symptoms, the development of a curative treatment is urgently required. Herein, we show an autoreactive immune cell-targetable approach using autoantigen-modified liposomes for the curative treatment of MS. In these experiments, experimental autoimmune encephalomyelitis (EAE) induced by autoantigenic myelin oligodendrocyte glycoprotein (MOG) peptide was used as a model of primary progressive MS, and MOG-modified liposomes encapsulating doxorubicin (MOG-LipDOX) were used as a therapeutic drug. The results showed that the progression of encephalomyelitis symptoms was significantly suppressed by MOG-LipDOX injection, whereas the other samples failed to show any effect. Additionally, invasion of inflammatory immune cells into the spinal cord and demyelination of neurons were clearly suppressed in the MOG-LipDOX-treated mice. FACS analysis revealed that the number of both MOG-recognizable $CD4^+$ T cells in the spleen was obviously decreased after MOG-LipDOX treatment. Furthermore, the number of effector Th17 cells in the spleen was significantly decreased and that of regulatory Treg cells was concomitantly increased. Finally, we demonstrated that myelin proteolipid protein (PLP)-modified liposomes encapsulating DOX (PLP-LipDOX) also showed the therapeutic effect on relapsing-remitting EAE. These findings indicate that autoantigen-modified liposomal drug produced a highly therapeutic effect on EAE by delivering the encapsulated drug to autoantigen-recognizable $CD4^+$ T cells and thus suppressing autoreactive immune responses. The present study suggests that the use of these autoantigen-modified liposomes promises to be a suitable therapeutic approach for the cure of MS.

1. Introduction

Autoimmune diseases develop due to abnormal immune response to autoantigens expressed in the body's own normal cells. Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) and is said to be an autoimmune disease elicited by CNS antigens such as myelin components, although the whole picture of MS pathogenesis has not yet been completely elucidated. Correspondingly, the use of steroids and some disease-modifying drugs (DMDs) to control the neurodegenerative symptoms and to reduce the relapse rate have become the mainstream approach for clinical treatment of MS; however, these treatments target only the symptoms and thus cannot be expected to result in a complete cure [1]. Furthermore, because of their non-specific immunosuppressive effect, MS patients frequently suffer side effects due to infections, such as progressive multifocal leukoencephalopathy (PML) [2,3] and this problem often limits treatment of the disease. Therefore, the development of a curative and safe treatment for MS is urgent goal.

Drug delivery system (DDS) is a powerful tool to achieve improvement of pharmaceutical properties and a therapeutic effect of ingredients for the treatment of various diseases including cancer [4], because it enables the delivery of a drug to target sites such as cancer tissue by means of guidable probe molecules and/or carrier nanoparticles. The liposome, a lipid-based nanoparticle, is widely used as a

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DDS carrier to improve the shortcomings of poor efficacy of drugs in the fields of both basic and clinical research [5]. For enhanced specificity of liposome targetability, modification of the liposomal surface with guidable molecules such as antibody and peptide is also a suitable technique; and many researchers have reported excellent therapeutic results by the use of ligand-modified liposomal drugs [6]. Regarding MS treatment, some research groups have already developed DDS drugs and succeeded in showing a prolonged blood concentration of ingredient drugs and a therapeutic effect on MS model animals [7,8]. On the other hand, for specific delivery of a drug to antigen-recognizing immune cells, we were the first to advocate a targeting strategy using liposomes modified with antigenic molecules and demonstrated the usefulness of allergen-modified drug-bearing liposomes for the treatment of allergy [9,10]. That is, ovalbumin (OVA)-modified liposomes encapsulating a cytotoxic drug or immunosuppressive drug strongly suppressed the production of anti-OVA IgE antibody in OVA-sensitized mice; and one of the targets of the liposomes was splenic B cells producing the antibodies [9,10].

To apply this targeting strategy for the treatment of MS, we presently developed drug-encapsulating liposomes surface-modified with an autoantigen to enable the targeting of immune cells involved in the progression of MS. In the experiments, we used experimental autoimmune encephalomyelitis (EAE) as a model of MS, since EAE mice display T cell-mediated inflammatory demyelination of neurons in the CNS and MS-similar symptoms such as axonal damage and paralysis [11]. As the autoantigen molecule, we used a myelin oligodendrocyte glycoprotein (MOG) peptide (residues 35–55, $MOG_{35–55}$) to induce primary progressive EAE and developed MOG-modified liposomes encapsulating doxorubicin (MOG-LipDOX) as a therapeutic drug. Then, we investigated the therapeutic effect of MOG-LipDOX on EAE and carried out a mechanistic study focused on splenic T cells involved in EAE pathogenesis. Finally, we examined the therapeutic effect of PLP-modified liposomal DOX (PLP-LipDOX) as a treatment for a relapsing-remitting model of EAE induced by a myelin proteolipid protein (PLP) peptide (residue 139–151, $PLP_{139–151}$).

2. Materials and methods

2.1. Materials

Syntheses of autoantigen peptides, MOG ($MOG_{35–55}$: MEVGWYRSPFSRVVHLYRNGK) and PLP ($PLP_{139–151}$: HSLGKWLGHPDKFC-NH_2), were outsourced to Medical & Biological Laboratories Co., Ltd. (Nagoya, Japan) or GenScript Japan Inc. (Tokyo Japan) and Peptide Institute, Inc. (Osaka, Japan), respectively. Dipalmitoylphosphatidylcholine (DPPC) and cholesterol (CHOL) were supplied from Nippon Fine Chemical Co. (Hyogo, Japan). Doxorubicin was purchased from Kyowa Kirin Co., Ltd. (Tokyo, Japan).

2.2. Animals

C57BL/6 and SJL/J female mice were respectively purchased from Japan SLC (Shizuoka, Japan) and Charles River Laboratories Japan, Inc. (Yokohama, Japan). All animal experiments were performed at the University of Shizuoka or Hamamatsu University School of Medicine and approved by the Animal and Ethics Committees of both universities. The animals were cared for according to the Animal Facility Guidelines of the both universities. MOG and PLP were used for pathological model preparations of primary progressive MS (PPMS) and relapsing-remitting MS (RRMS), respectively [12–14]. In brief, MOG (200 μg) or PLP (50 μg) mixed with complete Freund's adjuvant containing *Mycobacterium butyricum* (500 μg, Chondrex, Inc.) was subcutaneously injected into the back of C57BL/6 or SJL/J mice, respectively; and then pertussis toxin solution (Wako Pure Chemical Industries) was intravenously injected into the mice via a tail vein at days 0, 2, and 4 (200 ng/day).

2.3. Preparation of autoantigen-modified liposomes

Original liposome solutions were made by hydration of thin-layered lipids containing DPPC and CHOL (2: 1 as molar ratio) with 250 mM ammonium sulfate solution, freeze-thawed with a liquid nitrogen, sonicated for 5 min, and sized at 60 °C with a Lipex extruder attached to a 100-nm pore-size polycarbonate membrane filter. Then, the liposomes were dialyzed against water for at least 6 h to generate a pH gradient between the inner and outer liposomal layers. After ultracentrifugation of the liposomes, the liposomes were suspended in 300 mM HEPES buffer (pH 7.4). For drug encapsulation, DOX (2.4 mM) solution was added to the liposome solution (20 mM as DPPC); and mixing was carried out at 60 °C for 30 min. Unencapsulated DOX was removed by ultracentrifugation, and the pelleted liposomes were resuspended in 300 mM HEPES buffer (pH 7.4). For modification with autoantigen, MOG or PLP, DSPE-PEG-NHS (SUNBRIGHT DSPE-020GS, NOF Corporation) or DSPE-PEG-Mal (SUNBRIGHT DSPE-020MA, NOF Corporation), respectively, was used as the linker molecule. In brief, DSPE-PEG-NHS or DSPE-PEG-Mal was reacted at 4 °C for 2 h with MOG or PLP, respectively; and the mixture was then incubated with drug-encapsulated liposomes at 65 °C for 15 min to obtain autoantigen-modified liposomes. The particle size and ζ-potential of the liposomes were determined by dynamic light scattering (DLS) with the Zetasizer Nano ZS system (Malvern Instruments), and the quantification of MOG was done by measuring absorbance at the wavelength of 280 nm with an HPLC system (LaChrom Elite L-2000 series, Hitachi). To examine the stability of DOX-encapsulated liposomes, we incubated MOG-LipDOX for 0, 1, or 6 h in the presence of 90% fetal bovine serum (FBS, Thermo Fisher Scientific, Inc.); and after gel-filtration chromatography to remove the released DOX, the amount of DOX retained in the liposomes was measured with the HPLC system. To confirm the affinity of MOG-LipDOX for anti-$MOG_{35–55}$ antibody, we prepared CM5 sensor chips immobilized with purified mouse IgG1κ antibody (Control antibody, BD Biosciences) or mouse MOG monoclonal IgG1 antibody (anti-$MOG_{35–55}$ antibody, MyBioSource, Inc.) and performed binding analysis by using a Biacore 2000 (GE Healthcare) system.

2.4. Biodistribution assay

For preparation of radiolabeled liposomes, [^{3}H]cholesteryl hexadecyl ether (PerkinElmer Japan Co., Ltd.) was used as a probe molecule and mixed in the lipid/chloroform solution. Prepared [^{3}H]-labeled Cont-Lip or MOG-Lip (DPPC concentration: 1 mM) was intravenously injected into EAE mice via a tail vein (4 μCi/0.1 mL/mouse) at day 10. Three hours later, the mice were sacrificed; and the plasma and the organs (heart, lung, liver, spleen, kidney, brain, spinal cord, and inguinal lymph nodes) were collected. Then the samples were solubilized with Solvable™ (PerkinElmer) at 50 °C, decolorized with hydrogen peroxide and subsequently mixed with Hionic-Fluor™ (PerkinElmer). Thereafter, the radioactivity was measured by using a liquid scintillation counter (LSC-7400, Hitachi Aloka Medical, Ltd.). The total weight of plasma was assumed to be 4.38% of the body weight.

2.5. Intrasplenic distribution of liposomes

For preparation of fluorescently labeled liposomes, 3, 3′-dioctadecyloxacarbocyanine perchlorate (DiO, Invitrogen) was used as a fluorescent dye and mixed with lipids before lipid thin-layer formation. Prepared DiO-labeled Cont-Lip or MOG-Lip was intravenously injected into EAE mice at day 10 after immunization of them with MOG; and after 12 h, their spleens were perfused with an excess amount of PBS in order to remove the circulating liposomes in the bloodstream. Then, the spleens were dissected from the mice, embedded in optical cutting temperature (O.C.T.) compound (Sakura Finetek Japan Co., Ltd.), and frozen with dry ice. Next, ten-micrometer spleen sections were prepared with a cryostatic microtome (HM505E, MICROM), transferred to MAS-

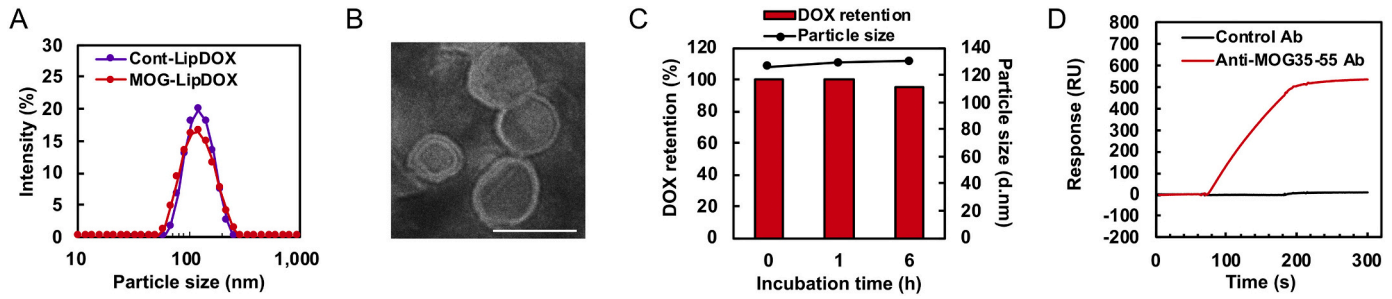

Fig. 1. Preparation of MOG-LipDOX.
(A) Particle-size distribution of Cont-LipDOX and MOG-LipDOX as analyzed by DLS. (B) TEM image of MOG-LipDOX. MOG-LipDOX was stained with ammonium molybdate and observed with a TEM system. The scale bar represents 200 nm. (C) Stability of MOG-LipDOX in FBS. MOG-LipDOX was incubated with 90% FBS for 1 or 6 h, and then the amount of DOX retained in the liposomes and the particle size were measured. (D) Ability of MOG-LipDOX to bind anti-$MOG_{35–55}$ antibody. Binding of MOG-LipDOX to control antibody or anti-$MOG_{35–55}$ antibody after incubation with FBS for 6 h was examined by using the Biacore system. Sample injection time: 2 min (from 60 to180 *sec* after start of analysis).

coated glass slides (Matsunami Glass Ind., Ltd.), fixed in 4% paraformaldehyde-PBS solution (Wako) for 15 min, and blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich Japan) for 1 h at room temperature. For staining of T cells, the sections were incubated with anti-CD3 (mouse) mAb-PE (EMD Millipore) for 2 h. After having been washed with PBS, the sections were incubated with DAPI (Thermo Fisher Scientific) for nuclear staining. The fluorescence was observed by use of a confocal laser-scanning microscope (N1R, Nikon).

2.6. Therapeutic experiments

In therapeutic experiments using primary progressive EAE mice, MOG-induced EAE mice were intravenously injected with MOG-LipDOX (0.01 or 0.05 mg/kg/day as DOX) via a tail vein on days 10, 12, 14, and 16 after immunization with MOG. For comparative experiments, EAE mice were intravenously injected with PBS, DOX, non-modified liposomal DOX (Cont-LipDOX), MOG-modified liposome without DOX (MOG-Lip), or MOG-LipDOX (0.1 mg/kg/day as DOX dosage) via a tail vein on days 10, 14, 18, and 22. For the treatment of relapsing-remitting EAE, PLP-induced EAE mice were intravenously injected with PBS, DOX, Cont-LipDOX, PLP-Lip or PLP-LipDOX (0.05 mg/kg/day as DOX dosage) via a tail vein on days 10, 14, 18 and 22 after immunization with PLP. Clinical EAE signs of the mice were monitored and scored according to the following 13 grades (0: No clinical signs; 0.5: Slightly limp tail; 1: Tip tail paralyzed; 1.5: Partially limp tail; 2: Completely limp tail; 2.5: Completely limp tail and weakness of hind legs; 3: Uncoordinated movement; 3.5: One hindlimb paralyzed; 4: Both hindlimbs paralyzed; 4.5: Both hindlimbs paralyzed and 1 forelimb paralyzed; 5: Both hindlimbs paralyzed and both forelimbs paralyzed; 5.5: Moribund state; 6: Death) by reference to previous report [15]. Body weight changes in the mice were also monitored.

2.7. Histological staining

Naive or EAE mice were treated with PBS, DOX, Cont-LipDOX, MOG-Lip or MOG-LipDOX at a dosage of 0.1 mg/kg/day on days 10, 14, 18, and 22. At day 24, their spinal cords were perfused with an excess amount of PBS, fixed with 4% paraformaldehyde solution by injecting it via the heart under isoflurane anesthesia, and collected. Then, the parts around the fifth lumbar vertebra were dissected and fixed in 10% formalin neutral buffer solution at 4 °C overnight. Preparation of paraffin section and subsequent histological staining were carried out at Advanced Research Facilities & Services, Hamamatsu University School of Medicine. In brief, for hematoxylin and eosin (HE) staining, freshly prepared Carrazzi's hematoxylin solution containing hematoxylin (Merck), aluminium potassium sulfate (Wako), sodium iodate (Wako), and glycerol (MUTO PURE CHEMICALS CO., LTD.); and eosin Y staining solution (Wako) were used. For Klüver-Barrera (KB) staining, Luxol fast blue stain solution (MUTO) and Cresyl violet stain solution (MUTO) were used. Stained sections were observed with an optical microscope (IX71, OLYMPUS) and the number of invasive immune cells at the dorsal side of the spinal cords was counted.

2.8. FACS analysis

EAE mice were treated with PBS or MOG-LipDOX (0.1 mg/kg/day as DOX) at days 10 and 14, and their spleens were harvested from the mice at day 16. The splenocytes were separated from the spleens in RPMI-1640 medium and passed through a 40 μm-mesh-sized BD cell strainer (Corning) to obtain single cells. After hemolyzing the preparation with ACK lysing buffer, the cells were blocked with both 3% BSA-PBS and Clear Back (MBL) and then labeled with I-Ab $MOG_{35–55}$ Tetramer-PE (MBL) and Anti-mouse CD4 mAb-FITC (MBL) to determine $MOG_{35–55}$-recognizing T cells. For determination of effector T cells, the splenocytes were probed with Mouse Th1/Th2/Th17 Phenotyping Kit (BD Pharmingen™) after incubation with ionomycin (500 ng/mL, Adipogen®), phorbol 12-myristate 13-acetate (PMA, 50 ng/mL, Adipogen®), and monensin (5 μg/mL, Cayman Chemical) in 5% FBS-containing RPMI1640 medium for 5 h at 37 °C. For determination of regulatory T (Treg) cells, the cells were labeled with both anti-FOXP3 (mouse) mAb-APC (Merk Millipore) and anti-mouse CD4 mAb-FITC. FACS measurements were carried out with a BD FACSCanto™ II system and the data were analyzed with a BD FACSDiva™ (BD) or a FlowJo™ (BD).

2.9. Statistical analysis

All data were analyzed with an unpaired Student's *t*-test, ANOVA followed by post hoc analysis using Tukey HSD, or Dunnett's test.

3. Results

3.1. Characteristics of MOG-LipDOX

We prepared MOG-Lip and MOG-LipDOX as autoantigen-modified liposome formulations and examined their physicochemical characteristics. Dynamic light-scattering (DLS) measurement and transmission electron microscope (TEM) observation showed that the liposomes were stably dispersed in aqueous solution, the mean particle sizes of MOG-Lip and MOG-LipDOX were 178 nm and 155 nm, respectively, a little bit larger than those of non-modified liposome (Cont-Lip) and Cont-Lip encapsulating DOX (Cont-LipDOX) due to attachment of MOG to the liposomal surface (Table S1, Fig. 1A, B). In addition, the encapsulated DOX in the MOG-LipDOX was quite stable in the presence of serum (Fig. 1C), indicating that MOG-LipDOX could certainly deliver the

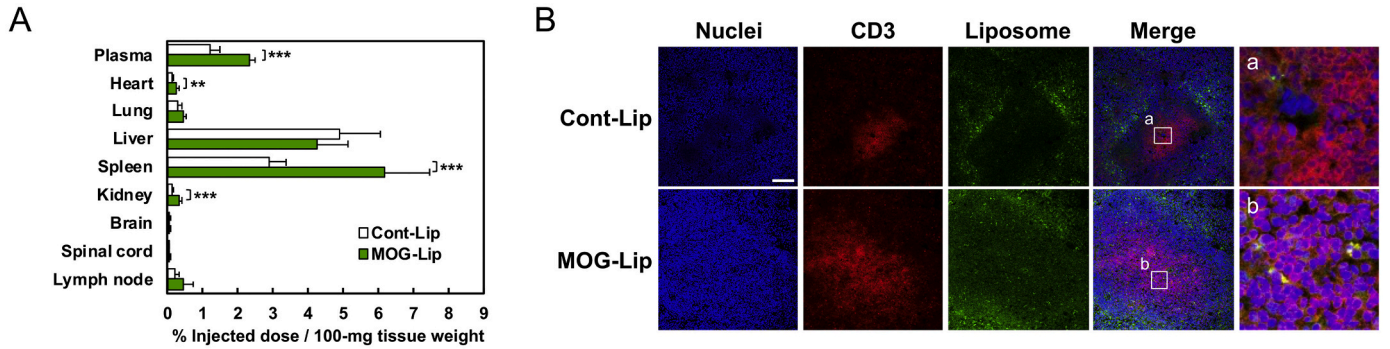

Fig. 2. Accumulation of MOG-LipDOX in splenic T cells.
(A) Biodistribution of MOG-Lip in EAE mice. [^{3}H]-labeled Cont-Lip or MOG-Lip was intravenously injected into EAE mice via a tail vein on day10 after MOG immunization and allowed to circulate for 3 h. Accumulation data represents the mean ± SD of percentage of injected dose per 100-mg tissue weight. Significant differences are shown with asterisks (**, $P < 0.01$; ***, $P < 0.001$, Student's *t*-test) (B) Intrasplenic distribution of MOG-LipDOX. DiO-labeled Cont-Lip or MOG-Lip (green) was intravenously injected into EAE mice via a tail vein on day10. After 3 h, 10-μm frozen sections of the spleen were prepared and probed with anti-CD3 antibody-PE (red) for T cell staining and DAPI (blue) for nuclear staining. Magnified images in each region are shown (a, b). Scale bars represent 100 μm.

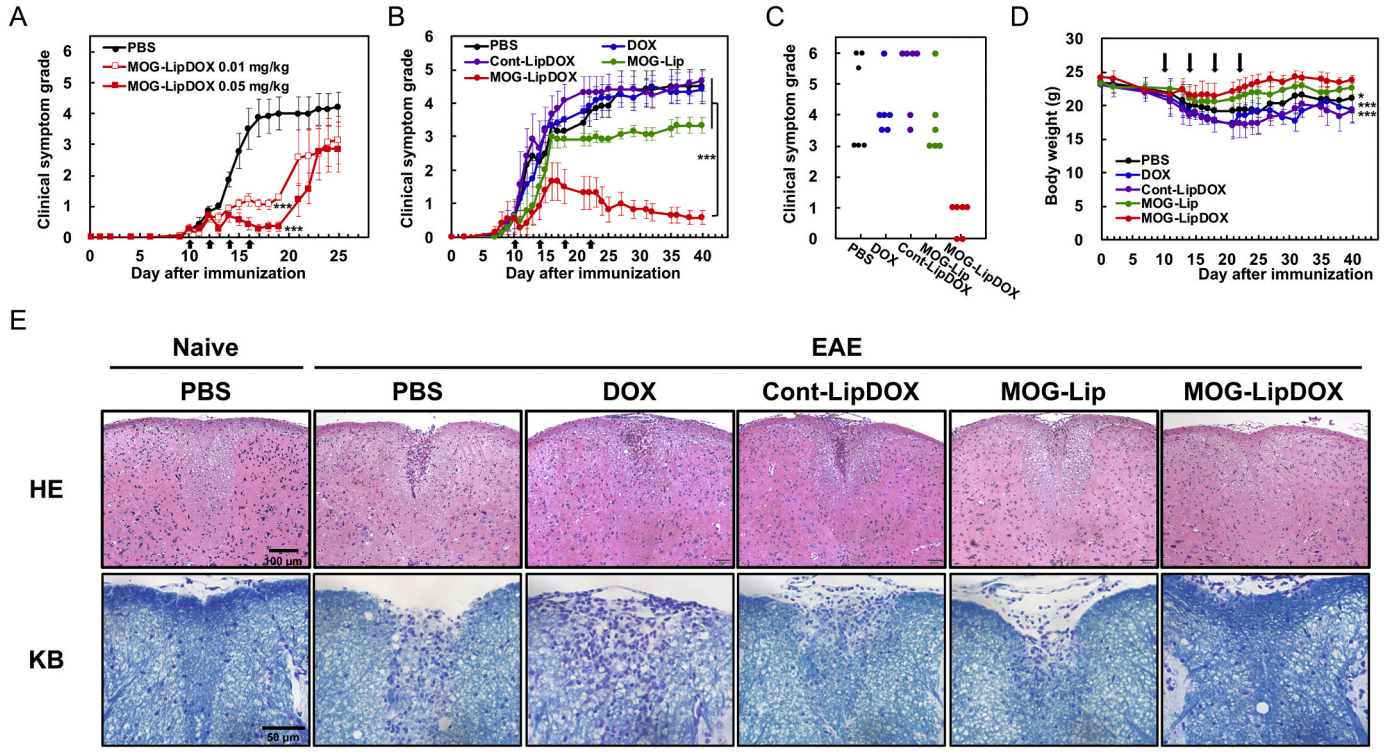

Fig. 3. Therapeutic effect of MOG-LipDOX on EAE.
(A) Dose-dependent suppression of EAE signs by MOG-LipDOX. EAE mice were intravenously injected with PBS or MOG-LipDOX (0.01 mg/kg/day or 0.05 mg/kg/day as DOX dosage) via a tail vein on days 10, 12, 14, and 16 (black arrows) after immunization with MOG. Clinical symptoms of EAE were monitored and scored based on the 13 grades described in Materials and Methods. Data represent the mean ± SEM. Significant differences (***, $P < 0.001$ vs. PBS, Tukey HSD) were shown only at day 19. (B) Comparison of therapeutic effect of PBS, DOX, Cont-LipDOX, MOG-Lip, and MOG-LipDOX on EAE. EAE mice were intravenously injected with samples via a tail vein at the DOX dosage of 0.1 mg/kg/day on days 10, 14, 18, and 22 (black arrows). Data represent the mean ± SEM. Significant differences were noted at day 40 (***, $P < 0.001$, Tukey HSD). (C) Long-term effect of MOG-LipDOX on EAE mice. The clinical symptoms were scored on day 101. (D) Body-weight change in EAE mice. Data represent the mean ± SD and black arrows indicate the days of drug injection. Significant differences (*, $P < 0.05$; ***, $P < 0.001$ vs. MOG-LipDOX, Dunnett's test) were shown only at day 40. (E) Histological observation of spinal cord of EAE mice. Spinal cords of naive or EAE mice treated with PBS, DOX, Cont-LipDOX, MOG-Lip or MOG-LipDOX were collected at day 24; and the paraffin sections were prepared. Hematoxylin and eosin (HE) staining and Klüver-Barrera (KB) staining were carried out to observe evidence of immune cell invasion and of demyelination of neurons, respectively.

encapsulated DOX to MOG-recognizing cells after systemic injection. Furthermore, MOG-LipDOX retained the ability to bind to anti-$MOG_{35–55}$ antibody but not to control antibody after incubation with FBS (Fig. 1D). These observations suggested that we had succeeded in preparing drug-encapsulating liposomes surface-modified with an autoantigen that could stably deliver the encapsulated drug to target cells.

3.2. Targetability of MOG-lip to T cells in the spleen

To find the target organ of the autoantigen-modified liposomes, we prepared radiolabeled Cont-Lip and MOG-Lip and quantitatively

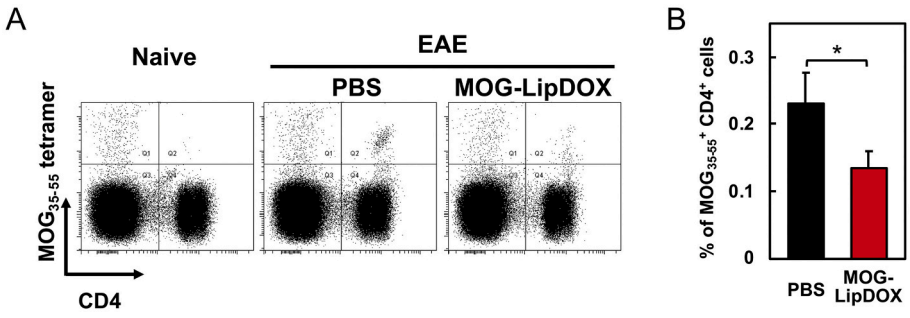

Fig. 4. FACS analysis of cell damage of MOG-recognizing T cells.
EAE mice were treated twice with PBS or MOG-LipDOX at a dosage of 0.1 mg/kg/day as DOX on days 10 and 14. On day 16, the collected splenocytes were probed with anti-CD4 antibody-FITC and I-Ab $MOG_{35–55}$ Tetramer-PE. Then, FACS analysis was performed to determine the percentage of numbers of $MOG_{35–55}$-recognizing $CD4^+$ T cells. Representative dot plots were shown (A) and is summarized in bar graphs (B). The data represent the mean ± SD ($n = 3$). Significant differences are shown with asterisk (*, $P < 0.05$, Student's *t*-test).

analyzed the biodistribution of these liposomes in EAE mice after systemic injection. Before the experiment, we confirmed that MOG-Lip specifically bound to anti-$MOG_{35–55}$ antibody (Fig. S1) but not to control antibody. The results of biodistribution assay indicated that the accumulation of MOG-Lip in some organs including spleen was significantly higher than that of Cont-Lip (Fig. 2A). Then, to examine the liposome distribution in the spleen in detail, we prepared fluorescently labeled liposomes and microscopically observed the localization of the fluorescence in the spleen. The results showed that MOG-Lip widely distributed in the spleen (Fig. 2B) and that a portion of them was localized in the CD3-positive T cell region (Fig. 2B (b)). On the other hand, Cont-Lip did not accumulate in the T cell region nor was it distributed around there (Fig. 2B (a)). These different observations indicate that the autoantigen-modified liposomes accumulated at splenic T cells as a consequence of the autoantigen having been displayed on the liposomal surface, suggesting that the targeted splenic T cells had the potential to recognize the autoantigenic MOG peptide.

3.3. Therapeutic effect of MOG-LipDOX on primary progressive EAE

To evaluate the therapeutic effect of autoantigen-modified liposomal drug on MS, we prepared MOG-induced EAE mice as a model of primary progressive MS (PPMS) and performed a therapeutic experiment with MOG-LipDOX. The injection of the samples was started when the symptoms of encephalomyelitis were observed in the MOG-EAE mice (first injection: day 10); and monitored the MOG-LipDOX-elicited improvement of the clinical score of EAE symptoms achieved by the liposome injections. The results indicated that the treatment with MOG-LipDOX significantly suppressed the EAE symptoms during the therapy, even when the injection dose of DOX was very low (Fig. 3A). To compare the therapeutic effect with other formulations and to confirm the effect at a higher dose, we carried out a similar therapeutic experiment with non-liposomal DOX, non-MOG-modified Cont-LipDOX, and non-DOX-encapsulated MOG-Lip. The result showed that an obvious therapeutic effect was observed only in the MOG-LipDOX-treated group (Fig. 3B). In contrast, DOX and Cont-LipDOX failed to show any effect, and the clinical symptom grade of these groups was similar to that of the PBS-treated mice. Surprisingly, the suppressive effect of MOG-LipDOX continued for over 100 days, and 2 mice in the MOG-LipDOX-treated group were completely cured (Fig. 3C). In addition, when the side effects of MOG-LipDOX were monitored, no change in body weight was observed in the MOG-LipDOX-treated mice (Fig. 3D); nor was any liver injury caused by the drug injection detected, which histology was similar to that seen for the PBS-treated EAE mice (Fig. S2). These results suggest that autoantigen-modified liposomal drug has a special therapeutic potential and that both autoantigen modification of the liposomal

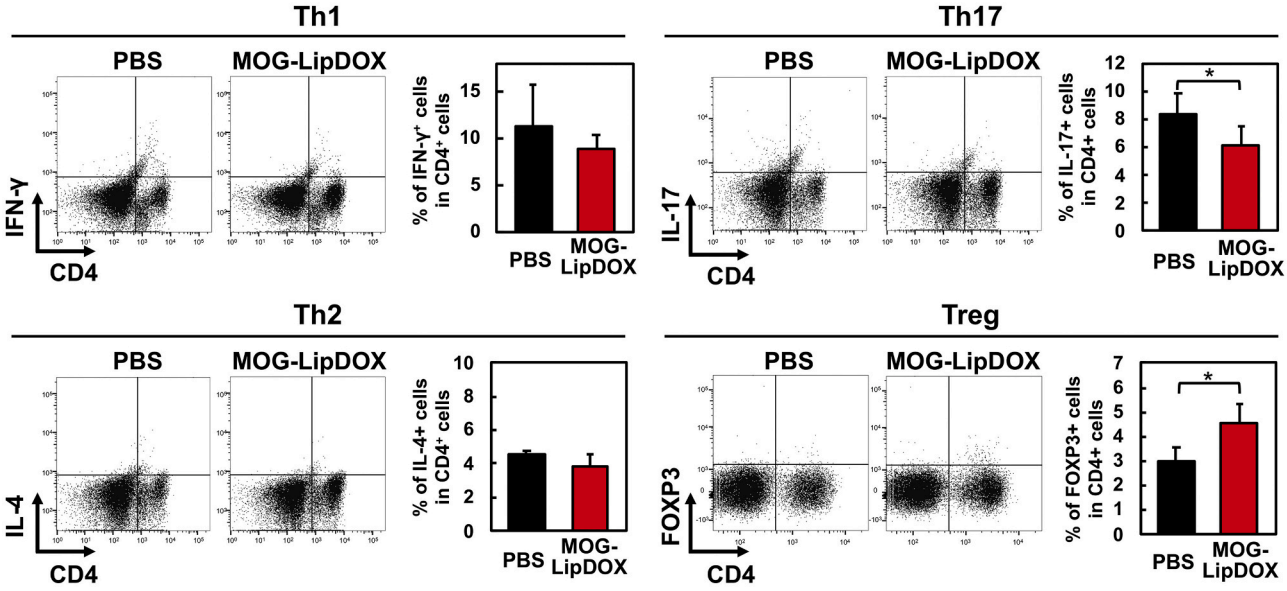

Fig. 5. Effect of MOG-LipDOX on splenic $CD4^+$ T cells.
Splenocytes were obtained from EAE mice treated with PBS or MOG-LipDOX (0.1 mg/kg/day as DOX, injected on days 10 and 14) on day 16 after immunization with MOG, and then activated by stimulation with ionomycin (500 ng/mL), PMA (50 ng/mL), and monensin (5 μg/mL) for 5 h at 37 °C. Lymphocytes were gated based on SSC/FSC gating. The cells were probed with anti-IFN-γ mAb -FITC, anti-IL-17 mAb-PE or anti-IL-4 mAb-APC, and anti-CD4 mAb PerCP-Cy5.5, for staining Th1 cells, Th17 cells, and Th2 cells, respectively. Treg cells were identified by probing the splenocytes with both anti-FOXP3 mAb-APC and anti-CD4 mAb-FITC. Each dot plot data is summarized in bar graphs. The data represent the mean ± SD. Significant differences are shown with asterisk (*, $P < 0.05$, Student's *t*-test).

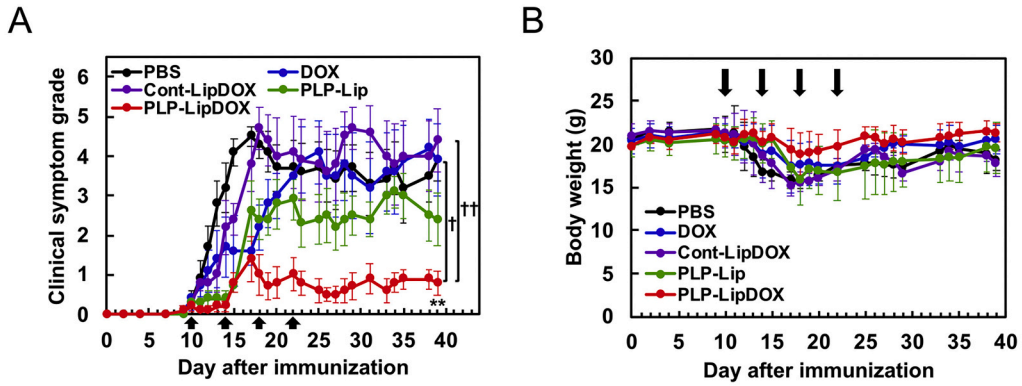

Fig. 6. Effect of PLP-LipDOX on relapsing-remitting EAE.
Relapsing-remitting EAE mice were prepared by immunization with $PLP_{139–151}$ peptide and then intravenously injected with PBS, DOX, Cont-LipDOX, PLP-Lip or PLP-LipDOX at a dosage of 0.05 mg/kg/day as DOX via a tail vein on days 10, 14, 18, and 22 (black arrows). Clinical symptoms of mouse motor dysfunction were monitored and scored based on the 13 grades described in Materials and Methods (A). Body weight of the mice was also measured (B). The summarized data represent the mean ± SEM of clinical score (A) or the mean ± SD (B). Significant differences were obtained at day 39 (**, $P < 0.01$ vs. PBS; †, $P < 0.05$ vs. DOX; ††, $P < 0.01$ vs. Cont-LipDOX, Tukey HSD).

surface and encapsulation of a drug into the liposomes are important to show its therapeutic effect.

3.4. Suppression of immune-cell invasion into the spinal cord

Demyelination of neurons in the CNS, caused by invading inflammatory immune cells, is a critical event for the development of MS and is frequently observed in not only MS patients but also EAE mice [16,17]. Therefore, we carried out histological observation of inflammatory immune cells and myelin sheath formation in the neurons of the spinal cord of EAE mice after the treatment with MOG-LipDOX. HE staining indicated that immune cells were not observed in the spinal cord of EAE mice treated with MOG-LipDOX, and the histological image was similar to that of the normal spinal cord (Fig. 3E). When the number of invasive immune cells at dorsal side of the spinal cords was counted, it was decreased in the MOG-LipDOX-treated spinal cords (Fig. S3), suggesting that invasion of the spinal cord by immune cells caused by EAE was completely suppressed by the treatment with MOG-LipDOX. In contrast, such invasion was observed in DOX- treated, Cont-LipDOX-treated, and MOG-Lip-treated mice. KB staining also revealed that the myelin sheaths were clearly observable at the edge of the spinal cord of EAE mice treated with MOG-LipDOX and that there was no evidence of nerve damage (Fig. 3E). These results suggest that treatment with MOG-LipDOX could prevent the demyelination of neurons caused by immune-cell invasion.

3.5. Cell damage of MOG-recognizing T cells by MOG-LipDOX

To elucidate the type of cells targeted MOG-LipDOX and to assess whether MOG-LipDOX could damage these MOG-recognizing cells or not, we performed FACS analysis of splenocytes harvested from EAE mice treated withMOG-LipDOX. As a result, the number of MOG-recognizing T cells in the spleen, which appeared by EAE induction, was obviously decreased by the treatment with MOG-LipDOX (Fig. 4A and B). Then, we examined the T cell damage effect after the treatment with PBS, DOX, Cont-LipDOX, MOG-Lip, or MOG-LipDOX. The results indicated that only MOG-LipDOX decreased the number of the splenic MOG-recognizing T cells (Fig. S4). These data mean that the target of MOG-LipDOX was the MOG-recognizing T cell, indicating that the lethal eradication of the cells by DOX was responsible for the potent therapeutic effect on EAE.

3.6. Effect of MOG-LipDOX treatment on splenic $CD4^+$ T cells

It is well known that activation of $CD4^+$ T cell subsets in secondary lymphatic organs including the spleen is strongly involved in the progression of MS and that especially the effector Th17 cell and regulatory Treg cells play an important role in it [18,19]. Therefore, we next examined the effect of MOG-LipDOX on splenic T cell subsets of Th1, Th2, Th17, and Treg cells by performing FACS analysis. Twice injections of MOG-LipDOX at the DOX dosage of 0.1 mg/kg/day to EAE mice resulted in changes in the number of T cell subsets in the spleen (Fig. 5): The number of $CD4^+IL\text{-}17^+$ Th17 cells was significantly decreased and that of $CD4^+IFN\text{-}\gamma^+$ Th1 cells tended to drop. The number of $CD4^+IL\text{-}4^+$ Th2 cells was not changed by the MOG-LipDOX injections. On the other hand, the number of $CD4^+FOXP3^+$ Treg cells was significantly increased. These observations indicate that the treatment with MOG-LipDOX suppressed the activity of Th17 cells involved in the progression of EAE and concomitantly promoted the production of Treg cells to suppressively regulate the onset of MS.

3.7. Therapeutic effect of PLP-LipDOX on relapsing-remitting EAE

Because most MS patients show repeated relapsing-remitting symptoms (RRMS) of neurological deficits and overcoming RRMS promises to have the strongest impact as a treatment goal of MS, we finally examined the therapeutic effect of autoantigen-modified liposomes encapsulating DOX on RRMS. To prepare an RRMS model, we immunized SJL/J mice with the autoantigenic $PLP_{139–151}$ peptide. For the therapy of PLP-EAE, we prepared $PLP_{139–151}$-modified liposomal DOX (PLP-LipDOX; Fig. S3, Table S2) as the therapeutic drug and recorded the clinical symptoms during the course of the experiment. The results indicated that 4 injections of PLP-LipDOX, given on days 10, 14, 18, and 22, showed strong suppression of the clinical EAE symptoms in PLP-EAE mice, whereas DOX or Cont-LipDOX treatment failed to have any effect (Fig. 6A). The treatment with PLP-Lip showed a small therapeutic effect, as similarly observed for the MOG-EAE therapy using MOG-Lip. Furthermore, body weight decrease caused by EAE induction was suppressed in PLP-LipDOX treated group (Fig. 6B). These findings suggest that an autoantigen-modified liposomal drug also has the potential to treat RRMS.

4. Discussion

Since overreaction of autoimmune T cells against myelin components in the CNS is critical to the onset, progression, and relapsing of MS [20], current MS treatment using DMDs is being done to prevent the T cells from autoimmune activation. For instance, INF-β formulations have been conventionally used for MS treatment and shown to have a protective effect on RRMS by suppressing T cell activation [21]. Also, fingolimod has been developed as an oral DMD that prevents the T cells from being transferred from secondary lymphatic organs to the blood

circulation via the action of the sphingosine-1-phosphate (S1P) receptor [22–25]. However, the immunosuppressive action of these DMDs is not specific for the immunity against the causative autoantigen; and so MS patients are more susceptible to infectious diseases such as PML caused by the systemic immunosuppression [26]. Furthermore, because DMDs are not acceptable to effect a complete cure of MS, these modalities need to be applied throughout the patients' lives. On the other hand, to expect an enhanced therapeutic effect on MS and to improve the patient's adherence, application of DDS technology to the development of MS drugs has been recently done, with beneficial results reported [27]. DDS carriers have been used to continuously maintain an adequate blood concentration of DMDs after their injection [7,8,28,29] or to effectively deliver drugs, vaccinal autoantigens or DNA vaccines to immune cells [30–32]. These DDS strategies promise a sustained effect of DMDs or potent vaccinal effect to suppress MS symptoms [33,34]. Our present strategy for MS treatment was not to use the autoantigen for vaccination, but rather to use it as a targetable probe of a DDS carrier to deliver the encapsulated drug to autoantigen-recognizing immune cells, since recognition of antigens by the antigen-recognizing immune cells in the body is quite high and specific. In other words, we speculated that the autoantigen-modified liposomes would be recognized by auto-reactive immune cells in an autoantigen-specific manner, thereby inhibiting self-reactive immune cells by delivering the cytotoxic agent to them, thus resulting in an MS cure. Biacore analysis revealed the specific binding of MOG-Lip to anti-$MOG_{35–55}$ antibody, as Cont-Lip did not bind to either control antibody or anti-$MOG_{35–55}$ antibody, whereas MOG-Lip bound to only anti-$MOG_{35–55}$ antibody (Fig. S1). This result suggests that autoantigenic $MOG_{35–55}$ peptide was conjugated on the surface of liposomes and that MOG-LipDOX had an affinity for antigen-recognizing molecules such as T cell receptors on T cells and on anti-$MOG_{35–55}$ antibody present on B cells in the body (Fig. 1D). Actually, we observed that MOG-Lip accumulated at T cell region in the spleen of EAE mice after systemic injection (Fig. 2B). The results of the therapeutic experiment indicated that MOG-LipDOX critically suppressed the clinical signs and showed a curative effect in MOG-EAE mice at 0.1 mg/kg dosage/day as DOX (Fig. 3B, C). DOX is generally used for cancer therapy at a dosage of approximately 10 mg/kg/day [35], and we used it at about one-hundredth of this dosage for EAE treatment in the present experiments. This remarkable effect means that our therapeutic strategy is very useful as well as safe without side effects. In fact, we could not see any side effects in the EAE mice after MOG-LipDOX treatment (Fig. 3D and S2). Interestingly, other DOX formulations of free DOX and Cont-LipDOX failed to show any therapeutic effect, despite the facts that the DOX dosage was the same and DOX itself possesses immunosuppressive potential. On the other hand, MOG-Lip showed a small suppressive effect compared with the non-treated group, suggesting that active delivery of autoantigen to autoantigen-recognizing cells by MOG-Lip would have a desensitizing effect. A previous study of ours using OVA as an exogenous antigen, demonstrated the treatment with OVA-Lip suppressed the production of anti-OVA IgE antibody in OVA-sensitized mice by inducing desensitization from OVA [36]. Santamaria et al. also reported that autoimmunogenic peptide-major histocompatibility complex (MHC)-based nanomedicine suppresses the symptoms in different autoimmune disease models including EAE by promoting the differentiation of disease-primed autoreactive T cells into the autoantigen-specific regulatory $CD4^+$ T cell type 1 cells [37–39]. Therefore, we presume that liposomes displaying the autoantigens on the surface worked in the same way as the peptide-MHC-based nanomedicine and showed weak suppression of EAE clinical symptom. However, MOG-LipDOX further suppressed the EAE symptoms compared with MOG-Lip despite the fact that the injection amount of MOG was the same between MOG-LipDOX and MOG-Lip, suggesting that drug encapsulation into autoantigen-modified liposomes is a key factor to show a strong therapeutic effect. Additionally, we also demonstrated the importance of autoantigen specificity for the therapeutic effect on EAE by an autoantigen-modified liposomal drug: We changed the autoantigen on the liposomal surface from the $MOG_{35–55}$ peptide to the $PLP_{139–151}$ one and compared the therapeutic effect of MOG-LipDOX with PLP-LipDOX on MOG-EAE mice. As the result, we found that the suppressive effect of MOG-LipDOX on the EAE clinical symptoms was much stronger than that of PLP-LipDOX (Fig. S4). We predict that strong cytotoxicity against autoantigen-recognizing immune cells brought by delivering the cytotoxic agent in autoantigen-modified liposome is important to show the antigen-specific immune suppression and subsequent curative effect on EAE. Actually, the treatment with MOG-LipDOX decreased the number of MOG-recognizing $CD4^+$ T cells in the spleen of MOG-EAE mice (Fig. 4). We further evaluated the potential therapeutic effect of the autoantigen-modified liposomal drug on RRMS and actually examined the therapeutic effect of PLP-LipDOX on PLP-induced EAE mice. These results indicated that PLP-LipDOX injected at low dosages significantly suppressed the clinical symptoms of PLP-EAE mice without any side effect (Fig. 6); and the effect was similar to that observed for therapy of MOG-EAE with MOG-LipDOX, suggesting our therapeutic strategy is also applicable to treat RRMS.

It is well known that helper T cell subsets play an important role in the progression of not only EAE but also MS and that especially Th1 and Th17 cells function as an effector to cause inflammation in the CNS and the subsequent onset of clinical symptoms [40,41]. Besides, the involvement of suppressor Treg cells in MS pathology has been elucidated [42], and treatment targeting the Treg cells has become a new approach for preventing MS progression [43]. Thus, we examined the impact of MOG-LipDOX treatment on T cell subsets. FACS analysis revealed that the treatment with MOG-LipDOX significantly reduced the number of Th17 cells (Fig. 5). Since Th17 cells are differentiated from antigen-recognizing naive $CD4^+$ T cells by being stimulated with proinflammatory cytokines such as TGF-β and IL-6 [44], we thought that eradication of MOG-recognizing $CD4^+$ T cells by MOG-LipDOX would result in a reduced number of Th17 cells. On the other hand, the number of Treg cells was concomitantly increased by the MOG-LipDOX treatment. Some researchers have already demonstrated that both Th17 cells and Treg cells share a common precursor cell (naive $CD4^+$ T cell). Autoimmunity is thought to be maintained by the balance of activity between effector Th17 cells and regulatory Treg cells, and a break in the Th17/Treg balance causes the development of autoimmune diseases including MS [45]. Furthermore, a challenge to control this balance for the treatment of autoimmune diseases has been started. [46,47]. These lines of evidence support our data that the MOG-LipDOX could return the Th17/Treg balance back to the normal state by directly affecting the MOG-recognizing $CD4^+$ T cells. Further experiments are needed to elucidate the whole mechanism of the therapeutic effect of MOG-LipDOX.

EAE is commonly used as an experimental model for MS and to evaluate the efficacy of developing drugs for MS therapy, because it shows pathological features such as inflammation, demyelination, and axonal loss in CNS and similar clinical symptoms to human MS. On the other hand, differences between EAE and human MS are often discussed, since the mechanism of MS onset is not fully elucidated and immune system of such inbred animals is different from that of human. However, glatiramer acetate, a random copolymer of four amino acids composed of glutamate (Glu), alanine (Ala), tyrosine (Tyr), and lysine (Lys) was originally discovered in the experiments using myelin basic protein (MBP)-induced EAE animal and has come to be used in clinical practice [48]. In addition, Haanstra et al. previously performed the induction of EAE with recombinant human MOG in non-human primates and succeeded to prepare EAE models that showed MS-like pathological features and produced anti-MOG antibodies [49]. These evidences support the significance of EAE use for developing MS drugs and further promise the feasibility of our research. MOG is thought as one of the causative autoantigens in MS and the other causative antigens including PLP are also found in MS patients [50]. In the present study, our therapeutic strategy showed the strong effect in a causative autoantigen-dependent

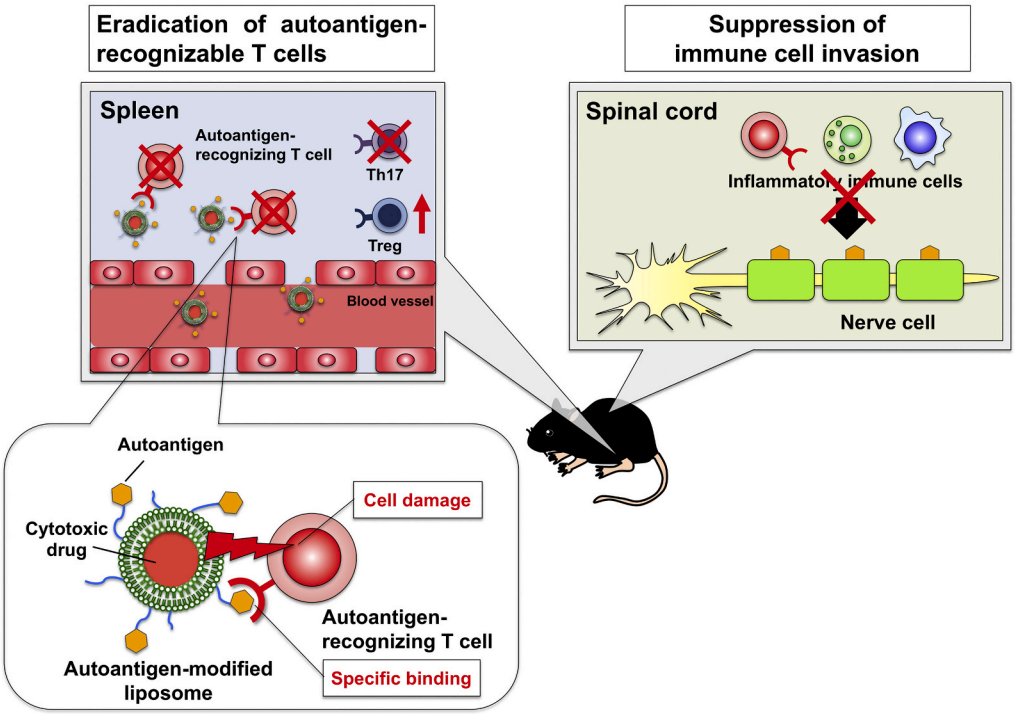

Fig. 7. Schematic of therapeutic strategy for the treatment of MS with autoantigen-modified drug-encapsulating liposomes.
Autoantigen-modified liposomes accumulate in the spleen after injection into MS model mice and are recognized by splenic autoantigen-recognizing T cells due to the autoantigen molecule on the liposomal surface. Then, the liposome-encapsulated cytotoxic drug lethally damages these T cells and subsequently causes a dynamic change in $CD4^+$ T cell subsets: The number of Th17 cells is decreased; and that of Treg, increased. Corresponding to this, invasion of immune cells into spinal cord is strongly suppressed; and neurodegeneration by the immune cells is prevented. Consequently, a long-term therapeutic effect on MS can be obtained.

manner. This means the therapy using MOG-LipDOX is not applicable to all MS patients. However, it is well known that autoantibody including anti-MOG antibody can be detected in MS patients [51]. We believe that our therapeutic strategy is applicable to MS patients in which the causative autoantigen has been identified by such autoantibody detection.

5. Conclusion

In summary, we presently demonstrated the usefulness of an autoantigen-modified liposomal drug for the treatment of MS. The autoantigen-modified liposomes delivered the encapsulated cytotoxic drug to the autoantigen-recognizing $CD4^+$ T cells in the spleen, lethally damaging them and resulting in the suppression of the onset of clinical symptoms caused by inflammatory neurological deficit in the CNS (Fig. 7). Besides, we also found that the treatment impacted the Th17/Treg axis and finally demonstrated that autoantigen-modified liposomal drug was applicable to not only PPMS but also RRMS. We strongly believe that our therapeutic strategy using autoantigen-modified liposomes will become a new therapy for MS and make a new avenue of hope for MS patients.

Declaration of Competing Interest

All authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

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