EBioMedicine 44 (2019) 50–59

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

EBioMedicine
Published by THE LANCET

Combination of apoptotic T cell induction and self-peptide administration for therapy of experimental autoimmune encephalomyelitis

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ARTICLE INFO

Article history:
Received 27 August 2018
Received in revised form 29 April 2019
Accepted 2 May 2019
Available online 13 May 2019

ABSTRACT

Background: Clinical trials on multiple sclerosis with repeated injections of monoclonal antibodies depleting $CD4^+$ T cells have not resulted in much success as a disease therapy. Here, we developed an immunotherapy for EAE in mice by combining a transient depletion of T cells together with the administration of neuron derived peptides.

Methods: EAE was induced in SJL and C57BL/6 mice, by proteolipid protein peptide $PLP_{139–151}$ (pPLP) and myelin-oligodendrocyte glycoprotein $MOG_{35–55}$ (pMOG) peptides, respectively. Anti-CD4 and anti-CD8 antibody were injected intraperitoneally before or after peptide immunization. EAE scores were evaluated and histology data from brain and spinal cord were analyzed. Splenocytes were isolated and $CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells were purified and cultured in the presence of either specific peptides or anti-CD3 antibody and proliferation of T cells as well as cytokines in supernatant were assessed.

Findings: This experimental treatment exhibited therapeutic effects on mice with established EAE in pPLP-susceptible SJL mice and pMOG-susceptible C57BL/6 mice. Mechanistically, we revealed that antibody-induced apoptotic T cells triggered macrophages to produce TGFβ, and together with administered auto-antigenic peptides, generated antigen-specific $Foxp3^+$ regulatory T cells (T_{reg} cells) *in vivo*.

Interpretation: We successfully developed a specific immunotherapy to EAE by generating autoantigen-specific T_{reg} cells. These findings have overcome the drawbacks of long and repeated depletion of $CD4^+$ T cells, but also obtained long-term immune tolerance, which should have clinical implications for the development of a new effective therapy for multiple sclerosis.

Fund: This research was supported by the Intramural Research Program of the NIH, NIDCR.

1. Introduction

$CD4^+$ T cell depletion by anti-CD4 antibody was first shown to be effective in prevention and treatment of mouse EAE three decades ago [1]. The simple and most plausible mechanism is that the $CD4^+$ T cell depletion induces immunosuppression. However, $CD4^+$ T cell depletion trials for patients with multiple sclerosis (MS) yield controversial results [2–4], although efficiently depleted 50–60% of the $CD4^+$ T cells. The discrepancy between mouse and human studies inspired us to re-examine the mechanism of $CD4^+$ T cell depletion therapy. Given $CD4^+$ T cell depletion may not be effective for the patients with MS, the depletion of $CD4^+$ T cells is unlikely the key mechanism for long-term suppression of mouse EAE [1,5]. A significant difference between the patients and mice was that excessive autoantigenic peptide was presented in the mice throughout the disease course, especially when the $CD4^+$ T cells were depleted, which suggested that autoantigenic peptide in combination with $CD4^+$ T cell depletion syntactically might exert the therapeutically effect.

Manipulation of $CD4^+CD25^+Foxp3^+$ T_{reg} cells hold promise for developing immunotherapy for autoimmune and autoinflammatory diseases [6–9]. Although many approaches have be proposed, most approaches are not able to manipulate T_{reg} cells to "treat" the established autoimmune diseases in experimental models or patients. We have

Abbreviations: αTGFβ, Anti-TGFβ; Del, T cell depletion antibodies; EAE, Experimental autoimmune encephalomyelitis; MOG, Myelin-oligodendrocyte glycoprotein; MT, *Mycobacterium Tuberculosis*; PLP, proteolipid protein; T_{reg}, regulatory T cells.

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https://doi.org/10.1016/j.ebiom.2019.05.005

Research in the context

Evidence before this study

$CD4^+$ T cell depletion by repetitive injection of specific antibody was first shown to be effective in prevention and reversal of EAE in mice back to three decades ago. However, translating these findings through experimental therapy in the clinical settings resulted in mixed results in attempt to treat patients with multiple sclerosis (MS). Anti-human CD4 antibody administration failed to establish high degree of efficacy for MS patients, although it efficiently depleted $CD4^+$ T cells.

Added value of this study

In this study we demonstrated that a combined treatment with transient depletion of T cells together with the administration of neuron derived peptides exhibited both preventive and therapeutic effects in mice with established EAE. The mechanism study revealed that antibody-induced apoptotic T cells triggered macrophages to produce TGFβ and together with administered autoantigenic peptides generated antigen-specific $Foxp3^+$ regulatory T cells (T_{reg} cells) *in vivo*.

Implications of all the available evidence

The results of this study provide an explanation as to why T cell deletion alone failed to efficiently cure the disease, and also provide a new approach to generate antigen specific T_{reg} cells *in vivo*.

recently [10] developed an approach to suppress established autoimmune EAE and Type I diabetes by induction of autoantigen-specific T_{reg} cells *in vivo*, which is accomplished by combination of transient B cell depletion or immune cell apoptosis with single and low dose γ-irradiation, plus auto-peptide administration [10]. However, it remains unknown whether *in vivo* induction of T cell apoptosis plus administration of autoantigenic peptide would have similar therapeutic effects on autoimmunity.

Here, we showed that induction of apoptotic T cells with anti-CD4 and CD8 antibodies followed by administration of auto-antigenic peptides significantly suppressed EAE in mice, which was achieved by the generation of autoantigen-specific T_{reg} cells *in vivo*. Furthermore, the tolerized mice show no compromised T cell response to bacterial antigens or overall T cell receptor stimulation. Our findings may have implications for the development of a similar immunotherapy for patients with MS.

2. Materials and methods

2.1. Mice

C57BL/6 and SJL mice were purchased from the Jackson Laboratory. Mice were maintained under specific pathogen free conditions according to the National Institutes of Health guidelines for the use and care of live animals.

2.2. Flow cytometry

Single-cell suspension was stained with the following flourochrome-conjugated antibodies; from eBioscience: IL-17 (clone ebio17B7), CD4 (clone RM4-4 and RM4-5), CD8b (clone H35-17.2), Foxp3 (clone FJK-16s), from Biolegend; CD11b (clone M1/70), F4/80 (clone BM8), LAP TGFβ1 (clone TW7-16B4), and from BD Biosciences; IFN-γ (clone XMG1.2). Foxp3 expression was examined using the mouse $CD4^+CD25^+$ Regulatory T cell Isolation Kit (Miltenyi Biotec). For intracellular cytokine measurement cells were incubated with PMA (5 ng/mL, Sigma), Ionomycin (250 ng/mL, Sigma) and GolgiPlug (1 μL/mL, BD Biosciences) to determine intracellular expression of IL-17 and IFN-γ. All samples were analyzed using FACS Calibur flow cytometer (BD Biosciences) and data were analyzed using Flowjo software.

2.3. MHC class II (IA^b) tetramer staining

The procedure for *ex vivo*-staining with MHC class II tetramer has been previously described in detail. Briefly, single cell suspensions were incubated at a density of 10^6 cells/mL with MOG_{38-49}/IA^b-tetramers (GWYRSPFSRVVH) in serum free DMEM at 4 °C/5% CO_2 for 16 h before cells were stained with indicated surface molecules.

2.4. Peptides

$PLP_{139-151}$(HSLGKWLGHPDKF), MOG_{35-55} (MEVGWYRSPFSRVVHLYRNGK) and $OVA_{323-339}$ (ISQAVHAAHAEINEAGR) were purchased from Invitrogen.

2.5. Cell isolation

$CD4^+$ T cells, $CD4^+CD25^+$ T cells, $CD4^+CD25^-$ T cells were isolated from spleens *via* either positive or negative selection using MACS isolation kits (Miltenyi Biotec) following the manufacturer's protocols. Briefly, $CD4^+CD25^-$ T cells were isolated by the Regulatory T cell Isolation Kit (Miltenyi Biotec). Non-$CD4^+$ T cells were isolated *via* negative selection by Regulatory T cell Isolation Kit (Miltenyi Biotec)(purity of cell separation was >90% each) and used as antigen presenting cells (APCs) after irradiation with 3000 rad of γ-irradiation (Gammacell 1000, Best Theratronics).

2.6. Proliferation assays and cytokine assays

Splenocytes were cultured at 37 °C in 5% CO_2 for 2–3 days with either soluble CD3-specific antibody (anti-CD3) (0.5 μg/mL) or MT (heat-killed *Mycobacterium tuberculosis*, H37RA, DIFCO) (50 μg/mL) or peptides (pMOG, pPLP) (0–50 μg/mL as indicated). We quantified cytokines in culture supernatants by ELISA; TNF-α, IL-6, and IFN-γ (BioLegend) and IL-17 (eBioscience). For the final 16 h of incubation, 1 μCi ^{3}H-thymidine was added to each well, and the incorporation of ^{3}H-thymidine was determined using a liquid scintillation counter Background was subtracted in ^{3}H-thymidine incorporation assay.

2.7. EAE induction, scoring, analysis and in vitro cell cultures

Peptide-induced EAE was induced in SJL mice and C57BL/6 mice as previously reported [11,12]. Individual mice were observed daily and clinical scores were assessed on a 0–5 scale as follows: 0, no abnormality; 1, limp tail or hindlimb weakness; 2, limp tail and hindlimb weakness; 3, hindlimb paralysis; 4, hindlimb paralysis and forelimb weakness; and 5, moribund. 7-wk-old male C57BL/6 mice were immunized subcutaneously with 200 μg/mouse of MOG_{35-55} peptide (pMOG) emulsified in complete Freund's adjuvant (CFA) (IFA supplemented with 300 μg/mL *Mycobacterium tuberculosis* (MT). 7-wk-old female SJL mice were immunized subcutaneously with 75 or 100 μg/mouse of pPLP emulsified in CFA (MT 300 μg/mouse). Mice also received 200 ng of *Bordetella pertussis* (List Biological Lab) i.p. on the day of immunization and 2 days later. At the end of each experiment spinal cords and brains were harvested and a part was fixed in neutral 10% formalin, extracted as well as embedded in paraffin blocks for Hematoxylin and eosin (H&E) staining. Cells were isolated from brains and spinal cords as previously reported (Perruche et al., 2008). Spleen was also harvested for further staining and culture. For cell cultures, splenocytes

were cultured at 37 °C in 5% CO_2 for 3 days with either soluble anti-CD3 (0.5 μg/mL) or MT (50 μg/mL) or peptides (pMOG, pPLP). After 3 days culture, cells were pulsed with 1 μCi [^{3}H] thymidine for 8-16 h. Radioactive incorporation was counted using a flatbed β-counter (Wallac). To examine the function of peptide-specific $CD4^+CD25^+$ T_{reg} cells in the spleen of mice, $CD4^+$, $CD4^+CD25^-$, and $CD4^+CD25^+$ T cells were MACS sorted and cultured with irradiated APCs from peptide-immunized EAE mice in the presence of either pPLP or pMOG (10 μg/mL), or anti-CD3 (0.5 μg/mL). After 3 days of culture supernatant and cells were collected for cytokine assays and determination of T cell proliferation.

2.8. Antibodies used for in vivo

Anti-CD4 (clone Gk1.5), anti-CD8a antibody (clone 53-6.72), anti-TGFβ antibody (clone 1D1.16.8) and mouse IgG1 (clone MOPC-21) were purchased from Bio X cell.

2.9. T cell depletion studies in EAE disease models

For EAE prevention studies SJL/J mice were either untreated or treated with anti-$CD4^-$ (100 μg/mouse) and anti-$CD8^-$ (50 μg/mouse) specific antibodies (T cell depletion antibody, once i.p. injection). Some mice were immediately injected intraperitoneally (i.p.) with pPLP or pOVA (25 μg/mouse) or PBS every other day for 16 days. All mice were immunized with pPLP and CFA (day 0). For EAE treatment studies, SJL mice were treated with anti-$CD4^-$ and anti-$CD8^-$ specific antibodies followed by i.p. injection of either 5 μg of pPLP, pOVA or PBS i.p. every other day from day 12 to day 26 before immunization with pPLP (day 0). In C57BL/6 mice, the same T cell depletion regimen was utilized but pMOG (10 μg/mouse) was administered *via* i.p. In some mice, anti-TGFβ or isotype control antibody (mIgG1) (200 μg/mice each day) were injected by i.p. administration on the same day (day 14) and one day after T cell depletion (day 15).

2.10. Statistical analyses

Group comparisons of parametric data were made by Student's *t*-test (unpaired two-tail). We tested data for normality and variance and considered a P value of <0.05 as significant.

3. Results

3.1. T cell depletion and autoantigenic peptide administration prevent EAE

We first tested the hypothesis of inducing tolerance by the apoptosis-antigen combination in a relapsing-remitting EAE model in SJL mice [12,13]. We used anti-CD4 and anti-CD8 depleting antibodies (Del) to induce T cell apoptosis [14,15], which depleted 90% of $CD4^+$ T cells for 3 weeks (Supplementary Fig. 1a). Given macrophages release TGFβ 12–24 h after phagocytosis of apoptotic cells [12], we injected pPLP from day 2 post deletion treatment to facilitate the apoptotic T cell-triggered macrophages to condition the immunosuppressive milieu by releasing TGFβ. We continued to administer the pPLP for 3 weeks to stimulate pPLP-specific T_{reg} cell generation. We then immunized the mice with pPLP and CFA to induce EAE (Fig. 1a, upper panel), with an typical acute phase of disease followed by relapsing-remitting EAE (Fig. 1a, lower panel). Strikingly, depletion of T cells followed by specific autoantigen pPLP administration not only significantly delayed the onset and suppressed the acute EAE, but also prevented relapse and attenuated the chronic EAE (Del/PLP) (Fig. 1a). However, injection of pPLP without T cell depletion exacerbated but not prevented EAE (Supplementary Fig. 1b), and depletion of T cells alone (Del/PBS) did not prevent EAE either (Fig. 1a). Moreover, depletion of T cells in combination with administration of the control peptide (chicken ovalbumin $OVA_{323-339}$) (Del/OVA) also failed to prevent the acute and chronic EAE (Fig. 1a).

Consistent with EAE disease scores, histological analysis of the central nervous system (CNS) revealed that the spinal cords and brains showed substantially less infiltration of inflammatory cells in Del/PLP-treated (tolerized) mice than those in the other groups (Fig. 1b). Immunophenotype analysis of $CD4^+$ T cells in the spinal cords showed that the frequencies of IL-17^+ (Th17) and IFN-γ^+ (Th1) $CD4^+$ T cells were lowest in tolerized mice among all groups (Fig. 1c, d). The frequency of $CD4^+$ $Foxp3^+$ T_{reg} cells in the tolerized mice was substantially higher compared to those with immunization alone, although it was not significantly different from those mice which received depletion treatment (Fig. 1c). Together, the ratio of Th17/T_{reg} cells and Th1/T_{reg} cells was lowest in tolerized mice among all the groups. Analysis of the spinal cords-infiltrated $CD8^+$ T cells revealed a reduction in IFN-γ^+ T cells only in tolerized mice (Fig. 1e). These data indicated an increased ratio of T_{reg} cells to T effector cells and favoring an immunoregulatory status in the CNS in tolerized mice treated with T cell apoptosis and pPLP administration (Del/PLP). *In vitro* re-stimulation of splenic T cells with pPLP showed a significant suppression of pPLP-specific T cell proliferation and IL-17, IFN-γ, and TNF-α production in the tolerized mice compared to untreated mice (Fig. 1f, g). However, $CD4^+$ T cells exhibited comparable T cell proliferation to *Mycobacterium tuberculosis* antigen (MT) among all groups (Supplementary Fig. 1c). Together, these data indicated that apoptotic depletion of T cells *in vivo* followed by pPLP injection induced an antigen-specific immunosuppressive state and prevented the development of EAE.

3.2. T cell depletion and autoantigenic peptide administration treat established EAE

We next utilized SJL mice with established EAE to explore the therapeutic potential of apoptosis-antigen therapy for EAE. We first induced EAE in SJL mice. At the peak of disease, mice were randomly assigned into one of five groups: untreated (PBS), pPLP (PLP) alone, anti-CD4 and anti-CD8- antibodies treated followed by injection of pPLP (αCD4/CD8 + PLP), control pOVA (αCD4/CD8 + OVA), or PBS (αCD4/CD8 + PBS) (Fig. 2a, upper panel). αCD4/CD8 + PLP-treated mice showed significantly lower disease scores and reduced relapse rate in chronic EAE than PBS, pPLP or αCD4/CD8 + OVA-treated mice (Fig. 2a). Unexpectedly, αCD4/CD8 + PBS-treated mice also showed lower disease scores [14,15] (Fig. 2a), which was in contrast to the exacerbation of EAE in the prevention experiments (Fig. 1a). Consistent with the disease score, the spinal cords and brain in αCD4/CD8 + PLP and αCD4/CD8 + PBS treated mice showed less inflammatory $CD4^+$ T cell infiltration (Supplementary Fig. 2a). Analysis of $CD4^+$ T cells in the CNS revealed that the frequency of $Foxp3^+$ T_{reg} cells was increased and the frequencies and numbers of IL-17^+ (Th17), IFN-γ^+ (Th1) or IL-17^+IFN-γ^+ double positive T cells were decreased in the tolerized mice compared to untreated (PBS) or pPLP-treated mice (Fig. 2b and Supplementary Fig. 2b). The decreased ratio of Th1 or Th17 to Treg cells in the CNS of αCD4/CD8 + PLP-treated mice indicated a favorable immunoregulatory status (Supplementary Fig. 2c). In spleens, $Foxp3^+$ T_{reg} cells were increased and Th17 cells were decreased in tolerized mice, yet the frequency of $CD4^+$ T cells and Th1 cells were comparable to untreated mice (Supplementary Fig. 2d–g). *In vitro* pPLP-specific T cell proliferation and inflammatory cytokines production were dramatically suppressed in tolerized mice compared to untreated mice (Fig. 2c, d). However, splenic T cells from PLP or αCD4/CD8 + OVA-treated mice showed no reduction in pPLP-specific inflammatory cytokines production (Fig. 2d). Importantly, the T cells in the tolerized spleens exhibited comparable levels of T cell proliferation to bacterial antigen (MT) or to anti-CD3 in all groups (Supplementary Fig. 2h,i).

We next utilized another EAE model induced by pMOG in C57BL/6 mice to confirm the therapeutic effects of apoptosis-antigen therapy for EAE. Similarly, αCD4/CD8 followed by pMOG injection (Del/MOG)

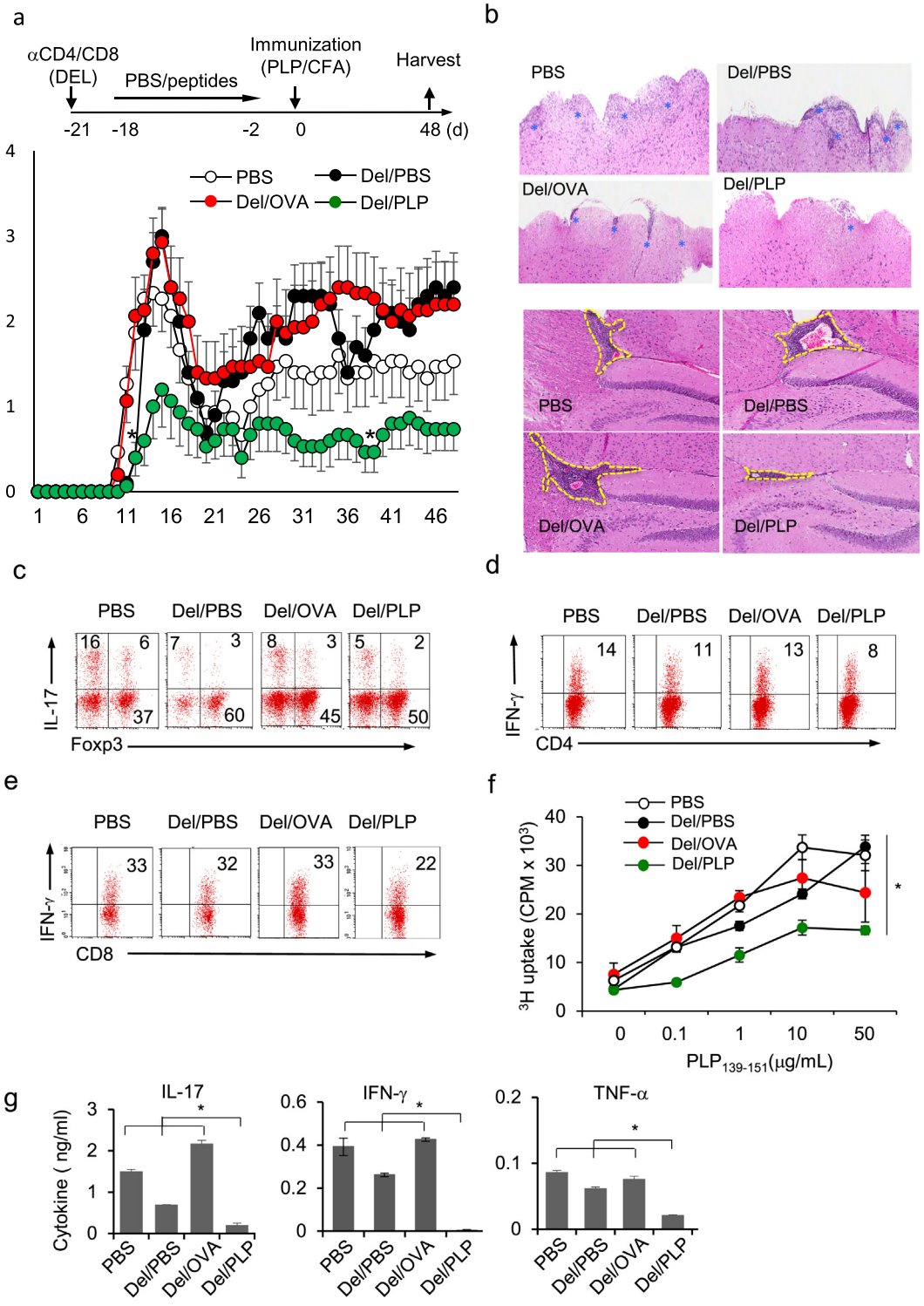

Fig. 1. Preventive effects of apoptosis-antigen treatment in EAE. 7 weeks old female SJL mice were treated with CD4- (Clone; Gk1.5) (100 μg/mouse) and CD8a- (Clone; 53–6.72) (50 μg/mouse) specific antibody (Deletion) 21 days before immunization, followed by pPLP peptide administration (25 μg/every other day for 16 days). Mice were sacrificed at day 48 after immunization. (a) *Upper panel*, the experimental scheme, *Lower panel*, the mean clinical score of EAE in SJL mice (mean ± s.e.m.). PBS (untreated control, $n = 10$ mice), Del/PBS (Deletion plus PBS, n = 10 mice), Del/PLP (Deletion plus pPLP administration, n = 10 mice), Del/OVA (Deletion plus pOVA administration, n = 10 mice). Pooled data of 3 different experiments. (b) Histological analysis of brain and spinal cord. Data are shown as H&E staining of formalin-fixed sections obtained from representative mice from each group. Blue dots or areas surrounded by yellow dashed lines indicate inflammatory infiltrates. (c, d) Flow cytometric analysis of $CD4^+$ IL-17^+, $CD4^+$ IFN-γ^+ and $CD4^+$ $Foxp3^+$ T cells in the spinal cords (cells were pooled in each group). (e) Flow cytometric analysis of $CD8^+$ IFN-γ^+ T cells in the spinal cords (cells were pooled in each group). (f) Splenocytes (pooled from each group) were stimulated by pPLP, and T cell proliferation was assessed by ^{3}H-thymidine incorporation (mean ± s.d. of triplicate measurements). (g) Protein levels of IL-17, IFN-γ, and TNF-α in the supernatants of the cultured splenocytes as described in (f) were measured by ELISA (mean ± s.d. of triplicate measurements). * $P < .05$ (PBS v.s. Del/PLP, Del/PBS v.s. Del/PLP and Del/OVA v.s. Del/PLP) determined by Student's *t*-test. (f-g) Data are representative of the three independent experiments.

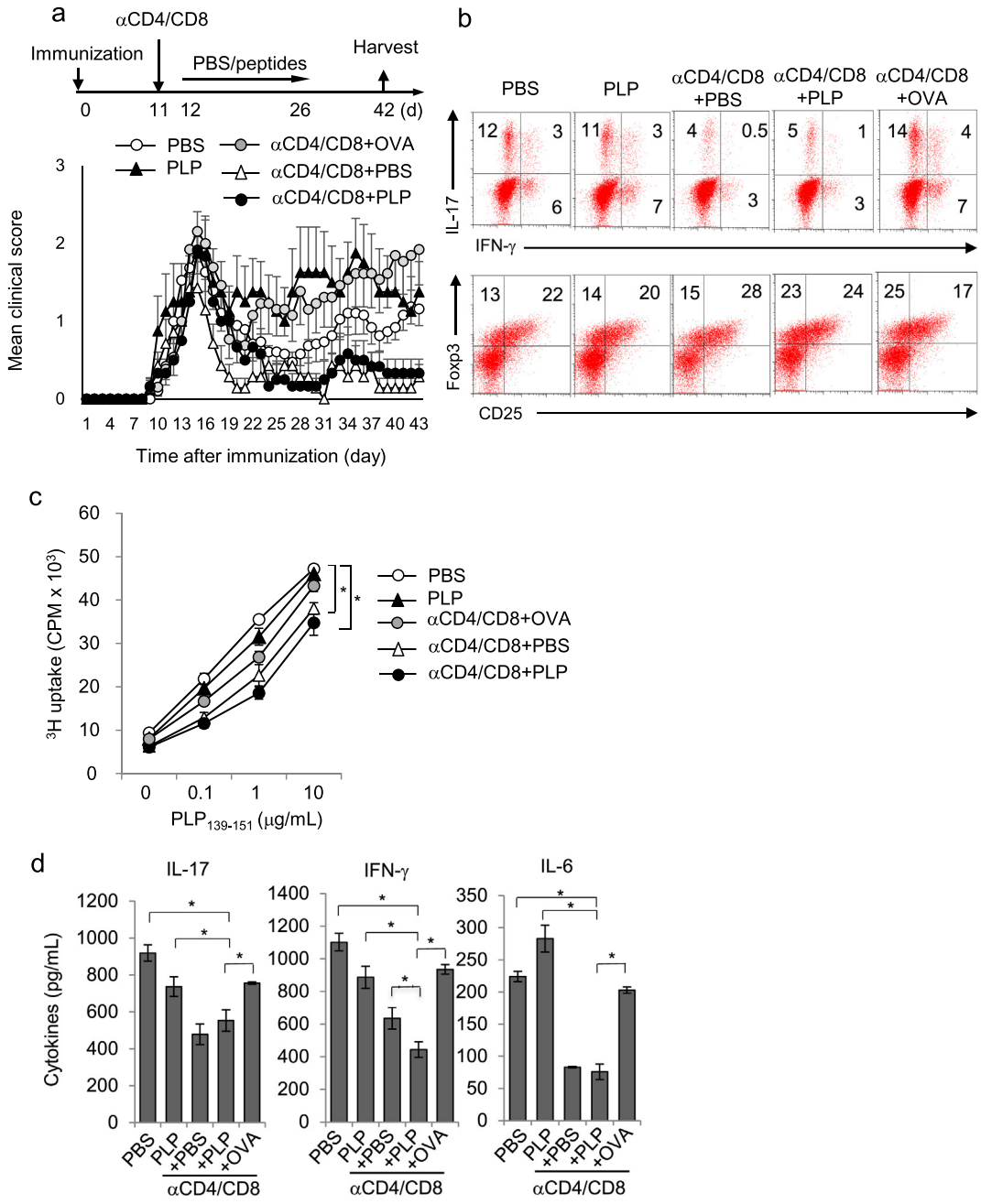

Fig. 2. Therapeutic effects of apoptosis-antigen treatment in EAE. 7 weeks old female SJL mice were immunized with pPLP peptide to induce EAE (day 0). Some mice were left untreated (PBS, $n = 3$) or treated with CD4- (Clone; Gk1.5) (100 μg/mouse) and CD8a- (Clone; 53–6.72) (50 μg/mouse) antibodies at day 11 after immunization with pPLP peptide. Mice treated with T cell depletion antibodies (αCD4/CD8) were either followed by i.p. injection of PBS (αCD4/CD8 + PBS, n = 3) or 5 μg of pOVA (αCD4/CD8 + OVA, n = 3) or 5 μg of pPLP (αCD4/CD8 + PLP, n = 3) every other day from day 12 to 26. Some mice were treated with 5 μg of pPLP every other day from day 12 to 26 alone (PLP, n = 3). (a) *Upper panel*, the experimental scheme; *Lower panel*, Clinical scores of EAE in SJL mice (mean ± s.e.m.,). (b) Flow cytometric analysis of $CD4^+IL\text{-}17^+$ or $CD4^+IFN\gamma^+$ or double-positive $CD4^+IL\text{-}17^+IFN\gamma^+$ (*upper panel*) or $CD4^+CD25^+Foxp3^+$ T_{reg} cells (*lower panel*) in the spinal cords (pooled in each group) at the end of the experiments (day 42). (c) Splenocytes (pooled from each group) were stimulated by pPLP peptide, and T cell proliferation was assessed by ^3H-thymidine incorporation method (mean ± s.d. of triplicate measurements). * $P < .05$ (PBS v.s. αCD4/CD8 + PLP, PBS v.s. αCD4/CD8 + PBS) determined by Student's *t*-test. (d) Protein levels of IL-17, IFN-γ, and IL-6 in the supernatants of the cultured splenocytes in (c) were measured by ELISA (mean ± s.d. of duplicate measurements). * $P < .05$ determined by Student's *t*-test. Data are representative of two experiments (SJL mice).

after the onset of EAE significantly suppressed the ensuing disease (Supplementary Fig. 3a). In spleens, pMOG-specific T cell proliferation and inflammatory cytokines production in tolerized mice were also inhibited compared to untreated mice (Supplementary Fig. 3b, c). Collectively, T cells apoptosis-autoantigen peptide administration, but not T cells apoptosis-foreign peptide administration, showed therapeutic potential in mice with established EAE.

3.3. A critical function of phagocytes in EAE suppression

We next explore the underlying mechanisms of the long-term EAE remission mediated by T cell depletion and self-peptide-mediated therapy. We hypothesized that professional phagocytes such as macrophages, played an essential roles in the tolerance induction by sensing and digesting apoptotic cells [16,17] . First, we pre-depleted phagocytes

with clodronate-loaded liposomes [12] before αCD4/CD8 and pMOG administration in C57BL/6 mice with established EAE. We showed that elimination of phagocytes reversed apoptosis-antigen-induced suppression of EAE (Supplementary Fig. 4a). We next pre-depleted phagocytes with clodronate-loaded liposomes before αCD4/CD8 and pPLP administration (Del/PLP) in unimmunized SJL mice, and then immunized mice with pPLP to induce EAE (Supplementary Fig. 4b, upper panel). Similarly, DEL/PLP + PBS-liposomes-treated (tolerized) mice showed significantly lowered disease scores compared to untreated (PBS) mice. Furthermore, pPLP-specific T cell proliferation and inflammatory cytokines production were significantly decreased in tolerized mice (Supplementary Fig. 4c, d). However, T cell depletion and pPLP administration plus *in vivo* depletion of phagocytes with clodronate-loaded liposomes (Del/PLP + Clodronate-liposome-treated mice) did not induce tolerance (Supplementary Fig. 4b, lower panel). Compatible with high disease activity in DEL/PLP + Clodronate-liposome-treated mice, pPLP-specific T cell proliferation and inflammatory cytokine production in these mice were higher compared to tolerized mice (Supplementary Fig. 4c, d). Collectively, these findings suggested that phagocytes are required for the T cell apoptosis-autoantigen therapy to induce tolerance in EAE.

3.4. TGFβ is key in T cell apoptosis plus autoantigen-mediated therapy of EAE

Since TGFβ is one of the primary cytokines produced by phagocytes upon digestion of apoptotic cells [12,16,17], we next determined the role of TGFβ in apoptosis-antigen-mediated suppression of EAE. We treated EAE mice at the peak of acute EAE with αCD4/CD8 and pMOG in the presence of anti-TGFβ neutralizing antibody (Del + pMOG +αTGFβ) or isotype control antibody (Del + pMOG+Control Ab). (Fig. 3a, upper panel). All mice with T cell depletion and pMOG injection showed rapid remission lasting about a week. However, relapse was observed in the mice treated with anti-TGFβ 10–14 days after the treatment, while the control antibody-treated (tolerized) mice remains in remission (Fig. 3a). We next examined the mRNA levels of TGFβ in the phagocytes (macrophages) after depletion therapy. As expected, mRNA levels of TGFβ1 and TGFβ2 of the peritoneal macrophages were significantly upregulated by T cell depletion therapy (Fig. 3b). Similarly, surface expression of LAP-TGFβ1 on the peritoneal macrophages was upregulated after depletion therapy (Fig. 3c). In spinal cords, the tolerized mice showed the lowest number of infiltrating cells among three groups of mice (Fig. 3d). The increased frequency of $Foxp3^+$ T_{reg} cells in the spleen of tolerized mice were partly reversed by anti-TGFβ injection (Fig. 3e). $MOG_{38–49}$ tetramer positive Treg cells significantly increased in the spinal cord tissues in tolerized mice compared to PBS group, which was decreased in mice treated with anti-TGFβ antibody (Fig. 3f, upper panel). Moreover, in $MOG_{38–49}$ tetramer positive $CD4^+$ T cells, both IFN-γ^+ and IL-17^+ cells were decreased in tolerized mice, but not in anti-TGFβ group (Fig. 3f, lower panel). In cell cultures, anti-TGFβ treatment reversed the pMOG-specific $CD4^+$ T cell proliferation and inflammatory cytokines IFN-γ and IL-17A secretion in tolerized mice (Fig. 3g, h). In contrast, anti-CD3 driven T cell proliferation in tolerized mice exhibited similar to or higher levels than those of untreated (PBS) mice (Fig. 3i). Furthermore, IL-10, another immunoregulatory cytokine produced by phagocytes after digesting apoptotic cell [18] seemed dispensable in the apoptosis-antigen-mediated therapy of EAE. Blockade with anti-IL-10 receptor antibody did not reverse the suppression of EAE in C57BL/6 mice induced by T cell depletion and pMOG injection (data not shown). Thus, these findings indicate that TGFβ but not IL-10 is crucial for the therapeutic effects on EAE by the apoptosis-antigen combination.

3.5. Induction and function of autoantigen-specific T_{reg} cells in tolerized mice

As TGFβ was found to be essential in mediating the therapeutic effects (Fig. 3), and TGFβ is critical for generating $Foxp3^+$ T_{reg} cells *in vitro* [19], we hypothesized that the apoptosis-antigen treatment induced antigen-specific $CD4^+Foxp3^+$ T_{reg} cells. Since $CD4^+CD25^{+-}$ $Foxp3^+$ T cells in SJL mice with established EAE are a pool of Treg cells recognizing many antigens, it was impossible to identify pPLP-specific T_{reg} cells with the markers of CD25 and Foxp3. We therefore developed an *in vitro* system to determine the presence of pPLP-specific T_{reg} cells. We isolated $CD4^+$ T cells as well as their $CD4^+CD25^-$ and $CD4^+CD25^+$ subsets from the spleens of EAE mice after apoptosis-antigen therapy, and examined the antigen-specific T cell proliferation and cytokine production by stimulation with pPLP and splenic antigen-presenting cells (APCs) isolated from the untreated immunized (PBS) mice. As controls, these T cell subpopulations were also stimulated with anti-CD3. The rationale for this approach was based on that the $Foxp3^+$ T_{reg} cell requires specific TCR stimulation to suppress effector T cells [20,21]. If pPLP-specific $Foxp3^+$ Treg cells were generated and served as suppressor T cells in the tolerized mice, a decreased $CD4^+$ T cell responses to pPLP in these mice compare to the responses in the untreated (PBS) mice would be presented. However, if $CD4^+CD25^{+-}$ $Foxp3^+$ T_{reg} cells were removed from $CD4^+$ T cells, the remaining $CD4^+CD25^-$ T cells in the tolerized mice would exhibit similar or higher T cell responses to pPLP. On the other hand, the same $CD4^+$ T cell subpopulations would exhibit no significant alterations of T cell responses to anti-CD3 antibody (non-specific TCR stimulation) compared to untreated control mice. Indeed, in T cell apoptosis-pPLP antigen treatment-mediated EAE prevention study (Fig. 1a), non-separated splenic $CD4^+$ T cells from Del/PLP-treated tolerized mice showed significantly decreased $CD4^+$ T cell proliferation to pPLP, but not to anti-CD3 stimulation (Fig. 4a,b). However, $CD4^+CD25^-$ T cells strikingly regained the proliferation potential to pPLP or anti-CD3 comparable with those from untreated mice (Fig. 4a, b). In contrast, $CD4^+$ T cells from Del/OVA or Del/PBS-treated mice showed no inhibition of pPLP-specific T cell responses, and proliferation of pPLP-specific $CD4^+CD25^-$ T cells was similar with those from untreated mice (Fig. 4a). These findings indicated that $CD4^+CD25^+$ T_{reg} cells inhibited pPLP-specific T cell proliferation only in Del/PLP-treated tolerized mice. Importantly, tolerized mice show similar levels of anti-CD3-driven T cell proliferation, suggesting that T cell depletion-pPLP (Del/PLP) therapy did not compromise overall immunity in preventive model of EAE.

We then investigated pPLP-specific T_{reg} cell generation in apoptosis-antigen-therapy in therapeutic model of EAE (Fig. 2a). Again, autoantigen-specific T_{reg} cells were generated in αCD4/CD8 + PLP or αCD4/CD8 + PBS-treated tolerized mice. pPLP-specific T cell proliferation was attenuated in splenic $CD4^+$ T cells in these tolerized mice, but not in αCD4/CD8 + OVA-treated mice (Fig. 4c). Again, the inhibition was completely restored when the $CD4^+CD25^+$ cells were removed in these tolerized mice (Fig. 4c). pPLP-specific IFN-γ and IL-17 production was also inhibited in splenic $CD4^+$ T cells in αCD4/CD8 + PLP-treated tolerized mice compared to untreated (PBS) control mice, but this inhibition was partially (Fig. 4e) or completely (Fig. 4f) restored to the levels of untreated mice when the $CD4^+CD25^+$ cells were removed in these tolerized mice. Tolerized mice showed similar levels of anti-CD3-driven T cell proliferation as untreated (PBS) mice or αCD4/CD8 + OVA-treated mice (Fig. 4d), suggesting that T cell depletion-pPLP (αCD4/CD8 + PLP) therapy did not compromise overall immunity in treatment model of EAE. Similarly, T cell depletion-pMOG therapy selectively suppressed MOG-specific T cell proliferation and IL-17 production but did not compromise overall immunity in treatment model of pMOG-induced EAE (Fig. 3g, h). Taken together, apoptosis-antigen treatment generated autoantigen-specific Treg cells in therapeutic and preventive models of EAE without compromising overall immunity.

4. Discussion

In this study, we have developed an approach to prevent mice from developing EAE, and more importantly suppress established EAE, by

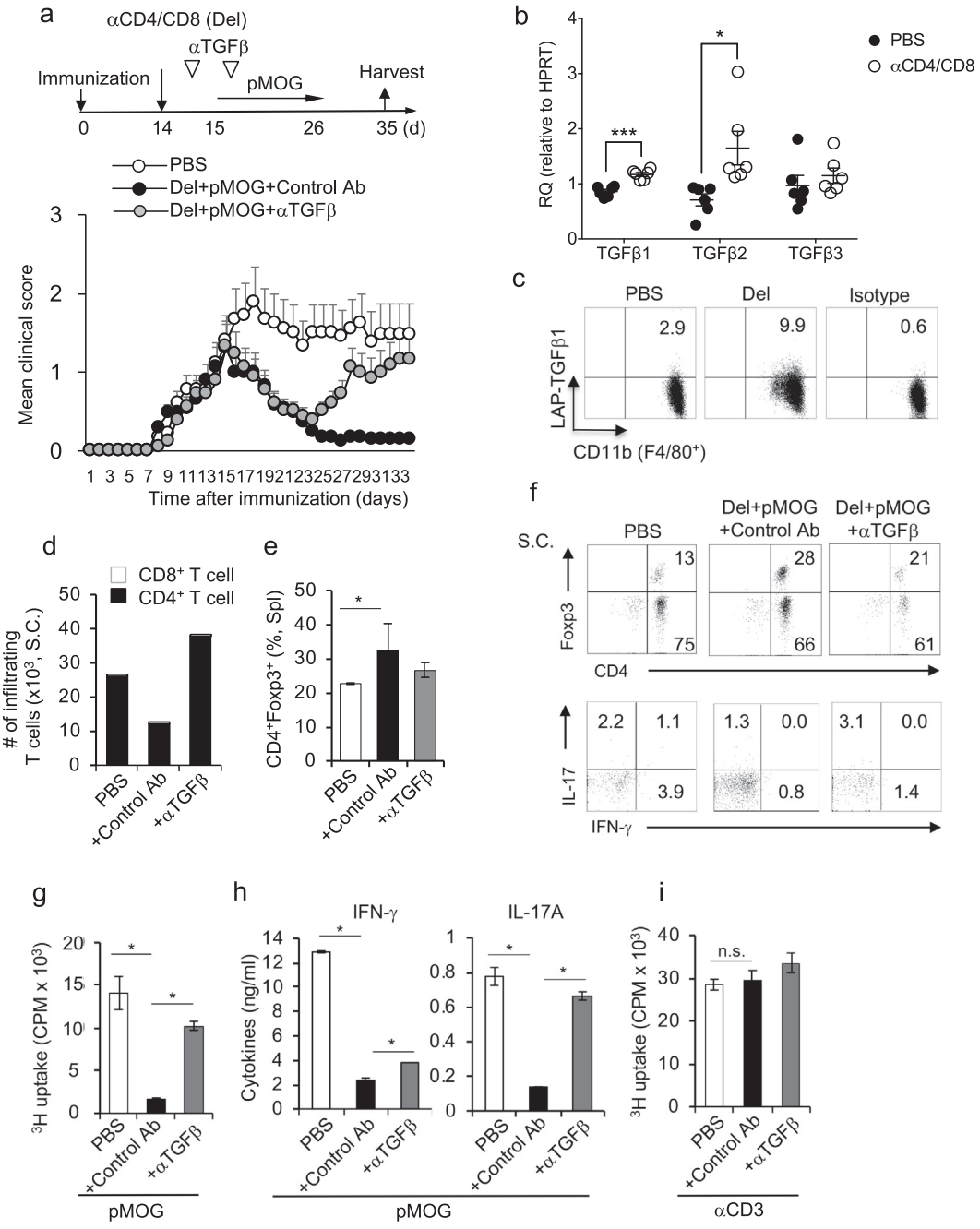

Fig. 3. TGFβ plays a key role in apoptosis-antigen combined therapy of EAE. 7 weeks old male C57BL/6 mice were immunized with pMOG to induce EAE (day 0) and were given CD4- (Clone; Gk1.5) (100 μg/mouse) and CD8a- (Clone; 53–6.72) (50 μg/mouse) antibodies (Del) at day 14 to induce T cell apoptosis. Mice were treated with i.p. injection of 10 μg of pMOG peptide every other day from day 15 to day 26 in the presence of either anti-TGF-β (αTGFβ)(200 μg/day/mouse) or isotype control antibody mouse IgG1 (control Ab) (200 μg/day/mouse) for twice from day 14 to 15 (indicated as invert opened trigons). (a) The clinical mean scores of EAE (mean ± s.e.m.). PBS (untreated control, $n = 8$ mice), Del + pMOG+ αTGFβ (T cell depletion plus pMOG plus anti-TGF-β antibody, n = 8 mice), and Del + pMOG+Control Ab (T cell depletion plus pMOG plus control antibody, n = 8 mice). (b) Gene expression of TGFβ1, TGFβ2 and TGFβ3 in the peritoneal macrophages. Cells were collected from untreated control mice (PBS) and mice receiving depletion therapy (Del) that were sacrificed 24 h after depletion therapy. (c) Protein level of LAP-TGFβ1 in the peritoneal macrophages of mice in the PBS group and Del group. (d) Total number of infiltrating T cells in the spinal cords of indicated groups. (e) The frequency of splenic $Foxp3^+$ T_{reg} cells. (f) The frequency of $MOG_{38–49}$ tetramer positive $Foxp3^+$ Treg cells (upper) or $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ T cell populations (lower) in the spinal cord of indicated group. (g-i) Splenocytes were pooled in each group and $CD4^+$ T cells were purified and stimulated by either pMOG (10 μg/mL) or anti-CD3 (0.5 μg/mL) in the presence of antigen presenting cells. T cell proliferation (g, i) was assessed by ^{3}H-thymidine incorporation (mean ± s.d. of triplicate measurements). The protein levels of pMOG-specific or anti-CD3-driven IFN-γ and IL-17A in the supernatant (h) were measured by ELISA (mean ± s.d. of duplicate measurements). * $P < .05$, *** $P < .001$ determined by Student's *t*-test. Data are representative of five independent experiments.

generating autoantigen-specific T_{reg} cells *in vivo* by combination of induction of T cell apoptosis and administration of autoantigenic peptides.

Our study highlighted several interesting points. *First,* T cell apoptosis is a key to initiating long-term immune tolerance. Our apoptosis process requires transient yet sufficient apoptosis of T cells *in vivo*. Depletion of $CD4^+$ T cells to suppress EAE was reported >20 years ago [1,15], but the underlying mechanisms were incompletely understood. The presumed mechanism was simply the depletion of effector T cells. However, long term-$CD4^+$ T cell depletion with anti-human chimeric CD4 antibody in clinical trials failed to receive significant therapeutic effects on MS patients [2]. Besides a more complex in MS compared with animal models, it is likely that the therapeutic effects on EAE models

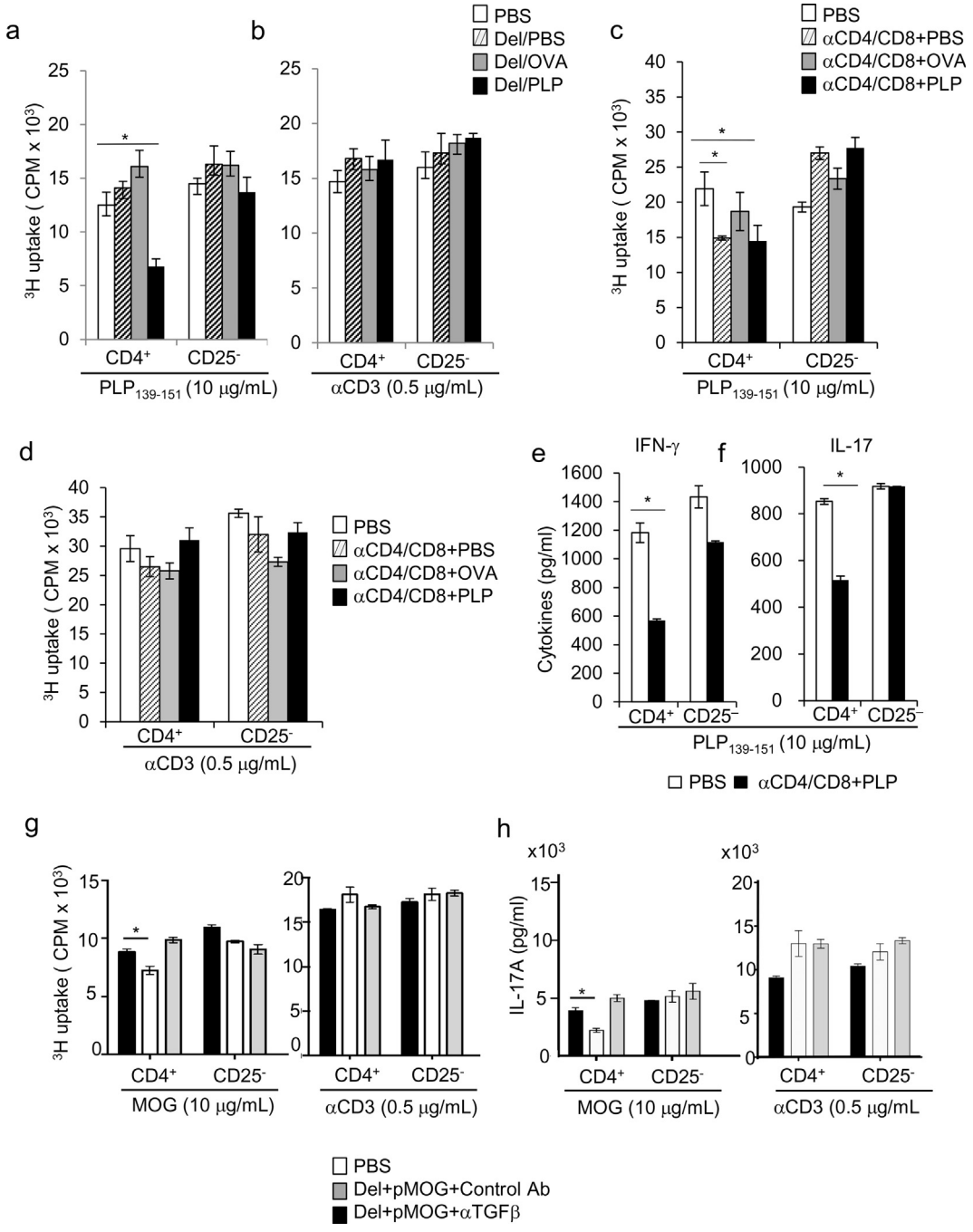

Fig. 4. Generation of antigen-specific CD4$^+$CD25$^+$ T$_{reg}$ cells in mice with long-term remission of EAE induced by apoptosis-antigen treatment. Splenocytes were isolated from the SJL/J mice as shown in Fig. 1a (a, b) and Fig. 2a (c–f). In each experiment, splenocytes were pooled from all of mice in each group and CD4$^+$, CD4$^+$CD25$^-$, and CD4$^+$CD25$^+$ T cells were purified and cultured with irradiated APCs obtained from untreated control (PBS) mice either in the presence of either pPLP peptide or anti-CD3 antibody. (a, b) pPLP-specific (a) or anti-CD3-driven (b) T cell proliferation in response to the different treatments as shown in Fig. 1a was assessed by ^{3}H-thymidine incorporation method (mean ± s.d. of triplicate wells). (c, d) pPLP-specific (c) or anti-CD3-driven (d) T cell proliferation in the indicated groups shown in Fig. 2a was assessed by ^{3}H-thymidine incorporation (mean ± s.d. of triplicate wells). (e, f) The supernatant levels of pPLP-specific IL-17 and IFN-γ of CD4$^+$ T cell and CD4$^+$CD25$^-$ T cell subsets in the indicated groups shown in Fig. 2a were determined by ELISA (mean ± s.d. of duplicate wells). T cell depletion-pMOG therapy selectively suppressed MOG specific cell proliferation (g) and IL-17 production (h), which disappeared in pMOG and anti-TGFβ treatment mice. *$P < .05$, determined by Student's *t*-test. Data are representative of two experiments.

were implicated with the mechanisms beyond the depletion of CD4$^+$ T cells. Our study suggests the depletion of T cells possibly serves as an initiator of a series of events toward long-term immune tolerance. Indeed, long-term immune tolerance was achieved even after T cells were recovered. The mechanisms of apoptosis-triggered tolerance reported here is also different from recent studies using non-depleting CD4-specific antibody [1,15] to block of CD4 molecules. Our study relied on the transient yet sufficient cell apoptosis to initiate the tolerance process, which generated autoantigen-specific T$_{reg}$ cells. It has been reported that antigen pulsed antigen-presenting cells (APCs) chemically fixed with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide could induce antigen-specific tolerance *in vivo*. The underlying mechanism however remains unknown [22]. Previously, we and others have reported that CD3 antibody is able to deplete large numbers of T cells and consequently induce long-term tolerance in experimental autoimmune disease and transplantation [12,23,24], although the clinical trials showed conflicting results [25,26]. However, CD3 antibody-mediated immune tolerance has potential adverse effects. First, it can transiently

yet powerfully engage TCR to promote T cells to release large amounts of proinflammatory cytokines including IFN-γ, TNF-α, and IL-6 *in vivo*, which potentially affect the consequential T cell differentiation even in the TGFβ-enrich environment *in vivo* [27,28]. Second, the CD3 antibody engages all TCR, which might theoretically promote T cells to differentiate into T_{reg} cells or other T cell subsets depending on the cytokine milieu, which might lead to T_{reg} cells lacking antigen specificity and potentially suppress essential T cell response such as anti-viral response. Our approach here overcomes those pitfalls and induce an immunologically quiescent microenvironment. Neither anti-CD4 nor anti-CD8 antibodies activated T cells to produce inflammatory cytokines. Indeed, a TGFβ-rich immunoregulatory milieu (discussed below) without inflammatory cytokines provide a precondition for generation of T_{reg} cells. The tolerance induction and maintenance are dependent on the reprogramming of the immune system, including the generation of antigen-specific T_{reg} cells, but not on the long-term depletion of T cells. This notion was further supported by our recent study in inducing and maintaining similar immune tolerance by depleting B cells or $CD8^+$ T cells without affecting $CD4^+$ T cells or non-discriminatively killing immune cells by single dose of irradiation plus adoptive transfer of exogenous normal macrophages [10].

Macrophages are key in mediating the long-term immune tolerance and therapy of EAE by T cell depletion and peptide combination therapy, which was implicated by experiments of depletion of endogenous phagocytes with clodronate-loaded liposomes [12] in tolerized mice induced by T cell depletion plus self-peptide treatment. It could be accomplished by professional phagocytes such as macrophages clearing apoptotic cells and consequently producing immunosuppressive cytokines that create and mediate the immunoregulatory milieu, which facilitates the generation of T_{reg} cells when the newly naïve T cells encounter specific antigen. Our findings not only help design an effective immunotherapy for autoimmune diseases and transplantation [29], but also provide an explanation for the reported enhanced effects of anti-tumor immunotherapy elicited by a high dose of systemic radiotherapy that may deplete professional phagocytes [30] .

TGFβ is the major player in antigen-specific T_{reg} cell generation. The evidence supporting this conclusion includes the fact that blockade of TGFβ *in vivo* reverses the tolerance effects. Although many cells can produce TGFβ *in vivo*, the macrophages may be the major cell subset [31] in apoptosis-mediated immune tolerance. Our data provide strong evidence that TGFβ is the primary driving force in generating antigen-specific T_{reg} cells that mediate the suppression of EAE and long-term immune tolerance in apoptosis-antigen combination therapy. It has been well recognized that TGFβ is the key cytokine of inducing $Foxp3^+$ T_{reg} cells (iT_{reg} cells) *in vitro*. Here we provide additional evidence that it does the same for iT_{reg} generation *in vivo*.

Apoptosis, phagocytes, and antigenic peptides are all required for the generation of antigen-specific T_{reg} cells *in vivo* in the mice with existing proinflammatory status. Induction of T cell apoptosis and presence of professional phagocytes (immunoregulatory milieu) without timely and adequate autoantigen exposure also fail to generate antigen-specific T_{reg} cells and suppress EAE. The proper autoantigenic peptides need to be introduced in a timely manner in which an immunoregulatory milieu was created by apoptosis-triggered phagocytes. This conclusion may provide an explanation for some published protocols in which administration of low amounts of peptide was able to induce antigen-specific tolerance in naïve mice [32–34], but not in mice or human with established disease [32,35]. In addition, the specificity of the antigenic peptides is also vital in this apoptosis- phagocyte- antigen mediated therapy of EAE. In fact, irrelevant control peptide (OVA) together with depletion antibody failed to suppress EAE. T cell depletion followed by administration of OVA theoretically induce OVA-specific T_{reg} cells, however, these cells may not migrate into CNS because of their antigen specificity, or even may not survive, expand, and suppress diseases in the absence of continuous OVA stimulation. Administration of OVA may also prevent naïve T cells from differentiating into neuron antigens-specific T_{reg} cells. Notably, unlike the prevention models, depletion of T cells in the presence of professional phagocytes (macrophages) without addition of exogenous self-peptides (*e.g.* pPLP or pMOG) in the treatment models (after immunization) did result in some suppression of EAE, although less effective than with exogenous self-peptide. At first glance, it seems that no antigen is needed for the tolerance induction. However, "treatment" models is different from "prevention" models in that there is target autoantigen present (pPLP or pMOG, which is mainly derived from the immunization step) during the tolerance induction phase/window in the former ("therapy"), but not in the later ("prevention") models. This phenomenon might be attributable to the fact that the mice with established EAE likely have some self-peptide present that was derived from the immunization step during the process of T_{reg} cell generation. Therefore, induction of autoantigen-specific T_{regs} still requires depletion antibody (cell apoptosis), phagocytes, and autoantigen. This finding may provide an explanation for the previous observations that long-term depletion of $CD4^+$ T cells alone could reverse the disease in EAE mice after immunization with peptides [1], but showed no significant suppression of human MS [3,4]. The peptides introduced in the immunization in EAE mice might provide sufficient antigens to drive auto-antigen-specific T_{reg} cell differentiation under the TGFβ-enrich environment. In contrast, the quantity of endogenous peptides in MS patients may not be sufficient to induce autoantigen-specific T_{reg} cells under the similar TGFβ-enrich milieu, which is need to be explore to facilitate the translation of these experimental approach to clinical settings.

Lastly and importantly, we discovered that T cell apoptosis-autoantigen combination treatment generated autoantigen-specific T_{reg} cells *in vivo*. These antigen-specific T_{reg} cells are the major force for inducing and maintaining long-term immune tolerance of EAE. In our functional assays, we use the feature that $Foxp3^+$ T_{reg} cells suppress target cells only after TCR stimulation by specific antigen [19,36]. We showed that in the presence of $CD4^+CD25^+$ T_{reg} cells, $CD4^+$ T cells from the tolerized mice showed attenuated proliferation and proinflammatory cytokine production to the target autoantigens. After the T_{regs} are removed, however, the remaining $CD4^+CD25^-$ T cells completely restored the T cell responses to the specific autoantigens. Strikingly, the generation and increase in the CNS autoantigen-specific T_{reg} cells in the EAE-suppressed mice dose not result in nonspecific overall immunosuppression to pan-TCR stimulation or MT antigen, which is consistent with our recent finding by induction of apoptosis of B cells for the treatment of EAE and type I diabetes [10] and might be a favorable approach to translate the findings into clinical practice.

In short, we have successfully generated autoantigen-specific T_{regs} *in vivo* in mice with established EAE and reversed EAE by induction of a transient T cell apoptotic depletion plus injection of autoantigenic peptides. The understanding and optimization of this process will help us "reprogram" the dysregulated immune responses in patients with MS to develop more specific immunotherapy.

5. Caveats and limitations

The conclusion of this study provides a new approach on how to generate autoantigen-specific T_{reg} cells in MS patients, by transient injection of anti-CD4 and anti-CD8 antibody, combined with the administration of disease specific peptides. However, our approach has several limitations. First, although CD4 T cell depletion were well-tolerated in several clinical trials, the safety profile of the combination of CD4 and CD8 cell depletion remains unknown. A controlled study is warranted to establish the safety of this depletion therapy. Second, a spectrum of autoantigens is presented in patients with MS, which is much more complicated than a single peptide induced EAE model. Therefore, a single peptide induced antigen-specific T_{reg} cell might not efficiently suppress MS in clinical settings. The major challenge in human MS is that the disease specific antigen is not clear and may not be a single antigen.

The pool, quantity and timing of peptide(s) administration might need to be optimized before applying this approach in clinical setting.

Although we did not observe a significant reversal of EAE suppression induced by T cell depletion and peptide administration by blockade of IL-10 signaling with anti-IL-10 receptor antibody, we cannot completely exclude the possibility that IL-10 producing T cells may also contribute to this tolerance process, which need to be further investigated. This only suggests a less important role for IL-10 in the generation of T_{reg} cells in this setting.

Author contributions

S.K, D.W, P.Z, designed and performed experiments, analyzed data and contributed to the writing of the manuscript. H.C, P.Z, and D.Z, performed experiments and contributed to the writing of the manuscript. J. L, L.C, T.M, H.N, R.W, and W.J, performed experiments. L.S provided crucial scientific input and designed experiments. W.J.C, conceived and supervised whole research, designed experiments and wrote the manuscript.

Disclosures

A patent application for the reported data is in progress by NIH, NIDCR Intramural Technology Transfer Office, which was filed by W.J.C., S·K, and P.Z. The authors declare no other competing financial interests.

Acknowledgement

This research was supported by the Intramural Research Program of the NIH, NIDCR. S.K and H.N are financially supported in part by JSPS research fellowship for Japanese Biomedical Researchers at NIH. D.Z and R. W are supported in part by Ph.D. Students Scholarship from The State Laboratory of Oral Disease, West China School of Stomatology, Sichuan University, China. The funders had no role in study design, data collection, data analysis, interpretation, or writing of the report.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.05.005.

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