Clinical & Experimental Immunology
The Journal of Translational Immunology
British Society for immunology
Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12756

Regulatory T and B lymphocytes in a spontaneous autoimmune polyneuropathy

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Accepted for publication 14 December 2015
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Summary

B7-2$^{-/-}$ non-obese diabetic (NOD) mice develop a spontaneous autoimmune polyneuropathy (SAP) that mimics the progressive form of chronic inflammatory demyelinating polyradiculoneuropathy (CIDP). In this study, we focused on the role of regulatory T cells (T_{regs}) and regulatory B cells (B_{regs}) in SAP. We found that deletion of B7-2 in female NOD mice led to a lower frequency and number of T_{regs} and B_{regs} in spleens and lymph nodes. T_{regs} but not B_{regs} suppressed antigen-stimulated splenocyte proliferation, whereas B_{regs} inhibited the T helper type 1 (Th1) cytokine response. Both T_{regs} and B_{regs} induced an increase in CD4$^+$interleukin (IL)-10^+ cells, although less effectively in the absence of B7-2. Adoptive transfer studies revealed that T_{regs}, but not B_{regs}, suppressed SAP, while B_{regs} attenuated disease severity when given prior to symptom onset. B cell deficiency in B cell-deficient (muMT)/B7-2$^{-/-}$ NOD mice prevented the development of SAP, which would indicate that the pathogenic role of B cells predominates over its regulatory role in this model. We conclude that B_{regs} and T_{regs} control the immunopathogenesis and progression of SAP in a non-redundant fashion, and that therapies aimed at expansion of B_{regs} and T_{regs} may be an effective approach in autoimmune neuropathies.

Keywords: Bregs, CIDP, co-stimulatory molecules, Guillain–Barré syndrome, Tregs

Introduction

Autoimmunity arises when there is a break in self-tolerance resulting in persistent autoreactive T cells and B cells. Tolerance to self-antigens is generated through two basic mechanisms: (a) elimination of self-reactive cells in thymus during selection and (b) generation of peripheral regulatory lymphocytes to control self-reactive cells that escape the thymus. Included in the complex network of regulatory T cells (T_{regs}) are CD4$^+$ T_{regs} [CD4$^+$CD25$^+$forkhead box protein 3 (FoxP3$^+$)], type 1 regulatory (Tr1) cells [interleukin (IL)-10^+, FoxP3$^-$], T helper type 3 (Th3) cells [transforming growth factor (TGF-β^+)], CD8$^+$ T_{regs}, CD1-restricted natural killer (NK) T cells and T cells with γ/δ receptors [1]. The identification of CD25 and subsequently FoxP3 as markers for CD4$^+$ T_{regs} has greatly facilitated studies of their crucial role in suppressing autoimmune diseases, their mechanisms of action and factors regulating their homeostasis [2–5]. Similar to T_{regs}, a variety of regulatory B cells (B_{regs}) has been described. Tedder and colleagues have identified a subset of IL-10-producing B cells (B10 cells) within the CD1d^{hi} CD5$^+$ B cell population that inhibit contact hypersensitivity responses [6]. B10 cells share certain phenotypical markers with B1-a cells, marginal zone (MZ) B cells and transitional 2-MZ precursor (T2-MZP) B cells, which are also capable of producing IL-10 [7].

CD4$^+$ T_{regs} suppress immune responses by modulation of antigen-presenting cell (APC) maturation and function, production of anti-inflammatory cytokines such as IL-10, TGF-β and IL-35, induction of effector T cell apoptosis and disruption of metabolic pathways [8]. B_{regs} promote T cell differentiation towards a regulatory phenotype instead of Th1 and Th17 cells [9,10]. B10 cells negatively regulate the ability of dendritic cells (DCs) to present antigens as well as suppress Th1 and Th17 responses in animal models of autoimmune diseases [11,12]. Similar to T_{regs}, some B_{regs} express TGF-β and IL-35 in addition to IL-10 [13]. Unlike T_{regs}, a B_{reg}-specific transcription factor has yet to be identified.

Most animal studies of the role of T_{regs} or B_{regs} in central nervous system (CNS) and peripheral nervous system (PNS) inflammation have been carried out in induced

experimental models such as autoimmune encephalomyelitis (EAE) and experimental autoimmune neuritis (EAN) [11,14–16]. The goal of this study was to investigate the role of $CD4^+$ T_{regs} and $CD1d^{hi}$ $CD5^+$ B_{regs} in a spontaneous autoimmune polyneuropathy (SAP) in B7-$2^{-/-}$ non-obese diabetic (NOD) mice. SAP is characterized by progressive weakness, electrophysiological findings of demyelinating features and axonal loss as well as presence of inflammatory cells in sections of sciatic nerves and dorsal root ganglia [17,18]. These findings mimic those of human chronic inflammatory demyelinating polyradiculoneuropathy (CIDP), although the latter can be relapsing–remitting or progressive. SAP is mediated by myelin P0-reactive Th1 cells and at least two epitopes are involved: P0 (180–199) and P0 (1–25) [17,19–21]. More recently, we have demonstrated that absence of B7-2 on DCs leads to impaired capacity to induce tolerance to P0, which can be restored by preconditioning with IL-10. Adoptive transfer (AT) of IL-10-conditioned DCs leads to increased $CD4^+$ T_{regs} and B10 cells *in vivo* [22]. Whether $CD4^+$ T_{regs} and B_{regs}/B10 cells share overlapping or distinct functions in SAP has not been investigated. For subsequent sections, $CD19^+CD1d^{hi}CD5^+$ B cells will be referred to simply as B_{regs}. B10 cells are defined as IL-10^+ B cells detected after 4–5 h exposure to lipopolysaccharide (LPS) and leucocyte activation cocktail as described originally [6]. Here, we provide evidence that B_{regs} and T_{regs} control the immunopathogenesis and progression of SAP in a distinct manner.

Materials and methods

Animals, clinical and electrophysiological assessment

Wild-type (WT) NOD, B7-$2^{-/-}$ NOD, FoxP3-enhanced green fluorescent protein-fused to cre recombinase sequence (eGFP-Cre) NOD mice (Jackson Laboratory, Bar Harbor, ME, USA), and B cell-deficient (muMT) NOD mice (generously provided by Dr A. Chervonsky, The University of Chicago) were housed and bred in pathogen-free conditions in the Animal Barrier Facility. All animal use procedures were conducted in strict accordance with the National Institutes of Health and University of Chicago institutional guidelines. In addition, muMT/B7-$2^{-/-}$ NOD mice were generated by crossing B7-$2^{-/-}$ NOD mice with muMT NOD mice. Female B7-$2^{-/-}$ NOD mice were used in this study unless stated otherwise. For clinical assessment, the following scale was used: 0, normal; 0·5, mild ruffled coat; 1, less active or flaccid tail; 1·5, one leg is curled in when held by tail; 2, mild paraparesis (both legs curled in); 2·5, drags one leg; 3, severe paraparesis (drags both legs); 3·5, severe quadriparesis; and 4, death. Grip strength testing consisted of five separate measurements in each of two trials per session using a grip strength meter (Columbus Instruments, Columbus, OH, USA). Results of two trials were averaged for each mouse per session. After the last grip strength measurement, electrophysiological studies of sciatic nerves were performed as described in our previous publications [22,23]. Latencies, conduction velocities and peak to peak amplitudes were measured. Results from stimulation of bilateral sciatic nerves were averaged for each animal, with '*n*' representing the number of animals in each study group.

In-vitro co-cultures and cell proliferation studies

Splenocyte proliferation was determined using [^{3}H]-thymidine incorporation assay as described previously [20]. Cells were stimulated with P0 (180–199) (20 μg/ml) or P0–ECD (20 μg/ml) for 72 h. On day 3, cultures were pulsed for 16 h with 1 μCi methyl-[^{3}H]-thymidine. The stimulation index was defined by counts per minute (cpm) in the presence of antigen divided by cpm in the absence of antigen. P0 peptide (180–199) and P0-ECD were purchased from Genscript (Piscataway, NJ, USA).

For T_{reg}-$CD4^+$ T cell and B_{reg}-$CD4^+$ T cell co-cultures, T_{regs} ($CD4^+CD25^+$) and B_{regs} ($CD19^+CD1d^{hi}CD5^+$) were sorted from splenocytes and lymph node (LN) cells using a BD fluorescence activated cell sorter (FACS)Aria cell sorter BD Biosciences (San Jose, CA, USA). T_{regs} were added to 5×10^4 effector T cells ($CD4^+CD25^-$ T cells from SAP mice) at varying ratios in the presence of 20 μg/ml P0 (180–189) and irradiated APCs (50,000) for 3 days in RPMI-1640 with 10% serum. B_{regs} were added to SAP $CD4^+CD25^-$ T cells at a 1 : 1 ratio. On day 3, co-cultures were pulsed for 16 h with 1 μCi methyl-[^{3}H]-thymidine for proliferation studies.

For T cell cytokine profile in co-culture studies, T_{regs} and B_{regs} were sorted from splenocytes and LN cells of 2-month-old WT and B7-$2^{-/-}$ NOD mice at 10 days post-immunization with 200 μg P0 (180–199). T_{regs} or B_{regs} were co-cultured with 5×10^4 effector T cells ($CD4^+CD25^-$ T cells from SAP mice) at a 1 : 1 ratio in the presence of P0 (180–199) and irradiated APCs (50,000). For B_{reg}-CD4 co-cultures, LPS (100 ng/ml) was also added. On day 3, leucocyte activation cocktail was added during the last 4 h prior to intracellular cytokine staining for flow cytometry.

Flow cytometry and intracellular cytokine staining

Single-cell suspensions from spleens and LNs were stained at 4°C using predetermined optimal concentrations of antibodies for 30 min. Cells with the forward- and side-scatter properties of lymphocytes were analysed using the Fortessa flow cytometer (BD Bioscience, San Jose, CA, USA). Background staining was assessed using isotype-matched control (Ctrl) antibodies. For intracellular cytokine staining, splenocytes (1×10^6/well) in 96-well plates were stimulated at 37°C in a humidified CO_2 incubator for 4 h with leucocyte activation cocktail (BD Pharmingen, San Jose, CA, USA). This was followed by staining for cell surface CD4 and intracellular interferon (IFN)-γ, IL-17 or IL-10

using the Intracellular Cytokine Staining Starter Kit (BD Pharmingen, San Diego, CA, USA). The percentage of IFN-γ-, IL-17- and IL-10-producing $CD4^+$ T cells was analysed by Fortessa flow cytometer and FlowJo software (TreeStar Inc., Ashland, OH, USA). For the detection of $CD4^+$ T_{regs}, splenocytes were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 and APC-conjugated anti-mouse CD25 antibodies, fixed, permeabilized and subsequently stained with phycoerythrin (PE)-conjugated anti-mouse FoxP3 antobody (eBioscience, San Diego, CA, USA).

With regard to B10 cells, splenocytes were incubated for 4 h in 96-well plates with LPS (10 μg/ml) in addition to leucocyte activation cocktail. Cells were then stained with V450-conjugated anti-mouse CD19 antibody followed by fixation and permeabilization using a Cytofix Kit prior to staining with PE-conjugated anti-mouse IL-10 antibody (BD Biosciences).

AT studies

A BD FACSAria cell sorter was used to sort $CD4^+$ $eGFP^+$ (T_{regs}), $CD4^+eGFP^-$ cells, B_{regs} ($CD19^+CD1d^{hi}CD5^+$) and non-B_{regs} ($CD19^+CD1d^-CD5^-$) from splenocytes and LN cells of 2-month-old *Foxp3-eGFP-Cre* NOD mice immunized with P0 (200 μg) followed by pertussis toxin (500 ng) on days 1 and 3 (killed at day 20). Approximately 1×10^6 sorted cells were injected via tail vein into 6-month-old female B7-$2^{-/-}$ NOD mice for suppression studies and 5-month-old female B7-$2^{-/-}$ NOD mice for prevention studies. Serial clinical assessments, grip strength measurements and electrophysiology were performed as described previously [22,24]. Animals were euthanized at the end of study duration for immunological studies.

Data analysis

Results from clinical severity, immunological studies, grip strength measurements and electrophysiology are expressed as mean ± standard error of the mean (s.e.m.). Statistical significance for these data was determined by analysis of variance (ANOVA) followed by Student's *t*-test and the Bonferroni method for multiple group experiments. Significance levels were set at $P < 0{\cdot}05$.

Results

Altered proportion and function of $CD4^+$ T_{regs} and B_{regs} in B7-$2^{-/-}$ NOD mice

B7-$2^{-/-}$ NOD mice usually start to develop progressive limb weakness ~ 6 months of age. By 8 months, 100% of female B7-$2^{-/-}$ NOD mice would exhibit clinical and electrophysiological findings of SAP [17]. In contrast, the incidence of SAP was lower in male B7-$2^{-/-}$ NOD mice (Fig. 1a,b). Flow cytometry was performed to investigate whether the development of SAP correlates with changes in the frequency/number of $CD4^+$ T_{regs} and B_{regs} in the spleen and peripheral LN (inguinal and axillary). Compared to the female WT NOD mice, there was a decrease in %$CD4^+$ T_{regs} in the spleen and LN of female B7-$2^{-/-}$ NOD mice at 8 months, while a reduction was noted only in the LN, but not in the spleen of male B7-$2^{-/-}$ NOD mice (Fig. 1c). In comparison, the percentage of B_{regs} was decreased in the spleen and LN of both female and male B7-$2^{-/-}$ NOD mice (Fig. 1d). However, the calculated number of T_{regs} and B_{regs} was reduced only in female but not in male B7-$2^{-/-}$ NOD mice due to a slightly higher number of total splenocytes and LN cells from the latter. There was no difference in the % $CD4^+$ T cells or $CD19^+$ B cells among the three groups (data not shown). In subsequent experiments, only female B7-$2^{-/-}$ and WT NOD mice were used.

We examined the ability of $CD4^+$ T_{regs} to suppress T cell proliferation at varying T_{reg}-$CD4^+$ T cell ratios (1 : 20, 1 : 5, 1 : 1). As shown in Fig. 2a, $CD4^+$ T_{regs} from 3-month-old WT and B7-$2^{-/-}$ NOD mice were equally effective in inhibiting the proliferation of effector T cells ($CD4^+CD25^-$ T cells from symptomatic SAP mice) in the presence of 20 μg/ml P0 (190–199). In contrast to T_{regs}, B_{regs} had no effect on T cell proliferation when cultured at a 1 : 1 ratio (Fig. 2b).

To increase the proportion of antigen-specific T_{regs} and B_{regs} for T cell cytokine experiments, T_{regs} and B_{regs} were sorted from splenocytes and LN cells of WT or B7-$2^{-/-}$ NOD mice (2 months) at day 10 post-immunization with P0 (180–199). At a 1 : 1 T_{reg} : CD4 ratio, T_{regs} had no effect on $CD4^+$IFN-γ^+ or $CD4^+$IL-17^+ T cells, but induced an increase in $CD4^+$IL-10^+ T cells, albeit to a lesser extent by B7-$2^{-/-}$ T_{regs} compared to WT T_{regs} (Fig. 2c). In comparison, both WT and B7-$2^{-/-}$ B_{regs} induced a decrease in $CD4^+$IFN-γ^+ T cells and an increase in $CD4^+$IL-10^+ T cells; the latter to a lesser extent by B7-$2^{-/-}$ B_{regs} compared to WT B_{regs}. The percentage of $CD4^+$IL-17^+ cells was not affected by the presence of B_{regs} in co-cultures (Fig. 2d). Thus, T_{regs} and B_{regs} from B7-$2^{-/-}$ NOD mice were less effective than those from WT NOD mice in inducing or maintaining $CD4^+$IL-10^+ T cells.

Efficacy of T_{regs} and B_{regs} at controlling early and late phases of SAP

To investigate the *in-vivo* effects of $CD4^+$ T_{regs}, we utilized *Foxp3-eGFP-Cre* NOD mice as the source of $CD4^+FoxP3^+$ ($eGFP^+$) and $CD4^+FoxP3^-$ ($eGFP^-$) T cells. Splenic $CD4^+eGFP^+$ cells were first confirmed to be > 95% $FoxP3^+$ by flow cytometry (data not shown). *Foxp3-eGFP-Cre* NOD mice immunized with P0 (200 μg/ml) were used as donor mice, which exhibited mild weakness with a clinical score of 1·5 ± 0·15 (n = 15). At day 20 post-immunization, animals were euthanized for sorting of T_{regs} and B_{regs} from splenocytes and LN cells. We compared the

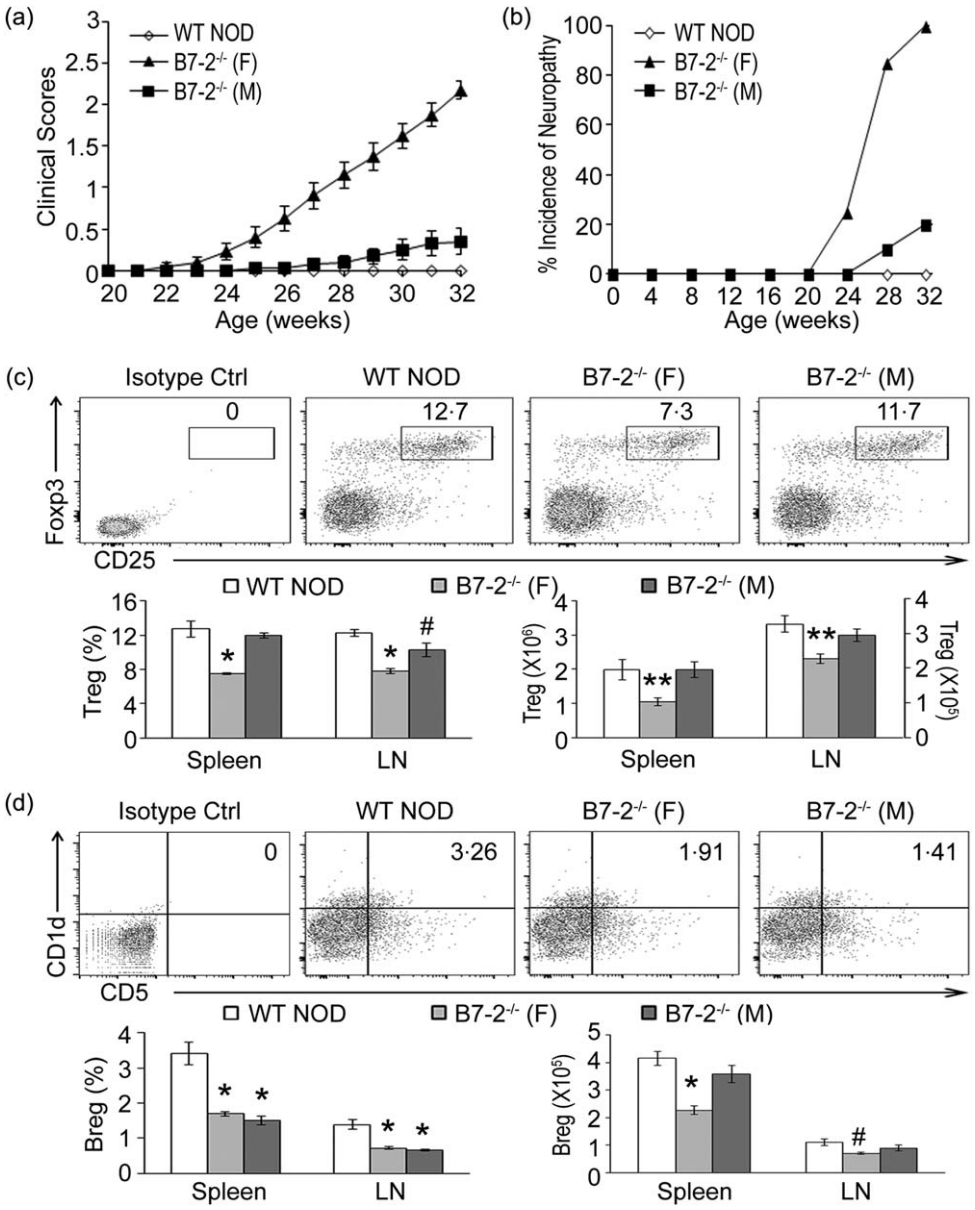

Fig. 1. Lower frequency and number of regulatory T cells (T_{regs}) and regulatory B cells (B_{regs}) in female B7-2$^{-/-}$ non-obese diabetic (NOD) mice compared to wild-type (WT) NOD mice. (a,b) Clinical severity and incidence of spontaneous autoimmune polyneuropathy (SAP) in female *versus* male B7-2$^{-/-}$ NOD mice ($n = 20$ for each group). (c). Examples of scatterplots showing decreased percentage of splenic CD4$^+$ T_{reg} [CD25$^+$forkhead box protein 3 (FoxP3)$^+$/CD4$^+$ T cells] in 8-month-old female B7-2$^{-/-}$ NOD mice but not in male B7-2$^{-/-}$ NOD mice. Total splenocytes or lymph node (LN) cells were gated on lymphocytes, followed by gating on CD4, and subsequent analysis for CD25 and FoxP3 expression. Lower panel: bar graphs indicate mean ± standard error of the mean (s.e.m.) percentage and number of CD4$^+$ T_{regs} in spleen and LN. *$P < 0{\cdot}0005$, #$P < 0{\cdot}03$; **$P < 0{\cdot}01$ ($n = 6$). (d) Examples of scatterplots showing decreased percentage of splenic B_{regs} (CD1d^{hi}CD5$^{+/}$CD19$^+$ B cells) in both female and male B7-2$^{-/-}$ NOD mice (8 months). Total splenocytes or LN cells were gated on lymphocytes, followed by gating on CD19 and subsequent analysis of CD1d and CD5 expression. Lower panel: bar graphs indicate mean ± s.e.m. percentage and number of B_{regs} in spleen and LN. *$P < 0{\cdot}002$; #$P < 0{\cdot}02$ ($n = 8$–9 for %B_{reg}, and $n = 3$–4 for B_{reg} number).

capacity of WT NOD T_{regs} and B_{regs} (1×10^6) to suppress SAP in AT experiments. Recipient female B7-2$^{-/-}$ NOD mice (6 months old) were divided into three groups: group A received PBS injections (no AT); group B received T_{regs} (CD4$^+$eGFP$^+$) designated as T_{reg} (AT); and group C received CD4$^+$eGFP$^-$ cells, designated as CD4$^+$eGFP$^-$ (AT). Clinical assessment (clinical score and grip strength measurements) revealed that the progression of SAP was halted by AT of T_{regs}, but not by CD4$^+$eGFP$^-$ T cells (Fig. 3a). Clinical stabilization was confirmed by

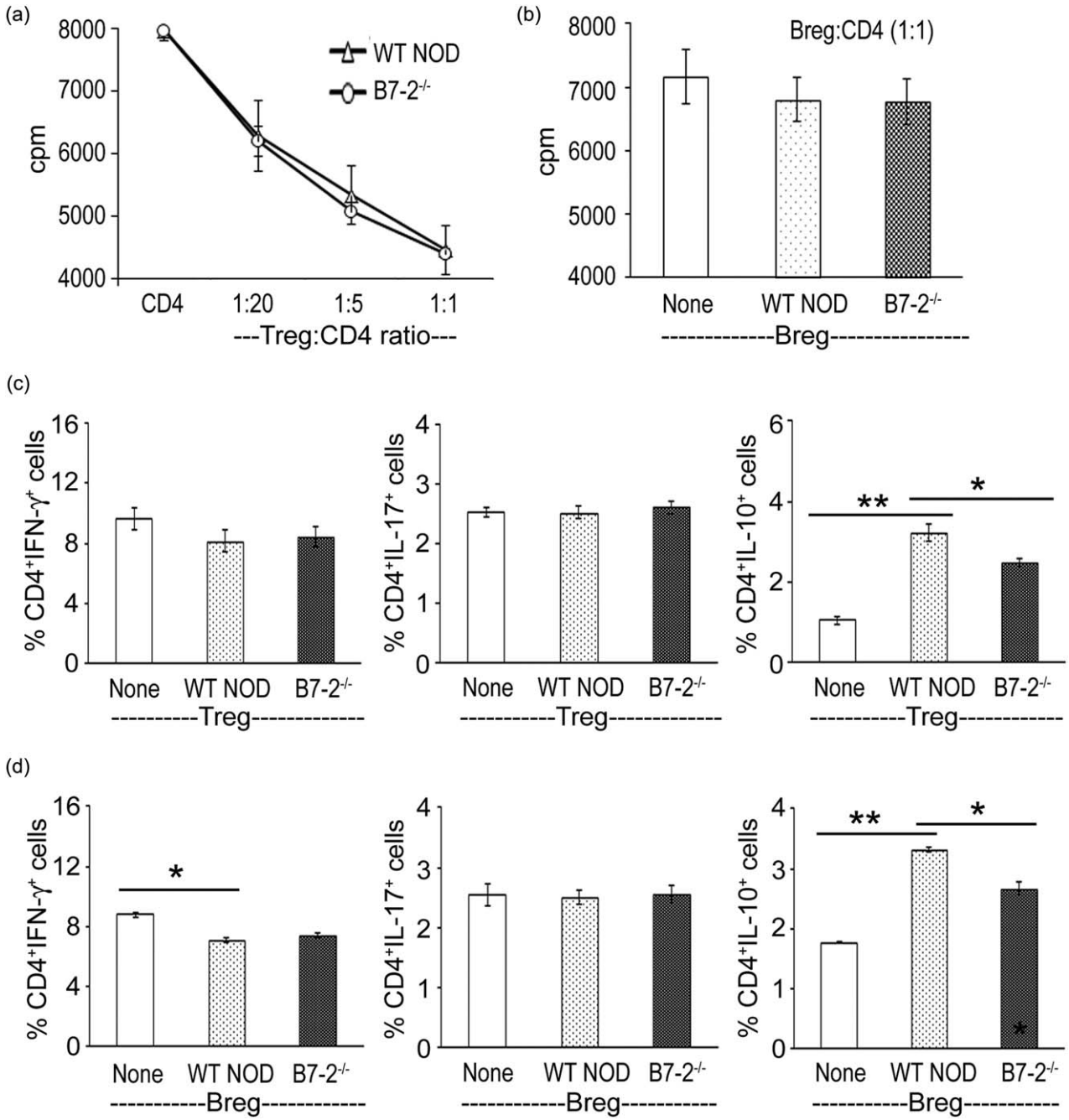

Fig. 2. Effect of $CD4^+$ regulatory T cells (T_{regs}) and regulatory B cells (B_{regs}) on T cell function *in vitro*. (a) Wild-type (WT) and B7-$2^{-/-}$ T_{regs} suppressed T cell proliferation with similar efficacy *in vitro*. $CD4^+CD25^+$ T_{regs} from 3-month-old female mice were co-cultured with $CD4^+CD25^-$ T cells from spontaneous autoimmune polyneuropathy (SAP) mice (8 months) in the presence of 20 μg/ml P0 (180–189) and irradiated antigen-presenting cells (APCs) (50 000) for 3 days. Proliferation was measured by $[^3H]$-thymidine incorporation ($n = 3$). (b) WT and B7-$2^{-/-}$ B_{regs} ($CD19^+CD1d^{hi}$ $CD5^+$) did not suppress the proliferation of $CD4^+CD25^-$ T cells at a 1 : 1 ratio ($n = 3$). (c) WT and B7-$2^{-/-}$ T_{regs} had no effect on $CD4^+$interferon (IFN)-γ or $CD4^+$interleukin (IL)-17^+ cells, but increased the percentage of $CD4^+IL\text{-}10^+$ T cells in co-cultures. Experimental conditions were the same as in (a), except that T_{regs} were obtained from WT or B7-$2^{-/-}$ NOD mice killed at 10 days post-immunization with P0 (180–199). T_{reg} : CD4 ratio was 1 : 1. $^*P < 0{\cdot}02$; $^{**}P < 0{\cdot}0005$ ($n = 3$). (d) WT and B7-$2^{-/-}$ B_{regs} induced a decrease in $CD4^+IFN\text{-}\gamma^+$ T cells and increase in $CD4^+IL\text{-}10^+$ T cells in co-cultures. B_{regs} sorted from immunized animals were co-cultured with $CD4^+CD25^-$ T cells at a : 1 ratio for 3 days in the presence of P0 (180–199), lipopolysaccharide (LPS) (100 ng/ml) and irradiated antigen-presenting cells (APCs) (50 000). $^*P < 0{\cdot}002$; $^{**}P < 0{\cdot}0001$ ($n = 3$).

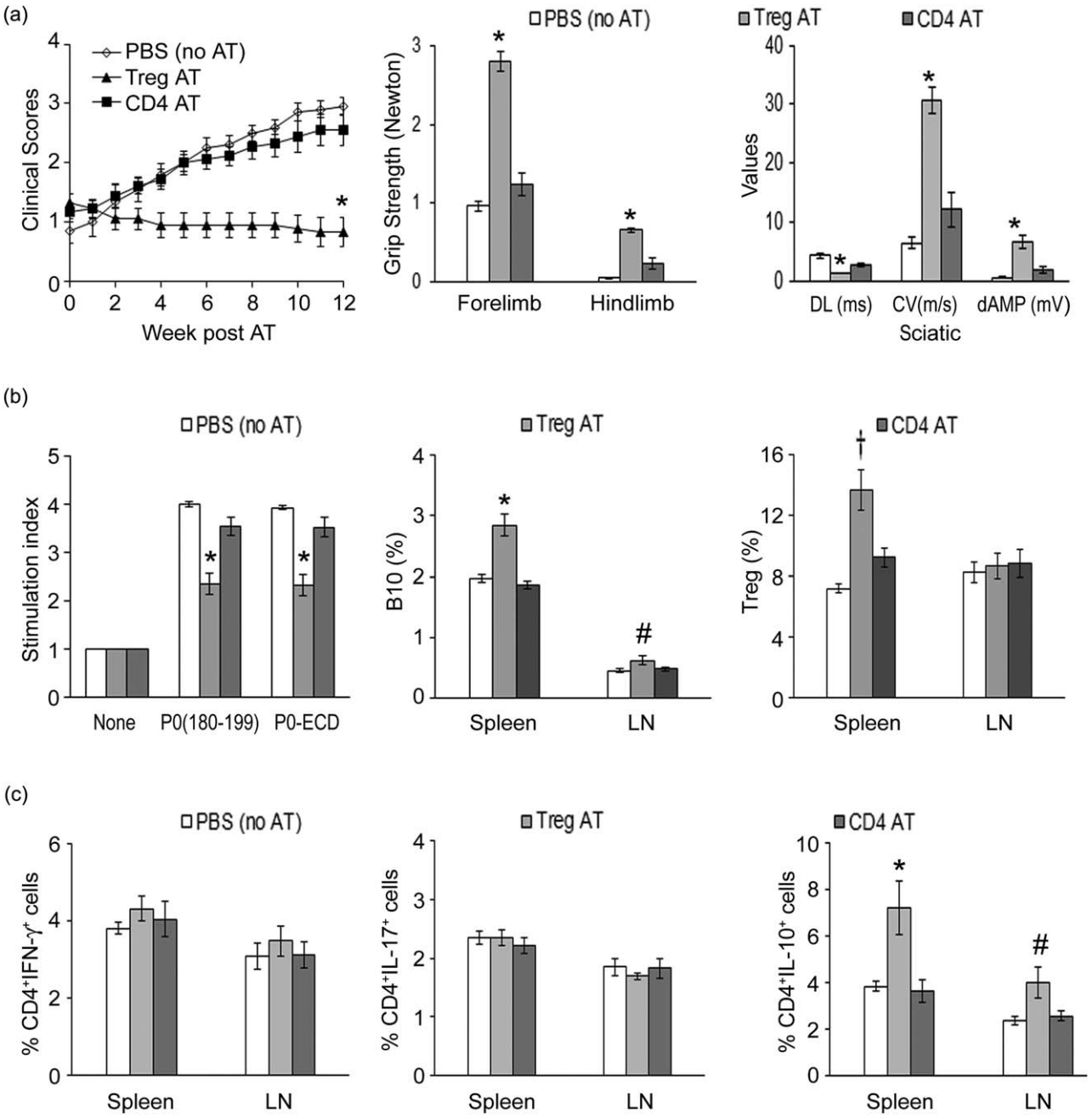

Fig. 3. Suppression of spontaneous autoimmune polyneuropathy (SAP) by adoptive transfer (AT) of wild-type $CD4^+$ regulatory T cells (T_{reg}) into symptomatic B7-$2^{-/-}$ non-obese diabetic mice (NOD) mice. (a) Clinical severity. $CD4^+$ enhanced green fluorescent protein ($eGFP^+$) (T_{reg}) and $CD4^+eGFP^-$ cells were sorted from splenocytes and LN cells of 2-month-old forkhead box protein 3 (FoxP3)-eGFP-Cre NOD mice at day 20 post-immunization with P0 (200 μg). Recipient mice (6-month-old female B7-$2^{-/-}$ NOD mice) were injected with 1×10^6 sorted cells via tail vein. Comparing T_{reg} (AT) *versus* $CD4^+eGFP^-$ (AT) or phosphate-buffered saline (PBS) (no AT), $^*P < 0{\cdot}0002$ (n = 9-10) for clinical scores (left panel), grip strength measurements (middle panel) and all parameters of sciatic motor responses (right panel). DL = distal latency; CV = conduction velocity; dAMP = distal amplitude. Grip strength measurements and sciatic nerve electrophysiology were performed at 12 weeks post-AT. (b) Splenocyte proliferation, B10 cells and T_{regs} at 12 weeks post-AT. Left panel: splenocyte proliferation based on [^{3}H]-thymidine incorporation. (Antigen): 20 μg/ml; treatment duration: 72 h. $^*P < 0{\cdot}0005$ (n = 9–10) comparing T_{reg} (AT) *versus* the other two groups. Middle panel: B10 cells. $^*P < 0{\cdot}0005$ for splenic B10 cells; $\#P < 0{\cdot}05$ for LN B10 cells (n = 6). Right panel: T_{regs}. $\dagger P < 0{\cdot}008$ (n = 6) for splenic T_{regs}. (c) T cell cytokine profile at 12 weeks post-AT. Comparing T_{reg} (AT) *versus* the other two groups, $^*P < 0{\cdot}01$ for splenic $CD4^+IL\text{-}10^+$ cells and # $P < 0{\cdot}04$ for LN $CD4^+IL\text{-}10^+$ cells (n = 6).

electrophysiological studies showing dramatic improvement in distal latencies, conduction velocities and distal amplitudes of sciatic motor response (Fig. 3a). In contrast to T_{regs}, AT of B_{regs} did not suppress disease in recipient SAP mice ($n = 5$) (Supporting information, Fig. S1).

Animals from AT experiments were euthanized at 12 weeks post-transfer (~9 months of age) for immunological studies. Splenocyte proliferation-induced by 20 μg/ml P0 (180–199) or P0-ECD was decreased in T_{reg} (AT) group compared to the other two groups. This was accompanied by increased B10 cells ($CD19^+IL10^+$) in the spleen and LN, and increased T_{regs} in the spleen of animals in T_{reg} (AT) group (Fig. 3b). There was no difference in the percentage of $CD4^+$IFN-γ^+ T cells or $CD4^+$IL-17^+ T cells among these three groups. However, there was an increase in $CD4^+$IL-10^+ T cells in the spleen and LN from the T_{reg} (AT) group (Fig. 3c).

Given that B_{regs} did not suppress SAP when AT was performed after disease onset, we examined whether transfer of these cells before the onset of symptoms would prevent the development of SAP or attenuate disease severity. Recipient B7-$2^{-/-}$ NOD mice (5 months old) were divided into three groups: group A received PBS injections (no AT); group B received B_{regs} ($CD19^+CD1d^{hi}CD5^+$), designated as B_{reg} (AT); and group C received $CD19^+$ $CD1d^-CD5^-$ cells, designated as non-B_{reg} (AT). As shown in Fig. 4a, AT of B_{regs} at 5 months could attenuate the disease severity based on clinical scores, grip strength measurements and electrophysiological parameters. In the B_{reg} AT group, there was attenuated antigen-specific splenocyte proliferation, increased percentages of both B10 cells and T_{regs} in the spleen and LN of recipient mice. There was no difference in the percentage of $CD4^+$IFN-γ^+ T cells or $CD4^+$IL-17^+ T cells among these three groups. However, there was an increase in $CD4^+$IL-10^+ T cells in the spleen and LN of B_{reg} (AT) group, but not in the other two groups.

To determine whether a positive regulatory or a negative regulatory role of B cells predominates in SAP, we compared the severity of neuropathy in B7-$2^{-/-}$ *versus* muMT/B7-$2^{-/-}$ NOD mice. Homozygous muMT mice lack mature B cells due to disruption of the gene encoding the heavy chain immunoglobulin (Ig)M [25]. Figure 5a confirms the absence of B cells ($CD19^+$) and IgM in muMT/B7-$2^{-/-}$ NOD mice. The latter exhibited a slightly higher frequency of $CD4^+$ T_{regs} in the spleen and LN compared to B7-$2^{-/-}$ NOD mice (Fig. 5b). $CD4^+$ T_{regs} from muMT/B7-$2^{-/-}$ mice were as effective as those from B7-$2^{-/-}$ mice in suppressing the proliferation of effector T cells from SAP mice (Fig. 5c). In addition, they were slightly more potent in shifting the cytokine profile from Th1 to $CD4^+IL10^+$ cells in co-cultures (Fig. 5d). As depicted in Fig. 5e, the lack of mature B cells prevented the development of SAP. Electrophysiological studies showed markedly improved conduction parameters, although a mild slowing of conduction velocity (CV) was still observed (Fig. 5f). These findings suggest that the pathogenic role of B cells predominates over its negative regulatory role in SAP, and that lack of B cells did not inhibit the generation or function of T_{regs}.

Discussion

B7-$2^{-/-}$ NOD mice exhibit enhanced autoreactivity to myelin P0 starting at 4 months, yet they do not develop weakness till ~6 months of age. The delayed onset suggests that the initial expansion of P0-reactive T cells is kept in check by regulatory mechanisms. The high incidence of SAP in female B7-$2^{-/-}$ NOD mice is associated with decreased frequency and number of T_{regs} and B_{regs} in the spleen and LN. In male B7-$2^{-/-}$ NOD mice, the number of T_{regs} and B_{regs} was not reduced significantly, as might have been expected from altered frequency of B_{regs} and LN T_{regs}. There is some evidence that both sex hormones and microbiota contribute to gender bias in autoimmunity in NOD mice [26]. Testosterone treatment induces expansion of T_{regs} and modulates FoxP3 expression [27]. Depletion of T_{regs} with anti-CD25 antibody in male B10.S mice prior to immunization with PLP139-151 results in increased susceptibility to EAE [28]. Earlier studies suggest that androgens can also induce immune deviation from a Th1 to a Th2 phenotype [29,30].

We found that $CD4^+$ T_{regs} but not B_{regs} suppressed T cell proliferation induced by myelin P0 (180–199). Conversely, B_{regs} were more effective than T_{regs} in attenuating the Th1 cytokine response, while both of them can induce an increase in $CD4^+$IL-10^+cells in co-cultures. *In-vitro* data indicate that the absence of B7-2 did not affect function of T_{regs} or B_{regs} in most assays except for diminished capacity to induce $CD4^+$IL-10^+ cells. IL-10 production in T cells is not limited to T_{regs}, Th2 cells and Tr1 cells, as Th1 and Th17 cells may produce IL-10 under certain circumstances as part of a negative feedback regulation of $CD4^+$ effector response [31]. Our findings on T_{regs} in B7-$2^{-/-}$ NOD mice are in agreement with those reported by other investigators [32]. It is well-recognized that the B7-1/B7-2:CD28/CTLA4 pathway plays a crucial role not only for optimal T cell activation but also for the homeostasis of T_{regs} [33,34]. B7-1 and B7-2 contribute equally to the development of T_{regs} in the thymus, whereas B7-2 appears more important than B7-1 in regulating the peripheral homeostasis of T_{regs} [35]. Whether B7 molecules are required for the generation of B_{regs} has not been as well investigated. One study reported that addition of B7-1/B7-2 blocking antibodies decreases the number of IL-10 producing B cells in co-cultures with primed myelin antigen-specific T cells lines, although the effect is not as robust as blocking the inducible co-stimulatory–inducible co-stimulatory ligand (ICOS–ICOSL) pathway [36]. Our finding that the frequency and number of B_{regs} is lower in the spleen and LN of female B7-$2^{-/-}$ NOD mice compared to WT mice suggests that B7-2 is important for the generation or maintenance of B_{regs}, although the exact mechanism remains to be determined.

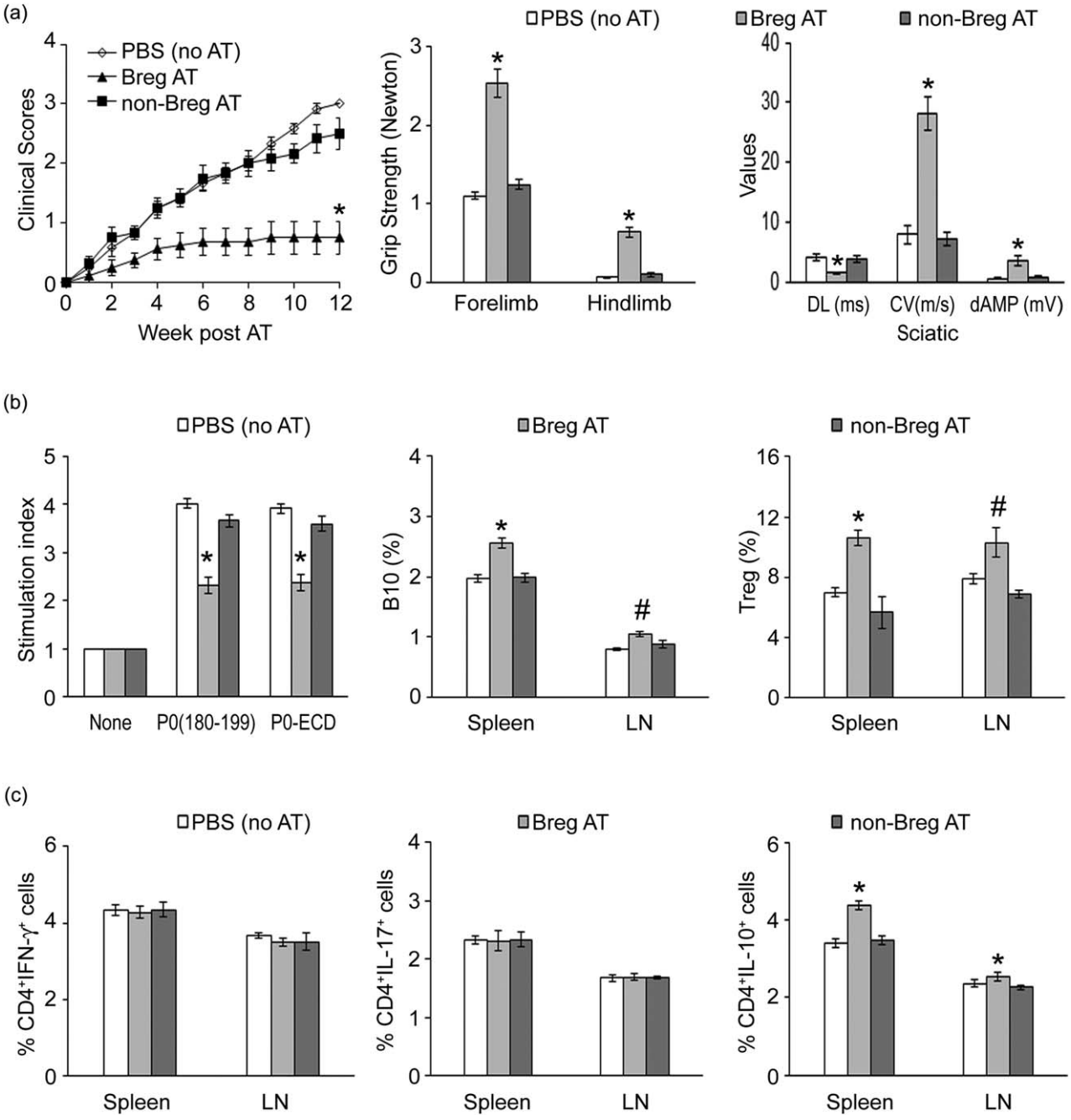

Fig. 4. Prevention of spontaneous autoimmune polyneuropathy (SAP) by adoptive transfer (AT) of wild-type regulatory B cells (B_{reg}) into asymptomatic B7-$2^{-/-}$ non-obese diabetic (NOD) mice. (a) Clinical severity. B_{regs} ($CD19^+CD1d^{hi}CD5^+$) and non-B_{regs} ($CD19^+CD1d^-CD5^-$) were sorted from splenocytes and lymph node (LN) cells of 2-month-old WT NOD mice at day 20 post-immunization with P0 (200 μg), and 1×10^6 sorted cells were injected via tail vein into 5-month-old female B7-$2^{-/-}$ NOD mice. Comparing B_{reg} (AT) *versus* non-B_{reg} (AT) or phosphate-buffered saline (PBS) (no AT), $^*P < 0{\cdot}0005$ ($n = 6$–8) for clinical scores (left panel), grip strength measurements (middle panel) and all the parameters of sciatic motor responses. (b) Splenocyte proliferation, B10 cells and regulatory T cells (T_{regs}) at 12 weeks post-AT. Left panel: splenocyte proliferation based on [^{3}H]-thymidine incorporation. [Ag]: 20 μg/ml; treatment duration: 72 h. $^*P < 0{\cdot}0002$ ($n = 6$–8). Middle panel: B10 cells. $^*P < 0{\cdot}0002$ for splenic B10 cells; $\#P < 0{\cdot}03$ for LN B10 cells ($n = 6$). Right panel: T_{regs}. $^*P < 0{\cdot}0002$ for splenic T_{regs}, $\#P < 0{\cdot}03$ for LN T_{reg} cells ($n = 6$). (c) T cell cytokine profile at 12 weeks post-AT. $^*P < 0{\cdot}0001$ ($n = 6$) for spleen and LN $CD4^+IL\text{-}10^+$ cells.

We found that SAP can be suppressed by AT of $CD4^+$ T_{regs} from mice immunized with P0. This therapeutic effect was associated with decreased antigen-specific splenocyte proliferation and with increased frequencies of B10 cells, $CD4^+IL\text{-}10^+$ cells and splenic $CD4^+$ T_{regs}. There were no changes in the frequencies of Th1 or Th17 cells in recipient

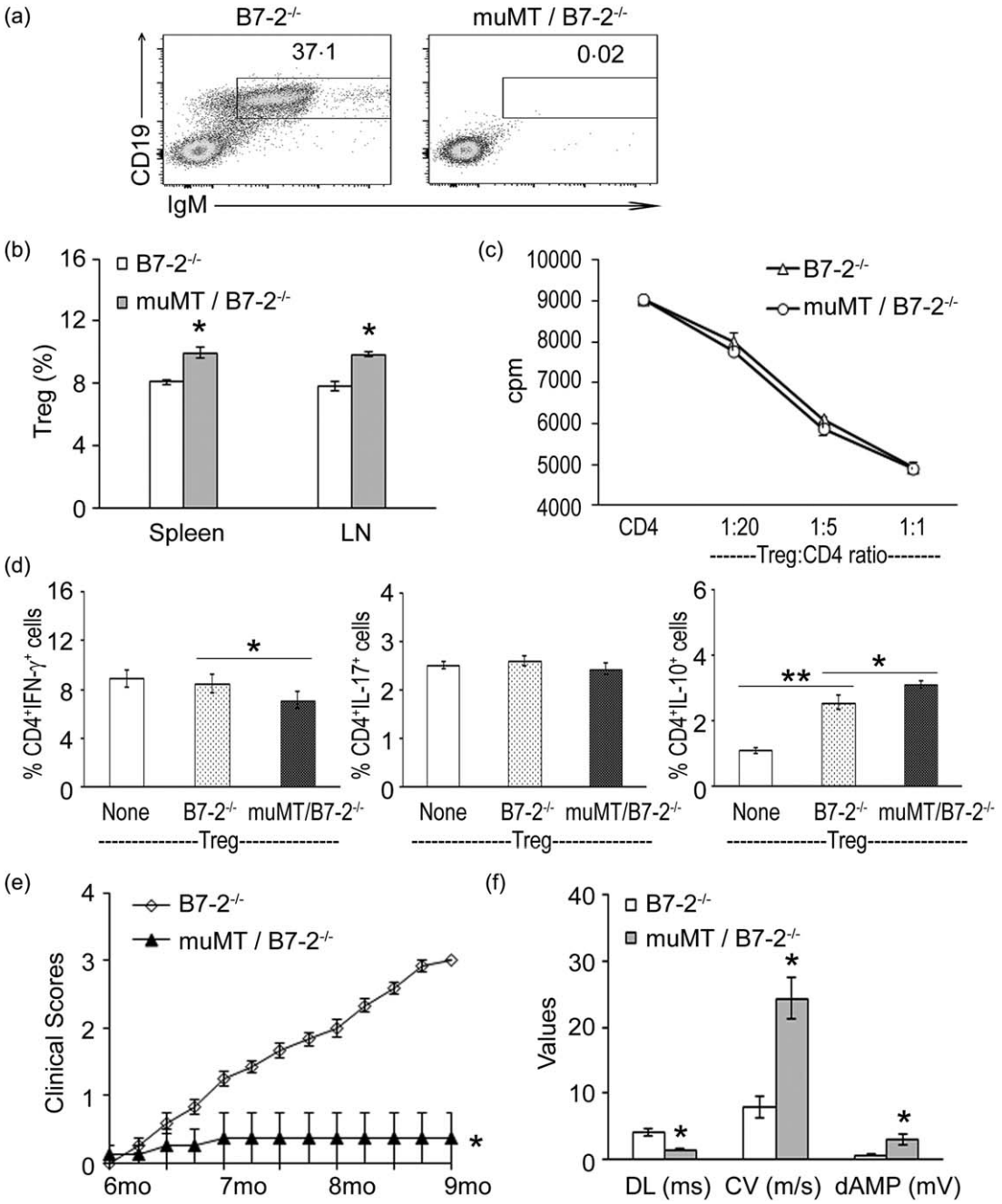

Fig. 5. Absence of B cells inhibits the development of spontaneous autoimmune polyneuropathy (SAP). (a) Examples of scatterplots confirming the lack of B cells [$CD19^+$immunoglobulin (Ig)M^+] in B cell-deficient (muMT)/B7-$2^{-/-}$ non-obese diabetic (NOD) mice. (b) Modest increase in the frequency of T_{regs} in muMT/B7-$2^{-/-}$ mice compared to that from B7-$2^{-/-}$ NOD mice. *$P < 0{\cdot}001$ ($n = 3$). (c) T_{regs} from B7-$2^{-/-}$ and muMT/B7-$2^{-/-}$ mice suppressed T cell proliferation with similar efficacy *in vitro* ($n = 3$). Experimental conditions were as described in Fig. 2a. (d) T_{regs} from muMT/B7-$2^{-/-}$ mice decreased the percentage of $CD4^+$interferon (IFN)-γ T cells, and led to a greater increase in the percentage of $CD4^+$interleukin (IL)-10^+ T cells in co-cultures when compared to T_{regs} from B7-$2^{-/-}$ mice. *$P < 0{\cdot}02$ and **$P < 0{\cdot}002$ ($n = 3$). Experimental conditions were as described in Fig. 2c. (e) Clinical severity.*$P < 0{\cdot}00001$ for B7-$2^{-/-}$ *versus* muMT/B7-$2^{-/-}$. (f). Sciatic nerve electrophysiology. *$P < 0{\cdot}003$ for all parameters comparing B7-2 KO *versus* muMT ($n = 5$–6).

mice. The role of T_{regs} has been demonstrated in EAE and EAN, where recovery correlates with the accumulation of T_{regs} within the CNS and sciatic nerves, respectively [15,37]. Depletion of $CD25^+$ T_{regs} inhibits the recovery, while AT of T_{regs} attenuates the disease severity and promotes recovery in EAE, which is IL-10-dependent [14,37–39]. Early depletion but not late depletion of T_{regs} with anti-CD25 antibodies leads to deterioration of EAN in

SJL mice and adoptive transferred neuritis [16]. That progression of SAP can be halted by transfer of T_{regs} after disease onset in our study would support the concept that the number of T_{regs} could determine whether inflammatory neuropathy would become chronic/progressive or monophasic with recovery. Reduced levels or impaired function of T_{regs} have been reported in Guillain-Barré syndrome (GBS) and CIDP, with improvement upon treatment with intravenous gammaglobulin noted in one GBS study [40–43].

Whether there is altered number or function of B_{regs} in GBS, CIDP or EAN has not been studied systematically. In experimental models, the regulatory role of B cells was supported by data showing failure of recovery from EAE in B cell-deficient mice, in mice with IL-$10^{-/-}$ B cells, or upon B cell depletion with anti-CD20 antibody prior to EAE induction [44–47]. Whether B_{regs} can prevent or suppress disease may vary depending on the experimental model. We found that B_{regs} prevent but do not suppress SAP, which is in agreement with work by other investigators showing that B_{regs} control the initiation but not the late phase of EAE [11]. Conversely, B_{regs} are capable of preventing and suppressing experimental autoimmune myasthenia gravis (EAMG), which may be due partly to its ability to suppress anti-AChR antibody production by B cells [48].

We found that AT of T_{regs} or B_{regs} into recipient mice led to expansion of both regulatory cell types. Whether the homeostasis or activation of T_{regs} and B_{regs} is influenced by each other has been an area of intense research. There is some evidence that $CD4^+$ T_{regs} induce and maintain B10 cells *in vitro* via CD5–CD72 interaction [49]. Conversely, some studies support the concept that B_{regs} are necessary for the generation and/or protective function of T_{regs} [50–52]. The regulatory function of B cells has been attributed to B7 molecules or to glucocorticoid-induced TNF receptor (TNFR) ligand [53–55]. Other studies revealed that B cells are not necessary for T_{reg} proliferation and accumulation in the CNS or for its suppressive function during EAE, in spite of lower frequency of $CD103^-$ T_{regs} in secondary lymphoid organs in B cell-deficient mice [56]. We found that the absence of B cells did not hamper the generation or maintenance of $CD4^+$ T_{regs} in NOD mice. To the contrary, there was a slightly higher frequency of $CD4^+$ T_{regs} in muMT/B7-$2^{-/-}$ NOD mice compared to B7-$2^{-/-}$ NOD mice. *In vitro*, $CD4^+$ T_{regs} from muMT/B7-$2^{-/-}$ NOD mice exhibited a greater capacity to shift cytokine profile from Th1 to $CD4^+$IL-10^+ T cells compared to those from B7-$2^{-/-}$ NOD cells, while both were equally effective in suppressing proliferation of SAP effector T cells.

That muMT/B7-$2^{-/-}$ NOD mice are protected against SAP is consistent with our data from B cell depletion with anti-CD19 antibody [24]. These findings indicate that B cells are critical APCs required for optimal stimulation of P0-reactive T cells. B cell-deficient mice are resistant to most, if not all, spontaneous autoimmune diseases such as diabetes or thyroiditis [57–59]. However, diabetes and thyroiditis can be induced in these mice by transient depletion of T_{regs} with anti-CD25 antibody, suggesting that T_{regs} control autoimmunity in the absence of B cells [59,60]. Recent studies revealed that T_{regs} from B cell-deficient mice and WT mice differ in their immunophenotypical properties, with the former being more effective at suppressing spontaneous autoimmune thyroiditis [61].

In summary, we found that: (1) lower frequency and number of both T_{regs} and B_{regs} were observed consistently in female B7-$2^{-/-}$ NOD mice, but less so in males; (2) T_{regs} and B_{regs} from B7-$2^{-/-}$ mice were less effective than those from WT mice in the induction or maintenance of $CD4^+$IL-10^+ cells; and (3) T_{regs} but not B_{regs} suppressed SAP when transferred after disease onset; however, B_{regs} were effective in controlling the early or initiation phase. These findings highlight the crucial role of B_{regs} and T_{regs} in maintaining immune homeostasis in SAP. Therapies aimed at expansion of these regulatory lymphocytes could be an effective approach in inflammatory neuropathies and other autoimmune diseases.

Acknowledgements

This work was supported by National Institute of Health Grant R01 NS064059 to B. Soliven and by a pilot grant from the GBS/CIDP Foundation International. We would like thank Danuta Dukala for her assistance in the early stage of the project. S. Q. designed and performed most of the experiments, J. R. S. and P. M. A. participated in some studies, B. S. supervised the study and wrote the paper together with S. Q.

Disclosure

The authors do not have any disclosures to declare.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Adoptive transfer of regulatory B cells (B_{regs}) after symptom onset did not attenuate the severity of spontaneous autoimmune polyneuropathy (SAP). Female B7-$2^{-/-}$ non-obese diabetic (NOD) mice (6 months old) were injected intravenously with 1×10^6 sorted wild-type (WT) B_{regs} or non-B_{regs} ($n = 5$). (a) Clinical scores. (b) Grip strength measurements.