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Oestrogen treatment of experimental autoimmune encephalomyelitis requires 17β-oestradiol-receptor-positive B cells that up-regulate PD-1 on $CD4^+$ $Foxp3^+$ regulatory T cells

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Summary

It is now well accepted that sex hormones have immunoregulatory activity and may prevent exacerbations in multiple sclerosis during pregnancy. Our previous studies demonstrated that oestrogen (17β-oestradiol; E_2) protection against experimental autoimmune encephalomyelitis (EAE) is mediated mainly through oestrogen receptor-α (ERα) and the membrane receptor G-protein-coupled receptor 30 (GPR30) and is abrogated in the absence of B cells and the co-inhibitory receptor, Programmed Death-1 (PD-1). To critically evaluate the cell source of the E_2 and PD-1 co-inhibitory pathways in EAE regulation, we assessed the requirement for ERs on transferred B cells and downstream effects on expression of PD-1/PD-ligand on $CD4^+$ $Foxp3^+$ regulatory T (Treg) cells in B-cell-replenished, E_2-treated B-cell-deficient ($\mu MT^{-/-}$) mice with EAE. The results clearly demonstrated involvement of ERα and GPR30 on transferred B cells that mediated the protective E_2 treatment effect on EAE and further showed an E_2-mediated B-cell-dependent up-regulation of PD-1 on $CD4^+$ $Foxp3^+$ Treg cells. These findings identify regulatory B-cell populations as key players in potentiating Treg-cell activity during E_2-mediated protection against EAE.

Keywords: experimental autoimmune encephalomyelitis; multiple sclerosis; oestrogen and receptors; programmed death-1/programmed death ligand; regulatory B cells.

Introduction

The increased incidence of multiple sclerosis (MS) in females has prompted extensive evaluation of possible gender-related mechanisms of disease induction and therapeutic strategies. One major conclusion from these studies is that increased levels of endogenous or exogenous sex hormones, including oestrogen (E_2), oestriol (E_3) and progesterone, can reduce lesion formation and severity of MS.[1–7] Our laboratory has focused on the ability of E_2 to induce protection against onset and severity of experimental autoimmune encephalomyelitis (EAE), an animal model of MS.[8,9] Collectively, our findings implicate regulatory B (Breg) cells,[10] oestrogen receptor-α (ERα)[11] and G-protein-coupled receptor 30 (GPR30)[12] and the programmed death-1 (PD-1)/programmed death ligand (PD-L) co-inhibitory pathway[13–16] as critical factors contributing to the E_2 protective mechanism. However, the cellular interactions that prevent or reduce EAE severity during E_2 treatment are not yet fully understood.

Our previous work demonstrated that B cells are indispensable for E_2-mediated protection against EAE.[10] The purpose of the current study was to establish the sufficiency of B cells and their oestrogen receptors to restore E_2-mediated protection against EAE and to evaluate downstream effects on infiltration of inflammatory cells into the central nervous system (CNS) and cellular expression of other critical regulatory molecules, including the PD-1 receptor and its ligands, PD-L1 and PD-L2. We therefore expanded upon our previous studies [10] that involved isolation and transfer of B cells from naive wild-type (WT) mice into recipient B-cell-deficient $\mu MT^{-/-}$ mice, in which signs of EAE disease were delayed but not reduced by E_2 treatment. Specifically, we transferred B cells from myelin oligodendrocyte glycoprotein (35–55) (MOG_{35-55})/complete Freund's adjuvant (CFA) immunized donor mice that were reported to have increased levels of

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Breg-cell subtypes and enhanced treatment effects on EAE.[17,18] The recipient $\mu MT^{-/-}$ mice were E_2-preconditioned a week before the B-cell transfers. Our results demonstrated clearly that passively transferred B cells from $MOG_{35–55}$/CFA-immunized WT donors, expressing ERα or GPR30, could restore E_2-mediated protection in B-cell-deficient $\mu MT^{-/-}$ mice, which resulted in significantly reduced infiltration of T cells, monocytes and dendritic cells into the brain, reduced CNS pathology and enhanced downstream induction of PD-1 by $CD4^+$ $Foxp3^+$ regulatory T (Treg) cells.

Materials and methods

Animals

Wild-type female C57BL/6 mice were purchased from Harlan Laboratories (Livermore, CA). The $\mu MT^{-/-}$ mice containing no B cells because of targeted disruption of the membrane exon of the immunoglobulin μ-chain gene were originally obtained from Jackson Laboratories (Bar Harbor, ME) and were bred at the Animal Resource Facility at the Portland Veteran Affairs Medical Center. The $ER\alpha^{-/-}$ mice on a C57BL/6 background, also bred in-house, were originally obtained from the Korach Laboratory (National Institutes of Health, Research Triangle Park, NC) and $GPR30^{-/-}$ mice were bred using in-house colonies. The generation of $ER\alpha^{-/-}$ [19] and $GPR30^{-/-}$ mice (also on a C57BL/6 background)[12] has been previously described. Female B6.129-*Esr2*^{tm1Unc} (Esr-2KO, $Esr2^{-/-}$, $ER\beta^{-/-}$) and $ER\alpha/ER\beta^{-/-}$ (Double-knockout) mice were a gift from Stephanie Murphy, PhD (Department of Anesthesiology and Perioperative Medicine, Oregon Health & Science University, Portland, OR, USA). All mice (on a C57BL/6 background) were used at 7–8 weeks of age and were housed in the Animal Resource Facility at the Portland Veterans Affairs Medical Center in accordance with institutional guidelines. The study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the Institutional Animal Care and Use Committee.

Donor mice

Female WT, $ER\alpha^{-/-}$, $ER\beta^{-/-}$, $ER\alpha/\beta^{-/-}$ and $GPR30^{-/-}$ mice that served as donors of B cells were immunized with 200 μg $MOG_{35–55}$ peptide (PolyPeptide Laboratories, San Diego, CA) in 200 μg CFA (H37Ra, Difco). Pertussis toxin was not given to ensure the retention of MOG-primed cells in the spleens.

Adoptive transfer of B cells

Spleens from MOG-immunized WT, $ER\alpha^{-/-}$, $ER\beta^{-/-}$, $ER\alpha/\beta^{-/-}$ and $GPR30^{-/-}$ mice were harvested on day 15 post-immunization (p.i.) and processed for B-cell isolation. Splenic $CD19^+$ B cells were purified using paramagnetic bead-conjugated antibodies from the CD19 cell isolation kit and subsequently separated by AutoMACS (Miltenyi Biotec, Auburn, CA). The positive fraction of the cells separated in this way were $CD19^+$ B cells with a purity of $\geq$ 95%. Approximately 10×10^6 purified splenic B cells from the donor mice were transferred intravenously into $\mu MT^{-/-}$ mice, on the same day as EAE induction.

Hormone treatment and induction of EAE in the recipient $\mu MT^{-/-}$ mice

Female $\mu MT^{-/-}$ mice (recipients) were implanted with 2·5 mg/60-day release E_2 pellets (Innovative Research of America, Sarasota, FL) or sham-treated (control) 1 week before B-cell transfer and immunization with 200 μg $MOG_{35–55}$ peptide (PolyPeptide Laboratories, San Diego, CA) in 200 μg CFA (H37Ra, Difco). The recipient mice received pertussis toxin (Ptx, List Biologicals, Campbell, CA) on the day of immunization (75 ng) and 2 days later (200 ng). All recipient mice were monitored daily for clinical signs of disease and scored using the following scale: 0 = no signs; 1 = limp tail or mild hind limb weakness; 2 = moderate hind limb weakness or mild ataxia; 3 = moderately severe hind limb weakness; 4 = severe hind limb weakness or mild forelimb weakness or moderate ataxia; 5 = paraplegia with no more than moderate forelimb weakness; and 6 = paraplegia with severe forelimb weakness or severe ataxia or moribund condition.

Histopathology

Intact spinal columns removed from recipient mice at the end of the study (i.e. day 20–28 p.i.) were fixed in 10% formalin. Dissected spinal cords were embedded in paraffin before sectioning. Sections were stained with haematoxylin & eosin to assess inflammatory lesions. Transverse sections were stained with a modified eriochrome cyanine protocol to assess the sparing of the white and grey matter (demyelination).[20] Slides were analysed by light microscopy.

Flow cytometry

Spleens and brains from control and E_2-treated $\mu MT^{-/-}$ mice were processed for lymphocyte isolation. Cells were stained with a combination of the following antibodies obtained from BD Bioscience (San Diego, CA): CD4 (L3T4), CD19 (1D3), CD1d (1B1), CD5 (53-7.3), PD-L1 (MIH5), PD-L2 (Ty25), CD11b (M1/70), CD11c (HL-3), CD45 (30-F11). The intracellular staining of Foxp3 (MF23) and PD-1 (J43) was completed following overnight incubation in fixation/permeabilization buffer

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(eBiosciences, San Diego, CA). Dead cells were gated out using propidium iodide discrimination. Cells were gated on CD19 to determine expression of the $CD1d^{high}$ $CD5^{+}$ and PD-L1 populations.

Intracellular interleukin-10 (IL-10) expression was also visualized by immunofluorescence staining and analysed by flow cytometry, as described in ref. 17. Briefly, isolated splenocytes were resuspended (2×10^6 cells/ml) in complete medium [RPMI-1640 medium containing 10% fetal calf serum, 200 μg/ml penicillin, 200 U/ml streptomycin, 4 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol (all from Life Technologies, Carlsbad, CA)] with lipopolysaccharide (10 μg/ml, *Escherichia coli* serotype 0111:B4; Sigma-Aldrich, St Louis, MO), PMA (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and Brefeldin A (1 μl/ml of medium; BD Biosciences) for 4 hr, in 24-well flat-bottom plates. For IL-10 detection, FcRs were blocked with mouse FcR monoclonal antibody (2.4G2; BD Biosciences) before surface staining. Stained cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions, and stained with allophycocyanin-conjugated mouse anti-IL-10 monoclonal antibody (JES5-16E3). Data were collected with CellQuest (BD Biosciences, San Jose, CA) and FCS Express (De Novo Software, Los Angeles, CA) software on a FACSCalibur (BD Biosciences). Absolute numbers were calculated from live-gated cells.

Statistical analysis

Data are reported using GRAPHPAD PRISM (v 4·0, San Diego, CA) and expressed as the mean ± SEM. Statistical significance for the disease course between recipient $\mu MT^{-/-}$ control and E_2-implanted mice was calculated using one-way analysis of variance with multiple comparison post-test (Bonferroni). The Student's *t*-test and the Kruskal–Wallis test (non-parametric analysis of variance) with Dunn's multiple comparison of means post-test were used for analysis of the cumulative disease index (CDI). Student's *t*-test was used to compare flow cytometry data between the recipient $\mu MT^{-/-}$ control and E_2-implanted mice. *P*-values $\leq$ 0·05 were considered significant.

Results

B cells from $mMOG_{35-55}$-immunized WT mice are sufficient to restore E_2-mediated protection in B-cell-deficient mice

Our previous work demonstrates that B cells are indispensable for E_2-mediated protection against EAE.[10] As demonstrated in ref. 10, our preliminary studies involved isolating B cells from naive WT mice and adoptively transferring into recipient $\mu MT^{-/-}$ mice that were either sham-treated or E_2-implanted a day after the transfers and MOG-immunized 7 days later. We demonstrated that the E_2-implanted $\mu MT^{-/-}$ mice that were B-cell recipients were significantly protected from EAE compared with the sham-treated recipients. However, this E_2-mediated protection, upon B-cell transfers from naive WT mice, was short-lived and the recipient mice lost protection from day 21 onwards. Previous studies by others indicated an enhanced ability of antigen-primed B cells to mediate protection against EAE.[17,18] Hence, as an extension to our previous studies, we evaluated whether B cells from $mMOG_{35-55}$-immunized WT mice were sufficient to restore E_2-mediated protection in E_2-preconditioned B-cell-deficient $\mu MT^{-/-}$ mice when transferred on the same day as immunization with $mMOG_{35-55}$/CFA/Ptx rather than 7 days before immunization, as done earlier. Briefly, 10 million B cells were transferred via tail vein injections into recipient $\mu MT^{-/-}$ mice that were either sham-treated or implanted with 2·5 mg per 60-day release E_2 pellets 7 days before the B-cell transfer and immunization protocol. The transferred B cells could be detected in the blood, inguinal lymph nodes and spleens until at least 15 days p.i. Disease course in the recipient $\mu MT^{-/-}$ mice was followed by monitoring changes in clinical EAE scores for 26–30 days p.i. to determine the sufficiency of B cells in E_2-implanted mice. Subsequently, the same protocol was followed for all the adoptive transfer experiments.

The control $\mu MT^{-/-}$ recipients of MOG-specific B cells from WT donors demonstrated an onset of EAE at day 11 p.i. and exhibited a steady increase in the disease scores with the peak of the disease at day 15 p.i. (Fig. 1a). However, the E_2-implanted $\mu MT^{-/-}$ recipients of MOG-specific WT B cells were significantly protected from EAE until day 24 p.i. with a loss of protection, here onwards, as compared with the control WT B-cell-recipient counterparts. These results were as per our expectation because the group of $\mu MT^{-/-}$ mice implanted with E_2 pellets was anticipated to mimic a WT scenario. That is, replenishment with WT B cells would enable E_2 protection against EAE compared with vehicle-treated $\mu MT^{-/-}$ mice which, despite receiving B cells, would not be protected from EAE (similar to the WT sham-treated mice). As demonstrated earlier, the E_2-implanted $\mu MT^{-/-}$ mice with no cell transfers exhibited a delay in the onset of EAE (day 15 p.i.), but completely lost E_2-mediated protection by day 18, with their disease scores being comparable to the respective sham-treated counterparts by day 19. The CDI and the peak of the disease of E_2-implanted $\mu MT^{-/-}$ recipients of WT B cells were also significantly lower than those of control recipients. Spinal cord sections were assessed for the extent of inflammation (haematoxylin & eosin staining) and demyelination (eriochrome staining) in the CNS of the control and E_2-implanted $\mu MT^{-/-}$ recipient mice. We demonstrated earlier[10] that spinal cord sections of E_2-implanted $\mu MT^{-/-}$ mice (receiving no B cells) demonstrated massive leucocyte

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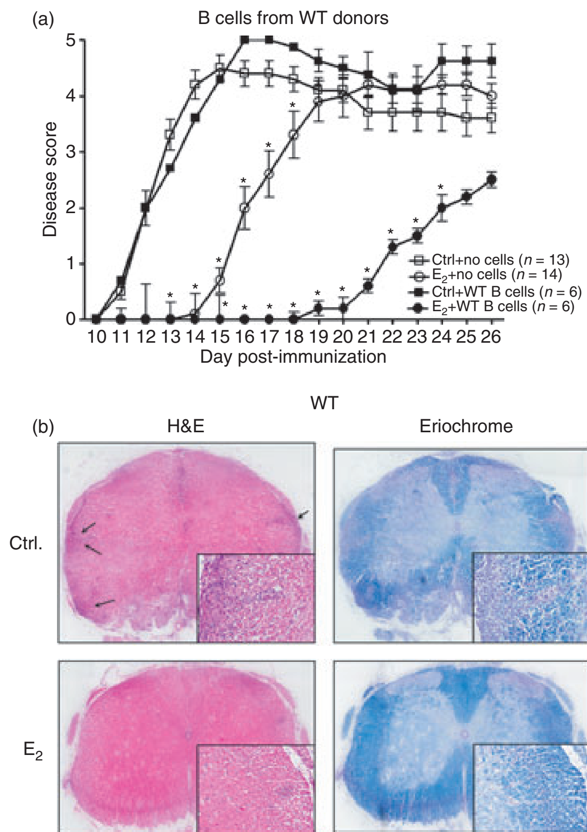

Figure 1. B cells are sufficient to restore 17β-oestradiol (E_2) -mediated protection in the B-cell-deficient mice. Female wild-type (WT) mice that served as donors of B cells were immunized with mouse myelin oligodendrocyte glycoprotein (35–55) ($mMOG_{35–55}$) peptide for 15 days in complete Freund's adjuvant (CFA) before collection of splenocytes and enrichment of B cells by MACS. Seven- to 8-week-old $\mu MT^{-/-}$ mice that served as recipients of B cells were either sham-treated (control) or implanted with E_2 pellets 1 week before transfer of 10 million B cells and immunization with $MOG_{35–55}$ peptide in CFA/pertussis toxin (Ptx). Recipient mice were monitored for signs of clinical experimental autoimmune encephalomyelitis over 26 days. Data presented are (a) mean daily disease scores from two independent experiments with three mice/group/experiment. $*P \leq 0{\cdot}05$, compared with the respective control mice (i.e. controls with no B cells and controls receiving WT B cells but no E_2) (one-way analysis of variance with Bonferroni post hoc test). (b) Histopathological evaluation of spinal cords of WT B-cell-recipient $\mu MT^{-/-}$ (sham and E_2-implanted) mice. Spinal cords from each group, collected on day 15 post-immunization, were fixed in PFA and embedded in paraffin. Transverse sections, 10 μm thick, from different regions of the spinal cord from each of the groups were stained with haematoxylin & eosin to enumerate infiltrating leucocytes and with Eriochrome cyanine to visualize the extent of demyelination. Arrows denote foci of inflammation; magnification was 50 × and 200 × (inset). Sections are representative of two experiments (n = 3/group/experiment).

infiltration with several foci of inflammation along with severe demyelination similar to control $\mu MT^{-/-}$ mice. Here, the control $\mu MT^{-/-}$ recipient mice demonstrated leucocyte infiltration along with demyelination. However, the spinal cord sections from the E_2-implanted $\mu MT^{-/-}$ recipient mice showed no obvious signs of inflammation and demyelination and appeared healthy (day 15 p.i.) (Fig. 1b), mimicking a phenomenon demonstrated by the WT E_2-treated mice.[8] Hence, the above results confirm that B cells are sufficient to restore E_2-mediated protection in the recipient $\mu MT^{-/-}$ mice and the protection is longer and more pronounced than that demonstrated by the E_2-implanted $\mu MT^{-/-}$ mice with no cell transfers.

Presence of ERα in B cells is indispensable for E_2-mediated protection against EAE

As B cells are known to express ERs, it was crucial to determine the contribution of these ERs to the protective function of B cells in E_2-related protection against EAE. To characterize the ERs involved in E_2-mediated protection, our laboratory has demonstrated earlier that the immunomodulatory effects of E_2 in EAE are dependent on ERα and not ERβ signalling.[11] Also based on our studies on the recently discovered membrane ER, GPR30,[12] it is likely that both GPR30 and ERα participate in E_2-mediated protection in an additive manner. In our recent studies,[10] we demonstrated that direct treatment of B cells with E_2 significantly reduced proliferation of $MOG_{35–55}$-specific T cells, suggesting a requirement for ERα on B cells. It was therefore important to ascertain the role of ERs on B cells as critical players responsible for mediating E_2-directed protection against EAE. We first assessed the role of ERα in B cells as a contributor to E_2-mediated protection in the recipient $\mu MT^{-/-}$ mice. B cells were obtained from MOG-immunized ER$\alpha^{-/-}$ (ERKO) donors, day 14 p.i., and transferred into sham-treated or E_2-implanted recipient $\mu MT^{-/-}$ mice. The E_2-implanted $\mu MT^{-/-}$ recipients of B cells from ER$\alpha^{-/-}$ mice lost protection on about the same day (day 16 p.i.) as the E_2-implanted $\mu MT^{-/-}$ mice with no cell transfers (Fig. 2a). They also demonstrated a significantly earlier onset compared with the E_2-implanted $\mu MT^{-/-}$ mouse recipients of WT B cells (Table 1). The CDI and peak of the disease for these E_2-implanted recipients of MOG-specific ER$\alpha^{-/-}$ B cells was also significantly higher compared with the E_2-implanted recipients of WT B cells and similar to the E_2-implanted $\mu MT^{-/-}$ mice with no cell transfers (Table 1). The spinal cord sections from the E_2-implanted ER$\alpha^{-/-}$ B-cell-recipient mice demonstrated equivalent leucocyte infiltration along with demyelination compared with the control recipients (Fig. 2b). These data indicate a critical role of ERα on the B cells in mediating the protective effects of E_2.

Upon transferring B cells from MOG-immunized ER$\beta^{-/-}$ (BERKO) donors, the E_2-implanted $\mu MT^{-/-}$ recipient mice had a significantly late onset compared with not only the sham-treated ER$\beta^{-/-}$ B-cell recipients

(Fig. 2a) but also the E_2-implanted $ER\alpha^{-/-}$ B-cell recipients (Table 1). The E_2-implanted $\mu MT^{-/-}$ recipients of B cells from $ER\beta^{-/-}$ mice lost protection at day 24 p.i., about the same day as the E_2-implanted recipients of B cells from WT donors. The day of disease onset and the disease peak were comparable with those of the E_2-implanted WT B-cell-recipient $\mu MT^{-/-}$ mice (Table 1). Spinal cord sections from E_2-implanted $ER\beta^{-/-}$ B-cell-recipient $\mu MT^{-/-}$ mice demonstrated fewer inflammatory foci and a lesser extent of demyelination (Fig. 2c). These data demonstrate the dispensable role of $ER\beta$ in B cells for E_2-mediated protection in the recipient $\mu MT^{-/-}$ mice, with observed protection potentially the result of residual functional $ER\alpha$ and GPR30 E_2 receptors.

Our studies in $GPR30^{-/-}$ mice[12] demonstrated that the mER, GPR30, is sufficient, yet not exclusively responsible, for the full E_2-mediated protection against EAE. The same study led us to conclude that that $ER\alpha$ and GPR30 work together additively to achieve optimal protection, and each receptor may adequately compensate the functional loss of the other. To verify the requirement of GPR30 on the B cells in E_2-mediated protection, we transferred B cells from MOG-immunized $GPR30^{-/-}$ donors into sham-treated or E_2-implanted $\mu MT^{-/-}$ mice on the day of induction of EAE in the recipient mice. Upon transferring B cells from MOG-immunized $GPR30^{-/-}$ donors, as expected, the control recipient $\mu MT^{-/-}$ mice had an early onset (day 10·5 p.i.); however, the E_2-implanted recipients had an onset of disease around day 16–17 p.i. (Fig. 2a). This trend of an early disease onset was similar to the E_2-implanted recipients of $ER\alpha^{-/-}$ B cells. However, the CDI and the peak of the disease in the E_2-implanted $GPR30^{-/-}$ B-cell-recipient $\mu MT^{-/-}$ mice were lower than those of the E_2-implanted $ER\alpha^{-/-}$ B-cell-recipient $\mu MT^{-/-}$ mice. The E_2-implanted recipients of $GPR30^{-/-}$ B cells lost protection from day 19 p.i. compared with the sham-treated recipients. Spinal cord sections from E_2-implanted $GPR30^{-/-}$ B-cell-recipient $\mu MT^{-/-}$ mice also demonstrated massive leucocyte infiltration with several foci of inflammation (indicated by arrows, Fig. 2d) along with severe demyelination similar to control recipient $\mu MT^{-/-}$ mice. These data indicate a partial role of GPR30 in B cells for E_2-mediated protection in recipient $\mu MT^{-/-}$ mice.

When B cells from MOG-immunized $ER\alpha/\beta^{-/-}$ (ERKO/BERKO) donors (which still express GPR30) were transferred into control and E_2-implanted $\mu MT^{-/-}$ mice, the E_2-implanted recipients had an onset similar to that of the E_2-implanted $ER\alpha^{-/-}$ and $GPR30^{-/-}$ B-cell recipients (Fig. 2a). The E_2-implanted recipients lost protection from day 19 p.i. onwards compared with the sham-treated recipients. The CDI and peak of disease in the E_2-implanted recipients of $ER\alpha/\beta^{-/-}$ B cells were similar to those of the E_2-implanted $ER\alpha^{-/-}$ and $GPR30^{-/-}$ B-cell recipients. The spinal cord sections of the E_2-implanted $ER\alpha/\beta^{-/-}$ also demonstrated leucocyte infiltration with several foci of inflammation and demyelination (Fig. 2e). Hence, these results confirm that GPR30 and $ER\alpha$ act in unison in E_2-mediated protection.

Donor B cells from $mMOG_{35–55}$-primed WT and different ER-deficient mice have similar regulatory markers

With the difference in the protection rendered by the B cells from WT and various ER receptor-deficient mice, we sought to determine whether the donor B cells attain a distinct phenotype in the presence of $MOG_{35–55}$ peptide, which causes them to elicit different levels of protection in the recipient $\mu MT^{-/-}$ mice. B cells from MOG-immunized WT, $ER\alpha^{-/-}$, $ER\beta^{-/-}$ and $GPR30^{-/-}$ donors were isolated on day 14 p.i. and characterized via flow cytometry for the presence of various regulatory markers. Our results[10] demonstrated that the requirement for B cells in E_2-mediated protection against EAE involved direct E_2 effects on Breg cells mediated through $ER\alpha$ and the PD-1/PD-L1 negative co-stimulatory pathway, indicating that B cells may mediate immunoregulation via these markers.

Figure 2. 17β-Oestradiol (E_2) -mediated protection against experimental autoimmune encephalomyelitis (EAE) is mediated through oestrogen receptor-α ($ER\alpha$) and G-protein-coupled receptor 30 (GPR30) on B cells. Female ER knockout mice (i.e. $ER\alpha^{-/-}$, $ER\beta^{-/-}$, $GPR30^{-/-}$ or $ER\alpha/\beta^{-/-}$ mice) that served as donors of B cells were immunized for 15 days with mouse myelin oligodendrocyte glycoprotein (35–55) ($mMOG_{35–55}$) peptide in complete Freund's adjuvant (CFA) before collection of splenic B cells for transfer. Seven- to 8-week-old $\mu MT^{-/-}$ mice that served as recipients of B cells were either sham-treated (control) or implanted with E_2 pellets 1 week before B-cell transfer and immunization with $MOG_{35–55}$ peptide in CFA/pertussis toxin (Ptx). Recipient mice were monitored for signs of clinical EAE for 26 days. Data presented are (a) mean daily EAE disease scores from two to four independent experiments in sham versus E_2-treated B-cell-deficient mice receiving 10 million B cells from $mMOG_{35–55}$-immunized $ER\alpha^{-/-}$, $ER\beta^{-/-}$, $GPR30^{-/-}$ and $ER\alpha/\beta^{-/-}$ mice with three or four mice/group/experiment. $^*P \leq 0·05$, compared with the respective control mice (i.e. sham-treated versus E_2-implanted $\mu MT^{-/-}$ mice receiving no cells and sham-treated versus E_2-implanted $\mu MT^{-/-}$ B-cell recipients) (one-way analysis of variance with Bonferroni *post hoc* test). Histopathological evaluation of spinal cords from (b) $ER\alpha^{-/-}$, (c) $ER\beta^{-/-}$, (d) $GPR30^{-/-}$ and (e) $ER\alpha/\beta^{-/-}$ B-cell-recipient $\mu MT^{-/-}$ mice (sham-treated and E_2-implanted, day 26 post-immunization). Spinal cords from each group were fixed in PFA and embedded in paraffin. Transverse sections, 10 μm thick, from different regions of the spinal cord from each of the groups were stained with haematoxylin & eosin to enumerate infiltrating leucocytes and with Eriochrome cyanine to visualize the extent of demyelination. Arrows denote foci of inflammation; magnification was 50 × and 200 × (inset). Sections are representative of two experiments (n = 3–4/group/experiment).

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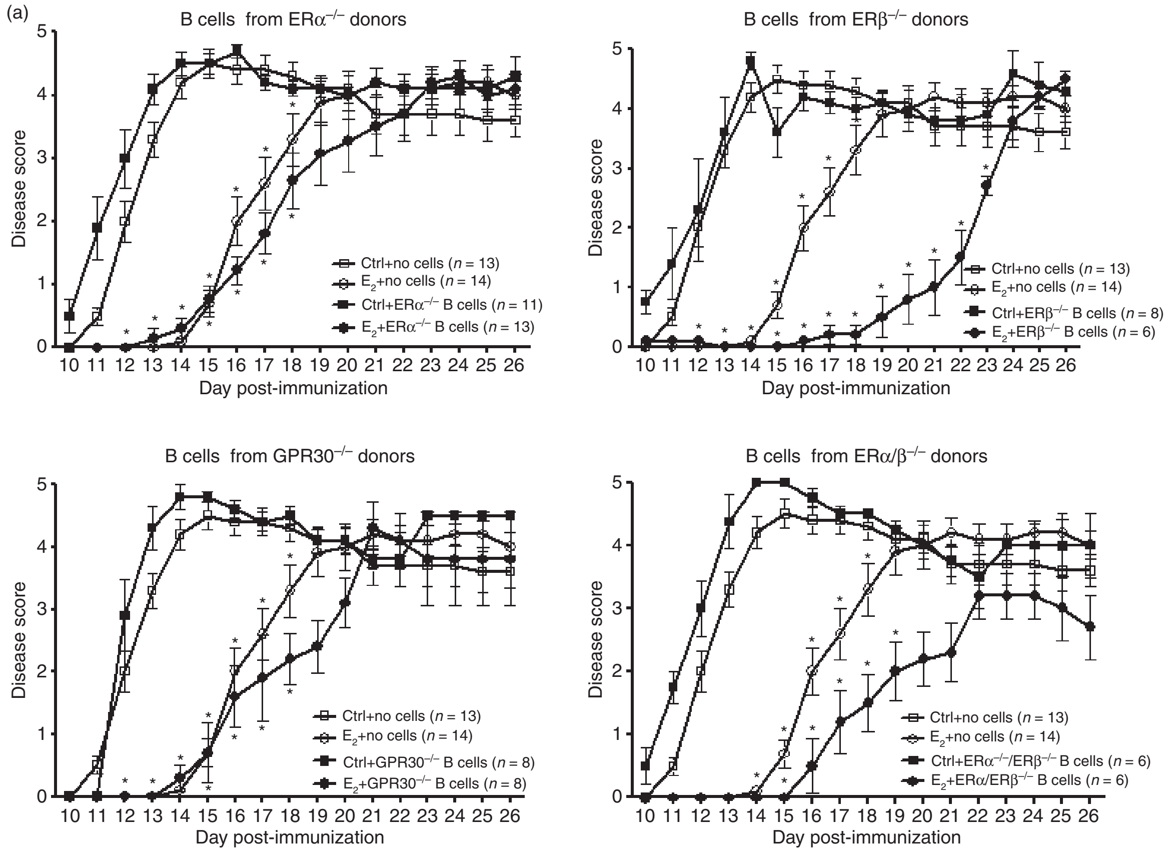
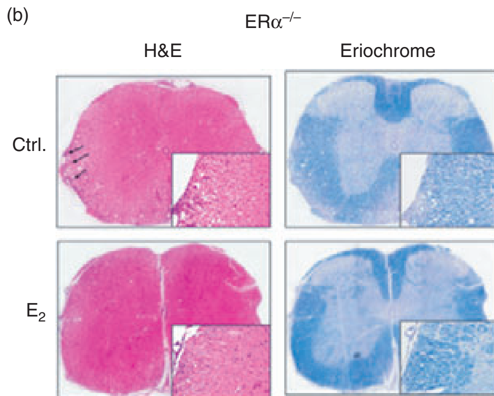
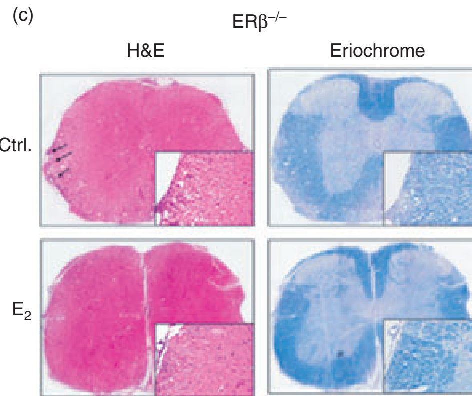
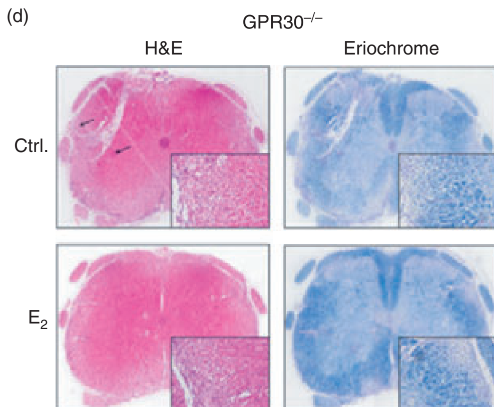

(a)
B cells from ERα−/− donors
B cells from ERβ−/− donors
B cells from GPR30−/− donors
B cells from ERα/β−/− donors
Disease score
Day post-immunization
Ctrl+no cells (n = 13)
E2+no cells (n = 14)
Ctrl+ERα−/− B cells (n = 11)
E2+ERα−/− B cells (n = 13)
Ctrl+ERβ−/− B cells (n = 8)
E2+ERβ−/− B cells (n = 6)
Ctrl+GPR30−/− B cells (n = 8)
E2+GPR30−/− B cells (n = 8)
Ctrl+ERα−/−/ERβ−/− B cells (n = 6)
E2+ERα/ERβ−/− B cells (n = 6)


(b)
ERα−/−
H&E
Eriochrome
Ctrl.
E2


(c)
ERβ−/−
H&E
Eriochrome
Ctrl.
E2


(d)
GPR30−/−
H&E
Eriochrome
Ctrl.
E2

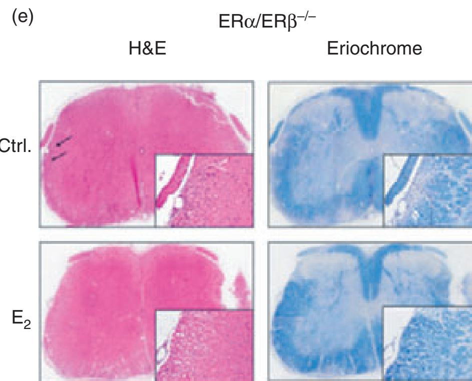

(e)
ERα/ERβ−/−
H&E
Eriochrome
Ctrl.
E2

Table 1. Clinical course of EAE in B-cell-recipient mMT$^{-/-}$ mice*

μMT$^{-/-}$ recipient mice	Incidence	Onset	Peak	CDI	Mortality
No cells Ctrl	13/13	11·6 ± 0·5	4·6 ± 0·1	43·8 ± 13·7	1
No cells E_2	13/14	16·4 ± 12·2[1]	4·7 ± 0·7	20·8 ± 10·9[1]	2
+ WT B cells Ctrl	6/6	12·2 ± 1·6	4·6 ± 0·9	53·7 ± 7·5	1
+ WT B cells E_2	4/6	20·6 ± 1·5[1]	2·6 ± 2·4	11·3 ± 10·4[1]	0
+ ER-α$^{-/-}$ B cells Ctrl	11/11	10·2 ± 3·0	4·7 ± 0·3	57·5 ± 15·7	0
+ ER-α$^{-/-}$ B cells E_2	13/13	16·3 ± 2·9[2]	4·5 ± 0·3[2]	30·3 ± 11·4[2]	0
+ ER-β$^{-/-}$ B cells Ctrl	8/8	11·6 ± 1·8	4·5 ± 0·4	53·3 ± 24·8	0
+ ER-β$^{-/-}$ B cells E_2	5/6	20·2 ± 2·4[1]	2·8 ± 1·9[1,3]	15·8 ± 14·8[1,3]	0
+ GPR30$^{-/-}$ B cells Ctrl	8/8	10·5 ± 0·7	5·0 ± 0·2	48·8 ± 14·4	0
+ GPR30$^{-/-}$ B cells E_2	6/8	16·7 ± 2·4[2]	3·3 ± 1·9[1]	23·6 ± 25·7[1]	1
+ ER-α/β$^{-/-}$ B cells Ctrl	6/6	10·5 ± 0·6	5·0 ± 0	58·8 ± 16·6	0
+ ER-α/β$^{-/-}$ B cells E_2	6/6	16·8 ± 1·0[1]	3·9 ± 1·6	20·2 ± 24·4[1]	2

*Statistical evaluation of EAE disease course for recipient mMT$^{-/-}$ Control and E2-implanted mice, including the Cumulative Disease Index (CDI), through day 26 post-immunization for the 2–4 experiments (n = 3–4 mice/group/experiment). The daily disease scores from pooled data and corresponding CDI data from each of the independent experiments (mean ± SEM) are presented. Additional information for the experiments is shown in Figs. 1 and 2.

CDI, cumulative disease index; Ctrl, control; E_2, 17β-oestradiol; ER, oestrogen receptor; GPR30, G-protein-coupled receptor 30.

[1]Significant ($P < 0·05$) as compared with the respective Control group.

[2]Significant ($P < 0·05$) as compared with the E_2-implanted wild-type B-cell-recipient group.

[3]Significant ($P < 0·05$) as compared with the E_2-implanted ERα$^{-/-}$ B-cell-recipient group.

Therefore, we assessed the donor B cells from different strains of mice for the percentage of PD-L1 and Breg cells (CD1d^{high} CD5^{+}) and for IL-10 production. As demonstrated in Fig. 3, the per cent expressions of each of these regulatory markers by the B cells from different strains were not significantly distinct from each other. However, as the donor B cells from different ER-deficient mice mediate different outcomes of protection against EAE, it implies that the donor B cells acquire regulatory properties in an E_2-rich milieu, which was provided in the E_2-implanted μMT$^{-/-}$ recipients.

Less infiltration of immune cells in CNS of E_2-implanted μMT$^{-/-}$ recipients of WT B cells

As the E_2-implanted μMT$^{-/-}$ recipients of WT B cells demonstrate protection from EAE compared with not only the sham-treated recipients but also the no transfer E_2-implanted μMT$^{-/-}$ mice, we characterized the immune cell trafficking into the CNS in these mice. Leucocytes isolated from brains of sham-treated (control) and E_2-implanted μMT$^{-/-}$ recipient of WT B cells were stained for several cell surface markers. Brains of E_2-implanted μMT$^{-/-}$ recipients not only demonstrated lower numbers of total leucocytes compared with control μMT$^{-/-}$ recipient mice (data not shown), but also had a significant decrease in percentages of CD4^{+} T cells (Fig. 4). Brains of E_2-implanted μMT$^{-/-}$ recipients also demonstrated significant decrease in percentages of infiltrating macrophages (CD11b^{+} CD45^{high}) and dendritic cells (CD11c^{+} CD45^{+}), which participate in disease pathogenesis through effects on blood–brain barrier permeability, antigen presentation and immune regulation[21] (Fig. 4). Hence, replenishment of B cells redeems the immunosuppressive and protective effects of E_2 in the μMT$^{-/-}$ recipient mice, as demonstrated by not only significantly less demyelination and fewer inflammatory foci (histology) but also less infiltration of immune cells into the CNS.

The E_2-implanted μMT$^{-/-}$ recipients of WT B cells have significantly increased percentages of PD-L1-expressing cells, PD-1 expression in Treg cells and CD1d^{high} CD5^{+} expression by the transferred B cells

Next, we assessed the induction of immune responses in the periphery (spleens) because it is known that after induction of immune responses in the periphery, pathogenic T cells migrate to the CNS to initiate an inflammatory process responsible for clinical signs of EAE.[22] To evaluate MOG-specific responses generated in spleens, mononuclear cells from sham-treated and E_2-implanted μMT$^{-/-}$ recipient mice were stained for the negative co-inhibitory markers, PD-L1 and PD-L2, and the expression of PD-1 on CD4^{+} Foxp3^{+} Treg cells. First, we also ascertained that the transferred B cells still survived in the recipients (Fig. 5a). Moreover, the per cent expression of total PD-L1 in the spleens of E_2-implanted μMT$^{-/-}$ B-cell-recipient mice was significantly higher than in the sham-treated recipients and the no-cell-transfer mice (Fig. 5b). There was no difference in the PD-L2 expression in the two groups of recipient mice (data not shown). Since E_2 induces an increase in the CD4^{+} Foxp3^{+} (Treg) cell population,[14,23] we evaluated the percentage of

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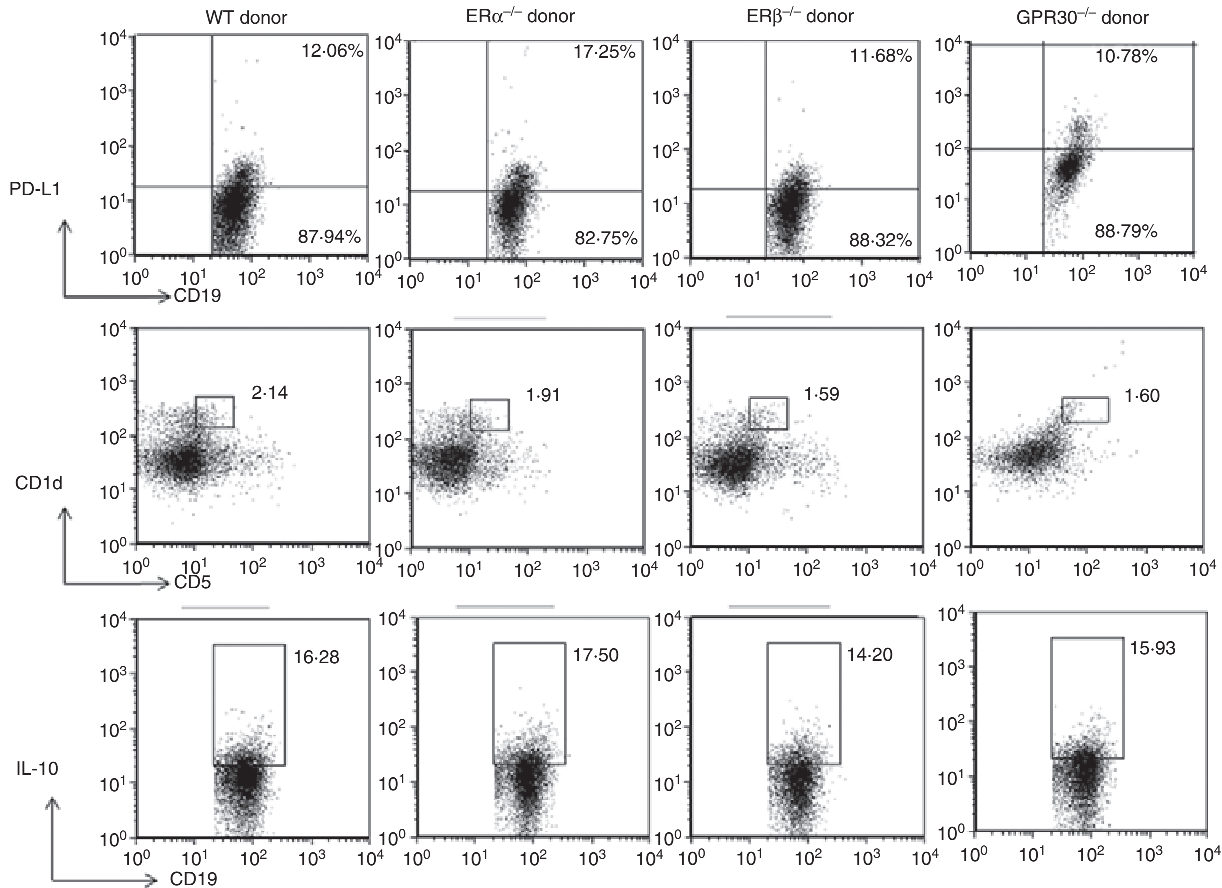

Figure 3. Donor B cells from mouse myelin oligodendrocyte glycoprotein (35–55) ($mMOG_{35–55}$) -primed wild-type (WT) and oestrogen receptor (ER) -deficient mice have similar regulatory markers. Mononuclear cells were isolated from the spleens of $mMOG_{35–55}$-immunized donor mice of different ER-deficient strains: i.e. WT, $ER\alpha^{-/-}$, $ER\beta^{-/-}$, and G-protein-coupled receptor 30 ($GPR30^{-/-}$) mice. On day 14 post-immunization, splenocytes were assessed for the expression of programmed death ligand 1 (PD-L1) on $CD19^+$ B cells, percentage of $CD1d^{high}$ $CD5^+$ B cells and interleukin-10 (IL-10) -producing B cells after lipopolysaccharide + PMA-ionomycin stimulation for 4 hr within the indicated gates among total $CD19^+$ B cells. Data represent PD-L1 expression, $CD1d^{high}$ $CD5^+$ expression and IL-10 expression on gated $CD19^+$ cells of total gated and live/fixed cells. Similar results were obtained when B cells from the donor mice were characterized in at least two independent experiments with (n = 2/strain/experiment).

Treg cells in the B-cell-transferred recipient mice. There was a trend towards an increased percentage of the $CD4^+$ $Foxp3^+$ population in the E_2-implanted $\mu MT^{-/-}$ B-cell-recipient mice but the increase was not statistically significant compared with the control $\mu MT^{-/-}$ B-cell-recipient mice, as demonstrated in Fig. 5(c). Our laboratory has earlier demonstrated the critical role of PD-1, a co-inhibitory receptor, in E_2-mediated protection against EAE.[13,14,24] Hence, we assessed the expression of PD-1 in the Treg-cell population ($PD\text{-}1^+$ $Foxp3^+$ $CD4^+$ cells) in the recipient mice. The E_2-implanted recipients of MOG-specific B cells indeed had significantly higher expression of PD-1 compared with the control recipients (Fig. 5d). The Breg-cell ($CD1d^{high}$ $CD5^+$) percentage among the transferred and surviving B cells was also calculated to quantify any differences between the sham-treated and E_2-implanted $\mu MT^{-/-}$ recipient mice. As indicated in Fig. 5(e), the percentage of Breg cells in the E_2-implanted recipients of WT B cells was significantly higher than that in the control recipients. These data demonstrate that B cells in an E_2-rich milieu attain regulatory properties and also that the transferred B cells induce immunoregulatory properties in other immune cells in the B-cell-deficient recipients.

Discussion

The results presented above demonstrate the requirement for expression of ERα or GPR30 by transferred B cells from $MOG_{35–55}$-immunized donors to restore responsiveness to E_2 therapy in $\mu MT^{-/-}$ mice developing EAE. Furthermore, the data confirm E_2-dependent B-cell involvement in inhibiting infiltration of $CD4^+$ T cells, monocytes and dendritic cells into the CNS and up-regulating PD-1 on $CD4^+$ $Foxp3^+$ Treg cells and PD-L1-expressing cells in recipient mice. These findings

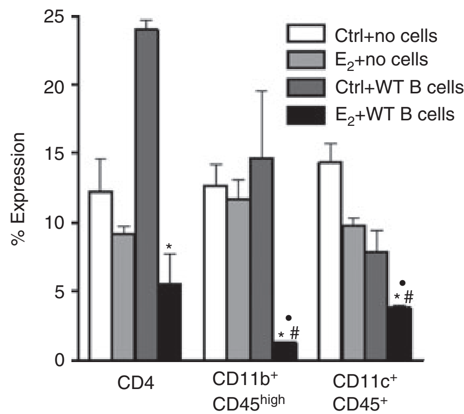

Figure 4. Less infiltration of immune cells in central nervous system of 17β-oestradiol (E_2) -implanted $\mu MT^{-/-}$ recipients of wild-type (WT) B cells. On day 15 post-immunization with mouse myelin oligodendrocyte glycoprotein (35–55) /complete Freunds' adjuvant/pertussis toxin, mononuclear cells isolated from brains of sham-treated versus E_2-implanted $\mu MT^{-/-}$ mice that received no cells or 10 million WT B cells were analysed by FACS for percentages of $CD4^+$ T cells, $CD11b^+$ $CD45^{high}$ macrophages and $CD11c^+$ $CD45^+$ dendritic cells. Frequencies of $CD4^+$ T cells, macrophages and dendritic cells were determined in individual brains and data indicate the percentages of total gated live leucocytes (n = 6). Data are pooled from two independent experiments (mean ± SEM). *$P \leq 0{\cdot}05$ compared with sham control $\mu MT^{-/-}$ mice that received WT B cells; #$P \leq 0{\cdot}05$ compared with the control $\mu MT^{-/-}$ mice that did not receive cell transfers and • $P \leq 0{\cdot}05$ compared with E_2-implanted $\mu MT^{-/-}$ mice that did not receive cell transfers (Student's t-test).

are important because they define the identity of immune cells involved in regulatory interactions that lead to protection against clinical and histological signs of EAE after treatment with E_2.

The model system used in the current study used transfer of B cells from MOG_{35-55}-immunized donors into sham or E_2-pretreated B-cell-deficient $\mu MT^{-/-}$ mice followed by EAE induction to establish the sufficiency of B cells in E_2 protection. This study design represents a departure from our previous protocols to take advantage of the reported increase in regulatory activity of using antigen-specific B cells for the transfers[17,18] and places the B cells into a pre-existing E_2-enhanced environment upon EAE induction. Although E_2 treatment of the B-cell-deficient $\mu MT^{-/-}$ mice does not inhibit EAE disease severity, it does delay onset of clinical signs by ~ 5 days, producing a later baseline (day 16) for comparison with B-cell-transferred recipients. This delay may be a result of the effects of E_2 on other cell types, which could include Treg cells,[21] myeloid cells and CNS cells.[25] To support this reasoning, studies demonstrate the link between B cells and $CD4^+$ $Foxp3^+$ Treg-cell homeostasis, wherein the adoptive transfer of naive WT splenic B cells into $\mu MT^{-/-}$ mice resulted in a significant increase in the numbers of Treg cells by day 10 post-transfer.[26] Likewise, in our study the E_2-implanted $\mu MT^{-/-}$ mice, with no cell transfers, demonstrated a higher percentage of $Foxp3^+$ Treg cells than the no cell-transfer control $\mu MT^{-/-}$ mice (Fig 5c), though this increase was not significant. Also, the E_2-implanted $\mu MT^{-/-}$ recipient mice transferred with WT MOG-specific B cells demonstrated a further increase in the Treg-cell percentages though it was not statistically significant. Another study indicates the crucial role for Treg cells in inhibiting late-phase EAE disease.[27] Our previous study also demonstrated reduced IL-17 production by the splenocytes of the E_2-implanted $\mu MT^{-/-}$ mice, suggesting a relatively less inflammatory environment in an E_2-rich milieu, even in the absence of B cells.[10] Therefore, we postulate that the delayed onset of disease in the $\mu MT^{-/-}$ mice, with no cell transfers, may be a result of the effects of E_2 on other cell types, such as Treg cells. The transfer of MOG-specific B cells from WT mice further delayed the onset of EAE by an additional 4 days (~ day 20) and inhibited both peak and cumulative disease severity scores.

The transfer of B cells from various ER knockout mice provided new and critical information regarding the direct effects of E_2 on the transferred regulatory B cells. Restoration of $\mu MT^{-/-}$ mice with B cells lacking ERα did not produce any clinical benefit, indicating a pivotal role for B-cell-expressed ERα. In contrast, restoration with B cells lacking ERβ but sufficient in ERα produced a similar effect to WT B cells. Interestingly, restoration with B cells lacking both ERα and ERβ or with B cells lacking the membrane GPR30 ER had a partial effect on EAE

Figure 5. 17β-Oestradiol (E_2) -implanted $\mu MT^{-/-}$ recipients of wild-type (WT) B cells have significantly increased percentages of splenic programmed death ligand 1 (PD-L1) -expressing cells, $PD\text{-}1^+$ $CD4^+$ $Foxp3^+$ regulatory T (Treg) cells and $CD1d^{hi}$ $CD5^+$ B cells. On day 15 post-immunization with mouse myelin oligodendrocyte glycoprotein (35–55) /complete Freunds' adjuvant/pertussis toxin, mononuclear cells isolated from spleens of control and E_2-implanted $\mu MT^{-/-}$ mice that received WT B cells and their no cell transfer counterparts were analysed for the presence of (a) B cells ($CD19^+$ cells), (b) total PD-L1-expressing cells, (c) total $Foxp3^+$ $CD4^+$ (Treg) cells, (d) $PD\text{-}1^+$ $CD4^+$ $Foxp3^+$ Treg cells and (e) $CD1d^{hi}$ $CD5^+$ $CD19^+$ B cells. Frequencies of B cells, total PD-L1-expressing cells, Treg cells, PD-1-expressing cells in the Treg population and regulatory B-cell subset ($CD1d^{hi}$ $CD5^+$ $CD19^+$ cells) were determined in individual spleens and data indicate the percentages of total gated live leucocytes (n = 6). Data are pooled from two independent experiments (mean ± SEM). *$P \leq 0{\cdot}05$ compared with sham control $\mu MT^{-/-}$ mice that received WT B cells; # $P \leq 0{\cdot}05$ compared with the control $\mu MT^{-/-}$ mice that did not receive cell transfers and • $P \leq 0{\cdot}05$ compared with E_2-implanted $\mu MT^{-/-}$ mice that did not receive cell transfers (Student's t-test).

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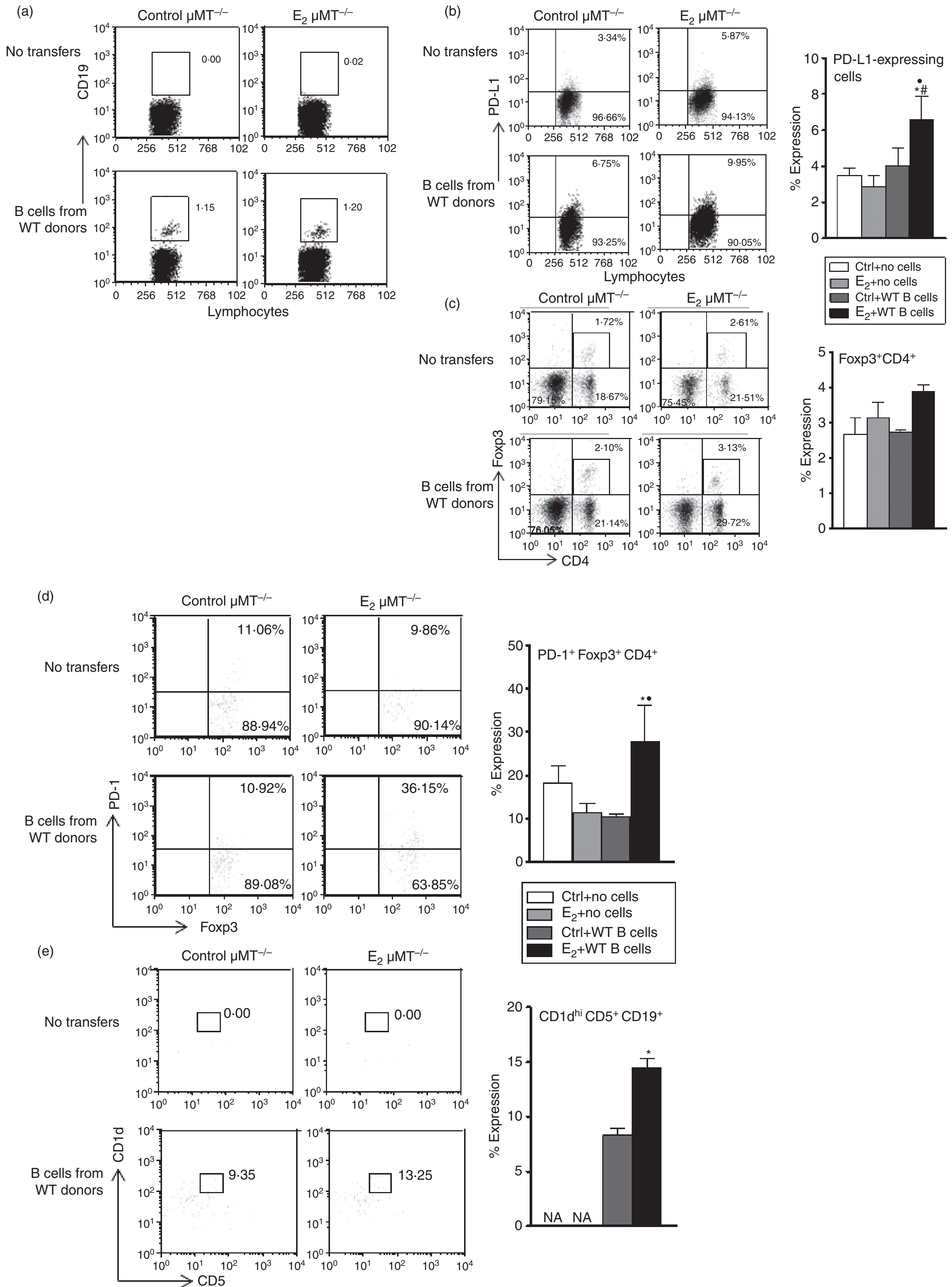
(a)
Control μMT–/–
E2 μMT–/–
No transfers
B cells from WT donors
CD19
Lymphocytes
0·00
0·02
1·15
1·20
(b)
PD-L1
3·34%
96·66%
5·87%
94·13%
6·75%
93·25%
9·95%
90·05%
PD-L1-expressing cells
% Expression
Ctrl+no cells
E2+no cells
Ctrl+WT B cells
E2+WT B cells
(c)
Foxp3
CD4
1·72%
79·15%
18·67%
2·61%
75·45%
21·51%
2·10%
76·05%
21·14%
3·13%
29·72%
Foxp3+CD4+
(d)
PD-1
Foxp3
11·06%
88·94%
9·86%
90·14%
10·92%
89·08%
36·15%
63·85%
PD-1+ Foxp3+ CD4+
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CD1d
CD5
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severity, implicating a role for GPR30 in the E_2 protective mechanism, albeit secondary to that of ERα. These findings put into perspective the crucial importance of ERα and GPR30 on B cells for E_2-dependent protection against EAE.

Our study further defined the downstream effects of transferred MOG-reactive B cells on E_2-dependent immunoregulatory mechanisms involving Treg cells, Breg cells and the PD-1/PD-L co-inhibitory pathway. The transferred WT B cells (i) strongly inhibited infiltration of $CD4^+$ T cells, monocytes/macrophages and dendritic cells into the CNS of recipient mice in the presence of E_2, similar to the pattern observed in E_2-treated WT mice; (ii) strongly increased expression of PD-1 from 11% to 28% in $CD4^+$ $Foxp3^+$ Treg cells in the presence of E_2; (iii) enhanced expression of PD-L1 but not PD-L2 on lymphocytes in both control and B-cell-transferred mice in the presence of E_2; and (iv) produced an expanded population of $CD1d^{high}$ $CD5^+$ regulatory B cells (increased from 9% to 13% of $CD19^+$ B cells) in the E_2-conditioned recipient mice. Although the above E_2-mediated effects were mediated by only 10 million B cells, it is possible that higher numbers of transferred MOG-reactive B cells might provide even more potent and longer-lasting protection against EAE.

These results suggest interactions between PD-L1 present on both the regulatory B-cell[10,16] and T-cell subsets (Fig. 5b) may activate and up-regulate expression of PD-1 on $CD4^+$ $Foxp3^+$ Treg cells (Fig. 5d) in the presence of E_2. Most of the protective effects of oestrogen appear to be mediated through ERα expressed by B cells, but as we have recently published,[12] the crucial up-regulation of PD-1 that potentiates suppressive activity of $CD4^+$ $Foxp3^+$ Treg cells may involve mainly GPR30. This scenario is both plausible and consistent with the idea that Breg cells (i.e. IL-10-producing B cells including the $CD1d^{high}$ $CD5^+$ regulatory B10 cells) have a protective role during EAE initiation, whereas Breg-potentiated Treg cells have a therapeutic role in the later stages after onset of clinical disease.[17]

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Disclosure

The authors have no conflict of interest to declare.

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