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Novel feedback loop between M2 macrophages/microglia and regulatory B cells in estrogen-protected EAE mice

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Abstract

Immunoregulatory sex hormones, including estrogen and estriol, may prevent relapses in multiple sclerosis during pregnancy. Our previous studies have demonstrated that regulatory B cells are crucial for estrogen-mediated protection against experimental autoimmune encephalomyelitis (EAE). Herein, we demonstrate an estrogen-dependent induction of alternatively activated (M2) macrophages/microglia that results in an increased frequency of regulatory B cells in the spinal cord of estrogen treated mice with EAE. We further demonstrate that cultured M2-polarized microglia promote the induction of regulatory B cells. Our study suggests that estrogen neuroprotection induces a regulatory feedback loop between M2 macrophages/microglia and regulatory B cells.

Keywords

Estrogen; EAE; Regulatory B cells; IL-10; M1/2 microglia

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Competing interests

The authors declare that they have no conflict of interest.

Authors' contributions

HO directed the research. GB designed the experiments and wrote the manuscript. JZ, HN, and GK carried out the experiments. AAV critically reviewed the manuscript. All authors critically reviewed data and edited the manuscript.

1. Introduction

Microglia and macrophages play a pathogenic role in multiple sclerosis (MS) (Sospedra and Martin, 2005). They are involved in surveying the tissue environment for damage or infections. In the central nervous system (CNS), microglia - which represent 5–12% of brain cells - have similar functions as macrophages in the periphery (Habib and Beyer, 2015; Tay et al., 2016). Experimental autoimmune encephalomyelitis (EAE), a well-established model of MS, is characterized by extensive lymphocytic infiltration into the CNS. Several reports have demonstrated that monocytes are involved in the exacerbation of EAE, with monocyte depletion resulting in a marked suppression of clinical disease (Bauer et al., 1995; Brosnan et al., 1981; Huitinga et al., 1990). It has been recently suggested that microglial activation may represent an initial step in EAE pathogenesis, wherein infiltrating monocytes may trigger EAE progression (Ajami et al., 2011). Depending on the type of stimulation, activated macrophages/microglia can express different cell surface markers and cytokine patterns, suggesting at least two opposing activation states: Classically activated macrophages (M1), present predominantly in the early stages of EAE, can prime inflammatory T cells and recruit them into the CNS. In contrast, alternatively activated macrophages (M2a, b & c), that appear later in the EAE disease course, inhibit inflammation and promote remyelination by phagocytosis of myelin debris and induction of oligodendrocyte differentiation (Gordon, 2003; Mikita et al., 2011; Miron et al., 2013; Voss et al., 2012).

Like many other autoimmune diseases, MS predominantly affects females. Of interest, pregnant women with MS may show clinical improvement during the third trimester, often followed by temporary post-partum exacerbations (Confavreux et al., 1998; Vukusic et al., 2004). These observations suggest that sex hormones, especially estrogen (E2) and estradiol (E3) that are dramatically increased during late pregnancy, may have immunoregulatory and possibly neuroprotective activities (Bebo et al., 2001; Polanczyk et al., 2006; Wang et al., 2009). We demonstrated previously that E2 (17 β -estradiol) could induce complete protection against EAE in WT C57BL/6 mice (Offner and Polanczyk, 2006; Polanczyk et al., 2003). The E2 protective mechanism involved the induction of CD1d^{hi}CD5⁺ and other IL-10 and IL-4 secreting regulatory B cells (Bregs) (Matsumoto et al., 2014; Matsushita et al., 2008; Ray et al., 2011) and was dependent upon the interaction of programmed death receptor 1 (PD-1) with its ligand, PDL1 (Bodhankar et al., 2013; Bodhankar et al., 2011; Zhang et al., 2015a). We further reported that Bregs could affect M2 microglia polarization by enhancing IL-4 secretion (Bodhankar et al., 2015). However, E2-dependent Breg–microglia interactions during MS/EAE have not yet been fully characterized.

Herein, we demonstrate that E2 can promote the M2 phenotype and inhibit M1 functions both in peripheral macrophages and in CNS microglia/macrophages during EAE. We further show that M2 polarized microglia enhance the induction of IL-10 producing Bregs, thus demonstrating a co-regulatory feedback loop between M2 cells and Bregs.

2. Materials and methods

2.1. Animals

Eight-week old female C57BL/6 wild-type mice were purchased from the Jackson Laboratory. IL-10 transcriptional reporter mice were obtained from Dr. Christopher Karp, Division of Molecular Immunology, University of Cincinnati College of Medicine, Cincinnati, Ohio. The generation and characterization of these mice has been described (Madan et al., 2009). Briefly, the IL10-GFP reporter mice have a floxed neomycin-IRES eGFP cassette (Mohrs et al., 2001) inserted between the endogenous stop site and the poly (A) site of Il10 to help track IL-10 producing cells in vivo. These mice (designated as Vert-X) are homozygous, develop normally and are viable and fertile without any obvious abnormal phenotype. The mice were housed in the Animal Resource Facility at the VAPHCS in accordance with institutional guidelines. This study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals and protocols were approved by the VAPHCS Animal Care and Use Committee.

2.2. Hormone treatment and induction of EAE

Female C57BL/6 wild-type mice were implanted subcutaneously with 2.5 mg/60-day release 17 β -estradiol pellets (Innovative Research of America, Sarasota, FL) or were sham-treated (control) one week prior to subcutaneous immunization at four sites on the flanks with 200 μ g mouse (m)MOG-35-55 peptide (PolyPeptide Laboratories, San Diego, CA) in 400 μ g Complete Freund's adjuvant comprised of Incomplete Freund's adjuvant (IFA, Sigma-Aldrich, St. Louis, MO) containing heat-killed *Mycobacterium tuberculosis* (Mtb, Difco, Detroit, MI). Mice received intraperitoneal injections of pertussis toxin (Ptx, List Biologicals, Campbell, CA) on the day of immunization (75 ng) and 2 days later (200 ng).

All mice were monitored daily for clinical signs of EAE and scored using the following scale: 0 = normal; 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. Mice were scored daily and were evaluated for incidence, day of onset, day of maximal clinical signs (peak) and for total disease score over the course of the experiment (Cumulative Disease Index, CDI). Mean $\pm$ SD were calculated for these parameters for each experimental group.

2.3. Isolation of leukocytes from spleen and spinal cord

Spleens were removed from euthanized animals under sterile conditions and single cell suspensions of leukocytes were prepared by disaggregation of the tissue through a 100 μ m nylon mesh (BD Falcon, Bedford, MA). Cells were washed once with RPMI 1640 supplemented with 10% heat-inactivated FBS (hiFBS; Thermo Scientific, Waltham, MA), then incubated with RBC Lysis Buffer (eBioscience, Inc., San Diego, CA) for 1 min to remove red cells, then washed in RPMI 1640 + hiFBS. Cells were enumerated at a 1:1 dilution with 0.2% trypan blue stain (Life Technologies, Grand Island, NY) using a Cellometer Auto T4 Cell Viability Counter (Nexcelom, Lawrence, MA), washed, and

resuspended at 10^7 cells/ml in stimulation medium (RPMI 1640 containing 10% FBS [Thermo Scientific, Waltham, MA], 1% sodium pyruvate [Life Technologies, Grand Island, NY], 1% L-glutamine [Thermo Scientific, Waltham, MA], and 0.4% β -ME [Sigma-Aldrich, St. Louis, MO]).

Spinal cords were passed through 100 μ m mesh screens and washed as above. Cells were resuspended in 80% Percoll (GE Healthcare, Pittsburgh, PA) then overlaid with 40% Percoll to establish a density gradient and centrifuged at 1600 rpm for 30 min following a previously described method (Campanella et al., 2002). Leukocytes were collected from the resultant interface, counted, and resuspended in stimulation medium for assay.

2.4. Flow cytometry

2.4.1. Antibodies—Leukocytes were labeled with a combination of the following antibodies obtained from BD Bioscience (San Jose, CA): APC CD19 (1D3), PE CD1d (1B1), PerCP CD5 (53–7.3), PerCP-Cy5.5 CD11b (M1/70), PE CD45 (30-F11), APC CD80 (16-10A1), APC LY6C, PE I-A^b (AF6-120.1), PE PDL1 (MIH5), from Ebioscience (San Diego, CA), APC CD206 (C068C2), APC NOS2 (CXNFT) from Biolegend (San Diego, CA) and PE CD9 (MZ3) and PE ARG1 from R&D systems (Minneapolis, MN).

2.4.2. Extracellular staining—Single cell suspensions were washed and resuspended in staining buffer (1 $\times$ PBS, 0.5% BSA [Sigma-Aldrich], 0.1% sodium azide [Sigma-Aldrich]). Fc receptors were blocked with anti-CD16/32 antibody (2.4G2, BD Biosciences) and cells were incubated with monoclonal antibodies (mAbs) listed above. Unbound mAbs were washed with staining buffer prior to four-color fluorescence flow cytometry analysis using a BD Accuri C6 flow cytometer (BD Biosciences).

2.4.3. Intracellular staining— 1×10^6 cells were incubated in 1 ml of stimulation medium (as above) with PMA (50 ng/ml, Sigma-Aldrich), Ionomycin (500 ng/ml, Sigma-Aldrich) and 1 μ l GolgiPlug (BD Biosciences, San Jose, CA, USA) protein transport inhibitor 4 h prior to immunostaining at 37 °C and 5% CO₂. Following incubation, Fc receptors were blocked with anti-CD16/32 monoclonal antibodies (mAb) (2.3G2; BD Biosciences) before cell surface staining. Cells were fixed and permeabilized with Fixation/Permeabilization buffer (BD Biosciences) according to the manufacturer's instructions. Permeabilized cells were washed with 1 $\times$ Perm/Wash Buffer (BD Biosciences) and were stained with anti-NOS2, anti-ARG1 and anti-CD206. Isotype-matched mAb served as negative controls to demonstrate specificity and to establish a background for the levels of NOS2 α , ARG1 or CD206 expression. All data were acquired and analyzed using the Accuri C6 (BD Biosciences) software included with the instrument.

2.5. RNA isolation and reverse transcription-polymerase chain reaction

Total RNA from sorted B cells and brains were isolated using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). cDNA was synthesized using the SuperScript II Reverse Transcriptase cDNA synthesis kit (Life Technologies, Grand Island, NY). Quantitative real time PCR was performed using the StepOne Plus Real-Time PCR System with TaqMan Gene Expression Array Plates for mouse immune response.

cDNA from three mice per group was quantified for gene array plates. Individual samples were analyzed in triplicate for Arg-1, CD206 and NOS2 (Life Technologies, Grand Island, NY). The GAPDH housekeeping gene was used as an endogenous control. Results were analyzed using Expression Suite Software (Life Technologies, Grand Island, NY).

2.6. B cell sorting

B cells were acquired from female C57BL/6 or IL-10 GFP mice. Splenic CD19⁺ B cells were purified using paramagnetic bead-conjugated antibodies (Abs) from the CD19 cell isolation kit and subsequently separated by AutoMACS (both from Miltenyi Biotec, Auburn, CA). The positive fraction of the cells thus separated were CD19⁺ B cells with a purity of 95%.

2.7. Primary CNS culture and purification of microglia

2.7.1. Tissue culture and enrichment for microglia—Mice were deeply anesthetized using 4% isoflurane inhaled anesthetic and maintained at 2.5% isoflurane during intracardiac perfusion with cold phosphate buffered saline (PBS, Fisher Scientific, Fair Lawn, NJ, USA). Brains were removed aseptically, olfactory bulbs and brain stem were removed and the remaining tissue washed twice in cold Hank's balanced salt solution lacking Mg²⁺, Ca²⁺ and phenol red (HBSS, Life Technologies, Grand Island, NY, USA).

A unicellular suspension of CNS tissue was obtained using the protocol and reagents supplied in the Neural Tissue Dissociation Kit P from Miltenyi Biotec (Auburn, CA, USA) with some modifications (Moussaud and Draheim, 2010). Briefly, tissues were disrupted mechanically on a gentleMACS tissue dissociator (Miltenyi Biotec) and incubated with the enzyme cocktails included with the kit. Following trituration using a P1000 pipette, the homogenate was passed through a 100 µm nylon mesh screen (Fisher Scientific) and washed in HBSS to quell enzymatic activity.

After disaggregation, total brain cells were prepared for culture based on a published method (Moussaud and Draheim, 2010). Myelin was removed by density centrifugation: 20% Percoll (GE Healthcare, Pittsburg, PA, USA) was overlaid with cold HBSS for 20 min at 200 ×g with no brake and then washed once in cold HBSS. Pelleted cells were resuspended in warmed Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 with Glutamax supplement (DMEM, Life Technologies) culture medium completed with 10% heat inactivated fetal bovine serum (FBS, GE Healthcare), 1% antibiotic-antimycotic (anti-anti, Life Technologies) and 5 ng/ml carrier-free recombinant mouse granulocyte macrophage colony-stimulating factor (GM-CSF, Life Technologies) and counted. 1.5×10⁶ cells were seeded into tissue culture grade poly L-lysine (L-lysine, Sigma-Aldrich, St. Louis, MO, USA) coated T75 cell culture treated flasks (Corning Life Sciences, Tewksbury, MA, USA) and placed in a 37 °C incubator with 5% CO₂ and 85% relative humidity. Culture supernatant was replaced twice weekly with 10 ml fresh completed medium until confluency of cells was observed, at approximately three weeks.

2.7.2. Harvest of microglia from enriched cultures—Upon confluence, loosely adherent cells were dislodged from culture flasks by agitation using an orbital shaker

(Benchmark, Edison, NJ, USA). Flasks were shaken at 260 rpm for 4 h and supernatants were harvested and centrifuged. Before the start of the in vitro co-culture experiments, cells were resuspended in culture medium free of GM-CSF, counted, and seeded into 24 well plates at a density of 2×10^5 cells per well and incubated at 37 °C and 5% CO₂ for 5 days prior to assay. Remaining cells were evaluated for purity and their phenotype was determined by flow cytometry. CD11b⁺CD45⁺ microglia comprised 95% of cells harvested in this manner.

2.8. BV2 cell culture

The murine microglial cell line BV2 cells were cultured in Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin and streptomycin). Cultures were incubated at 37 °C in 5% CO₂. Twenty four hours before stimulation, microglial cells were washed using phosphate-buffered saline and serum-free medium was added.

2.9. Cell stimulation and B cell-microglia co-culture

Primary microglial and BV2 line cells were treated with 1 nM or 1 mM ethanol soluble estrogen (Sigma-Aldrich) and stimulated by LPS (10 ng/ml) (Sigma-Aldrich) or by recombinant IL-4 (20 ng/ml) (Peprotech, Rocky Hill, NJ) for 24 h at 37 °C in 5% CO₂. After stimulation microglial cells were washed using phosphate-buffered saline and B cells were added in 1 ml of DMEM containing 10% FBS at a ratio of 1:1 (to primary microglia) or $1:1 \times 10^6$ cells (to BV2 cells). Cells were incubated for 48 h at 37 °C in 5% CO₂.

2.10. Statistical analysis

Data were reported using GraphPad Prism (v5.0, San Diego, CA) and expressed as the mean $\pm$ SD. Statistical significance for the disease course was calculated using the Mann-Whitney *U* test. Statistical significance for flow cytometry and real-time PCR data was calculated using Student's *t*-test or one way ANOVA with Tukey correction. *p*-Values ≤ 0.05 were considered to be significant (**p* ≤ 0.05 ; ***p* ≤ 0.01 ; ****p* ≤ 0.001).

3. Results

3.1. E2 treatment of EAE mice induces M2 phenotype in the CNS

We recently demonstrated that microglia cultures from E2 treated mice express higher levels of M2 markers compared to sham treated mice (Benedek et al., 2016). In order to further evaluate E2 effects on M1/M2 balance in EAE, female C57BL/6 WT mice were either sham treated or implanted with E2 pellets 7 days prior to immunization with mMOG-35-55 peptide/CFA/Ptx. As previously reported E2 implants induced complete protection against EAE in WT mice (Fig. 1A) and E2 treated mice had significantly less activated CD11b⁺CD45^{hi} cells in spinal cord compared to sham treated mice ($p < 0.001$) (Fig. 1B) on day 21 post-immunization (p.i). Analysis of M2a and M2c markers on CD11b⁺CD45^{hi} cells in spinal cord revealed a significant increase in Arg1⁺ and CD206⁺ expression levels ($p < 0.05$) (Fig. 1C & D). We further confirmed that the frequency of CD1d^{hi}CD5⁺ Bregs was significantly higher in the E2 treated mice, although the frequency of total B cells was significantly reduced in these mice ($p < 0.05$) (Supplementary Fig. 1). These data

demonstrate that estrogen protection against EAE results in an anti-inflammatory cellular phenotype in the CNS with increased frequencies of M2 macrophage/microglia and Bregs.

3.2. E2 treatment of EAE mice induces M2 phenotype in the periphery

We sought to further evaluate whether E2 treatment affects macrophage polarization in the periphery. We assessed M1 and M2 markers on CD11b⁺ cells in spleens of E2- or sham-treated mice on day 21 p.i. As demonstrated in Fig. 2A and C, CD11b⁺ cells in E2 mice expressed significantly lower levels of the M1 inflammatory marker, iNOS ($p < 0.05$) and a lower frequency of CD11b⁺LY6C^{hi} ($p < 0.05$) compared to sham treated mice. Interestingly, CD11b⁺ cells from E2 treated mice expressed higher levels of CD80 and MHC class II on their cell surface ($p < 0.001$ and $p < 0.05$, respectively) (Fig. 2B&D).

In addition, we observed significantly higher expression levels of M2 markers such as Arg1⁺ and CD206⁺ on peripheral CD11b⁺ cells from E2 treated mice compared to sham treated mice ($p < 0.05$) (Fig. 3A & B). CD11b⁺ cells from E2 treated mice also expressed significantly higher levels of PDL-1⁺ ($p < 0.05$) (Fig. 3C), not considered as a classical M2 marker. Furthermore, the frequency of CD11c⁺ CD103^{hi} dendritic cells, which were shown to induce regulatory T cells (Coombes et al., 2007), was also significantly higher in the spleen of E2 treated mice ($p < 0.05$) (Fig. 3D). Taken together, our data demonstrate that estrogen treatment can inhibit M1 peripheral polarization during EAE and promote M2 polarization and expression of other regulatory markers.

3.3. In-vitro treatment with E2 inhibits M1 polarization of microglial cells

The M2 phenotype in the CNS of E2 treated mice could be induced by estrogen that crosses the blood brain barrier and/or could be mediated by regulatory cells, such as Bregs that migrate into the CNS. In order to evaluate whether E2 directly affects cell polarization, we treated BV2 cells (a murine microglial cell line) with two concentrations of soluble E2 (1 nM or 1 mM) for 30 min or 6 h and measured the expression of iNOS, Arg1 and CD206 by real time PCR. As demonstrated in Fig. 4A, in-vitro treatment with soluble estrogen did not induce either an M1 or an M2 phenotype.

In order to evaluate whether E2 inhibits M1 polarization, BV2 cells were pretreated with 1 nM of soluble E2 for 10 min before stimulation with LPS for 30 min. While LPS treatment reduced the expression of CD206 and increased the expression of iNOS relative to controls ($p < 0.01$ and $p < 0.05$, respectively), the expression of these markers was restored to control levels when cells were pretreated with estrogen ($p < 0.05$ for both). Interestingly, in this experiment LPS stimulation nominally increased the expression of Arg1 in BV2 cells, although this change was not significant (Fig. 4B). Our data demonstrate that while in vitro estrogen does not induce an M2 phenotype in the BV2 microglial cell line, it does inhibit M1 polarization.

3.4. M2 microglia promote IL-10 producing B cells

We previously demonstrated that IL-10 producing Bregs promote an M2 phenotype in microglia (Bodhankar et al., 2015). However, the effects of M2 microglia on B cell phenotype have not been explored. To this end, we treated BV2 cells with either IL-4 or LPS

for 24 h to induce M2 or M1 microglia, respectively. M1 and M2 phenotypes were confirmed by gene expression analysis in which IL-4 treated cells expressed increased levels of Arg1 and LPS treated cells expressed increased levels of iNOS (Supplementary Fig. 2). To evaluate the effect of M1 or M2 microglia on IL-10 production from B cells, polarized BV2 cells were washed to remove residual IL-4 or LPS and co-cultured with isolated B cells from naïve IL-10 GFP female mice for 48 h in fresh medium. As demonstrated in Fig. 5A, M2 polarized cells significantly increased the frequency of CD19⁺CD9⁺IL-10⁺ cells compared to untreated (by about 33%; from 18% to 24%, respectively, $P < 0.01$) or LPS treated M1 BV2 cells (by about 50%; from 16% to 24%, respectively, $P < 0.01$), whereas the M1 polarized BV2 cells had nominally lower percentages of these cells compared to untreated control cells.

To further evaluate the microglia effect on B cells, we isolated and cultured primary microglia cells from EAE WT C57BL/6 mice (day 21 post-immunization), as described before (Benedek et al., 2016). Primary microglia were polarized with IL-4 or LPS for 24 h and co-cultured with isolated B cells from EAE WT C57BL/6 mice (day 21 post-immunization) for 48 h. Evaluation of Breg markers demonstrated a significant increase in the frequency of CD19⁺CD1d^{hi}CD5⁺ cells after co-culturing with IL-4 treated M2 microglia compared to B cells that were cultured with untreated (by about 45%; from 4.7% to 6.8%, respectively, $P < 0.05$) or LPS treated (by more than 100%; 3.3% to 6.8%, respectively, $P < 0.01$) microglia ($p < 0.05$ and $p < 0.01$, respectively, Fig. 5B). Moreover, the frequency of CD19⁺CD1d^{hi}CD5⁺ cells after co-culturing with M1 microglia was again nominally lower than that induced by control microglia. Taken together, our data demonstrate that M2 microglia enhance the frequency of IL-10⁺ Bregs, whereas M1 microglia do not.

4. Discussion

Sex hormones such as E2 and estriol have immunoregulatory activity and may prevent relapses in multiple sclerosis subjects during pregnancy. We and others demonstrated previously that E2 protection against EAE is mediated by T and B regulatory cells (Baker et al., 2004, Bebo et al., 2001, Bodhankar et al., 2011, Lelu et al., 2010, Offner and Polanczyk, 2006, Polanczyk et al., 2004, Polanczyk et al., 2003, Polanczyk et al., 2006, Subramanian et al., 2011). Recently, we demonstrated that E2 mediated protection against EAE is dependent on the presence of PDL1 expressing B cells (Bodhankar et al., 2013, Zhang et al., 2015b). We further showed that E2 treatment induced various Breg subtypes, including IL-10 producing cells expressing CD19⁺CD5⁺CD1d^{hi}, CD19⁺IRF-4⁺ and CD19⁺Tim-1⁺ markers, as well as CD19⁺CD138⁺CD44^{hi} and CD19⁺PDL1^{hi} cells (Benedek et al., 2016). In the current study we demonstrate that E2 treatment enhances the frequency of M2 macrophages and microglia in the CNS and in the periphery. We further demonstrate for the first time that M2 microglia can reciprocally enhance the frequency of IL-10 producing Breg cells.

Classically activated macrophages can prime T cells and recruit them into the CNS. These cells are present predominantly in the early stages of EAE. In contrast, alternatively activated macrophages that express high levels of CD206, CD163 and Arg-1 (Gordon, 2003, Mikita et al., 2011, Miron et al., 2013, Miron and Franklin, 2014) appear in the later stages of EAE and can inhibit inflammation and promote remyelination by phagocytosis of myelin

debris (Mikita et al., 2011, Miron et al., 2013, Voss et al., 2012). We have previously reported that isolated primary microglia from E2 treated mice express higher levels of M2 markers compared to sham treated mice (Benedek et al., 2016). Herein, we demonstrate that although the frequency of CD11b⁺CD45^{hi} cells in the CNS of E2-treated mice was significantly reduced compared to sham treated mice on day 21 post-disease induction, these cells from E2 treated mice expressed higher levels of M2 markers (CD206⁺ and Arg1⁺). This effect of E2 was observed also in spleens of E2 treated mice, in which CD11b⁺ cells exhibited significantly higher levels of M2 markers and lower expression levels of M1 pro-inflammatory genes such as iNOS. It is important to note that the frequency of CD80⁺ and MHC class II was up-regulated on CD11b⁺ cells from E2 treated mice compared to sham treated mice. This effect could be associated with increased expression of IFN- γ from B cells as we reported previously (Benedek et al., 2016). Thus, similar to B cells in E2 treated mice, the up regulation of PDL1, CD80 and MHC class II on CD11b⁺ cells might be mediated by IFN- γ (Nakaya et al., 2006). We also report that the frequency of CD11c⁺CD103^{hi} cells that were shown to induce regulatory T cells (Coombes et al., 2007), was significantly upregulated in E2 treated mice, consistent with our previous reports of E2-induced increases in CD4⁺FoxP3⁺ Tregs (Polanczyk et al., 2005). Women with MS often show clinical improvement during pregnancy, followed by temporary post-partum exacerbations (Confavreux et al., 1998, Vukusic et al., 2004). It was demonstrated that the M1/M2 balance of decidual macrophages changed during the progression of a normal pregnancy. As reviewed by Brown et al. and Nagamatsu et al., during the pre-implantation period, decidual macrophages are polarized toward an M1 phenotype. As the pregnancy progresses during the first trimester, decidual macrophages transition into a mixed M1/M2 population. After placental development, decidual macrophages are skewed toward an M2 phenotype until parturition (Brown et al., 2014, Nagamatsu and Schust, 2010). Furthermore, the increased expression of PDL1 on CD11b⁺ cells (and on CD19⁺ cells) is also in agreement with the important role of the PD1-PDL1 pathway in pregnancy-specific immune tolerance (Guleria et al., 2005, Petroff et al., 2003). In addition, it was shown that the frequency of IL-10-producing B cells (B10) increases during normal pregnancy (Fettke et al., 2014, Jensen et al., 2013). Hence, these findings suggest that the anti-inflammatory M2 phenotype is positively associated with estrogen levels.

The effect of sex hormones on microglia activation was studied extensively (Baker et al., 2004, Drew et al., 2003, Habib and Beyer, 2015, Villa et al., 2016). Interestingly, it was suggested that early stage treatment of EAE with estrogen receptor α (ER α , ESR1) ligands have an anti-inflammatory protective effect (Spence and Voskuhl, 2012), whereas estrogen receptor β (ER β , ESR2) ligands could protect against EAE in a later stage of the disease by affecting microglia and dendritic cells (Itoh et al., 2016).

Our in vivo EAE data clearly demonstrated that E2 treatment enhanced an M2 phenotype in macrophages and microglia compared to sham treated mice. In vitro treatment of the BV2 microglial cell line with soluble estrogen did not induce an M2 phenotype, but rather reversed and limited the M1 phenotype induced by LPS. These results are similar to the work of Toniolo et al. which reported that in vitro treatment with estrogen rescued the M2 phenotype after M1 polarization, but did not increase it over baseline expression (Toniolo et al., 2015). In addition, it was previously reported that the dampening of LPS-stimulated NO

production in microglia by estrogen was mediated through estrogen receptor β (ER β) (Baker et al., 2004). This receptor was also found to negatively regulate microglia secretion of proinflammatory factors in EAE (Saijo et al., 2011). Thus, it seems plausible that the in vivo M2 polarization of macrophage and microglia is not directly induced by estrogen.

Although the M1/M2 activation state in microglia is controversial (Ransohoff, 2016), it is widely accepted that microglial cell subtypes can promote either demyelination or remyelination (Miron and Franklin, 2014). As the effects of Bregs on macrophage/microglial polarization have advanced, the reciprocal effect of polarized microglia on Bregs has not yet been fully explored. We have previously demonstrated that LPS stimulated microglia isolated from naïve mice or microglia isolated from middle cerebral artery occlusion (MCAO) mice significantly reduced the expression of pro-inflammatory factors in the presence of Bregs (Bodhankar et al., 2015). This effect was more pronounced in female compared to male mice. Furthermore, IL-10 secreting B cells increased the expression of CD206 on microglia post-MCAO by cell-cell interactions. It is thus possible that while estrogen inhibits M1 polarization of microglia, other regulatory cells such as Bregs induce the M2 polarization. In addition, Wong et al., reported that B1 cells could inhibit M1 polarization of macrophages and promote M2 polarization (Wong et al., 2010).

While estrogen treatment was shown to induce regulatory T cells, its protective effect during EAE is mediated and dependent on B cells (Bodhankar et al., 2011). Hence, we focused again at B cell – M2 cell interactions. The frequency of Bregs in the CNS of estrogen treated mice increased compared to sham treated mice, while the frequency of total B cells was reduced in E2 treated mice. Herein, we demonstrate for the first time that M2 polarized microglia increased the frequency of IL-10 secreting Bregs, whereas M1 polarized microglia had no significant effect. Of note, we also identified IL-10⁺ B cells as CD9⁺ cells as described by Sun (Sun et al., 2015). These data demonstrate a novel feedback loop between M2 microglia and Bregs, suggesting that during EAE estrogen might induce M2 macrophage - Breg interactions in the periphery and that the increased frequency of Bregs in the CNS might be the result of Breg migration into the CNS and/or conversion of effector B cells to IL-10 secreting Bregs by M2 microglia. Furthermore, it is possible that in sham treated mice inflammatory M1 microglia might suppress IL-10 production from Bregs. Additional studies are required in order to evaluate the molecular mechanism of this feedback loop. As induction of M2 cells by B cells is contact dependent, the reciprocal cellular effect could be also dependent on the same receptor-ligand interaction, such as PD1-PDL1. However, we cannot exclude the possibility that Bregs are induced by anti-inflammatory factors that are secreted by the M2 cells, which conceivably might also affect the vascular endothelium and stabilize the blood-brain barrier. Recently, it was shown that the anti-inflammatory cytokine, IL-35, could induce the development of Bregs (Wang et al., 2014). Furthermore, it would be interesting to determine whether M2 cells could induce regulatory T cells as well.

5. Conclusions

In summary, our present study demonstrates that estrogen treatment protects against EAE by promoting M2 polarization of macrophage/microglia and these cells enhance the frequency

of Bregs. Taken together with our previous report, our observation highlights a novel regulatory feedback loop between regulatory B cells and M2 macrophage/microglia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jneuroim.2016.12.018>.

Fig. 1.

Elevated expression of M2 markers on CD11b⁺ cells in spinal cord of sham- and E2-treated female C57BL/6 mice with EAE. A) 8 week old female C57BL/6 mice were implanted with sham or E2 pellets one week prior to EAE induction by injection of mouse (m)MOG-35–55 peptide/CFA/Ptx. Daily mean scores were analyzed by Mann Whitney *U* test. **p* < 0.05. B) Frequency of CD11b⁺CD45^{hi} cells in spinal cord on Day 21 (*n* = 12 each group). Mean values ± SD ****p* < 0.001, Student's *t*-test. Right panels: representative dot-plots. C&D) Arg1⁺ and CD206⁺ intracellular expression of CD11b⁺CD45^{hi} cells in spinal cord (left panels). Mean values ± SD. **p* < 0.05, Student's *t*-test. Representative histograms of mean fluorescence intensity (MFI)(right panels).

Fig. 2. Reduced expression of M1 markers on CD11b⁺ cells in spleen of sham- and E2-treated female C57BL/6 mice with EAE (Day 21). Expression levels of M1 markers on CD11b⁺ cells ($n = 12$ each group). A & B) iNOS⁺ intracellular expression and CD80⁺ cell surface expression, mean fluorescence intensity (MFI). Right panels: Representative histograms and mean values $\pm$ SD. C&D) LY6C⁺ and I-A/I-E⁺ frequencies. Mean values $\pm$ SD. * $p < 0.05$, *** $p < 0.001$, Student's t -test.

Fig. 3.

Elevated expression of M2 markers on CD11b⁺ cells in spleens of sham- and E2-treated female C57BL/6 mice with EAE (Day 21). Expression levels of M2 markers on CD11b⁺ cells ($n = 12$ each group). A & B) Arg1⁺ and CD206⁺ intracellular expression, mean fluorescence intensity (MFI). Right panels: Representative histograms and mean values $\pm$ SD. C) PDL1 frequency. Mean values $\pm$ SD. D) CD11c⁺CD103^{hi} frequency. Mean values $\pm$ SD. * $p < 0.05$, *** $p < 0.001$, Student's t -test.

Fig. 4.

In-vitro E2 treatment inhibits M1 polarization of microglial cells. A) BV2 microglia line cells were treated with ethanol soluble E2 (1 nM or 1 mM) for 30 min or for 6 h. Relative expression of mRNA of CD206, Arg-1, and iNOS was analyzed by real-time PCR ($n = 3$ each group). B) BV2 microglia line cells were treated with 10 ng/ml LPS and 1 nM ethanol soluble E2 for 30 min. Relative expression of mRNA of CD206, Arg-1, and iNOS relative to GAPDH was analyzed by real-time PCR ($n = 3$ for each group). Mean values $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, one way ANOVA followed by Tukey post hoc test.

Fig. 5.

M2 but not M1 microglia promote IL-10 producing B cells. A) BV2 line cells were polarized to an M1 or M2 phenotype with 10 ng/ml LPS or 20 ng/ml rIL-4 treatment for 24 h. Polarized microglia were co-cultured for 24 h at 37 °C with isolated B cells from naïve IL-10 GFP female mice and IL-10⁺ frequency was assessed on CD19⁺CD9⁺ cells ($n = 4$ for each group). B) Primary microglia isolated from female C57BL/6 mice with EAE were polarized to an M1 or M2 phenotype by 10 ng/ml LPS or 20 ng/ml rIL-4 treatment for 24 h. Polarized microglia were subsequently co-cultured for 24 h at 37 °C with isolated B cells from female C57BL/6 mice with EAE and CD1d^{hi}CD5⁺ frequency was assessed on CD19⁺CD9⁺ cells ($n = 4$ for each group). Mean values $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, one way ANOVA followed by Tukey post hoc test.