

# PD-L1 is Required for Estrogen-induced Protection Against Severe EAE in IL-10 Deficient Mice

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## Research Article

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# Abstract

## Background

IL-10 knockout (KO) mice can be protected against experimental autoimmune encephalomyelitis (EAE) with low-dose estrogen (E2) treatment similar to wild type (WT) mice, indicating that IL-10 is not required for E2-induced EAE protection. Our previous study demonstrated that E2 treatment induced an increase in programmed death ligands 1 (PD-L1) and 2 (PD-L2) on monocytes and macrophages in the periphery and within the CNS. In this study, we selectively inhibited the function of PD-L1 and PD-L2 to evaluate their critical role in maintaining E2-induced protection against EAE in IL-10-KO mice.

## Methods

This study used female IL-10 KO mice pre-treated with either E2 or sham pellets seven days prior to induction of EAE and subsequently treated with Vehicle or antibodies to PD-L1, PD-L2 or respective isotype controls. Mice were scored daily for EAE severity over 21 days post-immunization for EAE induction. Cells from the spleen and brain were evaluated by flow cytometry.

## Results

Differences in EAE severity were assessed in E2 and sham pre-treated IL-10-KO mice treated with  $\alpha$ -PD-L1 or  $\alpha$ -PD-L2 antibodies over the course of disease compared to treatment with Vehicle or anti-isotype antibodies. The results revealed real-time development of severe EAE in E2-pre-treated IL-10-KO mice treated with  $\alpha$ -PD-L1 but not  $\alpha$ -PD-L2 antibodies, mediated in part by increased percentages of activated CD74<sup>+</sup>CD11b<sup>+</sup> myeloid cells in spleen and brain as well as splenic B-cells, T-cells and CD73<sup>+</sup> cells.

## Conclusion

These results demonstrate unequivocally that PD-L1 but not PD-L2 was required to retain the inhibitory effects of E2 on clinical EAE scores in female IL-10-KO mice and further implicate the emergence of the MIF/CD74 axis as a contributing pathogenic mechanism.

## Background

Multiple sclerosis (MS) is a devastating progressive demyelinating disease of the CNS that predominantly affects females (Ben-Nun et al. 2014). Women with MS often experience clinical remissions during pregnancy, followed by relapses post-partum (Abramsky 1994; Confavreux et al. 1998; Whitacre 2001). These are striking clinical events that may be related to hormone levels, particularly 17 $\beta$ -estradiol (estrogen = E2). Estrogen can modulate both innate and adaptive immunity (Khan and Ansar

Ahmed 2015) and in MS, levels of E2 and progesterone are inversely associated with circulating levels of tumor necrosis factor (TNF) and interferon gamma (IFN $\gamma$ ) (Trenova et al. 2013). Treatment with low doses of E2 in an MS animal model, experimental autoimmune encephalomyelitis (EAE), can prevent disease development (Bebo et al. 2001; Ito et al. 2001) and a medium dose is sufficient to protect ovariectomized mice from developing EAE (Haghmorad et al. 2016). E2 does not directly engage encephalitogenic T-cells (Polanczyk et al. 2004), but blocks EAE indirectly by upregulating expression of the PD-1/PD-L1/2 checkpoint inhibitor pathway (Polanczyk et al. 2006) through estrogen receptor alpha (ER $\alpha$ ) (Polanczyk et al. 2003; Morales et al. 2006) on antigen presenting cells (APC), including macrophages, B-cells, and dendritic cells.

Although B-cells were initially thought to play a pathogenic role in EAE (Wolf et al. 1996; Cross et al. 2001; Du and Sriram 2002; Fillatreau et al. 2002), studies examining B-cell function revealed a protective role for B-cells in reducing EAE progression. B-cell deficient ( $\mu$ MT $^{-/-}$ ) or B-cell depleted (anti-CD20 antibody) mice develop a more severe form of EAE compared to WT or naïve mice (Matsushita et al. 2008; Zhang et al. 2015b). Subsequent studies revealed a small yet potent population of regulatory B-cells (Breg), called B10 cells, that can protect mice from EAE (Fillatreau et al. 2002; Zhang et al. 2015a, b; Benedek et al. 2016, 2017) in part through secretion of IL-10 (Matsushita et al. 2010). IL-10 is a potent anti-inflammatory cytokine produced by several immune cell types that can influence the differentiation of T helper (Th) cells, inhibit Th $_1$  and Th $_{17}$  cells, inhibit major histocompatibility complex (MHC) class II expression and increase antibody production by B-cells (Saxena et al. 2015). EAE is more severe in IL-10 knockout (KO) mice compared to wild type (WT) mice, whereas mice that overexpress IL-10 are protected from developing EAE (Bettelli et al. 1998; Cua et al. 1999). Additionally, Breg cells can induce IL-10 producing Treg cells *in vivo* that help to protect mice from active EAE.

E2 pre-treatment protects WT mice from developing EAE, in part by inducing a variety of Bregs, including B10, that have increased expression of PD-L1 and PD-L2 (Zhang et al. 2015a). Of interest, PD-1 KO mice were not protected from EAE by treatment with E2, suggesting one or both of its ligands may be critical for E2-mediated protection (Polanczyk et al. 2007). Further studies demonstrated that unlike PD-L2 KO mice that were protected from EAE after E2 treatment, PD-L1 KO mice developed severe EAE. This suggests PD-L1 is the predominant PD-1 ligand involved in E2 protection during EAE in WT mice (Bodhankar et al. 2013). But IL-10 secreting Bregs may also contribute to E2 protection from EAE (Zhang et al. 2015b), although IL-10 itself may not be critical since IL-10 KO mice remain protected from EAE after E2 pre-treatment.

Our previous study revealed that E2 treatment of IL-10 KO mice with EAE significantly increased activity in the PD-L1/2 pathway, increased CD73 expression and showed increases in the percentage of CD4 $^+$ CD25 $^+$ FoxP3 $^+$  Treg cells in the CNS. Of these possibilities, we believe that the previous data best support the emergence of PD-L1 as the dominant compensatory component that retains E2 protection against EAE in IL-10 KO mice. Using antibodies specific for PD-L1, PD-L2 and respective isotype controls as well as Vehicle to treat E2 primed IL-10-KO mice, we here determined that neutralization of PD-L1 but

not PD-L2 fully abrogated the protective E2 effect, thus demonstrating conclusively that PD-L1 is necessary and sufficient to retain E2-mediated protection in the absence of IL-10.

## Methods

### Animals:

Female wild type C57BL/6 mice and female IL-10 KO (B6.129P2-*Il10*<sup>tm1Cgn</sup>/J) mice (8–10 weeks old) were purchased from The Jackson Laboratory (Sacramento, CA). Mice were housed together by strain and treatment group. Mice were given access to food and water *ad libitum* and kept on a 12h light/dark cycle. This study was conducted in accordance with NIH guidelines for the use of experimental animals and the VAPORHCS Animal Care and Use Committee approved protocols.

### E2 pellet implantation and induction of EAE:

All mice were treated and immunized as previously described (Seifert et al. 2019). Briefly, mice were implanted subcutaneously with 2.5mg/60-day release 17 $\beta$ -estradiol pellets (Innovative Research of America, Sarasota, FL) or sham treated one week prior to immunization. The 2.5mg E2 pellet produces 1,500-2,000pg/ml of E2 in the serum, which approaches pregnancy serum levels of E2 (5,000–10,000pg/ml) that is EAE protective (Bebo et al. 2001). Mice were then immunized with 200 $\mu$ g mouse MOG-35-55 peptide (PolyPeptide Laboratories, San Diego, CA) in 200 $\mu$ g Complete Freund's adjuvant [Incomplete Freund's adjuvant (IFA, Sigma-Aldrich, St. Louis, MO) with heat-killed *Mycobacterium tuberculosis* (Mtb, Difco, Detroit, MI)] subcutaneously along the flanks at four sites. Additionally, mice were administered 75ng of pertussis toxin (Ptx, List Biologicals, Campbell, CA) via an intraperitoneal (i.p.) injection on the day of immunization and 200ng i.p. two days later. All mice were monitored daily for weight loss and clinical signs of EAE disease. Mice were 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; 6 = paraplegia with severe forelimb weakness or severe ataxia or moribund condition. The cumulative disease index (CDI) is the sum of the daily scores for each mouse from day 8 to day 21 post-immunization.

### Leukocyte preparation from the spleen and brain:

All tissues were collected from mice 21 days post-immunization. Spleens were passed through a 100 $\mu$ m nylon mesh filter (BD Falcon, Bedford, MA) into RPMI 1640 to create a single cell suspension. Red cells were lysed with 1x red cell lysis buffer (eBioscience, Inc., San Diego, CA) and the cell suspension subsequently washed with RPMI 1640. Cells were counted using a hemocytometer. After counting, cells were centrifuged and resuspended in staining buffer (PBS with 0.1% NaN<sub>3</sub> and 1% BSA) for staining.

Brains were passed through 100 $\mu$ m mesh screens and washed as stated above. Cells were resuspended in 80% Percoll (GE Healthcare, Pittsburgh, PA) then overlaid with 40% Percoll to establish a density

gradient and centrifuged at 1,600 rpm for 30 min following a previously described protocol (Campanella et al. 2002). Leukocytes were collected from the resultant interface, counted, and resuspended in staining buffer for staining.

## Flow cytometry:

Cells were resuspended at a concentration of  $1 \times 10^6$  cells/ml in staining buffer. All cells were stained for extracellular markers after being blocked with rat anti-mouse CD16/CD32 Mouse BD Fc Block™ (BD Bioscience, San Jose, CA). After blocking, cells were incubated with fluorescently tagged antibodies and protected from light. All samples were then run on a BD Accuri™ C6 (BD Bioscience) with a four color (FITC, PE, PerCP Cy5.5, and APC) fluorescence flow cytometry analysis.

The following antibodies were used: CD11b (M1/70), CD19 (1D3), CD4 (RM 4–5), CD45 (30-F11), CD74 (In-1) (BD Pharmingen, Franklin Lakes, NJ), CD73 (TY/11.8) (Biolegend, San Diego, CA), PD-L1 (BE0101), IgG2b isotype control for PD-L1 (rat anti-keyhole limpet hemanocyanin, BE0090), PD-L2 (BE0112) and IgG2a isotype control for anti-PD-L2 (rat anti-trinitrophenol, BE0089) (Bio X Cell, Inc., Lebanon, NH).

## Statistics:

Data were analyzed using Prism software (GraphPad Software, La Jolla, CA) using the Mann-Whitney U test for determining significance for disease course. All other data were analyzed using ANOVA with a Fisher's Least Significant Difference post-hoc test or Student's t-test when appropriate. A p value of  $\leq 0.05$  was considered significant. Data are represented as the mean  $\pm$  standard error of the mean (SEM) and figures were created with Prism. All analyses were carried out in blinded fashion.

## Results

*PD-L1 but not PD-L2 is required for E2-induced protection against severe EAE in IL-10 deficient mice.*

Initial studies were carried out in WT and IL-10-KO female mice receiving E2 pellets seven days prior to the induction of EAE and further treatments on days 1, 7 & 14 post-EAE induction with Vehicle (Veh) or antibodies to PD-L1 ( $\alpha$ -PD-L1), PD-L2 ( $\alpha$ -PD-L2) or their respective isotype controls ( $\alpha$ -ISO). Mice were scored for 21 days post-immunization to determine if neutralization of PD-L1 or PD-L2 abrogated E2-induced protection against EAE in IL-10-KO vs. WT mice. As is shown in **Fig. 1a**, treatment of E2-primed *WT mice* with Veh,  $\alpha$ -PD-L1 or  $\alpha$ -ISO resulted in near complete protection of all treatment groups over the 21-day scoring period. In contrast, treatment of E2-primed *IL-10-KO mice* with Veh or  $\alpha$ -ISO resulted in moderate to low clinical signs of EAE (consistent with lack of IL-10 mediated suppression), whereas treatment with  $\alpha$ -PD-L1 rapidly reversed the E2-mediated protection beginning on Day 13 and resulting in significantly more severe clinical EAE over days 17–21 than E2-primed WT mice treated with  $\alpha$ -PD-L1 as well as E2-primed IL-10-KO mice treated with Veh- or  $\alpha$ -ISO (**Figs. 1b and 1c**). In a parallel experiment shown in **Fig. 2**, E2-primed WT mice treated with Veh,  $\alpha$ -ISO and  $\alpha$ -PD-L2 again showed near complete protection against EAE over a 21 day scoring period, whereas E2-primed IL-10-KO mice treated with Veh or  $\alpha$ -ISO developed moderate to background levels of EAE as above. However, unlike E2-primed IL-10-KO

mice treated with  $\alpha$ -PD-L1, those treated with  $\alpha$ -PD-L2 did not exceed the EAE background levels observed in the Veh- or  $\alpha$ -ISO-treated groups (**Fig. 2b and 2c**).

These data demonstrated that functional PD-L1 but not PD-L2 is required to sustain E2-induced protection against EAE in mice lacking IL-10. We thus focused our subsequent studies *on  $\alpha$ -PD-L1 treated E2-primed vs. Sham primed IL-10-KO mice* in order to clearly identify E2-associated differences compared to E2 untreated control mice over a 21-day observation period. Female IL-10-KO mice were primed with E2 pellets or sham treatment seven days prior to EAE induction and subsequently treated on days 1, 7 and 14 with Veh,  $\alpha$ -PD-L1 or  $\alpha$ -ISO after immunization to induce EAE as above. As shown in **Fig. 3a**, *sham-primed* IL-10-KO mice treated with Veh or  $\alpha$ -ISO developed typically severe EAE over the 21-day observation period (peak scores  $\sim$  4.5–5.0) with a transient reduction in EAE scores in the  $\alpha$ -PD-L1 treatment group on days 13–15 post-induction. In contrast, *E2-primed* IL-10-KO mice treated with  $\alpha$ -PD-L1 developed severe EAE in real time beginning on Day 15 (concurrent with the third  $\alpha$ -PD-L1 injection) indistinguishable from all of the Sham pre-treatment groups by Day 18 after disease induction but significantly greater than E2-primed IL-10-KO mice treated with Veh or  $\alpha$ -ISO (**Figs. 3b and 3c**). To validate effects of  $\alpha$ -PD-L1 treatment, our FACS evaluation of IL-10-KO spleen cells documented an E2-dependent reduction of PD-L1<sup>+</sup> cells in the E2 vs. Sham Veh,  $\alpha$ -ISO and  $\alpha$ -PD-L1 treatment groups (**Fig. 3d**). These results clearly demonstrate that PD-L1 is a critical E2-induced compensatory component required for inhibition of EAE in IL-10-KO mice.

*Treatment with  $\alpha$ -PD-L1 increased percentages of CD11b<sup>+</sup> CD45<sup>hi</sup> cells in the brain and CD11b<sup>+</sup>, CD4<sup>+</sup> and CD19<sup>+</sup> cells in the spleen of E2-primed IL-10-KO mice concordant with increasing EAE severity over time.*

Local activation of resident microglia, recruitment of infiltrating macrophages into the CNS and enhancement of T and B-cells numbers all can enhance EAE disease severity (Wiedrick et al. 2021). FACS analysis demonstrated that  $\alpha$ -PD-L1 treatment of E2-primed IL-10-KO mice (that resulted in increased EAE severity) resulted in an increased percentage of activated CD11b<sup>+</sup>CD45<sup>hi</sup> but not resting CD11b<sup>+</sup>CD45<sup>low</sup> microglia/macrophages in the brain (**Fig. 4a & 4b**) and a parallel increase in CD11b<sup>+</sup> cells in the spleen (**Fig. 4c**) with no other changes observed in the indicated control groups. Moreover, there were significantly increased percentages of CD4<sup>+</sup> T-cells and CD19<sup>+</sup> B-cells in the spleens of E2-primed IL-10-KO  $\alpha$ -PD-L1 treated mice vs. E2-primed IL-10-KO Veh and  $\alpha$ -ISO treated mice (**Figs. 4d & 4e**). These findings support the contention that loss of PD-L1 mediated E2-associated protection against EAE in IL-10-KO mice is strongly reflected by increased numbers of activated macrophages, T-cells and B-cells in brain and spleen.

*Increased percentage of CD74<sup>+</sup> cells in brain and spleen mice upon treatment with  $\alpha$ -PD-L1 reflects loss of E2-mediated protection against EAE in IL-10-KO mice.*

Upregulation of CD74 in lymphoid tissues and CNS is an indicator of increased inflammation and EAE disease severity induced through the MIF/CD74 axis (Benedek et al. 2013). As shown in **Fig. 5a**, FACS

analysis revealed ~ 20% CD74<sup>+</sup> cells in brains of Sham Veh and Sham ISO treated mice that developed severe clinical EAE, but a significantly lower percentage of CD74<sup>+</sup> cells (~ 3%,  $p < 0.01$ ) in E2-protected Veh and  $\alpha$ -ISO treated groups that developed only mild EAE. In contrast, both Sham- and E2-primed groups treated with  $\alpha$ -PD-L1 had equivalent ~ 20% levels of CD74<sup>+</sup> cells and severe EAE, clearly demonstrating that the increase in CD74<sup>+</sup> cells after  $\alpha$ -PD-L1 treatment reflected the loss of E2 protection against EAE. A similar pattern of responses was observed in the spleen although the inhibitory effect of E2 on CD74 expression in the Veh and ISO control groups was less pronounced (**Fig. 5b**).

*Increased E2-dependent expression of CD73<sup>+</sup> cells in spleens of IL-10-KO Veh and  $\alpha$ -ISO mice is further enhanced after  $\alpha$ -PD-L1 treatment of both Sham- and E2-primed treatment groups.*

CD73 is an IL-10 independent immune regulatory receptor expressed on Bregs and other cells that mediates immunosuppression by upregulating adenosine production (Kaku et al. 2014). Our previous study demonstrated that CD73 expression was increased on both Sham- and E2-primed splenic Breg cells from IL-10-KO vs. WT mice and that there was a significant increase in CD73 expression in spinal cords of E2-primed vs. Sham-primed IL-10-KO mice. Here, we further demonstrate that in the spleen, increased CD73 expression in IL-10-KO mice is greater in E2- vs. Sham-primed groups and that even higher expression occurs in both groups after treatment with  $\alpha$ -PD-L1 (Fig. 6). This result confirms that increased CD73 expression in IL-10-KO mice is enhanced after E2 priming and further shows that this increase could be further enhanced after antibody neutralization of PD-L1.

## Discussion

IL-10 is a key immunoregulatory cytokine in EAE and MS. Mice lacking IL-10 (eg., in IL-10 KO mice) develop more severe clinical and histological signs of EAE, whereas increased expression of IL-10 reduces EAE severity (Bettelli et al. 1998; Cua et al. 1999). Our prior studies demonstrated that E2 pre-treatment of WT mice developing EAE can lower circulating levels of the signature disease promoting cytokine, TNF- $\alpha$ , resulting in complete prevention of clinical signs and CNS lesions characteristic of severe EAE (Ito et al. 2001; Liu et al. 2002). Furthermore, this E2-induced protection was mediated in large part through the induction of Breg subsets (Matsushita et al. 2010; Bodhankar et al. 2013; Korniotis et al. 2016; Pennati et al. 2016), most of which secrete copious levels of IL-10 (Candando et al. 2014). However, similar to WT mice, *E2-pre-treated IL-10-KO mice* remained largely protected against EAE, indicating that IL-10 was not critical for E2-induced protection.

This surprising result raised the question as to what other E2-induced factors could potentially compensate for IL-10 for protecting IL-10-KO mice against EAE? Our recent study reported that E2 treatment of IL-10-KO mice: 1) significantly increased activity in the PD-1/PD-L1/2 pathway; 2) increased the expression of CD73 in the inflamed CNS which could increase production of the anti-inflammatory molecule, adenosine; and 3) decreased CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T-cells in the CNS. Moreover, IL-10-KO mice treated with E2 down-regulated several pro-inflammatory cytokines and chemokines not previously implicated in WT mice with EAE.

In the current study, we focused on the increased activity of the PD-1/PD-L1/2 pathway as the most likely compensatory mechanism. Our approach was to evaluate differences in EAE severity in E2 or sham pre-treated IL-10-KO mice treated with  $\alpha$ -PD-L1 or  $\alpha$ -PD-L2 antibodies over the course of disease compared to treatment with Vehicle or anti-isotype antibodies. The results revealed real-time increases in EAE severity from background levels to severe disease in E2-pre-treated IL-10-KO mice treated with  $\alpha$ -PD-L1 but not  $\alpha$ -PD-L2 antibodies. These findings thus demonstrated unequivocally that PD-L1 but not PD-L2 was required to retain the inhibitory effects of E2 on clinical EAE scores in IL-10-KO mice.

This striking reversal of E2-mediated protection after treatment with  $\alpha$ -PD-L1 was reflected by changes in key cell populations in both CNS and spleen. In CNS, there were increased percentages of activated inflammatory CD11b<sup>+</sup>CD45<sup>hi</sup> microglia and/or infiltrating monocytes as well as a markedly increased percentage of CD74<sup>+</sup> cells compared to the E2 Veh and E2 ISO treatment groups. In spleen, there were decreased levels of CD4<sup>+</sup> T-cells, CD19<sup>+</sup> B-cells and CD11b<sup>+</sup> myeloid cells in E2 Veh treated groups with minimal EAE severity, but no decrease in T and B-cells and even an increase in myeloid cells in the  $\alpha$ -PD-L1 treated group. Enhanced expression of CD74 in CNS is a strong indicator of increased EAE severity mediated through its ligands, MIF-1 and MIF-2 (Benedek et al. 2013) *that are here implicated for the first time* as potential downstream components of an E2-induced compensatory pathway.

The ability of PD-L1 to suppress T-cell activity independent of IL-10 activity was reported previously by Oldstone and colleagues in studies evaluating persistence of lymphocytic choriomeningitis virus (LCMV) (Brooks et al. 2008). This study found that antibody blockade of IL-10 or PD-L1 individually enhanced T-cell function and reduced viral titers and that blockade of both factors together led to significantly greater suppression of chronic LCMV infection than blockade of either factor alone. To the extent that PD-L1 blocks requisite T-cell activity for inducing EAE or reduces protective LCMV immunity, neutralizing PD-L1 resulted in starkly opposing outcomes (worse EAE vs. more viral protection) even though the mechanistic T-cell inhibitory function of PD-L1 remained the same.

The demonstration that PD-L1 is a required factor in maintaining E2-induced inhibition of EAE in IL-10-KO mice does not discount the potential inhibitory roles of the other compensatory factors mentioned above. Although the role of Treg cells was not addressed, our current data show that E2 pre-treatment increases CD73 expression in spleens of all IL-10-KO treatment groups, particularly those primed with E2 and treated with  $\alpha$ -PD-L1, thus confirming and extending our previous report. These data suggest that although CD73 may act in some way to regulate EAE induction and/or severity, it does not appear to be a critical component of E2-mediated EAE suppression, *since blockade of PD-L1 increased rather than decreased the inhibitory potential of CD73*. However, proof of requisite function will require neutralization of these factors similar to the approach used here for such identification of PD-L1.

## Conclusion

The results of our study demonstrate the emergence and critical role played by PD-L1 in maintaining E2-mediated protection against EAE in IL-10-KO mice.

# Declarations

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## Competing interests

The authors declare that they have no competing interests.

## Author Contributions

HO contributed to experimental design and wrote and reviewed the manuscript. DL performed experiments, collected and analyzed data, and helped with manuscript preparation. RMR and AAV reviewed and helped with manuscript preparation. All authors read and approved the final manuscript.

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Ethics approval and consent to participate

All applicable international, national and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors.

## Consent to publish

Not applicable

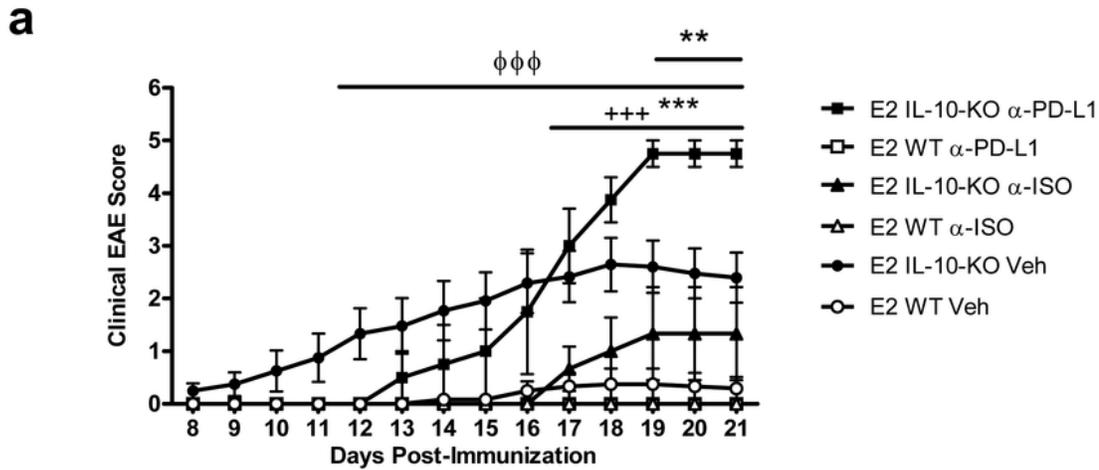
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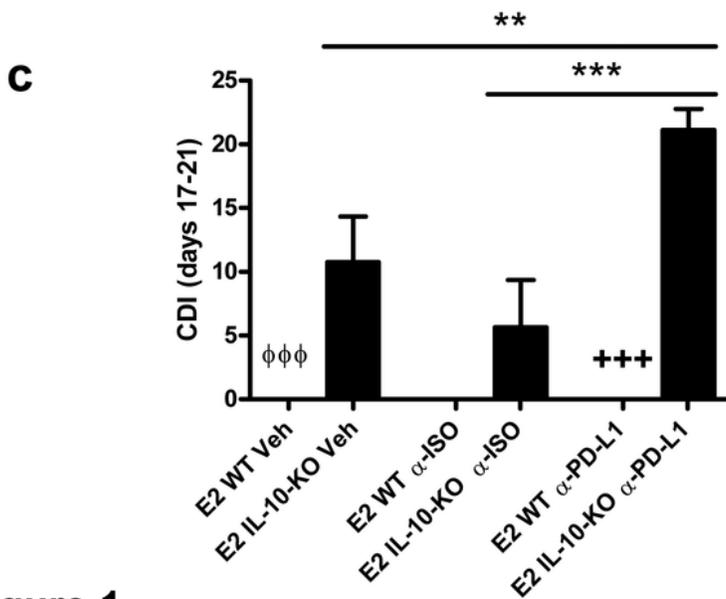
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## Figures



**b**

Cohort	Incidence	CDI	Onset	Peak
E2 WT Veh (n=6)	0/6	0.00 $\pm$ 0.00	0.0 $\pm$ 0.0	0.00 $\pm$ 0.00
E2 IL-10-KO Veh (n=6)	4/6	10.75 $\pm$ 3.59	14.30 $\pm$ 1.25	2.42 $\pm$ 0.84
E2 WT PD-L1 ISO (n=6)	0/6	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
E2 IL-10-KO PD-L1 ISO (n=6)	2/6	5.67 $\pm$ 3.70	17.00 $\pm$ 0.00	1.33 $\pm$ 0.88
E2 WT $\alpha$ -PD-L1 (n=6)	0/6	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00
E2 IL-10-KO $\alpha$ -PD-L1 (n=6)	4/6	21.13 $\pm$ 1.66	15.75 $\pm$ 0.95	4.75 $\pm$ 0.25



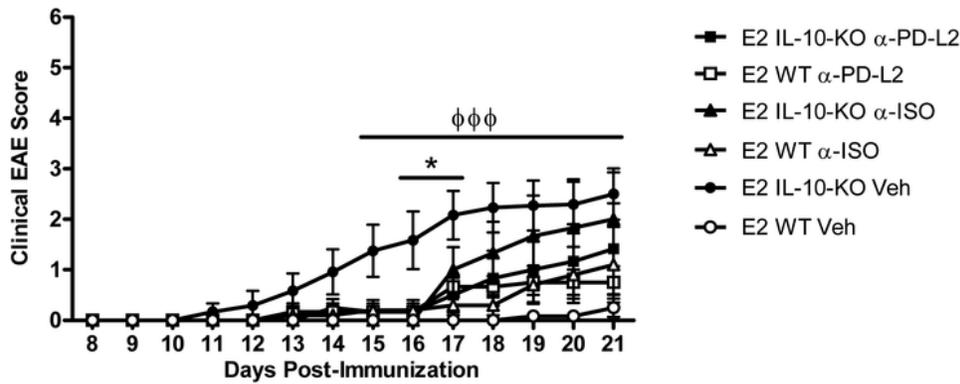
**Figure 1.**

Figure 1

**E2-primed IL-10-KO mice treated with *anti-PD-L1* lose protection against EAE.** EAE scores (CDI) were compared between E2-primed WT and E2-primed IL-10-KO mice after treatment with Vehicle (Veh), anti-isotype Ab ( $\alpha$ -ISO) or anti-PD-L1 Ab ( $\alpha$ -PD-L1). **a)** EAE disease scores on Day 21 post-immunization (PI) were low to moderately higher in E2-primed IL-10-KO mice vs. E2-primed WT mice after treatment with Vehicle [ $\phi\phi\phi$ ,  $p < 0.001$ , IL-10-KO Veh (n=6) vs WT Veh (n=6), (days 12-21)] or  $\alpha$ -ISO (NS). In contrast, the

E2-primed IL-10-KO mice treated with  $\alpha$ -PDL-1 had increased CDI scores beginning on Day 13 that became significant over days 17-21 [(+++p<0.001 E2-primed *IL-10-KO*  $\alpha$ -PD-L1 group (n=4) vs. E2-primed *WT*  $\alpha$ -PD-L1 group]. **b)** Table shows EAE metrics for each group. **c)** CDI scores of E2-primed IL-10-KO mice for Days 17-21 post-immunization showed significantly *increased* severity of EAE in the  $\alpha$ -PD-L1 treated group compared to Veh- (\*\*p<0.01, days 17-21) and  $\alpha$ -ISO (\*\*p<0.001, days 17-21) treated groups. Data are represented as mean  $\pm$  SEM. Phi ( $\phi$ ) denotes differences between E2-primed WT vs. IL-10-KO vehicle-treated groups. Plus sign (+) denotes differences between E2-primed WT vs. IL-10-KO  $\alpha$ -PD-L1-treated mice.

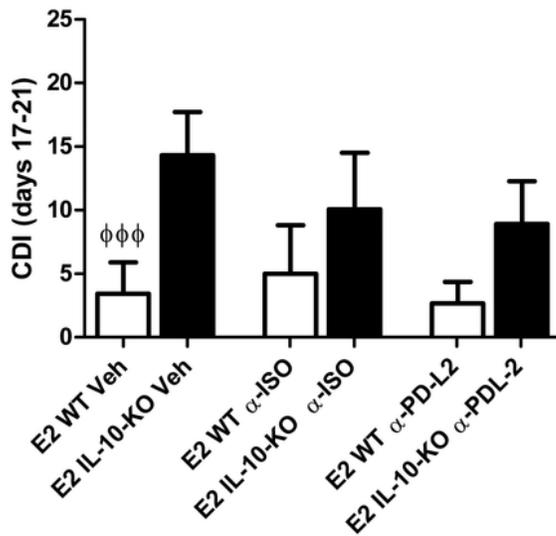
**a**



**b**

Cohort	Incidence	CDI	Onset	Peak
E2 WT Veh (n=6)	3/6	3.42 ± 2.50	17.00 ± 2.08	0.92 ± 0.55
E2 IL-10-KO Veh (n=6)	5/6	14.33 ± 3.39	9.60 ± 0.98	3.46 ± 0.74
E2 WT PD-L2 ISO (n=6)	3/6	5.00 ± 3.82	11.70 ± 2.03	1.20 ± 0.85
E2 IL-10-KO PD-L2 ISO (n=6)	4/6	10.04 ± 4.44	14.25 ± 2.25	2.46 ± 0.98
E2 WT α-PD-L2 (n=6)	5/6	2.67 ± 1.71	13.20 ± 2.06	1.00 ± 0.37
E2 IL-10-KO α-PD-L2 (n=6)	5/6	8.92 ± 3.36	11.00 ± 2.71	2.58 ± 0.83

**c**



**Figure 2.**

Figure 2

**E2-pretreated IL-10-KO mice treated with *anti-PD-L2* remain protected against EAE.** E2 treatment protects IL-10-KO mice from developing EAE, but unlike the effects of anti-PD-L1, the protective effects were unaffected by the administration of anti-PD-L2. **a)** Clinical disease scores were significantly higher in IL-10-KO mice compared to WT mice when either group was primed with E2 and later treated with vehicle (days 17-21; φφφ= $p < 0.001$  IL-10-KO vs. WT), with lesser effects after treatment with α-PD-L2 or α-ISO.

b) Table shows EAE metrics for each group. c) Within the IL-10-KO groups, the mice treated with  $\alpha$ -PD-L2 had lower EAE scores than Veh or  $\alpha$ -ISO-treated groups on days 17-21. Data are represented as mean  $\pm$  SEM. Phi ( $\phi$ ) denotes differences between E2-primed WT vs. IL-10-KO Veh-treated mice.

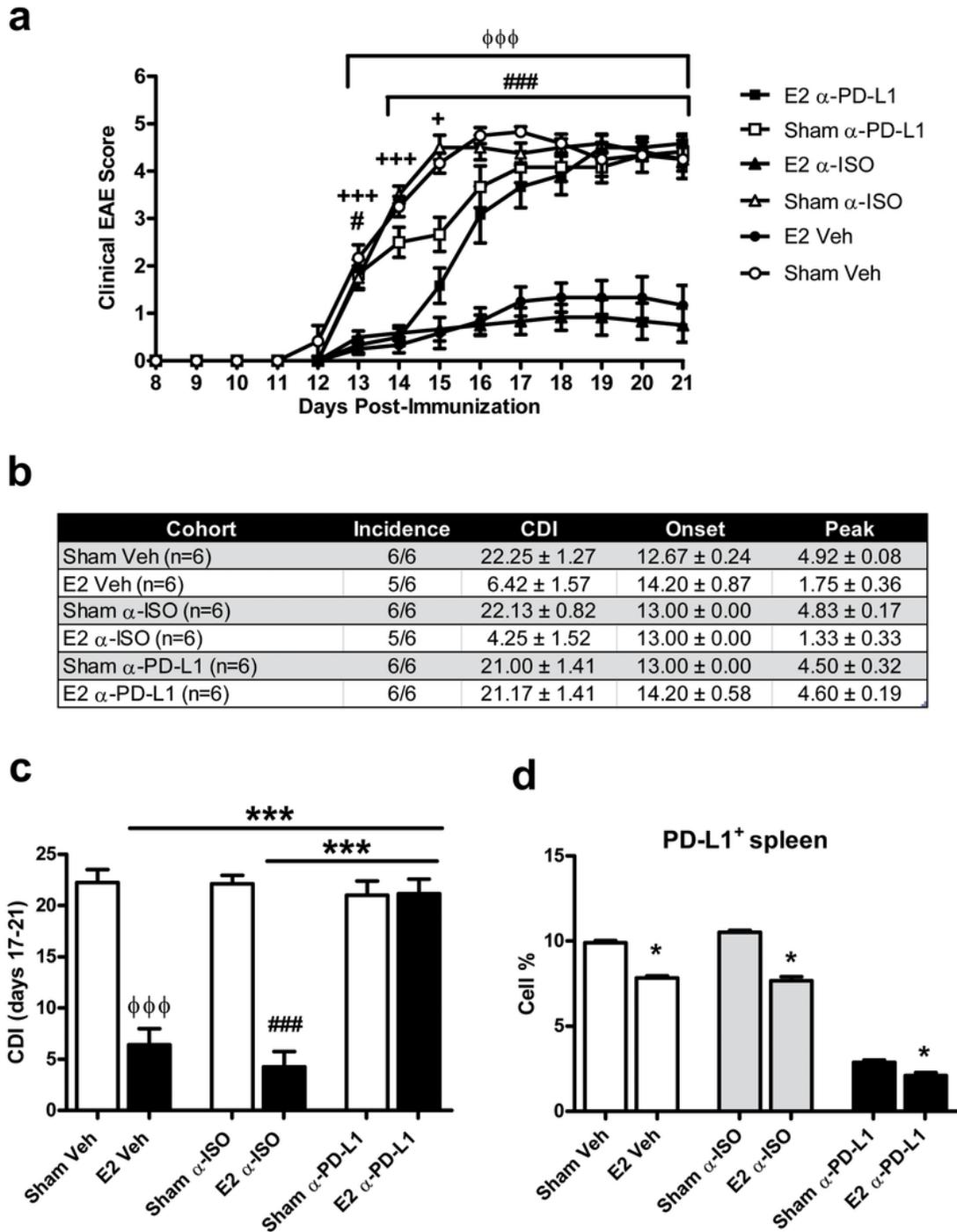
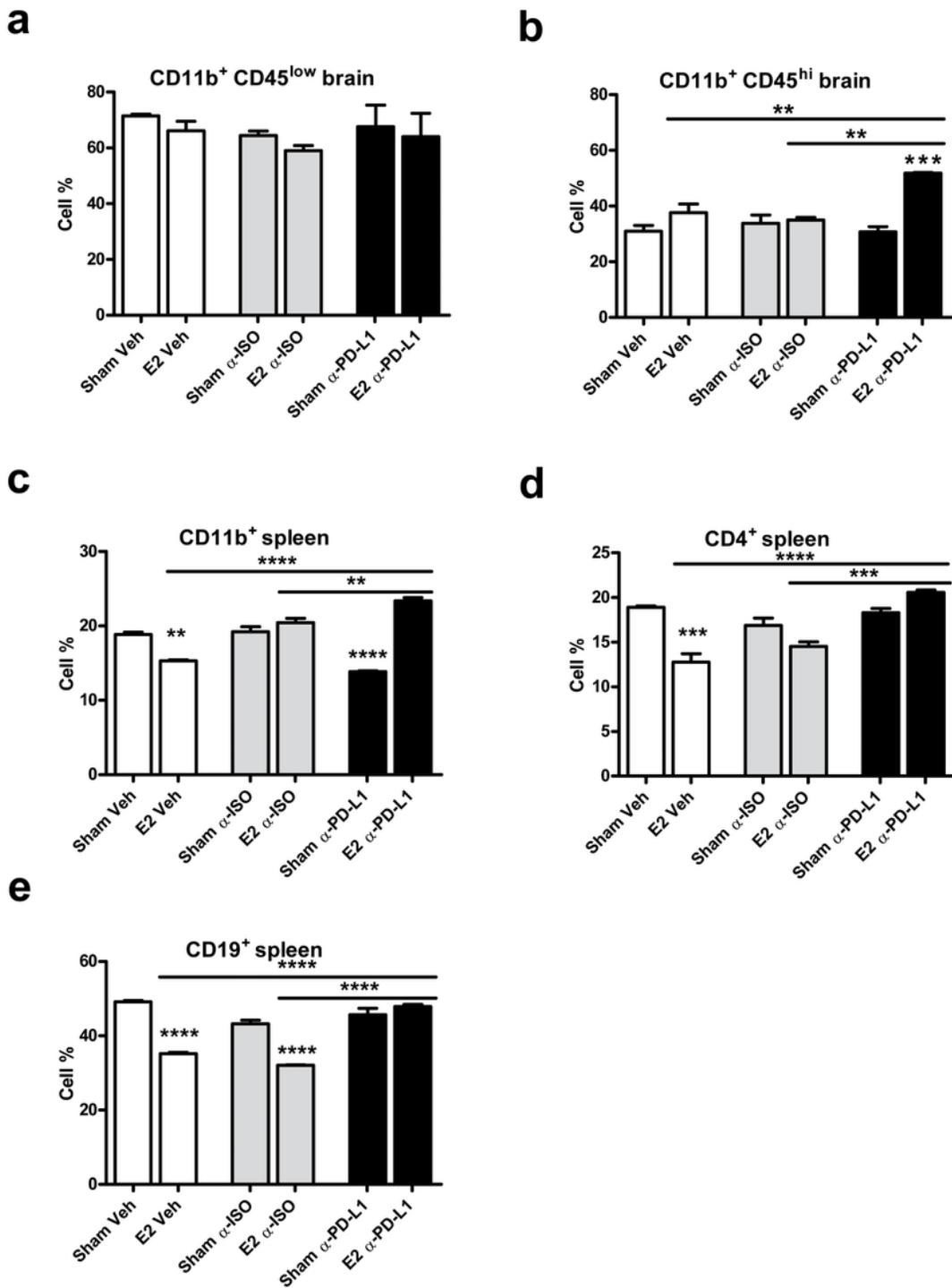


Figure 3.

Figure 3

**E2 pretreatment significantly protects IL-10-KO mice from developing EAE through a PD-L1-dependent mechanism.** EAE scores (CDI) were compared between E2- vs. Sham-primed IL-10-KO mice after treatment with Vehicle (Veh), anti-isotype Ab ( $\alpha$ -ISO) or anti-PD-L1 Ab ( $\alpha$ -PD-L1). **a)** Clinical disease scores were significantly lower for IL-10-KO E2- vs. Sham-primed Veh and IL-10-KO E2- vs. Sham-primed  $\alpha$ -ISO treated groups starting on day 13 post-immunization and remaining significantly lower through day 21 post-immunization ( $\varphi\varphi\varphi$   $p < 0.001$  E2 Veh vs. Sham Veh;  $###$   $p < 0.001$  E2  $\alpha$ -ISO vs. Sham  $\alpha$ -ISO). In contrast, E2-primed IL-10-KO mice treated with  $\alpha$ -PD-L1 had lower scores than Sham-primed mice over days 13-15 ( $+++$   $p < 0.05-0.001$ ) but then had strongly increased CDI scores that were essentially identical to the Sham  $\alpha$ -PD-L1 group on Days 16-21. **b)** Table shows EAE metrics for each group. **c)** Bar graph shows higher CDI scores in the E2  $\alpha$ -PD-L1 group than in the E2 Veh or E2  $\alpha$ -ISO groups ( $p < 0.001$  for both), thus confirming loss of E2-mediated protection [e.g. low to high CDI scores) was due to blockade of PD-L1 in E2-primed IL-10-KO mice. **d)** E2-treated mice had a lower percentage of PD-L1<sup>+</sup> cells in the spleen in all three treatment groups ( $*p < 0.05$ ), thus confirming mAb targeting of PD-L1. Data are represented as mean  $\pm$  SEM. Phi ( $\varphi$ ) denotes CDI differences between E2-primed WT vs. IL-10-KO vehicle-treated groups. Hashtag (#) denotes CDI differences between E2-primed WT vs. IL-10-KO  $\alpha$ -ISO treated mice. Plus sign (+) denotes CDI differences between E2-primed WT vs. IL-10-KO  $\alpha$ -PD-L1-treated mice.

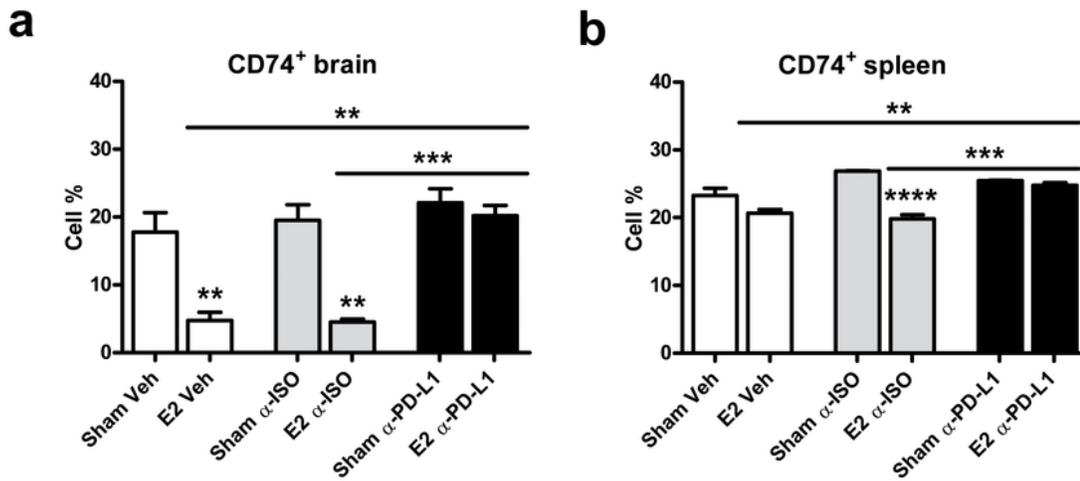


**Figure 4.**

Figure 4

Effects of E2 and anti-PD-L1 treatment differ in myeloid vs. T- and B-cell populations in spleen vs. brain of IL-10-KO mice with EAE. **a)** The resting CD11b<sup>+</sup>CD45<sup>low</sup> subpopulation from E2 pretreated macrophage/microglial cells from Veh,  $\alpha$ -ISO and  $\alpha$ -PD-L1 groups vs. the corresponding Sham treated groups showed no differences in brain, suggesting no E2- or PD-L1-dependent effect on expression of these markers. **b)** Similarly, the activated CD11b<sup>+</sup>CD45<sup>hi</sup> subpopulation of E2 pretreated Sham and  $\alpha$ -ISO

treated groups showed no E2-dependent changes. However, the E2 pretreated mice injected with  $\alpha$ -PD-L1 had a significantly higher percentage of CD11b<sup>+</sup>CD45<sup>hi</sup> brain cells than Sham-treated  $\alpha$ -PD-L1 treated mice ( $p < 0.001$ ) as well as significant increases vs. both E2 Veh and E2  $\alpha$ -ISO groups ( $p < 0.01$ ), indicating that the enhancing effect of  $\alpha$ -PD-L1 treatment on CD11b<sup>+</sup>CD45<sup>hi</sup> cells could be E2 dependent. **c)** The percentage of CD11b<sup>+</sup> spleen cells in the E2 Veh group was significantly inhibited vs. the Sham Veh group ( $p < 0.01$ ), indicating an E2-dependent inhibition that was not evident in brain CD11b<sup>+</sup>CD45<sup>hi</sup> cells. Similar to brain cells, however, the percentage of CD11b<sup>+</sup> cells in spleen was also significantly higher in the E2  $\alpha$ -PD-L1 group vs. the Sham  $\alpha$ -PD-L1 group ( $p < 0.0001$ ) as well as in the E2-treated Veh and  $\alpha$ -ISO groups ( $p < 0.0001$  and  $p < 0.01$ , respectively). **d)** The percentages of splenic CD4<sup>+</sup> T-cells and **e)** splenic CD19<sup>+</sup> B-cells in the E2-primed Veh groups were significantly reduced vs. the Sham-primed Veh groups ( $p < 0.001$  and  $p < 0.0001$ , respectively), as was the CD19<sup>+</sup> E2  $\alpha$ -ISO vs. Sham  $\alpha$ -ISO group ( $p < 0.0001$ ), thus indicating an E2-dependent effect on both T- and B-cell percentages. However, unlike the splenic CD11b<sup>+</sup> cells, the percentages of E2  $\alpha$ -PD-L1 treated CD4<sup>+</sup> T-cells and CD19<sup>+</sup> B-cells were not significantly different from the respective Sham  $\alpha$ -PD-L1 groups, but *were* increased vs. the E2 Veh and E2  $\alpha$ -ISO treated groups ( $p < 0.001$ ). Data are represented as mean  $\pm$  SEM.

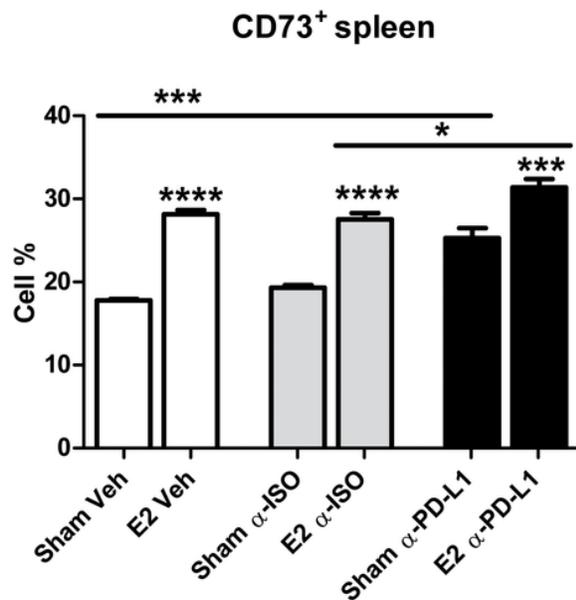


## Figure 5.

### Figure 5

**E2-dependent inhibition of CD74 expression is reversed in α-PD-L1 treated IL-10-KO mice with EAE. a)** The CD74<sup>+</sup> cell percentages in the brain were significantly lower in E2- vs. Sham-pretreated mice further treated with Veh or α-ISO injections (\*\*p<0.01), thus indicating an E2 dependent effect. However, there was no difference in E2- vs. Sham-primed mice later treated with α-PD-L1, which reversed the E2 inhibitory effect. Moreover, the E2 α-PD-L1 treated group was significantly greater than the E2 Veh (p,0.01) and E2

$\alpha$ -ISO ( $p < 0.001$ ) treated groups, confirming reversal of the E2 inhibitory effect. This pattern closely matched the clinical CDI outcome shown in **Fig. 3c. b)** In the spleen, the pattern of inhibition of CD74<sup>+</sup> cells was similar but less pronounced than in the brain, with E2 primed mice later treated with  $\alpha$ -ISO having a significantly lower percentage of splenic CD74<sup>+</sup> cells than Sham pretreated  $\alpha$ -ISO control mice ( $p < 0.001$ ), but no difference between the E2- vs. Sham-primed groups later treated with  $\alpha$ -PD-L1. Again, the E2  $\alpha$ -PD-L1 treated group had a significantly greater percentage of CD74<sup>+</sup> cells than the E2 Veh ( $p < 0.01$ ) and E2  $\alpha$ -ISO ( $p < 0.001$ ) treated groups, confirming reversal of the E2 inhibitory effect. Data are represented as mean  $\pm$  SEM.



**Figure 6.**

**Figure 6**

**E2-dependent enhancement of CD73 expression is further amplified in  $\alpha$ -PD-L1 treated IL-10-KO mice with EAE.** All three treatment groups of E2-primed mice (Veh,  $\alpha$ -ISO &  $\alpha$ -PD-L1) had elevated percentages of splenic CD73<sup>+</sup> cells vs. Sham treated groups. (\*\*\*\*= $p < 0.0001$ ; \*\*\*= $p < 0.001$ ), thus indicating E2-dependent enhancing rather than E2-dependent inhibitory effects. Moreover, the percentages of CD73<sup>+</sup> cells were

further amplified after E2  $\alpha$ -PD-L1 vs. E2  $\alpha$ -ISO treatment (\*= $p$ <0.05) and after Sham  $\alpha$ -PD-L1 vs. Sham Veh treatment ( $p$ <0.01). Data are represented as mean  $\pm$  SEM.