

CD44 Expression and Sex Modulate the Severity of Murine Experimental Autoimmune Encephalomyelitis

Sujata Kar^{1,2}, Janet Y. Lee^{1,3}, Jordi Esparza^{1,4}, Kelly Flynn^{1,5,6} and Joseph A. Madri^{1,6*}

¹Department of Pathology, Yale University School of Medicine New Haven CT. 06520

²Present Address: Department of Physiology, College of Medical Sciences-Teaching Hospital, Bharatpur, Chitwan, Nepal. 44200

³Present Address: Adult and Pediatric Endocrinology Fellow Physician at UCSF, 505 Parnassus Ave., San Francisco, CA 94143

⁴Present Address: Takeda Vaccines Pte Ltd, Singapore, 21 Biopolis Road Nucleos North Tower Level 4, Singapore 1385

⁵Present Address: The University of Wisconsin-Madison, School of Veterinary Medicine, 2015 Linden Dr. Madison, WI 53706

⁶co-senior authors

***Corresponding author:** Joseph A. Madri, Ph.D., M.D, Department of Pathology, Yale University School of Medicine, 310 Cedar Street Lauder Hall, Rm L115A, P.O. Box 208023, New Haven, CT 06520-8023; E-mail: joseph.madri@yale.edu

Received Date: May 21, 2019 **Accepted Date:** June 27, 2019 **Published Date:** June 29, 2019

Citation: Sujata Kar (2019) CD44 Expression and Sex Modulate the Severity of Murine Experimental Autoimmune Encephalomyelitis. *J Neurophysiol Neurol Disord* 5: 1-12.

Abstract

In our murine model of experimental autoimmune encephalomyelitis (EAE), when male and female mice were separately assessed, CD44-KO males exhibited greater disease scores compared to CD44-KO females, consistent with sex differences noted in other strains and in the MS population. In contrast, WT males and females exhibited no sex differences in their responses to EAE induction. Our findings may suggest a potential dynamic inter-relationship between CD44 and estrogen, with each capable of modulating the other and each being able to affect changes in T-cell subset ratios, namely Th1, Th2, and Treg cells, affecting cytokine profiles and the onset and severity of EAE, in part in a CD44- and sex-specific pattern. This intersection of the matrix (CD44) and gender-endocrinology (estrogen) affecting the immune system and autoimmunity may have important implications in our understanding of EAE and MS progression and severity and enhance our understanding and treatment of breast cancer patients with MS treated with estrogen inhibitors.

Keywords: Murine Autoimmune Encephalomyelitis, CD44, Estrogen, Sex, Th1 Cells, Th2 Cells, and Treg Cells

Introduction

Multiple Sclerosis (MS) is a chronic demyelinating autoimmune disease caused by a dysregulation of the pro- and anti-inflammatory arms of the immune system manifested in the central nervous system (CNS) [17]. Murine Experimental Autoimmune Encephalomyelitis (EAE) is an established animal model of MS in which multiple cell types, adhesion molecules, and stromal molecules participate in complex interactions, resulting in distinct but overlapping symptomologies in various strains as well as sexes [6, 7, 8, 18, 23]. Several published reports have described CD44 as a modulator of murine EAE [19]. CD44 expression has been associated with T helper (Th) Th1, Th2, FoxP3 and regulatory T cell (Treg) expression and function [25, 4, 11, 20, 35] and expression of select cytokines [34]. These studies suggest that CD44 can have protective and/or enhancing roles in inflammation due to dynamic, complex interactions among several organ systems depending upon the species and strain studied, the eliciting agent and environmental setting [3, 13]. While the sex effects associated with MS and EAE are well documented, the underlying mechanisms at play are complex [39, 42, 46]. One driver of sex differences in disease severity, estrogen (E2), has been known and studied in both humans and animal models [1, 14, 17, 27, 30, 31, 36, 39, 40, 47]. Pregnancy in MS patients is associated with a lower risk of progression and rate of exacerbation [1]. These beneficial effects, that are reversed postpartum, are associated with a shift towards a Th2 cytokine profile. Recently, it has been found that treatment of relapsing-remitting MS patients with dimethyl fumarate, which has shown efficacy in phase III trials reduced memory T cells and shifted the ratios between Th1/Th17 and Th2, favoring Th2 cells [36, 55].

Although linkages between CD44 and E2 and E2 receptor have been documented in breast and colon cancer [5, 16] there is scant evidence that the two physically or functionally interact to alter each other's behavior. There are multiple polymorphisms and complex alternative splicing in CD44 and recent studies have demonstrated interactions between CD44 and E2 responses [12, 29, 45], in addition to the multiple known signaling pathways impacted by CD44 [41] and E2 [22, 51]. As such, it is possible that CD44 and E2, both known to affect immune responsiveness in MS and EAE, could be capable of modulating each other's immune responses differentially in males and females.

In this report, we observed increased EAE severity in

CD44KO mice with CD44-KO males exhibiting significantly greater disease compared to CD44-KO females, consistent with sex differences noted previously in mouse models [20, 21] and in the human population [52]. Our studies support the concept of a potential interrelationship between CD44 (in light of its complex, dynamic alternative splicing, post-translational modification, and their effects) and E2 or other gender-related hormones, with each capable of modulating the other and each being able to affect changes in the ratios of T- cell subsets, namely Th1 (pro-inflammatory), Th2 (anti-inflammatory) cells and Tregs, affecting the onset and severity of MS and EAE, in part in a sex-specific pattern.

Materials and Methods

Mice and Reagents

C57BL/6 wild type (WT) and CD44-KO (B6.129(Cg) Cd44bn/Hbg/J) male and female mice between 8 to 10 weeks of age were obtained from The Jackson Laboratory, Bar Harbor, ME, and maintained in the animal facilities at Yale University School of Medicine according to Yale University and NIH guidelines.

Ethical Assurance

The authors are in compliance with the US National Research Council's Guide for the Care and Use of Laboratory Animals, the US Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and Guide for the Care and Use of Laboratory Animals. All animal procedures followed these guidelines for animal care and safety and were approved by the Animal Care and Use Committee of Yale University (Approved Protocol # 07366).

Murine MOG35-55 peptide was synthesized by the W.M. Keck Biotechnology Resource Laboratory at Yale University. Antibodies and cytokines were purchased from BD Bioscience (anti-CD3, anti-CD28, CD4, CD25), R&D Systems (IL-2, IL-4, CCR3, CCR7, CXCR3), eBioscience (IL-12, anti-IL-4, anti-IFN γ , anti-IL-12), and In Vitrogen (Alexa fluor-conjugated secondary antibodies).

Induction of EAE

EAE induction was performed as described previously [20, 21]. Mice were assessed daily and graded for clinical signs as follows: 0, no disease; 1, flaccid tail; 2, hind-limb weakness; 3, hind-limb paralysis; 4, hind and fore-limb weakness/paralysis; 5, moribund. The data illustrated is comprised of several pooled independent experiments totaling 226 mice. In each independent experiment, all groups (WT M, WT F, KO M, KO F) were

present. No significant variation/differences were observed in disease scores of similarly named groups between individual experiments.

Th1 and Th2 polarization

Easy Sep Mouse CD4⁺ T cell Enrichment Kit (Stem cell Technologies, Vancouver) was used to isolate CD4⁺ cells from total splenocytes of EAE mice following manufacturer's instructions. Isolated CD4⁺ cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 at 1 µg/ml each. Th1 and Th2 polarization were performed according to standard methods [2, 44] using IL-2, IL-12 and anti-IL-4 for Th1 and IL-2, IL-4, anti-IFN γ and anti-IL-12 for Th2.

Flow Cytometry

Splenocytes were prepared for FACS as described earlier [20, 21]. For CD4, CD25, FoxP3 staining, fixation, and FoxP3 staining was done using FoxP3 Staining kit (eBioscience) as per the manufacturer's instructions, and fluorescently conjugated CD4 and CD25 antibodies. Flow cytometric analysis was performed using FACS Calibur and Flow Jo software (BD Bioscience).

Transmigration assay

Splenic CD4⁺ T cells were isolated and polarized to Th1 or Th2 cells as above. Transmigration of Th1 and Th2 cells from WT and CD44-KO mice were assessed using TranswellTM System (BD Bioscience). 1.5×10^6 cells were added to the upper chamber in DMEM and allowed to transmigrate through bare membrane or collagen- or fibronectin-coated 3µm pore inserts. SDF-1 was used as a chemoattractant in case of bare membrane and CCL19 and/or CCL21 were used otherwise. Transmigrated cells were collected from the lower chambers after 12 hours and counted (Flynn et al. 2013a).

Zymography

MMP-9 levels in Th1 and Th2 differentiated cells were assessed by zymography [20] using cell lysates. Triplicate samples were tested in independent zymography.

Immunofluorescence

Briefly, deeply anesthetized mice were perfused intracardially as described [20] and inguinal lymph nodes were removed. Lymph nodes were processed, cut into 2µm thin sections, and washed and blocked at room temperature as

described (12). Sections were incubated with the primary antibody CCR3 (Santa Cruz) or CCR7 (eBioscience) or CXCR3 (Santa Cruz) or FoxP3 (BD Bioscience) or MMP-9 (BD Bioscience) for 2 hr at RT followed by washing and incubation with fluorescently conjugated and relevant secondary antibodies (In vitrogen).

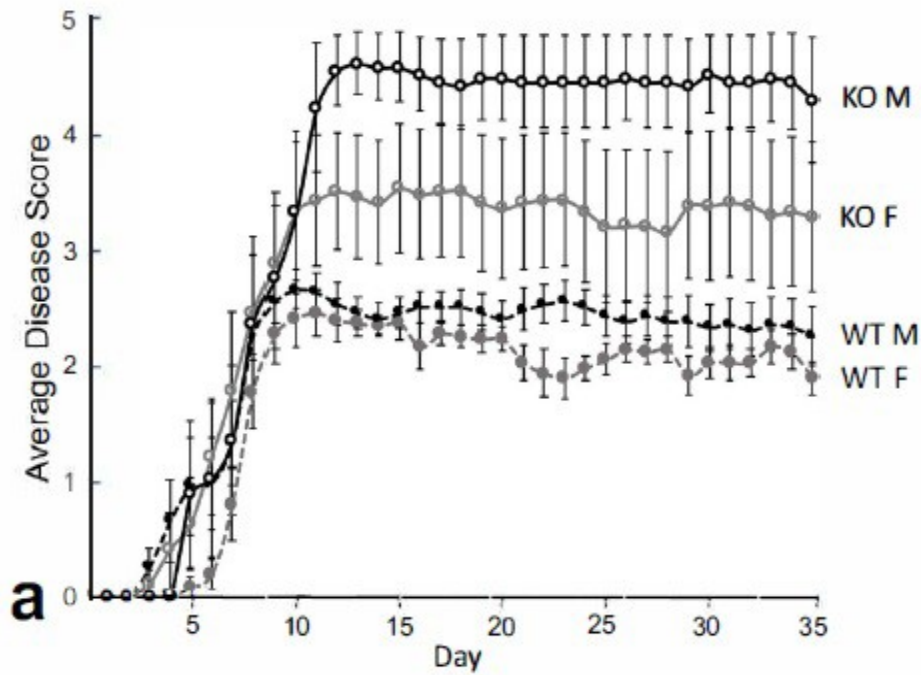
Restimulation of Splenocytes and Cytokine ELISA

Isolated splenocytes (3.5×10^6 cells per well) were cultured with 50 µg/ml MOG35-55 in RPMI-1640 medium supplemented with 10% FBS in 24-well plates. The supernatant was collected after 72 hrs. Cytokine ELISAs were performed using eBioscience kits as per the manufacturer's instructions. Tests for each sample were performed in triplicate.

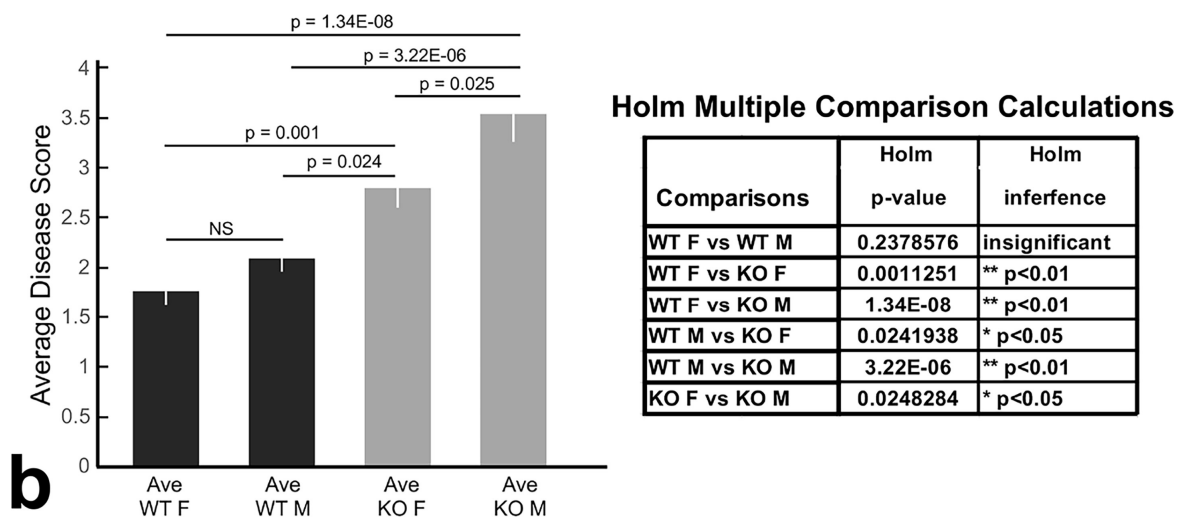
Statistics

Statistical significance was ascribed to data that achieved $P < 0.05$ (expressed as means \pm SE) using StatView and Prism8. Specifically, one-way ANOVA with post-hoc Holm-Bonferroni multiple comparisons tests were used to evaluate the statistical significance of WT & CD44-KO disease scores (Figure. 1). Survival data (Figure. 1c) was assessed using Kaplan-Meier analysis and log-rank test. A Student's 2 samples independent t-test was used to ascertain significance between the two conditions in T lymphocyte subtype numbers and percent transmigration in Figure 2 and the cytokine levels in T lymphocyte cultures in Figure 3.

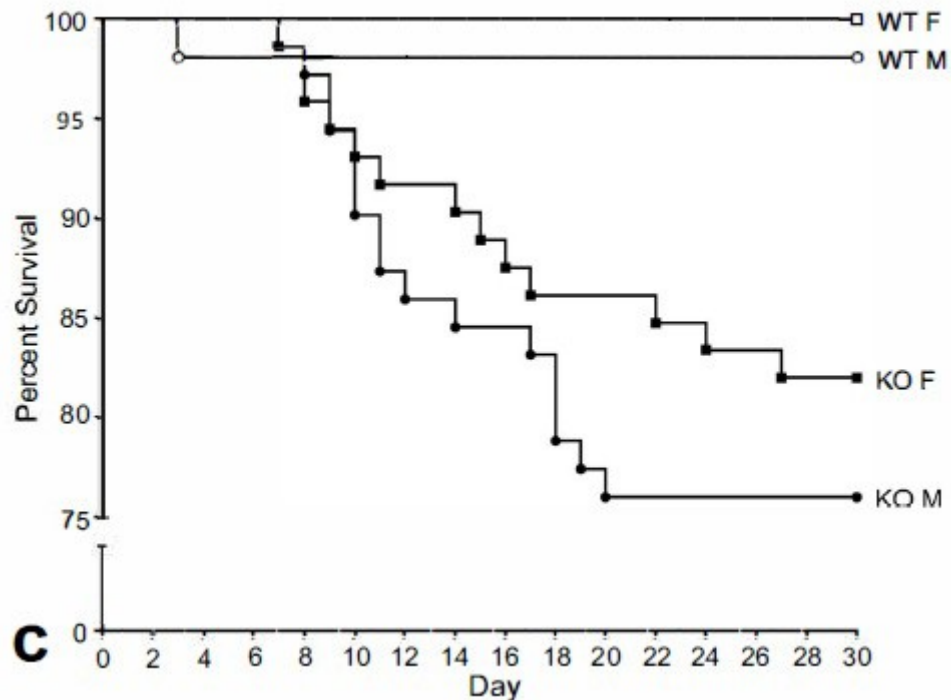
Figure 1. a-c. CD44-KO mice suffer from increased EAE disease severity compared to WT mice and CD44-KO.



a. Average daily clinical disease scores of WT male, WT female, CD44-KO male and CD44-KO female mice following induction of EAE. Mortality scores were included in average scores from the time of death throughout the study. Data are expressed as means +/- standard errors.



b. Average clinical disease score over the course of the disease was calculated by averaging the disease scores experienced by each mouse over the 30 day study period and is then represented as a mean per group. Analysis of the average clinical scores (Holm-Bonferroni multiple comparisons test) revealed significant increases in the disease severity of the CD44-KO male and CD44-KO female animals compared to the WT male and WT female animals as well as a significant increase in disease score of CD44-KO male mice compared to CD44-KO female mice. No statistically significant differences were noted when between WT males and females.



c. Percent of survival data derived from WT and CD44-KO male and female cohorts. Log Rank (Mantel-Cox) revealed that all WT groups were significantly different compared to the CD44-KO groups. No statistically significant differences were found between the WT male and female cohorts nor between CD44-KO male and female cohorts, despite a modest reduction in percent survival of the CD44-KO male cohort.

The data illustrated in figure 1 is representative of several pooled independent experiments totaling 226 mice.

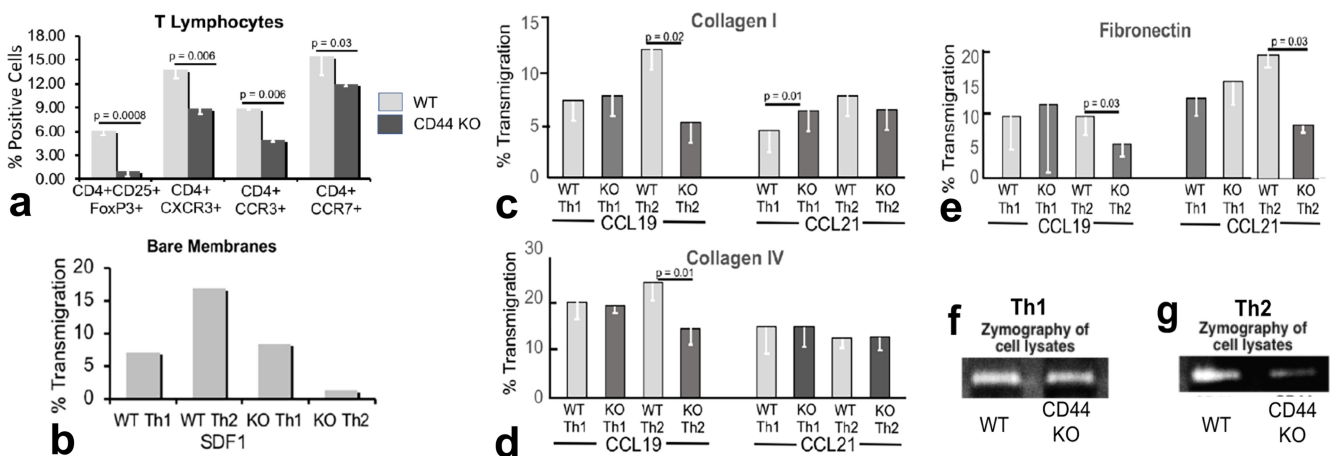


Figure 2. a-g. CD44-KO mice exhibit lower numbers of T cell subsets, decreased transmigration of Th2 cells and decreased Th2 MMP-9 activity

a. CD44-KO mice express lower numbers of CD4+CD25+FoxP3+ (Treg), CD4+CXCR3+ (Th1), CD4+CCR3+ (Th2) and CD4+CCR7+ (TCM & TEM) cells compared to WT, expressed as averages of triplicate FACS analyses.

b. Transmigration of polyclonally activated WT and CD44-KO Th1 and Th2 cells through bare 3 μ m pore membranes, expressed as averages of three independent assays using SDF-1 as chemoattractant. There were similar percent transigrations, of WT and CD44-KO Th1 cells, while CD44-KO Th2 cells were observed to exhibit decreased% transmigration compared to WT Th2 cells.

c-e. Transmigrations of WT and CD44-KO Th1 and Th2 cells through (c) collagen type I-, (d) collagen type IV- and (e) fibronectin-coated 3 μ pore membranes, expressed as averages of three independent assays using CCL19 and CCL21 as chemoattractants. The analysis revealed similar percent transmigrations when comparing WT and CD44-KO Th1 cells on all three coatings with CCL19 as a chemoattractant. In contrast, when using CCL19 as a chemoattractant, decreased CD44-KO Th2 cell migration was noted compared to WT Th2 cells. When CCL21 was used as the chemoattractant WT Th1 cell transmigration through collagen I coated membranes was decreased compared to CD44-KO Th1 cells. Additionally, on fibronectin-coated membranes CD44-KO Th2 cell transmigration was noted to be decreased compared to WT Th2 cells.

f & g. Representative zymographies illustrating decreased MMP-9 enzymatic activity in CD44-KO Th2 cell lysates compared to WT Th2 cell lysates (g), while no changes in MMP-9 enzymatic activity were noted in WT or CD44-KO Th1 cell lysates (f).

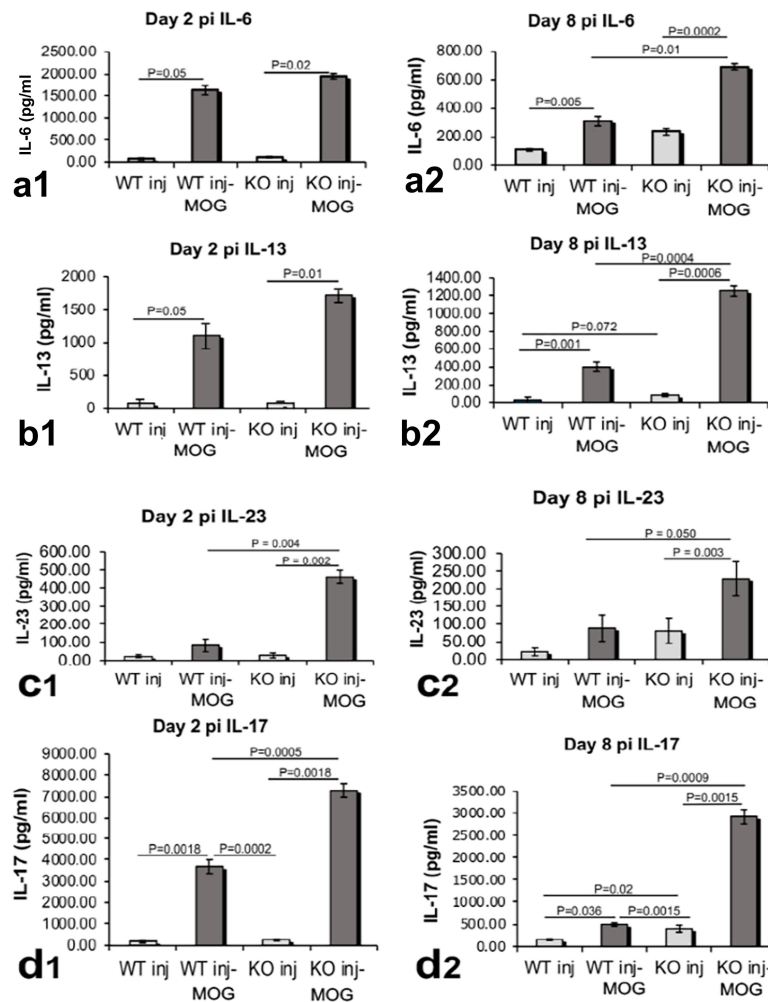


Figure 3. a1-d2. CD44 KO MOG-induced mice produce increased. Pro-inflammatory cytokines.

CD44-KO mice produce increased amounts of pro-inflammatory and anti-inflammatory cytokines. "WT inj" and "KO inj" means injected with pertussis toxin (PT) only, but not with MOG; "WT inj MOG" and "KO inj-MOG" means injected with PT and MOG (the complete course)

a1 & 2 IL-6. By days 2 (a1) and 8 (a2), IL-6 levels are noted to be increased in both WT inj-MOG and KO inj-MOG mice compared to the PT only mice. Additionally, at day 8 (a2) the KO inj-MOG mice exhibit increased IL-6 compared to the WT inj-MOG- injected mice.

b1 & 2 IL-13. Similarly, by days 2 (b1) and 8 (b2) IL- 13 levels are noted to be increased in both WT inj-MOG and KO inj-MOG mice compared to the PT only mice. Additionally, at day 8 (b2) the KO-inj-MOG mice exhibit increased IL-13 compared to the WT inj-MOG mice.

c1 & 2 IL-23. The expression level of IL-23 by KO inj-MOG groups were higher than those observed in the WT inj-MOG groups at 2 (c1) and 8 (c2) days. Also, IL-23 levels were increased in KO inj-MOG compared to KO inj at days 2 and 8, but the WT inj-MOG was not significantly increased over the WT inj mice.

d_{1&2} IL-17. The expression level of IL-17 in the KO inj-MOG groups was higher than those observed in the WT inj-MOG groups at 2 (d1) and 8 (d2) days. Additionally, at 8 days, IL-17 levels were higher in the KO “inj” groups compared to the WT “inj” groups. Again, by days 2 and 8 IL-17 levels are noted to be increased in both WT inj-MOG and KO inj-MOG mice compared to the PT only mice.

Results

EAE Severity and Survival in CD44-KO and WT mice

Consistent with our previous data [21], CD44-deficient mice (CD44-KO) (n= 125) experienced more severe disease than WT mice (n = 101) (**Figure 1a & b**). Interestingly, CD44-KO males (n = 63) exhibited significantly higher disease scores compared to CD44-KO females (n = 62) (**Figure 1a & b**). In contrast, no significant differences were observed between WT males (n = 42) and WT females (n = 59) (**Figure 1a & b**). The p-values are listed in figure 2b. Specifically, post hoc Holm-Bonferroni multiple comparisons analysis revealed significant differences between the KO female and male groups and the WT female and male groups, in addition, the KO female and male groups also displayed significant differences.

Analysis of survival revealed no differences between the WT male and WT female cohorts (**Figure 1c**). Within the CD44-KO cohort, there was a modest reduction in the survival of the CD44-KO male group compared to the CD44-KO female cohort (Figure 1c). Log Rank (Mantel-Cox) analysis of all groups together showed both WT groups to be significantly different from both CD44-KO cohorts (Chi-square = 52.68, df = 5, P < 0.0001). In contrast, the survival of CD44-KO males vs. CD44-KO females and WT males vs. WT females were not statistically different (Chi-squared: 0.7552 and 1.385, respectively; P-value: 0.3792 and 0.2393, respectively).

CD4⁺ T lymphocytes subsets in CD44-KO and WT mice following MOG challenge in order to elucidate the cause(s) of the differences in disease severity between WT and CD44-KO mice we assessed splenic CD4⁺ T cell subsets using FACS analysis. Robust decreases in CD4⁺CD25⁺FoxP3⁺ T lymphocytes were noted in the CD44-KO mice compared to the WT mice (WT: KO ratio = 5.6:1). In contrast, only modest decreases were noted in the CD4⁺CXCR3⁺; CD4⁺CCR3⁺ and CD4⁺C- CR7⁺ populations from CD44-KO mice compared to WT counterparts (WT:KO Ratios: 1.54:1, 1.8:1 and 1.3:1 respectively) (Figure 2a). Reduced *in vivo* expression, via immunofluorescence, of FoxP3, CXCR3, CCR3, CCR7 and MMP-9 in peripheral lymph nodes was also

observed in CD44-KO mice compared to WT mice (Figure 4).

Transmigrations of CD44-KO and WT Th1 and Th2 Cells

To investigate whether T lymphocyte subset migration may be involved in the altered populations found in the CD44-KO mice, we assessed transmigrations of differentiated WT and CD44-KO Th1 and Th2 cells. As illustrated in **Figure 2b** there were no observable differences between WT Th1 and CD44-KO Th1 transmigration across a naked membrane. However, there were reductions observed in the transmigration percentages of CD44-KO Th2 cells compared to WT Th2 cells.

To better characterize this observed difference, the transmigrations were assessed through collagen type I, collagen type IV and fibronectin coated membranes. Using CCL19 as a chemoattractant, the transmigration of CD44-KO Th2 cells was significantly reduced compared to WT Th2 cells on all substrates (**Figure 2c, d, e**). When using CCL21, decreased CD44-KO Th2 transmigration, relative to WT Th2, was only observed through fibronectin (Figure 2e). No observable differences were noted between WT and CD44-KO Th1 cells regardless of membrane substrate in the presence of CCL19 (Figure 2c, d, e). Using CCL21, the transmigration of CD44-KO and WT Th1 cells was similar through collagen IV and fibronectin (Figure 2d, e), but increased CD44-KO Th1 transmigration was observed through collagen I (Figure 2c).

MMP-9 activities in WT and CD44-KO cells during EAE

Given its well-characterized role in lymphocyte migration [15, 57], we evaluated MMP-9 activity as a possible explanation for the decreased transmigration of CD44-KO Th2 cells through ECM-coated membranes.

Interestingly, while lysates of purified Th1 cells derived from both WT and CD44-KO mice exhibited similar MMP-9 enzymatic activities, Th2 lysates derived from CD44-KO mice displayed decreased MMP-9 activity compared to Th2 cells derived from WT mice (**Figure 2f & g**).

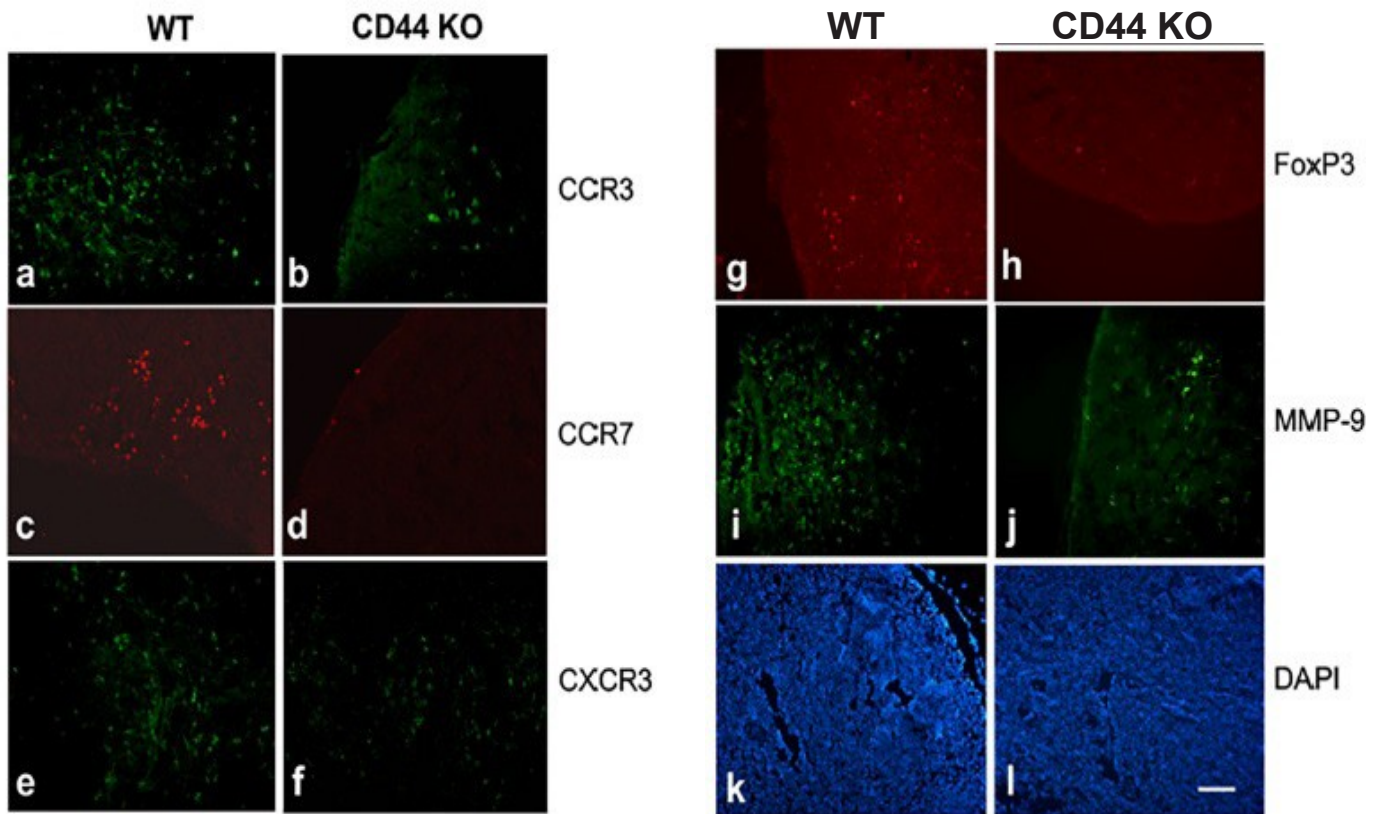


Figure 4. a-l. Peripheral lymphatic tissues from CD44-KO mice exhibit lower levels of CCR3, CCR7, CXCR3, Foxp3, and MMP-9.

Representative immunofluorescence micrographs of lymph nodes illustrating decreased CCR3 (a & b), CCR7 (c & d), CXCR3 (e & f), FoxP3 (g & h) and MMP-9 (I & j) in CD44-KO animals compared to WT animals. Representative DAPI stains (i & j). Scale Bar = 50µm.

Immunofluorescence

In-vivo expression, via immunofluorescence, of CCR3, CCR7, CXCR3, FoxP3 and MMMP-9 in peripheral lymph node tissues were also observed to be reduced in peripheral lymph nodes of CD44-KO mice compared to WT mice (**Figure 4**).

Selected inflammatory cytokine profiles in CD44-KO and WT mice during EAE We next assessed how the observed differences in cell populations between WT and CD44-KO mice correlated with inflammatory cytokine expression. Following MOG- challenge, IL-6, IL-13, IL-23, and IL-17 were increased in the CD44-KO groups compared to the WT groups. Specifically, in IL-6 and IL-13 at day 8 (**Figure 3a₂**) and in IL- 23 and IL- 17 at days 2 and 8, (**Figure 3c_{1,2} and 3d_{1,2}**). Additionally, increased IL-13 and IL-17 at day 8 (**Figure 3b₂, d₂**) were noted in the CD44-KO mice injected with pertussis toxin only (inj) compared to the WT mice injected with pertussis toxin only (inj). Similar cytokine expression was observed from nave WT and CD44-KO mice (IL-6, IL-17, IL- 23) in our previous study [20].

Discussion

The importance of T lymphocyte subset dynamics has been demonstrated in the treatment of relapsing-remitting multiple sclerosis [35, 55]. One MS drug that blunts relapsing-remitting MS, dimethyl fumarate, has been found to mod- ulate Th1/Th2 ratios in favoring Th2 cells [35, 55]. In our study, CD44 deficiency led to a dramatic decrease in CD4+CD25+ FoxP3+ cells (Treg) and modest decreases in CD4+CXCR3+ (Th1), CCR3+ (Th2) and CCR7+ (TCM & TEM) subsets altering the Th subset ratios. CD44-KO mice also produce Th2 effectors that exhibit reduced MMP-9 activity and a corresponding reduction in migratory capacity. The loss of this protective cell type (Th2) as well reduced Treg populations may contribute to a more severe disease index in CD44-KO mice.

CD44 expression has correlated directly with driving Th cells toward a Th2 phenotype [58] and a degree of suppressor activity [19]. while its interaction with heparan can upregulate Th2 cytokines and ameliorate EAE [9]. Expression of CD44 with other molecules on Th2 cells can promote greater Th2 adhesion and migration and greater inflammation in

other inflammatory murine models [31]. These studies and our data confirm roles for CD44 in Treg and Th2 cell behavior in various inflammatory models, with some studies demonstrating pro-inflammatory effects of Th2 cells [31], while other studies [9] and our data supporting suppressive Th2 effects, consistent with Th2's role is dependent upon the mouse strain, model studied, eliciting agent and environmental context.

The decreases in Treg and Th2 cells observed in CD44KO mice during EAE is consistent with increased levels of IL-6, IL-13, IL-23 and IL-17 produced by CD44-KO splenocytes following MOG stimulation. Additionally, increased IL-13 and IL-17 production in CD44-KO mice following priming with pertussis is consistent with elevated baseline immune responsiveness in the absence of CD44 and may also reflect vascular permeability changes previously noted in CD44-KO mice [21].

Together these data support a role for CD44 in modulating T-cell subset numbers and ratios with CD44 deficiency reducing Th2 and Treg numbers, leading to increased levels of pro-inflammatory cytokine expression and increased disease severity. The absence of CD44 would abrogate CD44-dependent signaling with known immunomodulatory molecules (hyaluronan, MMP-9, TGF β , among others) in many cell types [10, 11]. This loss would likely alter immune cell ability to adhere to and transmigrate through the endothelium and adjacent ECM [48] and could possibly affect their proliferative and apoptotic rates as well [20, 53, 54]. Deficiency in CD44 resulted in a profound decrease in Treg and altered Th1/Th2 ratios, as well as decreased Th2 transmigration. These findings could be secondary to changes in T-cell apoptosis, proliferation, adhesion and/or trans migratory abilities. Further, the increased cytokine production by CD44-KO cells is consistent with the modified T-cell subsets, resulting in an increased proinflammatory environment and increased disease index. Given the known roles of estradiol (E2) in modulating autoimmune diseases during pregnancy [3, 6, 14, 36] and that E2 protects against EAE [3], we evaluated male and female mice separately following EAE induction. The CD44-KO male mice exhibited statistically greater disease scores than CD44-KO female mice. In contrast, although there was a trend of decreased survival of CD44-KO males compared to CD44-KO females, no significant differences were found in post-hoc analysis. This suggests that sex, in the absence of CD44, is associated with increased disease severity, but only a trend in increased mortality of EAE.

Serum E2 levels are reported to be approximately nine times higher in adult female C57BL/6 mice than in males [37]. In our study, WT males and females did not exhibit appreciable differences in disease despite having different E2 levels, while CD44-KO males were more affected than CD44-KO females and both experienced more severe disease than WT cohorts. In addition to sex differences in E2 expression, there are differences in progesterone, testosterone, dehydroepiandrosterone, and androstenedione [37]. Although we have no definitive mechanism elucidating the difference in disease between CD44-KO males and females, given the multiple alternative splice variants and polymorphisms in CD44 and recent studies demonstrating interactions between CD44 and E2 responses [12, 45], as well as the various signaling pathways impacted by CD44 [41] and E2 [22, 51], we postulate that CD44 deficiency alters hormonal effects and profiles between males and females creating the observed sex differences in disease severity. Alternatively, the presence or absence of CD44 could impact the T cell profiles in a manner independent of E2 levels, with the presence of CD44 being dominant over the E2 level in the WT animals, in contrast to the KO mice in which its absence would permit the increased E2 levels in the KO females to dominate, compared to the lower E2 levels in the KO males. Additionally, perhaps even the reduced E2 expression in the WT males still exceeds a critical threshold that maintains the protective effects of E2 in the disease process. In this scenario, the loss of CD44 could then further reduce E2 expression (perhaps beneath a critical threshold) or alter how the limited E2 in the male can interact with its receptor or other downstream signaling components. To further elucidate the underlying mechanism(s) future studies could include determining the expression levels of E2 and other hormones and dynamic subcellular localization of E2 receptor (ER) α in WT and CD44-KO male and female mice and/or expressing alternatively spliced isoforms and the cytoplasmic domain of CD44 in CD44-KO mice to determine possible effects on EAE severity and the dynamics of ER α expression and subcellular localizations.

Pregnant mice and mice treated exogenously with E2 exhibited enhanced expression of FoxP3 and increased Treg functional suppression [40] and reduced EAE clinical severity [27, 47]. In these models, alleviation of severity was associated with favorable changes in cytokine production (enhanced production of anti-inflammatory cytokines and decreased the Th1/Th17 cytokines) and increases in percentages of Th2 and Treg cells. Similarly, E2 treatment of PBMCs isolated from MS patients and healthy controls favorably affected the expression

of inflammatory and anti-inflammatory cytokines, driving the response towards regulatory pathway [30]. These protective effects of E2 have been demonstrated to be dependent on ER signaling and specifically on ER α [14, 36]. These studies clearly illustrate a potent E2 effect on T- cell subsets and its potential role in determining the sex effects observed in MS and EAE.

In light of the effects of CD44 and E2 on T-cell subsets, we examined the literature describing interactions between E2, ER, and CD44. In ovarian cancer cells, CD44 forms a complex to initiate downstream signaling that ultimately promotes transcriptional activation of ER α [12]. In cervical adenocarcinoma cells, expression of a CD44 isoform is upregulated by E2 [29] while in human breast cancer cells a phytoestrogen attenuates ER α induced CD44 expression [45]. Thus, we postulate that the absence of CD44 in the CD44-KO male population coupled with a low E2 baseline expression (0.3pg/ml) [37] compared to female mice results in a more profound change in T cell subsets (specifically Th2 and Treg), which is blunted or abrogated by the presence of higher E2 levels (2.7 pg/ml) [37] in the CD44-KO female population.

Given our results and the various studies showing an effect CD44 and/or E2 on immune responsiveness in MS and EAE, we postulate that these two molecules could be capable of modulating each other's immune responses differentially in males and females. As such, our results may be suggestive of a potential dynamic interrelationship between CD44 and E2, with each capable of modulating the other and each able to affect changes in the ratios of T-cell subsets, namely Th1 (pro-inflammatory), Th2 (anti-inflammatory) cells and Tregs, affecting the severity of MS and EAE, in part in a sex-specific pattern and possibly affecting breast cancer patients with MS treated with E2 inhibitors [24]. This dynamic intersection of the matrix (CD44) and gender-endocrinology (E2) affecting the immune system and autoimmunity may have important implications in our understanding of EAE and MS progression and severity and enhance our understanding and treatment of breast cancer patients with MS treated with E2 inhibitors.

* Supported in part. By USPHS Grants R37-HL28373, ROI HL-51018, PO1- NS003447381 and a generous gift from Joseph & Lucille Madri to JAM.

References

1. Al-Shammri S, Rawoot P, Azizieh F, AbuQoora A, Hanna M, et al. (2004) Th1/Th2 cytokine patterns and clinical profiles during and after pregnancy in women with multiple sclerosis. *J Neurol Sci* 222:21-27.
2. Annunziato F, and Maggi L (2017) *Methods in Molecular Biology*. New York: Springer Science+Business Media.
3. Apeltgren LD, Bailey DL, Fouts RL, Short N, Evans GF, et al. (1996) The effect of a selective estrogen receptor modulator on the progression of spontaneous autoimmune disease in MRL lpr/lpr mice. *Cellular Immunology* 173: 55-63.
4. Baaten BJ, Li CR, Deiro MF, Lin MM, et al. (2010) CD44 regulates survival and memory development in Th1 cells. *Immunity*. *Immunity* 32: 104-115.
5. Bagnoli F, Oliveira VM, Silva MA, Taromaru GC, et al. (1992) The interaction between aromatase, metalloproteinase 2,9 and CD44 in breast cancer. *Rev Assoc Med Bras* 56: 472-477.
6. Bebo BF, Fyfe-Johnson A, Adlard K, Beam AG, et al. (2001) Low- dose estrogen therapy ameliorates experimental autoimmune encephalomyelitis in two different inbred mouse strains. *Journal of Immunology* 166:2080-2089.
7. Benedek G, Zhang J, Nguyen H, Kent G, Seifert HA, et al. (2017) Estrogen protection against EAE modulate the microbiota and mucosal-associated regulatory cells. *J Neuroimmunol* 310: 51-59.
8. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, et al. (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441: 235-238.
9. Bitan M, Weiss L, Reibstein I, Zeira M, Fellig Y, Slavin S, et al. (2010) Heparanase upregulates Th2 cytokines, ameliorating experimental autoimmune encephalitis. *Mol Immunol* 47: 1890-1898.
10. Bollyky PL, Falk BA, Long SA, Preisinger A, et al. (2009) CD44 costimulation promotes FoxP3+ regulatory T cell persistence and function via the production of IL-2, IL-10, and TGF-beta. *Immunol* 183: 2232–2241.
11. Bollyky PL, Lord JD, Masewicz SA, Evanko SP, et al. (2007) Cutting edge: high molecular weight hyaluronan promotes the suppressive effects of CD4 CD25 regulatory T cells. *J Immunol* 179: 744–747.
12. Bourguignon LY, Gilad E, Rothman K, and Peyrollier K (2005) Hyaluronan-CD44 interaction with IQGAP1 promotes Cdc42 and ERK signaling, leading to actin binding, Elk-1/estrogen receptor transcriptional activation, and ovarian cancer progression. *J Biol Chem* 280:11961-11972.

13. Chitralla KN, Guan H, Singh NP, Busbee B, Gandy A, et al. (2017) CD44 deletion leading to attenuation of experimental autoimmune encephalomyelitis results from alterations in gut microbiome in mice. *Eur J Immunol* 47: 1188-1199.
14. Elloso MM, Phiel K, Henderson RA, Harris HA, and Adelman SJ (2005) Suppression of experimental autoimmune encephalomyelitis using estrogen receptor-selective ligands. *J Endocrinol* 185: 243-252.
15. Esparza J, Kruse M, Lee J, Michaud M, and Madri JA (2004) MMP-2 null mice exhibit an early onset and severe experimental autoimmune encephalomyelitis due to an increase in MMP-9 expression and activity. *FASEB J* 18: 1682-1691.
16. Fang YJ, Zhang L, Wu XJ, Lu ZH, Li JB, Ou QJ, et al. (2012) Impact of ER β and CD44 expression on the prognosis of patients with stage II colon cancer. *Tumour Biol* 33: 1907-1914.
17. Figueiredo AS, and Schumacher A (2016) The T helper type 17/regulatory T cell paradigm in pregnancy. *Immunology* 148: 13-21.
18. Filippi M, Bar-Or A, Piehl F, Preziosa P, Solari A, et al. (2018) Multiple sclerosis. *Nat Rev Dis Primers* 4: 43.
19. Firan M, Dhillon S, Estess P, and Siegelman MH (2006) Suppressor activity and potency among regulatory T cells are discriminated by functionally active CD44. *BLOOD* 107: 619-627.
20. Flynn K, Michaud M, Canosa S, and Madri JA (2013) CD44 Regulates Vascular Endothelial Barrier Integrity via a PECAM-1 Dependent Mechanism. *Angiogenesis* 16: 689-705.
21. Flynn K, Michaud M, and Madri JA (2013) CD44 Deficiency Contributes to Enhanced Experimental Autoimmune Encephalomyelitis: A Role in Immune Cells and Vascular Cells of the Blood Brain Barrier. *Amer J Pathol* 182: 1322-1336.
22. Fuentes N, Silveyra P (2019) Estrogen receptor signaling mechanisms. *Adv Protein Chem Struct Biol* 116: 135-170.
23. Glatigny S, and Bettelli E (2018) Experimental Autoimmune Encephalomyelitis (EAE) as Animal Models of Multiple Sclerosis (MS). *Cold Spring Harb Perspect Med* 8.
24. Gonzalez GA, Hofer MP, Syed YA, Amaral AI, et al. (2016) Tamoxifen accelerates the repair of demyelinated lesions in the central nervous system. *Sci Rep* 6: 31599.
25. Guan H, Nagarkatti PS, and Nagarkatti M (2011) CD44 Reciprocally regulates the differentiation of encephalitogenic Th1/ Th17 and Th2/regulatory T cells through epigenetic modulation involving DNA methylation of cytokine gene promoters, thereby controlling the development of experimental autoimmune encephalomyelitis. *J Immunol* 186.
26. Guan H, Nagarkatti PS, and Nagarkatti M (2009) Role of CD44 in the differentiation of Th1 and Th2 cells: CD44 deficiency enhances the development of Th2 effectors in response to sheep RBC and chicken ovalbumin. *J Immunol* 183: 172-180.
27. Haghmorad D, Amini AA, Mahmoud iMB, et al. (2014) Pregnancy level of estrogen attenuates experimental autoimmune encephalomyelitis in both ovariectomized and pregnant C57BL/6 mice through the expansion of Treg and Th2 cells. *J Neuroimmunol* 277: 85-95.
28. Hofstetter H, Gold R, and Hartung HP (2009) Th17 cells in MS and experimental autoimmune encephalomyelitis. *Int MS J* 16: 12-18.
29. Ibrahim EM, Stewart RL, Corke K, et al. (2006) Upregulation of CD44 expression by interleukins [1, 4, and 13] transforming growth factor-beta1, estrogen, and progesterone in human cervical adenocarcinoma cell lines. *Int J Gynecol Cancer* 16: 1631-1642.
30. Javadian A, Salehi E, Bidad K, Sahraian MA, and Izad M (2014) Effect of estrogen on Th1, Th2 and Th17 cytokines production by proteolipid protein and PHA activated peripheral blood mononuclear cells isolated from multiple sclerosis patients. *Arch Med Res* 45: 177-182.
31. Katoh S, Kaminuma O, Hiroi T, Mori A, Ohtomo T, et al. (2011) CD44 is critical for airway accumulation of antigen-specific Th2, but not Th1, cells induced by antigen challenge in mice. *European Journal of Immunology* 41:3198-3320.
32. Kebir H, Kreymborg K, Ifergan I, Dodelet-Devillers A, et al. (2007) Human TH17 lymphocytes promote blood-brain barrier disruption and central nervous system inflammation. *Nat Med* 13: 1173-1175.
33. Liu T, Soong L, Liu G, König R, and Chopra AK (2009) CD44 expression positively correlates with Foxp3 expression and suppressive function of CD4⁺ Treg cells. *Biol Direct* 4: 40.
34. Macdonald DC, Leir SH, Brooks C, et al. (2003) CD44 isoform expression on colonic epithelium mediates lamina propria lymphocyte adhesion and is controlled by Th1 and Th2 cytokines. *Eur J Gastroenterol Hepatol* 15: 1101-1110.
35. Mills EA, Ogrodnik MA, Plave A, and Mao-Draayer Y (2018) Emerging Understanding of the Mechanism of Action for Dimethyl Fumarate in the Treatment of Multiple Sclerosis. *Front Neurol* 9: 5.
36. Morales LB, Loo KK, Liu HB, Peterson C, et al. (2006) Treatment with an estrogen receptor alpha ligand is neuroprotective in experimental autoimmune encephalomyelitis. *J Neurosci* 26: 6823-6683.
37. Nilsson ME, Vandenput L, Tivesten Å, Norlén AK, et al. (2015) Measurement of a Comprehensive Sex Steroid Profile in Rodent Serum by High-Sensitive Gas Chromatography-Tandem Mass Spectrometry. *Endocrinology* 156:2492-2502.

38. Nishimura T, Katoh S, Mori A, Ohtomo T, et al. Critical role of CD44 in antigen-induced Th2- but not Th17-mediated murine airway inflammation. *Allerg Int* 65: S59-61.
39. Papenfuss TL, Rogers CJ, Gienapp I, et al. (2004) Sex differences in experimental autoimmune encephalomyelitis in multiple murine strains. *J Neuroimmunol* 150: 59-69.
40. Polanczyk MJ, Hopke C, Huan J, Vandenbark AA, and Offner H (2005) Enhanced FoxP3 expression and Treg cell function in pregnant and estrogen-treated mice. *J Neuroimmunol* 170: 85-92.
41. Ponta H, Sgerman L, and Herrlich PA (2003) From adhesion molecules to signaling regulators. *Nature Reviews Molecular Cell Biology* 4: 33-45.
42. Reddy J, Waldner H, Zhang X, Illes Z, et al. (2005) Cutting edge: CD4+CD25+ regulatory T cells contribute to gender differences in susceptibility to experimental autoimmune encephalomyelitis. *J Immunol* 175: 5591-5595.
43. Seifert HA, Benedek G, Nguyen H, Kent G, et al. (2017) Estrogen protects both sexes against EAE by promoting common regulatory cell subtypes independent of endogenous estrogen. *Metab Brain Dis* 32: 1747-1754.
44. Sekiya T, and Yoshimura A (2016) In Vitro Th Differentiation Protocol. *Methods Mol Biol* 183-191.
45. Shi J, Chen Y, Chen W, Tang C, Zhang H, et al. (2018) Isobavachalcone sensitizes cells to E2-induced paclitaxel resistance by down-regulating CD44 expression in ER+ breast cancer cell. *J Cell Mol Med* 22: 5220-5230.
46. Sinha S, Kaler LJ, Proctor TM, et al. (2008) IL-13- mediated gender difference in susceptibility to autoimmune encephalomyelitis. *J Immunol* 180: 2679-2685.
47. Tiwari-Woodruff S, and Voskuhi RR (2009) Neuroprotective and anti-inflammatory effects of estrogen receptor ligand treatment in mice. *Journal of the Neurological Sciences* 286: 81-85.
48. Tsuneki M, and Madri JA (2014) Adhesion Molecule-Mediated Hippo Pathway Modulates Hemangioendothelioma Cell Behavior. *Mol Cell Biol* 34: 4485-4499.
49. Tsuneki M, and Madri JA (2015) CD44 influences fibroblast behaviors via modulation of cell-cell and cell-matrix interactions, affecting Survivin and Hippo pathways. *Journal of Cellular Physiology* 231: 731-743.
50. Tsuneki M, and Madri JA (2014) CD44 regulation of endothelial cell proliferation and apoptosis via modulation of CD31 and VE-cadherin expression. *J Biol Chem*: 5357-5370.
51. Valéra MC, Fontaine C, Dupuis M, et al. (2018) Towards optimization of estrogen receptor modulation in medicine. *Pharmacol Ther* 189: 123-129.
52. Weber JD, Smith E, Berry-Kravis E, et al. (2019) Voice of People with Fragile X Syndrome and Their Families: Reports from a Survey on Treatment Priorities. *Brain Sci* 92: pii: E18.
53. Wittig BM, Johansson B, Zöllner M, Schwärzler C, et al. (2000) Abrogation of experimental colitis correlates with increased apoptosis in mice deficient for CD44 variant exon 7 (CD44v7). *J Exp Med* 191: 2053-2064.
54. Wittig BM, Stallmach A, Zeitz M, and Günther U (2003) Functional involvement of CD44 variant 7 in gut immune response. *Pathobiology* 70: 184-189.
55. Wu Q, Wang Q, Mao G, Dowling CA, et al. (2017) Dimethyl Fumarate Selectively Reduces Memory T Cells and Shifts the Balance between Th1/Th17 and Th2 in Multiple Sclerosis Patients. *J Immunol* 198: 3069-3080.
56. Xue L, Liang H, and Jiang X (2011) Up-regulation of CD44 expression by interleukin-13 in a murine model of asthma. *Mol Med Rep* 4: 1233-1237.
57. Yakubenko VP, Lobb RR, Plow EF, and Ugarova TP (2000) Differential induction of gelatinase B (MMP-9) and gelatinase A (MMP-2) in T lymphocytes upon alpha (4) beta (1)-mediated adhesion to VCAM-1 and the CS-1 peptide of fibronectin. *Exp Cell Res* 260: 73-84.
58. Yang M, Liu Y, Ren G, Shao Q, Gao W, et al. (2015) Increased expression of surface CD44 in hypoxia-DCs skews helper T cells toward a Th2 polarization. *Sci Rep* 5: 13674.

Submit your manuscript to a JScholar journal and benefit from:

- ¶ Convenient online submission
- ¶ Rigorous peer review
- ¶ Immediate publication on acceptance
- ¶ Open access: articles freely available online
- ¶ High visibility within the field
- ¶ Better discount for your subsequent articles

Submit your manuscript at
<http://www.jscholaronline.org/submit-manuscript.php>

lial cell proliferation and apoptosis via modulation of CD31