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

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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-IFNγ, anti-IL-12), and In Vitrogen (Alexa fluor-conjugated secondary antibodies).

Induction of EAE

EAE induction was performed as described pre- viously [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 an- ti-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- IFNγ 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 Trans well^{TM} System (BD Bioscience). 1.5x10^{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).

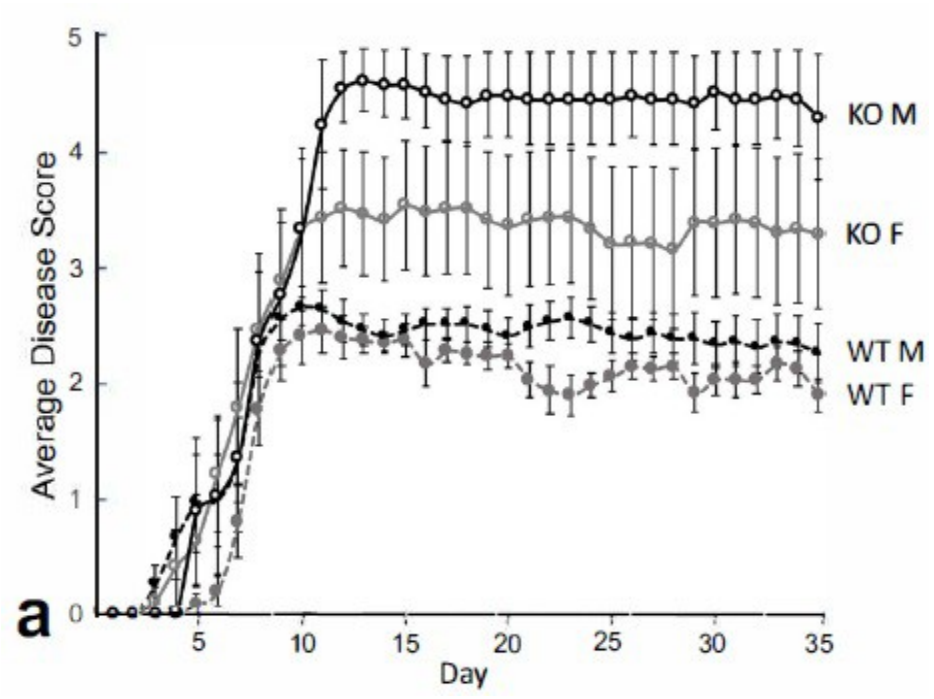
Restimulation of Splenocytes and Cytokine ELISA

Isolated splenocytes (3.5x106 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.

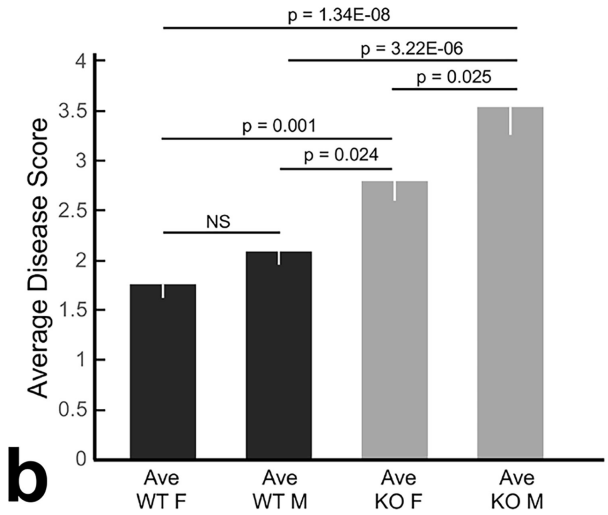
Statistics

Statistical significance was ascribed to data that achieved $P < 0.05$ (expressed as means +/- 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.



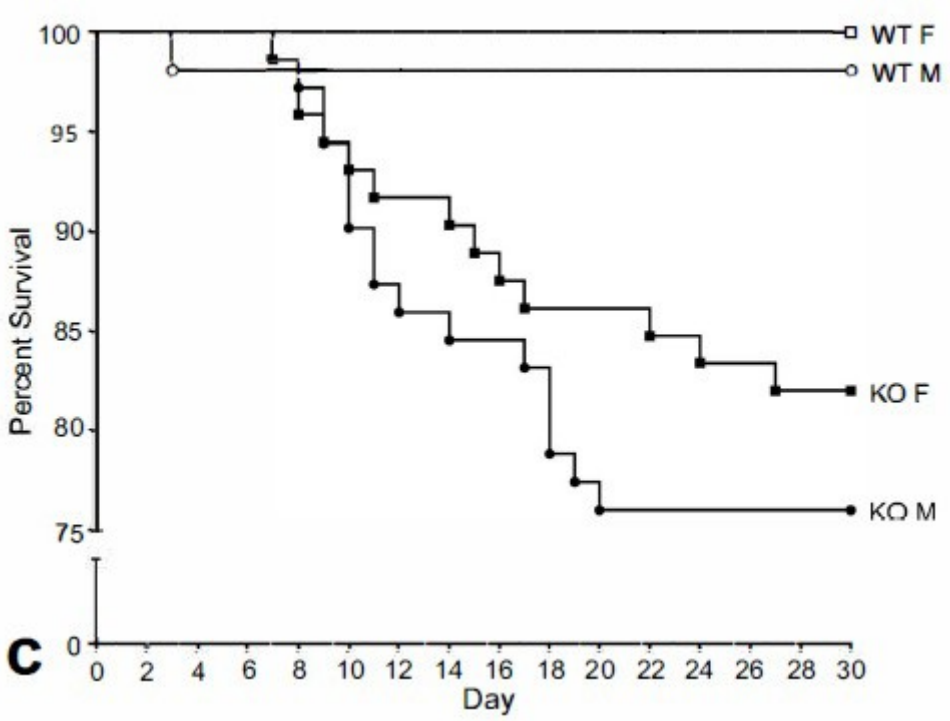
a. Average daily clinical disease scores of WT male, WT female, CD44-KO male and CD44-KO female mice following induction of EAE. Mortality scores were included in average scores from the time of death throughout the study. Data are expressed as means +/- standard errors.



Holm Multiple Comparison Calculations

Comparisons	Holm p-value	Holm inferfence
WT F vs WT M	0.2378576	insignificant
WT F vs KO F	0.0011251	** p<0.01
WT F vs KO M	1.34E-08	** p<0.01
WT M vs KO F	0.0241938	* p<0.05
WT M vs KO M	3.22E-06	** p<0.01
KO F vs KO M	0.0248284	* p<0.05

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.

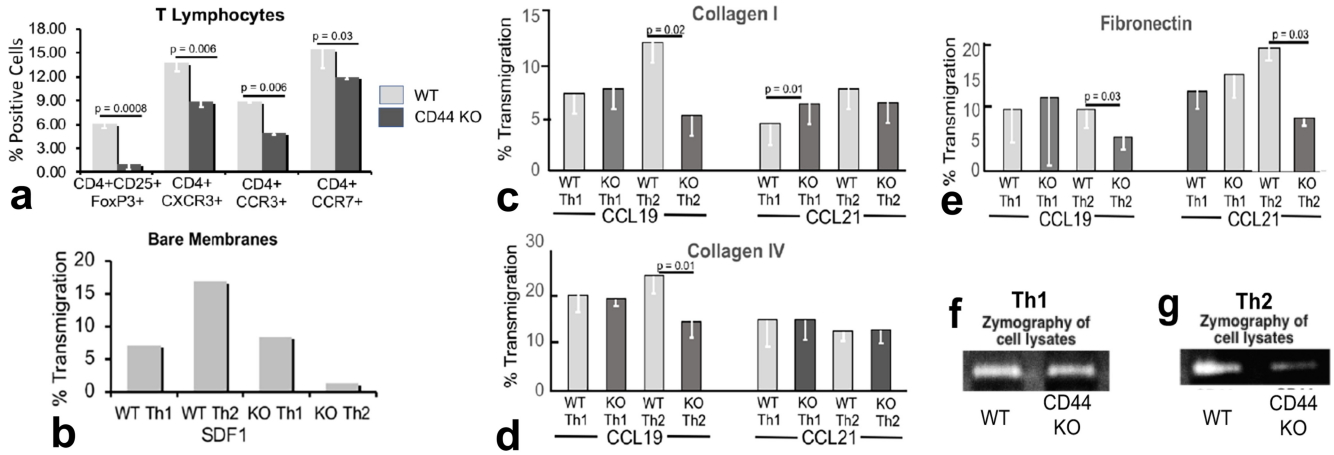


Figure 2. a-g. CD44-KO mice exhibit lower numbers of T cell subsets, decreased transmigration of Th2 cells and decreased Th2 MMP-9 activity

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 μm pore membranes, expressed as averages of three independent assays using SDF-1 as chemoattractant. There were similar percent transmigrations, 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 3μ 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).

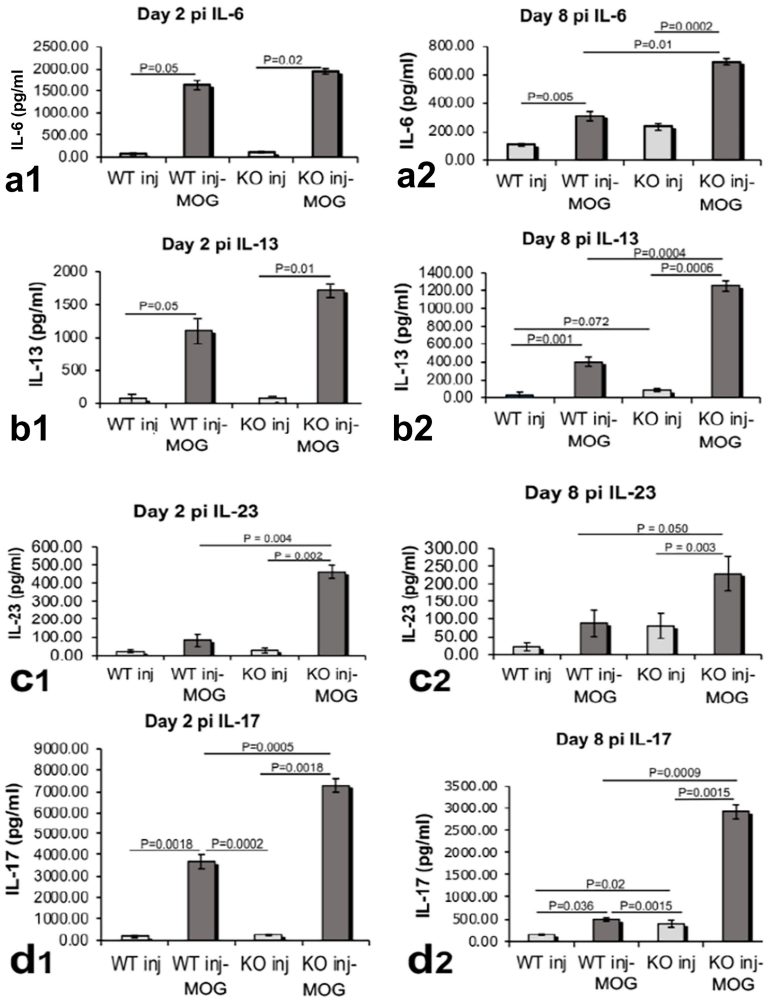


Figure 3. a1-d2. CD44 KO MOG-induced mice produce increased. Pro-inflammatory cytokines.
CD44-KO mice produce increased amounts of pro-inflammatory and anti-inflammatory cytokines. "WT inj" and "KO inj" means injected with pertussis toxin (PT) only, but not with MOG; "WT inj MOG" and "KO inj-MOG" means 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 dis- ease 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 be- tween 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 fe- male 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 WTand 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 (Figure2c).

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**).

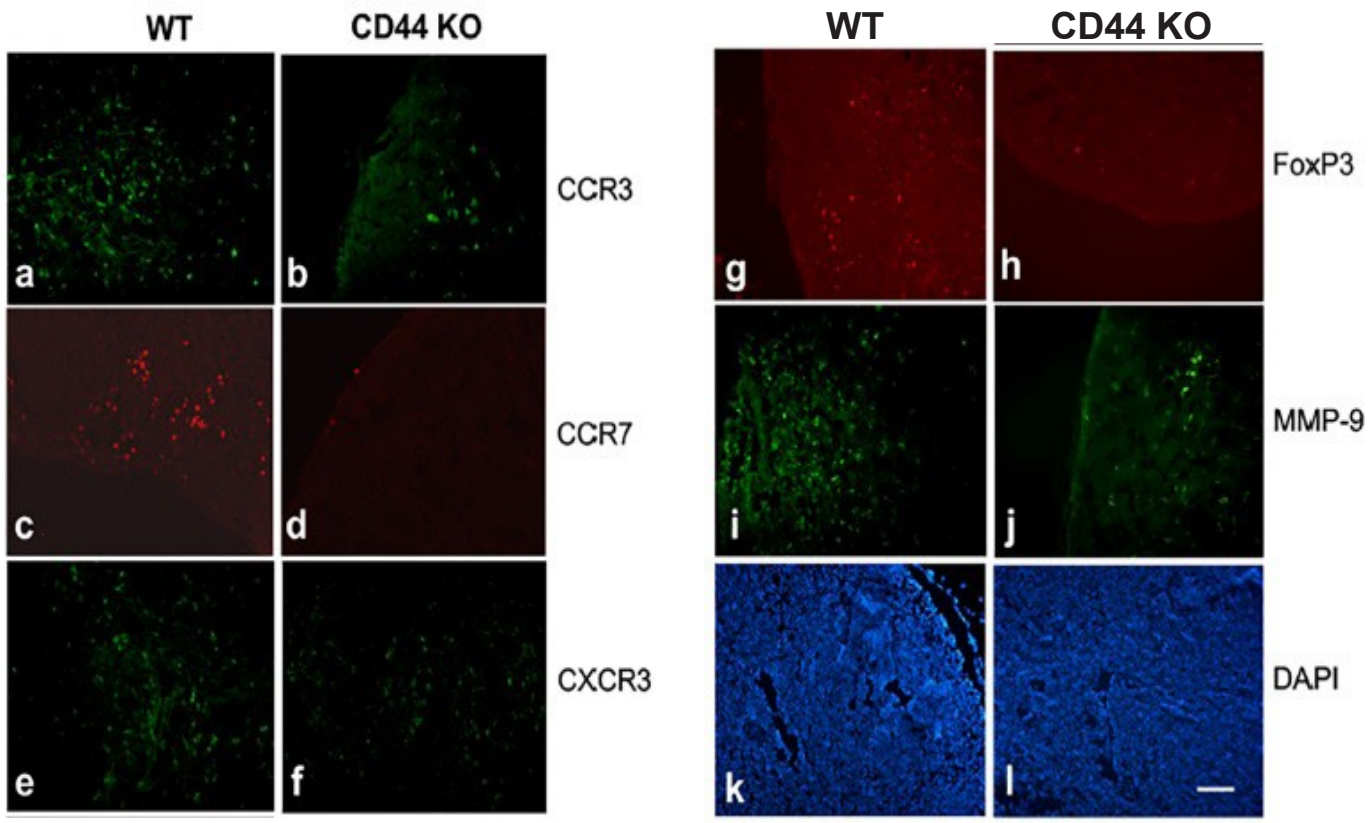


Figure 4. a-l. Peripheral lymphatic tissues from CD44-KO mice exhibit lower levels of CCR3, CCR7, CXCR3, Foxp3, and MMP-9.

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 3a$_2$**) 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 CD-44KO 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, TGFβ, 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 transmigratory 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 dy- namic 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 ERα 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 pro-duction 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 ERα [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 ERα [12]. In cervical adenocarcinoma cells, expression of a CD44 isoform is upregulated by E2 [29] while in human breast cancer cells a phytoestrogen attenuates ERα 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 under- standing and treatment of breast cancer patients with MS treated with E2 inhibitors.

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