

## ER $\beta$ and ER $\alpha$ Differentially Regulate NKT and V $\gamma$ 4<sup>+</sup> T-cell Activation and T-regulatory Cell Response in Coxsackievirus B3 Infected Mice

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### Abstract

**Objectives:** Coxsackievirus B3 (CVB3) induced myocarditis is sex dependent with males developing more severe disease than females. Previous studies had shown that sex-associated hormones determine the sex bias with testosterone and progesterone promoting myocarditis while estrogen (E2) is protective. There are two major estrogen receptors: estrogen receptor alpha (ER $\alpha$ ) and estrogen receptor beta (ER $\beta$ ). The goal of the current study was to determine the relative role of these receptors to myocarditis susceptibility and the mechanism of their action.

**Methods:** Female C57Bl/6 wild-type mice and C57Bl/6 mice deficient in ER $\alpha$ , or ER $\beta$  were infected intraperitoneally with 10<sup>2</sup> plaque forming units CVB3. After 7 days, hearts were evaluated for virus titers by plaque forming assay and myocardial inflammation. Lymphoid cells either from the spleen or infiltrating the heart were characterized by labeling with antibodies including CD4, CD25, FoxP3, IFN $\gamma$ , IL-4, CD11b, CD1d, V $\gamma$ 4, TCR $\beta$ , or with CD1d-tetramer and evaluated by flow cytometry. To confirm that signaling through distinct estrogen receptors controlled myocarditis susceptibility and T-regulatory cell response, male C57Bl/6 mice were treated with the ER $\alpha$ -specific agonist, propyl pyrazole triol (PPT), ER $\beta$  agonist, diarylpropionitrile (DPN), or 17- $\beta$ -estradiol (E2) as a non-specific estrogen receptor agonist.

**Results:** Myocarditis, cardiac virus titers, and CD4<sup>+</sup> Th1 (IFN $\gamma$ ) bias were increased in infected ER $\alpha$ KO and decreased in infected ER $\beta$ KO mice compared to C57Bl/6 controls. CD4<sup>+</sup>Th1 bias and myocarditis severity correlated inversely with numbers of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T regulatory cells which were decreased in ER $\alpha$ KO and increased in ER $\beta$ KO mice. Increased T-regulatory cells corresponded to a preferential activation of natural killer T (NKT) cells in ER $\beta$ KO mice. Male C57Bl/6 mice treated with DPN showed increased myocarditis while those treated with PPT and E2 showed decreased myocarditis corresponding to either decreased (DPN) or increased (PPT/E2) T-regulatory cell responses in male C57Bl/6 mice. DPN and PPT treatment had no effect on T-regulatory cell responses in NKT KO or  $\gamma$ 4KO mice.

**Conclusion:** These results demonstrate that ER $\alpha$  and ER $\beta$  both modulated CVB3 myocarditis susceptibility but in opposite directions and that their predominant effect is mediated through their ability to alter NKT and V $\gamma$ 4<sup>+</sup> innate T cell responses in the infected host. It is these innate T cells which positively or negatively modulate T-regulatory cell responses.

**Keywords:** Coxsackievirus B3; Myocarditis; Estrogen receptor alpha; Estrogen receptor beta; T-regulatory cells; CD4<sup>+</sup> cells; Immunosuppression

### Introduction

Myocarditis is an inflammation of the myocardium which often follows microbial infections. While somewhat controversial, many clinical studies report an increased incidence of viral myocarditis in men compared to women, although women are more susceptible during pregnancy [1-3]. Mice infected with coxsackievirus B3 (CVB3) develop myocarditis with many similar characteristics to the human disease. The inflammatory infiltrate is predominantly comprised of mononuclear cells and inflammatory cells are intimately associated with necrotic myocytes while adjacent myocytes appear unharmed [4,5]. Male and pregnant female mice are highly susceptible to CVB3 induced myocarditis while virgin females are more resistant [6-10]. Sex associated hormones control myocarditis since castration of males is protective while restoration of testosterone increases susceptibility [11-13]. Furthermore, treating male mice with 17- $\beta$ -estradiol (E2) significantly suppresses myocarditis while treatment of females with androgen enhances myocarditis [14]. The effects of E2 on both innate and adaptive immunity have been extensively studied and show a wide range of hormonal effects. The generally accepted estrogen effects include increasing immunoglobulin synthesis [15]; suppressing both T and B cell lymphopoiesis [16]; enhancing dendritic cell differentiation and antigen presentation [17]; suppressing TNF $\alpha$  and IL-6 expression

[18,19]; while increasing IL-4 and IFN $\gamma$  production [20,21]; inhibiting B cell apoptosis [22]; and promoting FoxP3<sup>+</sup> T regulatory cell development [23,24]. Although the above effects are generally agreed upon by the majority of investigators, divergent reports exist. For example, while most published studies demonstrate a positive effect on IFN $\gamma$  production by E2, some studies show either no effect on the level of this cytokine [21] or increased expression [25]. Thus, the effects of E2 might not be absolute but depend upon various factors including hormone concentration, cell type/tissue involved, or unknown variables.

There are two known estrogen receptors (ER $\alpha$  and ER $\beta$ ) which bind with equivalent specificity and affinity to estrogen response elements (ERE) in gene promoter regions leading to modulation of

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gene expression [26]. ER $\alpha$  and ER $\beta$  have wide tissue distribution with ER $\alpha$  primarily found in the uterus, liver, kidney and heart while ER $\beta$  is primarily found in ovary prostate, lung, central nervous system, gastrointestinal tract, and bladder. Lymphocytes, macrophages and dendritic cells co-express both ER $\alpha$  and ER $\beta$  [27] but ER $\alpha$  primarily controls E2 modulation of dendritic cell maturation, T cell cytokine production and immunoglobulin response [21,28,29]. In contrast, signaling through ER $\beta$  up-regulates inducible nitric oxide synthase (iNOS) and nitric oxide generation while ER $\alpha$  suppresses this response [30]. Several important studies now show that where ER $\alpha$  and ER $\beta$  are co-expressed in the same cell, these receptors may exert opposing effects on gene expression and thus counter-balance each other [25,26,31,32].

Previous studies have also demonstrated that selective generation of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T regulatory cells prevent CVB3 induced myocarditis and that E2 treatment of infected mice promotes T regulatory cell activation [33]. This observation is consistent with previous published reports showing the same phenomenon [24,34]. However, the use of estradiol in the previous studies fails to distinguish between the hormonal signaling between the ER $\alpha$  and ER $\beta$  forms as the hormone binds to and causes signal transduction through both receptors. The goal of the current study was to determine whether there was a distinctive role of each receptor on CVB3 myocarditis and on immunoregulation. As shown here, the study found that in the CVB3 induced myocarditis model, signaling through the ER $\alpha$  is responsible for T regulatory cell response while signaling through ER $\beta$  inhibits T regulatory cell activation. The significance of this observation may be that selective activation of individual hormonal receptors may identify a potential therapeutic approach.

## Materials and Methods

### Mice

Female C57Bl/6 ER $\alpha$ KO (B6.129P2-Esr1<sup>tm1Ksk</sup>/J), female C57Bl/6 ER $\beta$ KO (B6.129P2-Esr2<sup>tm1Unc</sup>/J), male C57Bl/6 GFP-FoxP3 (B6.Cg-Foxp3<sup>tm2Tch</sup>/J), male and female C57Bl/6J mice, and C57Bl/6  $\delta$  KO (B6.129P2-Tcra<sup>tm1Mom</sup>/J) 4-7 weeks of age, were purchased from Jackson Laboratories, Bar Harbor ME. Ja18 deficient were backcrossed for more than 10 generations to the C57Bl/6 background and were kindly supplied by Dr. Jon Boyson (University of Vermont, Burlington VT). These animals lack natural killer T cells (NKT KO) [35]. All experimental groups contained 4-6 mice each. All of the studies have been reviewed and approved by the University of Vermont Institutional Animal Care and Use Committee.

### Virus

The H3 variant of CVB3 was made from an infectious cDNA clone as described previously [36].

### Infection of mice

Mice were injected intraperitoneally (i.p.) with 10<sup>2</sup> plaque forming units (PFU) virus in 0.5 ml PBS. Animals were killed when moribund or 7 days after infection. Controls were uninfected mice which were killed at the same time as infected animals.

### Organ virus titers

Hearts were aseptically removed from the animals, weighed, homogenized in RPMI 1640 medium containing 5% fetal bovine serum (FBS), L-glutamine, streptomycin and penicillin. Cellular debris was removed by centrifugation at 300  $\times$  g for 10 min. Supernatants were diluted serially using 10-fold dilutions and titered on Hela cell

monolayers by the plaque forming assay [37].

### Hormone treatment

17- $\beta$ -estradiol (Sigma Chemical Co., St. Louis, MO) was initially (120  $\mu$ g/ml) diluted in 100% ethanol then diluted to 400 ng/ml in corn oil. Mice were injected subcutaneously (s.c.) with either 200 ng/mouse estradiol or ethanol/corn oil on days -4, 0 and +4 relative to infection. Mice treated with hormone included male C57Bl/6, NKTKO and V $\gamma$ 4KO animals.

### Estrogen receptor agonists

The ER $\alpha$  selective agonist, propyl pyrazole triol (PPT), and the ER $\beta$  selective agonist, diarylpropionitrile (DPN), were purchased from Tocris Co, Ellisville MO, initially dissolved in DMSO, then diluted 1:10 in corn oil to inject 0.05 mM/kg body weight (19.8 mg/kg). Mice were injected s.c. with the agonists or DMSO/corn oil vehicle on days -4, 0 and +4 relative to infection [38]. Mice treated with hormone included male C57Bl/6, NKTKO and V $\gamma$ 4KO animals.

### Histology

Hearts were fixed in 10% buffered formalin for 48 h, paraffin embedded, sectioned and stained by hematoxylin and eosin. Image analysis of cardiac inflammation was done as described previously [36].

### Isolation of Inflammatory cells from the heart

The protocol for isolating inflammatory cells infiltrating the hearts of CVB3 infected mice has been published previously [39]. Hearts were perfused with 10 ml PBS, removed, minced finely then subjected to a 10 min digestion with 0.4% collagenase II (Sigma Chemical Co., St. Louis MO) and 0.25% pancreatin (Sigma) at 37°C and removal of the supernatant to a tube containing 10% FBS. The remaining tissue was pressed through a fine mesh screen to release additional lymphoid cells. The large cellular debris was allowed to settle and the cell suspension containing the inflammatory cells was added to the cells released by digestion and layered on Histopaque (Sigma-Aldrich, St. Louis MO) and centrifuged at 300  $\times$  g for 25 min. The cells at the interface were retrieved, and washed in PBS-2% FBS.

### Flow cytometry

Details for flow cytometry and intracellular cytokine staining have been published previously [40]. Unless otherwise indicated, all antibodies were obtained from BD Biosciences, BD Sciences, Fair Lawn, NJ. As indicated in the text, lymphoid cells used for flow cytometry were either isolated from the heart as described above, or were isolated from spleens of mice. When isolated from the spleen, spleens were pressed through fine mesh screens to form single cell suspensions and the suspensions were layered on Histopaque and centrifuged at 300  $\times$  g for 25 min. The cells at the interface were retrieved, and washed in PBS-2% FBS. For intracellular cytokine staining, 10<sup>6</sup> lymphoid cells were cultured for 4 h in RPMI 1640 medium containing 5% FBS, 10  $\mu$ g/ml Brefeldin A (BFA), 50 ng/ml phorbol myristate acetate (PMA), and 500 ng/ml ionomycin (Sigma). After culture, the cells were washed in PBS-1% bovine serum albumin (BSA; Sigma) containing BFA, and labeled with PerCP-Cy5.5-anti-CD4 (clone GK1.5) for 30 min, washed, then fixed in 2% paraformaldehyde for 10 min. The cells were resuspended in PBS-BSA containing 0.5% saponin, Fc Block and 1:100 dilutions of PE-anti-IFN $\gamma$  (clone XMG 1.2), Alexa 647-anti-IL-4 (clone 11B11), or PE and Alexa 647- rat IgG1 (clone R3-34) and incubated for 30 min on ice. The cells were washed once in PBS-BSA-saponin and once in PBS-BSA, then resuspended in 2% paraformaldehyde. FoxP3 labeling was

done using the eBioscience kit according to manufacturer's directions. Cells were labeled with Alexa647 anti-CD4, FITC-rat-anti-mouse CD1d (clone 1B1) and PerCP-Cy5.5 anti-CD25 (clone PC61) in PBS-1% BSA containing FcBlock, washed, fixed and permeabilized, then incubated with PE-anti-FoxP3 (clone FJK-16s, eBiosciences, San Diego CA) and FcBlock overnight at 4°C. Cells were washed and resuspended in PBS-2% paraformaldehyde. Additional cells were labeled with APC-Cy7 anti-CD11b (clone M1/70), FITC-anti-CD1d in PBS-BSA on ice for 30 min, washed and resuspended in 2% paraformaldehyde. To evaluate natural killer T (NKT) and V $\gamma$ 4<sup>+</sup>T cells, inflammatory cells isolated from hearts as described above were stained with PE-mCD1d-tetramer (NIH Tetramer facility Yerkes) and FITC-anti-TCR $\beta$  (clone H57-597) for NKT cells or with PE-anti-V $\gamma$ 4 (clone UC3) and FITC anti-CD69 (clone H1.2F3) for V $\gamma$ 4<sup>+</sup> cells. Cells were incubated for 30 min, washed and resuspended in 2% paraformaldehyde for analysis. Cells were analyzed using a Coulter Epics Elite flow cytometer with a single excitation wavelength (488 nm) and band filters for FITC (525 nm), PerCP-Cy5.5 (695/40 nm), and PE (575 nm). The excitation wavelength for Alexa 647 is 643 nm and a band filter of 660/20 nm. The excitation wavelength for APC-Cy7 was 595 nm and a band filter of 650 nm. The cell population was classified for cell size (forward scatter) and complexity (side scatter). At least 10,000 cells were evaluated. Positive staining was determined relative to isotype controls.

### Isolation of mouse T-regulatory cells

Isolation of CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells from the spleen was performed using the EasySep<sup>™</sup> Mouse CD4<sup>+</sup>CD25<sup>+</sup> Regulatory T Cell Isolation Kit (StemCell Technologies, Vancouver, Canada) according to manufacturer's directions.

### Adoptive transfer of CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells into ER $\alpha$ KO recipients

CD4<sup>+</sup>CD25<sup>+</sup> T regulatory cells were isolated from C57Bl/6, ER $\alpha$ KO and ER $\beta$ KO female mice infected 7 days earlier with 10<sup>2</sup> PFU CVB3 as described above. Cells were resuspended in PBS, and 0.2 mL containing either 1.0 × 10<sup>4</sup> or 1.0 × 10<sup>5</sup> cells was injected through the tail vein of anesthetized female ER $\alpha$ KO recipient mice on the same day that the recipient mice were infected with 10<sup>2</sup> PFU CVB3. Recipient mice were killed 7 days later.

### Statistics

Data was analyzed for skewness and kurtosis using the SPSS for Windows program (Version 11.0. Chicago, IL: SPSS, Inc.; 2001) and showed that variance was not normally distributed for several groups. Statistical analysis was done by the nonparametric Mann-Whitney test using SPSS for Windows. Threshold for significance was 0.05 or better.

## Results

### ER $\alpha$ Protects against CVB3 induced myocarditis

To determine whether estrogen-dependent protection requires either ER $\alpha$  or ER $\beta$ , wild-type C57Bl/6, ER $\alpha$ KO, and ER $\beta$ KO female mice were infected with CVB3 and evaluated for myocarditis, and cardiac virus titers 7 days later (Figure 1). Neither C57Bl/6 nor ER $\beta$ KO mice developed myocarditis (Figures 1A and 1F) while ER $\alpha$ KO mice were significantly more susceptible to myocarditis (Figures 1A and 1E; p<0.01). Cardiac virus titers were also significantly increased in infected ER $\alpha$ KO (p<0.01) mice and significantly decreased in infected ER $\beta$ KO (p<0.05) females (Figure 1B). Spleen cells were labeled with anti-CD11b and anti-CD1d and evaluated by flow cytometry. Increased

expression of CD1d on monocytes as determined by mean fluorescence intensity (MFI) staining was observed in ER $\alpha$ KO and decreased MFI staining was observed in ER $\beta$ KO infected mice (Figure 1C; p<0.05).

### Effect of ER $\alpha$ and ER $\beta$ in IFN $\gamma$ and IL-4 expression by CD4<sup>+</sup> cells

C57Bl/6, ER $\alpha$ KO and ER $\beta$ KO females were infected with CVB3 and killed 7 days later. Spleen cells were activated with PMA and ionomycin in the presence of brefeldin A, then labeled with antibody to CD4 and intracellularly with antibodies to IFN $\gamma$  and IL-4 (Figure 2). Representative flow diagrams of cytokine expression from an individual mouse from each group are provided in Figure 2A and the summary of cytokine expression by all mice in each group are given in Figures 2B and 2C. IL-4 expression is significantly reduced in infected ER $\alpha$ KO mice (p<0.05) and increased in ER $\beta$ KO (p<0.01) females. IFN $\gamma$  expression is significantly increased in ER $\alpha$ KO mice but decreased in ER $\beta$ KO animals (p<0.05 for both).

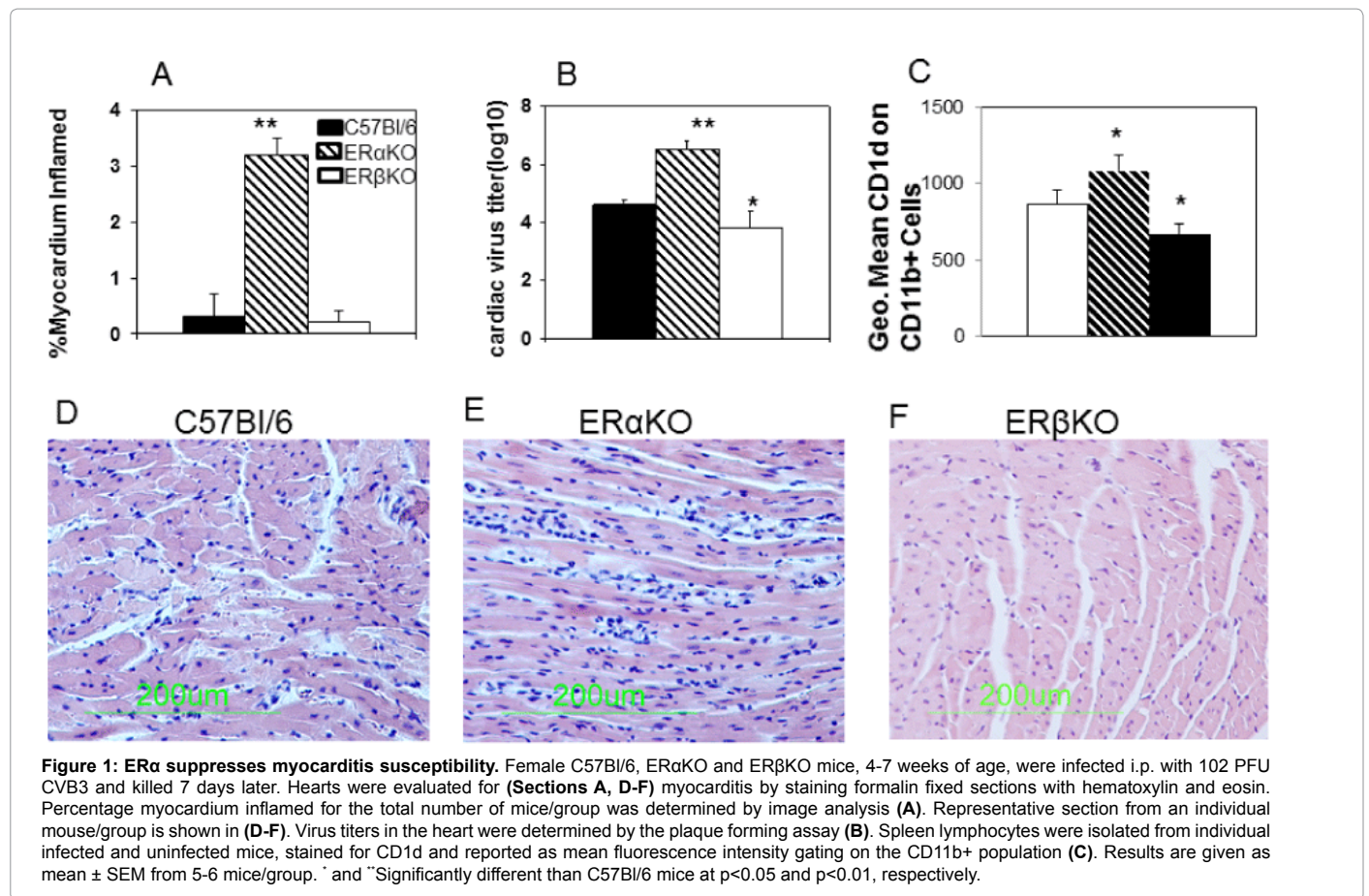
### ER $\alpha$ promotes CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T regulatory cell response during CVB3 infection

Prior studies found that females preferentially activate T regulatory cells when infected with CVB3 [33] and that this was mediated through the effects of estradiol. To determine whether the distinct estrogen receptors have different effects on T regulatory cell responses, spleen cells from C57Bl/6, ER $\alpha$ KO and ER $\beta$ KO females infected 7 days previously with CVB3 were gated on the CD4<sup>+</sup> cell population and evaluated for CD25 and FoxP3 expression (Figure 3). Approximately 24% of CD4<sup>+</sup> cells in infected C57Bl/6 mice are CD25<sup>+</sup>FoxP3<sup>+</sup> compared to only 15% of CD4<sup>+</sup> cells from infected ER $\alpha$ KO mice indicating that significantly fewer T regulatory cells are generated in ER $\alpha$ KO mice (Figure 3B; p<0.05). No significant difference was observed in T regulatory cell numbers in infected ER $\beta$ KO mice. CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T cells express higher levels of CD1d than CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> non-regulatory T cells (Figures 3A and 3C; p<0.05). However, when the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells were evaluated for CD1d expression from the three different CVB3 infected mouse groups, ER $\beta$ KO mice showed significantly increased MFI staining for CD1d than either C57Bl/6 or ER $\alpha$ KO females (Figure 3C; p<0.05). Since prior studies demonstrated CD1d expression levels by T regulatory cells correlated to their immunosuppressive activity *in vivo* [39], CD4<sup>+</sup>CD25<sup>+</sup> cells were isolated from spleens of C57Bl/6, ER $\alpha$ KO and ER $\beta$ KO female mice 7 days after CVB3 infection and either 10<sup>4</sup> or 10<sup>5</sup> cells were injected i.v. into female ER $\alpha$ KO mice on the same day as CVB3 infection. The recipients were killed 7 days later and evaluated for myocardial inflammation (Figure 4). Compared to infected ER $\alpha$ KO mice not receiving cells, both recipient groups given C57Bl/6 and ER $\alpha$ KO CD4<sup>+</sup>CD25<sup>+</sup> T cells showed modest but not statistically significant suppression of myocarditis especially with 50,000 cells transferred. However, CD4<sup>+</sup>CD25<sup>+</sup> cells from ER $\beta$ KO donor mice were substantially more immunosuppressive (p<0.05).

### Different effect of ER $\alpha$ and ER $\beta$ on NKT and V $\gamma$ 4 T cell infiltration into the CVB3 infected heart

During CVB3 infections, innate T cells (NKT and  $\gamma\delta$  T cells) differentially control T regulatory cell activation [39,41,42]. Therefore, inflammatory lymphoid cells were isolated from hearts of female C57Bl/6, ER $\alpha$ KO and ER $\beta$ KO mice 7 days after CVB3 infection evaluated for activated V $\gamma$ 4<sup>+</sup>CD69<sup>+</sup> T cells (Figure 5C) or NKT cells using the mCD1d-tetramer and antibody to TCR $\beta$  (Figure 5B). As shown in Figure 5A, ER $\alpha$ KO mice had few or no NKT cells infiltrating the hearts but had dramatically increased numbers of V $\gamma$ 4<sup>+</sup> cells



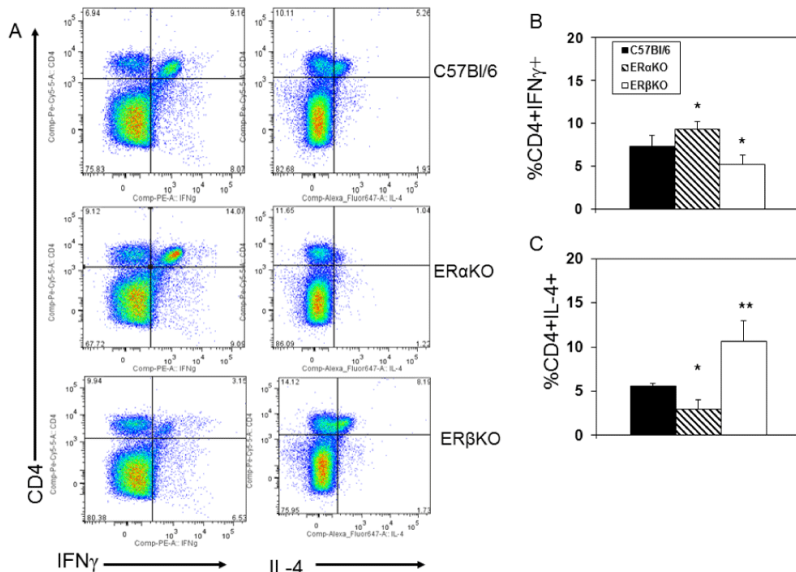


compared to C57Bl/6 females (approximately 7-fold increase compared to C57Bl/6, p<0.01). ER $\beta$ KO females showed slightly increased numbers of NKT (1.5 fold increase; p<0.05) and decreased V $\gamma$ 4+ cells (p<0.05). To determine if the predominant immune modulatory role of estrogen receptors is mediated through these innate T cell mediators, initial studies confirmed that the receptor specific agonists PPT (ER $\alpha$ ) and DPN (ER $\beta$ ) replicated results using knockout mice. Male C57Bl/6 GFP-FoxP3 Tg mice were injected with PPT or DNP at 0.05 mM/kg on days -4, 0 and +4 relative to infection with CVB3. Male mice were also injected s.c. with estradiol (E2) as a non-specific ER agonist. Treatment of male mice with either E2 or PPT significantly reduced mortality (Figure 6A; p<0.05) and myocarditis (Figure 6B; p<0.05) while treatment with DPN actually promoted myocarditis (p<0.05). T regulatory cells were increased in males treated with PPT (p<0.01) and E2 (p<0.05) but were not increased by DPN treatment (Figure 6C). Finally, C57Bl/6,  $\gamma$  $\delta$ KO and NKT KO mice were treated with either vehicle, PPT, DNP or E2 and infected with CVB3 (Figure 7). While treating C57Bl/6 mice with PPT or E2 caused substantial increases in T regulatory cell responses compared to vehicle control (4-fold and 3-fold increase; p<0.01 and p<0.05), NKT KO or  $\gamma$  $\delta$ KO mice showed no difference between the treatment groups although NKTKO mice had fewer T regulatory cells while  $\gamma$  $\delta$ KO mice had greater numbers of T regulatory cells than C57Bl/6 mice.

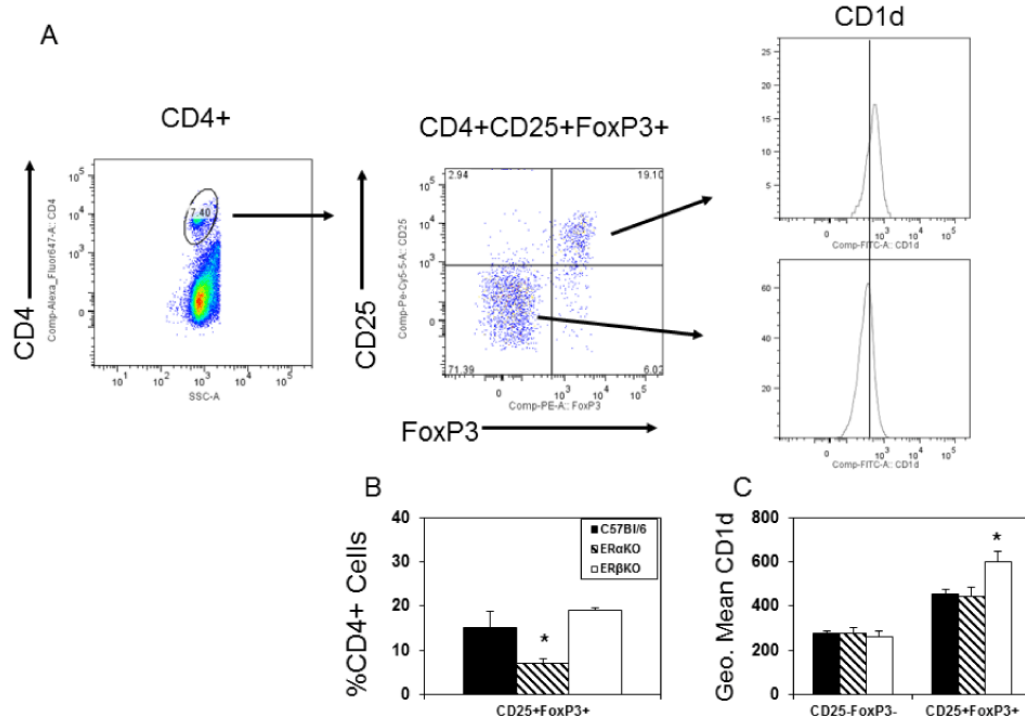
## Discussion

This paper shows that ER $\alpha$  and ER $\beta$  differentially regulate both myocarditis susceptibility and T regulatory cell responses primarily though their impact on the innate effector T cell populations NKT,

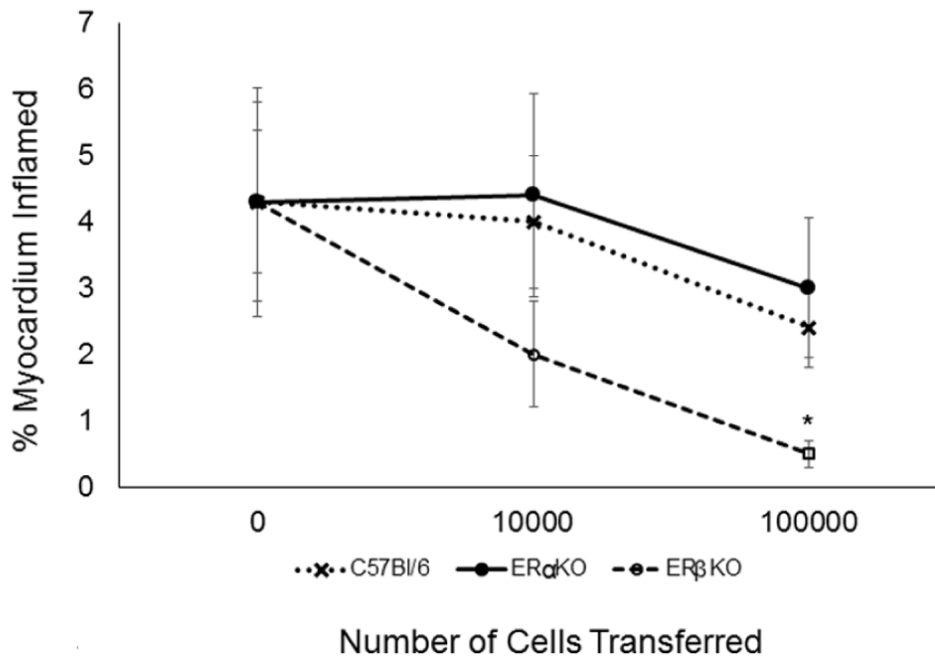
and V $\gamma$ 4+ cells. Previous studies in male mice showed that NKT cells preferentially activate T regulatory cells during CVB3 infections whereas T cells expressing the V $\gamma$ 4 T cell receptor selectively abrogate immunosuppression by killing the CD1d+ CD4+CD25+FoxP3+ cell population [39,41-43]. Furthermore, studies showed that protection in females from CVB3-induced myocarditis was estrogen-dependent and was mediated through preferential activation of T regulatory cells in this sex [33,44]. However, the earlier study did not evaluate how the different estrogen receptors (ER $\alpha$  and ER $\beta$ ) might selectively impact either CVB3 pathogenicity or T-regulatory cell responses. Sex hormones bind to specific nuclear and membrane-associated protein receptors resulting in diverse and often contradictory effects in innate and adaptive immunity. Evidence that membrane estrogen receptors (mER) are derived from the same genes as nuclear ERs comes from studies where deletion of the ER $\alpha$  or ER $\beta$  gene eliminates the function of both types of receptors [45]. A separate estrogen receptor is G protein-coupled ER (GPER), which is a 7-transmembrane protein that is unrelated to ER $\alpha$  or ER $\beta$ . Cells may express different combinations of the various ERs which may result in complex signaling patterns [46]. Among T cell populations, CD4+ cells in general express high levels of ER $\alpha$  but minimal or no ER $\beta$  [47]. However, this is not true for CD4+CD25+FoxP3+ T regulatory cells which express both ERs with high levels of ER $\beta$  (at least in patients with multiple sclerosis) [48]. Although this might have indicated that signaling through ER $\beta$  should preferentially promote immunosuppression through T regulatory cell activation, other studies have directly implicated ER $\alpha$  in T regulatory cell responses. One mechanism by which ER $\alpha$  might enhance T regulatory cell responses may be through direct upregulation of FoxP3



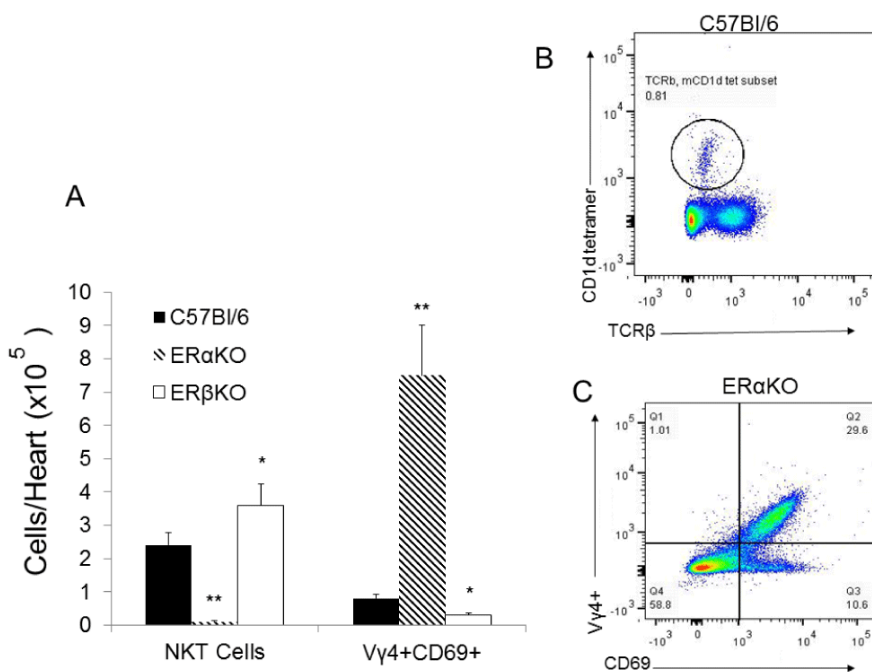
**Figure 2:** ER modulates IL-4 and IFN $\gamma$  expression by CD4<sup>+</sup> T-cells. Spleen cells from C57Bl/6, ER $\alpha$ KO and ER $\beta$ KO mice infected 7 days earlier with 10<sup>2</sup> PFU CVB3 were cultured for 4 h in medium containing PMA, ionomycin and brefeldin A, labeled with antibody to CD4, fixed, permeabilized, and labeled with antibodies to IFN $\gamma$  and IL-4. Cells were evaluated by flow cytometry. (A) Representative flow diagrams from an animal in each group; (B) Mean (%) of cells staining positive for CD4 and IFN $\gamma$   $\pm$  SEM. (C) Mean (%) cells staining positive for CD4 and IL-4  $\pm$  SEM. Groups consisted of 5-6 mice each. \* and \*\* Significantly different than C57Bl/6 mice at p<0.05 and p<0.01, respectively.



**Figure 3:** ER $\alpha$  promotes CD4<sup>+</sup> T regulatory cell response. Spleen cells from C57Bl/6, ER $\alpha$ KO and ER $\beta$ KO mice infected 7 days earlier with 10<sup>2</sup> PFU CVB3 were labeled with antibodies to CD4, CD1d and CD25, fixed, permeabilized and labeled with antibody to FoxP3 for flow cytometry analysis. (A) Cells were gated on the CD4<sup>+</sup> cell population from an infected C57Bl/6 mouse, and then evaluated for CD25 and FoxP3 expression. The CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> and CD4<sup>+</sup>CD25<sup>-</sup>FoxP3<sup>-</sup> subpopulations were evaluated for CD1d expression. The line in the CD1d graph at far right indicates mean intensity fluorescence for the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> population and indicates that the CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> cells express higher levels of CD1d. (B) Summary of percentage of CD4<sup>+</sup> cells which are CD25<sup>+</sup>FoxP3<sup>+</sup> for 5-6 mice/group (mean  $\pm$  SEM). (C) Summary of mean fluorescence intensity of staining for CD4<sup>+</sup> cells which are either CD25<sup>-</sup>FoxP3<sup>-</sup> or CD25<sup>+</sup>FoxP3<sup>+</sup> for 5-6 mice/group (mean  $\pm$  SEM). \*Significantly different than C57Bl/6 mice at p<0.05.

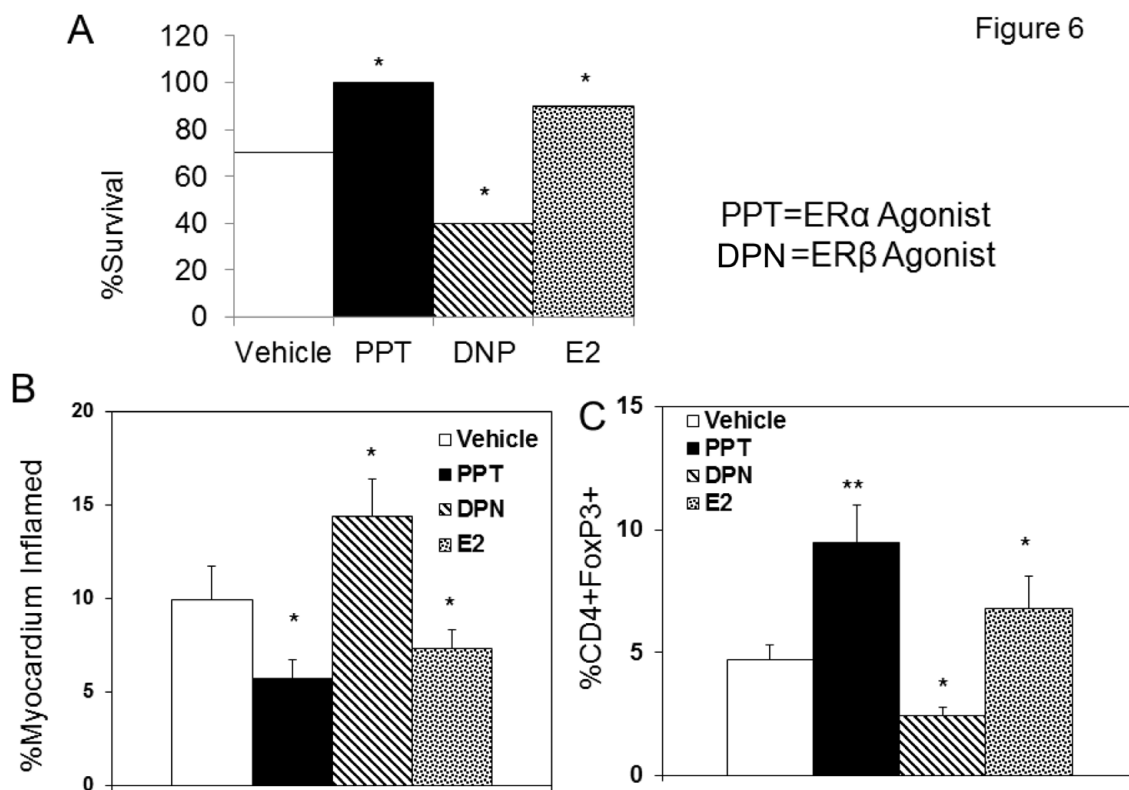


**Figure 4:** Adoptive Transfer of CD4<sup>+</sup>CD25<sup>+</sup> cells into CVB3 infected ER $\alpha$ KO female recipients. C57Bl/6, ER $\alpha$ KO and ER $\beta$ KO female mice were infected with 10<sup>2</sup> PFU CVB3 and killed 7 days later. CD4<sup>+</sup>CD25<sup>+</sup> T-cells were isolated from the spleen and 0, 10<sup>4</sup> or 10<sup>5</sup> cells were injected i.v. into infected female ER $\alpha$ KO recipients on the same day as infection. Recipients were killed 7 days later and evaluated for myocarditis. Results represent mean  $\pm$  SEM of 4-6 mice/group. \*Significantly different than C57Bl/6 mice at p<0.05.



**Figure 5:** Estrogen receptor determines innate T-cell response in the heart. Female C57Bl/6, ER $\alpha$ KO and ER $\beta$ KO mice were infected with CVB3 and killed 7 days later. Hearts were removed and infiltrating inflammatory cells were recovered and evaluated for natural killer T (NKT) cells using PE-mCD1d tetramer and FITC-anti-TCR $\beta$ . T-cells expressing the V $\gamma$ 4 T-cell receptor (TCR) were identified by labeling cells with PE-anti-V $\gamma$ 4 and FITC-anti-CD69. Summary of NKT and V $\gamma$ 4<sup>+</sup> cells in the different infected mouse strains is given in (A). Representative flow diagrams for NKT (B) and V $\gamma$ 4<sup>+</sup> (C) cells are provided. Results are given as mean  $\pm$  SEM from 5-6 mice/group. \* and \*\*Significantly different than C57Bl/6 mice at p<0.05 and p<0.01, respectively.

## Estrogen Receptor Signaling in CVB3 Infected Male Mice



**Figure 6:** Treatment of male mice with either E2 or the ER $\alpha$  agonist protects against myocarditis and stimulates T regulatory cell response. Male C57Bl/6 GFP-FoxP3 Tg mice, 4-7 weeks of age, were injected s.c. with 0.05 mM/kg ER $\alpha$  agonist (PPT), ER $\beta$  agonist (DPN) or 200 ng/mouse E2 on days -4, 0, or +4 relative to infection with 102 PFU CVB3 on day 0. Control animals were injected s.c. with DMSO/corn oil only (vehicle). Groups contained 10 mice each. (A) Animal survival by day 7 after infection, (B) Percentage (%) of myocardium inflamed as determined by image analysis of hematoxylin and eosin stained sections, and (C) Percentage (%) of spleen cells which are positive for CD4 and the GFP-FoxP3. Results are given as mean  $\pm$  SEM. \* and \*\*Significantly different than mice given vehicle at  $p < 0.05$  and  $p < 0.01$ , respectively. Results are from 5-6 mice/group.

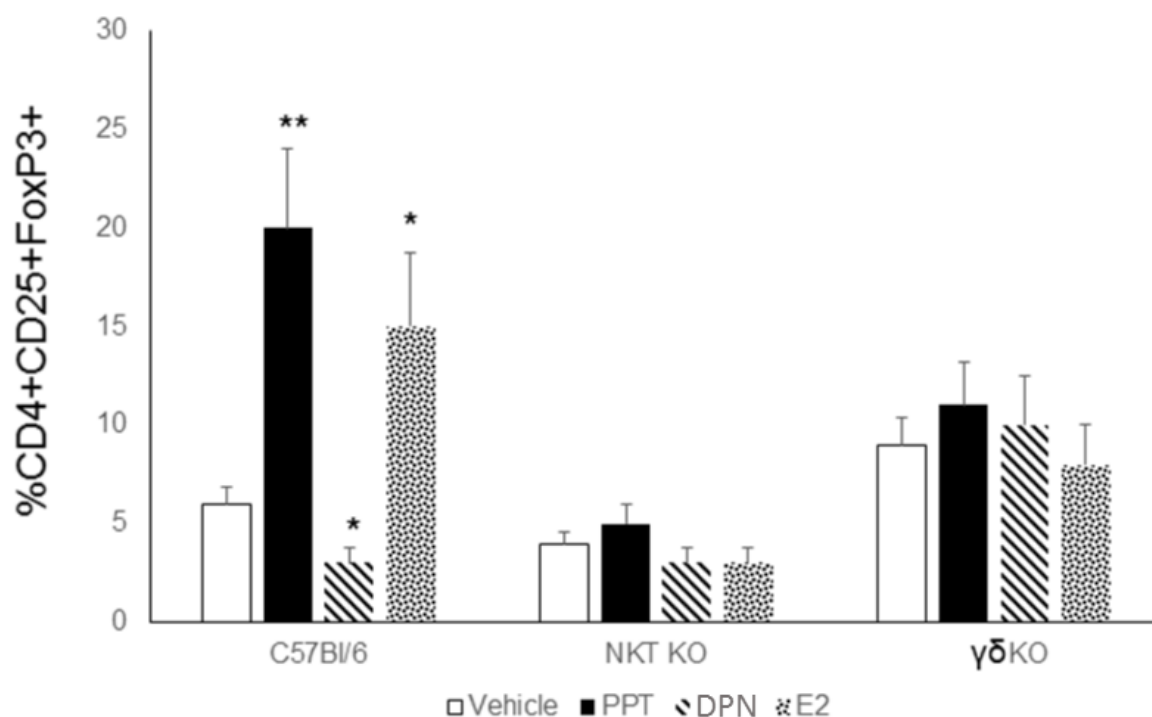
expression [34], although other mechanisms are also likely including impaired antigen presentation [23].

The significance of the current communication is that it provides another unique mechanism for hormonal control of T regulatory cell modulation different than those previously described. This study confirms that signaling through ER $\alpha$  promotes T regulatory responses leading to suppression of Th1 cell responses and protection from myocarditis during CVB3 infections of female mice. However, the effect does not appear to be mediated primarily through a direct effect on the T regulatory cell population since there was no increase in T regulatory cells in NKTKO or  $\gamma\delta$ KO mice treated with PPT, the ER $\alpha$  agonist. If the effect of estrogen signaling is solely to upregulate FoxP3 expression in the CD4<sup>+</sup>CD25<sup>-</sup> cell population, lack of these innate T cells should not have hampered this response. The key may be in the level of CD1d expression which is substantially increased in mice lacking ER $\alpha$ . Prior studies have shown that CD1d expression levels are substantially higher in the myocarditis susceptible male than the myocarditis resistant female mice [49]. This suggests that ER $\alpha$  signaling either directly or indirectly influences CD1d expression. This could be either through hormone receptor binding to the CD1d promoter or through hormonal influence on the cytokine environment and antigen

presenting cell activation altering interaction of macrophage/dendritic cells with the innate T cell subsets. Estrogen enhances dendritic cell maturation and antigen presentation resulting in increased MHC and accessory molecule expression, which may lead to stronger T cell-APC interactions [50]. Also sex hormones affect TLR expression and signaling. This may alter the cytokine environment in which the adaptive immune response develops [51,52].

Besides generating greater numbers of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> T regulatory cells in CVB3 infected ER $\beta$ KO mice, the suppressive activity of these cells is substantially increased compared to T regulatory populations from C57Bl/6 or ER $\alpha$ KO animals (Figure 4) on a per cell basis. Whether this indicates that these are a distinct subpopulation of T regulatory cells or whether activation of T regulatory cells through factors produced by NKT cells results in distinct gene expression profiles is currently unclear. Future studies will need to delineate why signaling through ER $\beta$  selectively allows activation of  $\gamma\delta$ T cells whereas signaling through ER $\alpha$  selectively allows activation of NKT cells. Also, it is not clear where this signaling is important. One might assume that there might be differential estrogen receptor expression on the innate T cell effectors which control their activation profiles. Alternatively, and especially since CD1d expression levels on CD11b<sup>+</sup> cells is significantly





**Figure 7:** ER $\alpha$  induction of T regulatory cells depends upon NK T-cells. Male C57Bl/6, NKT KO (Ja18KO) and  $\gamma\delta$ KO mice were treated with 200 ng/mouse 17- $\beta$ -estradiol or 0.05 mM/kg of the ER $\alpha$  and ER $\beta$  agonists (PPT and DPN) s.c., or with corn oil/DMSO (vehicle) as a control and infected with  $10^2$  PFU CVB3. Mice were killed 7 days after infection and spleens were evaluated for T-regulatory cells. Results are given as mean  $\pm$  SEM. \* and \*\*Significantly different than mice given vehicle at  $p < 0.05$  and  $p < 0.01$ , respectively. Results are from 5-6 mice/group.

modulated through signaling by the different receptors (Figure 1), the effect may be indirect and mediated through interaction of either NKT or  $\gamma\delta$ T cells with macrophage using the CD1d on the macrophage cell surface. Understanding the complexities of how estrogen signaling affects immune regulation during infection can have long-reaching impact not only for CVB3 and myocarditis, but might be relevant to other infectious, immunologic and autoimmune diseases as well.

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