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## Gender-specific differences in PPAR $\gamma$ regulation of follicular helper T cell responses with estrogen

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Peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), a master regulator of adipocyte differentiation, has recently been connected with effector T cells, though its role is still not clear. Here, we investigated the roles of PPAR $\gamma$  in follicular helper T (T<sub>FH</sub>) cell responses regarding gender specificity. NP-OVA immunization in female but not male CD4-PPAR $\gamma$ <sup>KO</sup> mice induced higher proportions of T<sub>FH</sub> cells and germinal center (GC) B cells following immunization than were seen in wild type mice. Treatment with the PPAR $\gamma$  agonist pioglitazone significantly reduced T<sub>FH</sub> cell responses in female mice while pioglitazone and estradiol (E2) co-treatment ameliorated T<sub>FH</sub> cells and GC responses in male mice. E2 treatment significantly enhanced PPAR $\gamma$  expression in male T cells, while T cell activation in the estrus but not in the diestrus stage of the menstrual cycle of females was inhibited by pioglitazone, suggesting that an estrogen-sufficient environment is important for PPAR $\gamma$ -mediated T cell regulation. These results demonstrate gender-based differences in sensitivities of PPAR $\gamma$  in T<sub>FH</sub> responses. These findings suggest that appropriate function of PPAR $\gamma$  is required in the regulation of female GC responses and that therapeutic strategies for autoimmune diseases using PPAR $\gamma$  agonists need to be tailored accordingly.

PPAR $\gamma$  is a transcription factor and a master regulator of adipocyte differentiation<sup>1–5</sup>. It is activated by ligands such as 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>)<sup>6,7</sup> and 13-hydroxyoctadecadienoic acid (13-HODE)<sup>8</sup>, which are derived from eicosanoids including prostaglandin D<sub>2</sub> or fatty acid metabolites<sup>9</sup>. Thiazolidinediones (TZDs) such as pioglitazone, rosiglitazone, ciglitazone, and troglitazone are synthetic ligands for PPAR $\gamma$ <sup>10</sup>, and have been approved for use in the treatment of type 2 diabetes mellitus<sup>11</sup>. These ligands effectively inhibit NF- $\kappa$ B function to regulate inflammation and inflammatory diseases<sup>12</sup>.

PPAR $\gamma$  has been highlighted in T cell responses and autoimmune diseases and PPAR $\gamma$  ligand treatment has been shown to inhibit effector T cell functions *in vitro* and *in vivo*. Ciglitazone and 15d-PGJ<sub>2</sub> inhibited T cell proliferation and IL-2 production<sup>13</sup>. We previously reported that pioglitazone inhibited human memory T cell responses in a model of arterial grafts<sup>14</sup>. Pioglitazone was also reported to selectively regulate Th17 cell responses to ameliorate experimental autoimmune encephalomyelitis (EAE)<sup>15</sup>. More recently, PPAR $\gamma$ -deficient Treg cells showed an impaired ability to migrate to visceral adipose tissue on a high-fat diet<sup>16</sup> and failed to regulate effector T cell functions and development of colitis and GVHD<sup>17,18</sup>. In contrast, another study showed that PPAR $\gamma$  contributed to the development of colitis in a lymphopenic environment<sup>19</sup>. Thus, there are still some controversies regarding the role of PPAR $\gamma$  in T cells.

Previously, we reported that female CD4-PPAR $\gamma$ <sup>KO</sup> mice have spontaneous autoimmune phenotypes with increased T<sub>FH</sub> cells and GC reactions<sup>20</sup>. Here, we observed that male CD4-PPAR $\gamma$ <sup>KO</sup> mice do not develop autoimmune phenotypes and hypothesized that there are gender-based differences in PPAR $\gamma$  regulation of T<sub>FH</sub> cell responses. In this report, we demonstrate that an estrogen-sufficient environment promotes PPAR $\gamma$  activity to regulate T<sub>FH</sub> responses and we also suggest that PPAR $\gamma$  is more necessary in females to regulate effector T cell responses than it is in males.

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## Results

**Female CD4-PPAR $\gamma$ <sup>KO</sup> but not male mice induce a higher proportion of T<sub>FH</sub> cells and GC B cells.** Previously, we reported that female CD4-PPAR $\gamma$ <sup>KO</sup> mice spontaneously develop autoimmune phenotypes with an increase in T<sub>FH</sub> cells with GC responses<sup>20</sup>. However, interestingly, 6-month to 1-year-old male CD4-PPAR $\gamma$ <sup>KO</sup> mice did not show spontaneous autoimmune phenotypes including the proportion of activated T cells (Supplementary Fig. S1a,b), autoantibodies in the serum (Supplementary Fig. S1c,d), and glomerulus inflammation (Supplementary Fig. S1e,f), which are typical indicators of systemic autoimmune diseases. In addition, there was no difference in the proportion of T<sub>FH</sub> cells in the spleen (Supplementary Fig. S1g,h). These results prompted us to consider the possibility of gender-based differences in phenotypic sensitivity of PPAR $\gamma$  deletion in T cells. To determine gender-based differences in T<sub>FH</sub> responses to antigen immunization in CD4-PPAR $\gamma$ <sup>KO</sup> mice, we first immunized six- to eight-week-old mice with NP-OVA (4-Hydroxy-3-nitrophenyl-ovalbumin) and analyzed T<sub>FH</sub> cells and GC responses in the draining lymph node seven days after the immunization (Fig. 1A). CXCR5 and Bcl-6 double-positive cells gated on CD4<sup>+</sup>CD44<sup>high</sup> were identified as T<sub>FH</sub> cells<sup>21–23</sup>. The proportion and the absolute cell number of T<sub>FH</sub> cells was significantly increased in female CD4-PPAR $\gamma$ <sup>KO</sup> mice compared with the wild-type littermate control group, while there was a significant decrease in T<sub>FH</sub> cells in the draining lymph node of male CD4-PPAR $\gamma$ <sup>KO</sup> mice (Fig. 1B,C, Supplementary Fig. S2a). In addition, female CD4-PPAR $\gamma$ <sup>KO</sup> mice displayed a significantly increased population and the cell number of GL-7<sup>+</sup>CD95<sup>+</sup> GC B cells, while male CD4-PPAR $\gamma$ <sup>KO</sup> mice showed a population of GC B cells smaller than that of the control group (Fig. 1D,E, Supplementary Fig. S2b). These data demonstrate that there are gender-specific effects of PPAR $\gamma$  deletion in T cells, and PPAR $\gamma$  is required to prevent T<sub>FH</sub> responses in female but not in male mice.

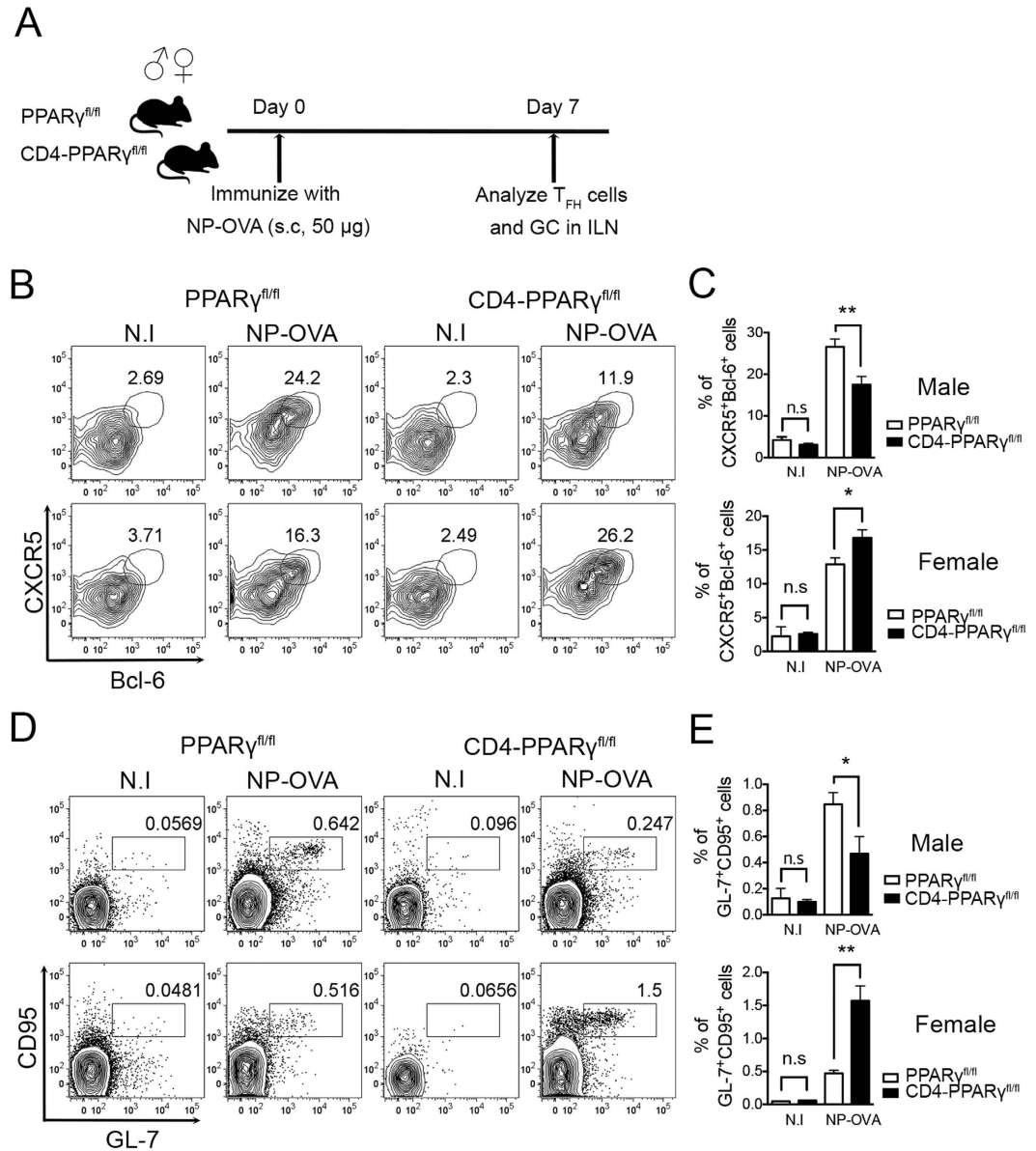
### PPAR $\gamma$ agonist pioglitazone treatment reduces T<sub>FH</sub> responses in female but not in male mice.

To determine whether PPAR $\gamma$  agonist pioglitazone treatment also shows gender-based differences in its effects on the induction of T<sub>FH</sub> cells and GC responses, six- to eight-week-old C57BL/6 mice were immunized with SRBC or NP-OVA, 10 mg/kg of pioglitazone was administered intra-peritoneally once a day from day 1 to day 6, and T<sub>FH</sub> cells and GC responses were analyzed in the spleen or draining lymph node at day 7 (Fig. 2A). We observed that pioglitazone treatment significantly inhibited the proportion of T<sub>FH</sub> cells in the spleen compared to the DMSO-treated control group only in female mice, while no effect was observed in the males (Fig. 2B,C). The population of GC B cells was also significantly diminished by pioglitazone treatment in females, whereas it was not affected in male mice (Fig. 2D,E). Frozen spleen tissue was sectioned and stained to determine the T cell zone (anti-CD4-APC, blue), B cell zone (anti-IgD-PE, red), and GCs (PNA, green). The number of GCs was significantly reduced in female mice, but not in males following pioglitazone treatment (Fig. 2F,G). These observations are consistent with those in a NP-OVA immunization model showing that pioglitazone treatment inhibited a proportion of T<sub>FH</sub> cells and GC cells of the draining lymph node only in female mice (Fig. 2H–K). These results collectively demonstrate that the stimulation of PPAR $\gamma$  via its ligand also has gender-based differences in effects in that it significantly inhibits T<sub>FH</sub> responses only in female mice but is not effective in males.

**Pioglitazone and estradiol co-treatment in males reduces T<sub>FH</sub> responses.** From the gender-specific results regarding T<sub>FH</sub> responses with PPAR $\gamma$  deletion in T cells or stimulation of PPAR $\gamma$  by its ligand following immunization, we hypothesized that estrogen in the female might be important for this action of PPAR $\gamma$ . We first measured the basal PPAR $\gamma$  mRNA expression levels in male and female MACS-purified naive T cells (CD4<sup>+</sup>CD62L<sup>high</sup>) and also determined whether E2 treatment enhances PPAR $\gamma$  levels in male T cells. From the results, we found that PPAR $\gamma$  mRNA was expressed at significantly higher levels in female T cells than in males while male T cells had higher levels of PPAR $\alpha$  and comparable expression of PPAR $\delta$  (Fig. 3A). Interestingly, 5 nM E2 treatment of male T cells augmented PPAR $\gamma$  mRNA expression compared with DMSO-treated T cells, while no altered expression was found in E2-treated female T cells (Fig. 3B) suggesting that there is some positive feedback action of estrogen on PPAR $\gamma$  expression. To investigate the synergistic role of E2 and pioglitazone, six- to eight-week-old male C57BL/6 mice and CD4-PPAR $\gamma$ <sup>KO</sup> mice were immunized with NP-OVA and treated with either 60  $\mu$ g of E2 and/or 10 mg/kg of pioglitazone once a day from day 1 to day 6, then T<sub>FH</sub> cells and GC responses in the draining lymph node were analyzed seven days after immunization (Fig. 4A). *In vivo* administration of E2 for six days results in significantly increased PPAR $\gamma$  mRNA expression in the spleen of male mice which is comparable level in estrus cycle of female mice (Supplementary Fig. S3). Only co-treatment with pioglitazone and E2, and not either treatment by itself, significantly inhibited the proportion of T<sub>FH</sub> cells in the lymph node compared to the other groups in male mice (Fig. 4B,C). The proportion of GC B cells was also significantly reduced by pioglitazone and E2 co-treatment (Fig. 4D,E). The lack of any effect of this co-treatment in CD4-PPAR $\gamma$ <sup>KO</sup> mice suggests that the co-treatment effect is dependent on PPAR $\gamma$  action. These results collectively suggest that E2 enhances PPAR $\gamma$  sensitivity in male mice for the regulation of T<sub>FH</sub> responses.

### Pioglitazone inhibits T cell activation in the estrus but not in the diestrus stage of the menstrual cycle in females.

Due to the dynamic estrogen cycle in females, we hypothesized that PPAR $\gamma$  sensitivity in T cells might also differ during the menstrual cycle of female mice. We isolated splenocytes during the estrus and diestrus stages and then stimulated the cells with anti-CD3 and CD28 antibodies followed by pioglitazone treatment to determine if the differential PPAR $\gamma$  sensitivity depends on estrogen level (Fig. 5A). The levels of activation markers, CD25 and CD69, in CD4<sup>+</sup> T cells were significantly reduced by pioglitazone treatment in the cells only at estrus cycle but not at diestrus cycle (Fig. 5B,C). In addition, production of IFN- $\gamma$  and IL-2 by activated splenocytes was also significantly decreased by pioglitazone only at the estrus stage of the cycle (Fig. 5D). These results seem to correlate with PPAR $\gamma$  expression level since the cells from the estrus stage have higher expression levels of PPAR $\gamma$  than cells from the diestrus stage (Fig. 5E). As consistent with previous results, pioglitazone treatment could not inhibit T cell activation in male splenocytes (Supplementary Fig. S4a–c). Taken



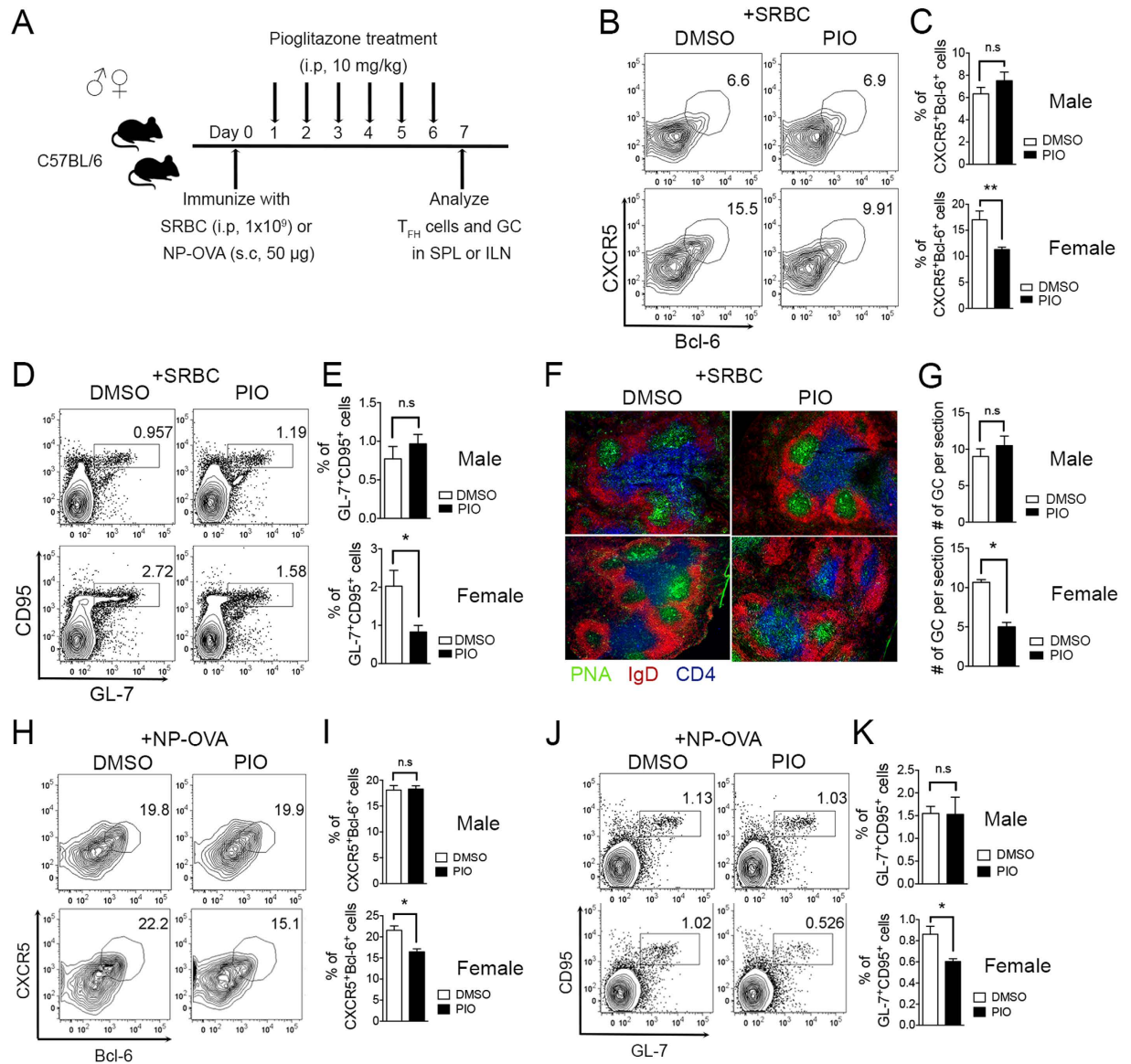
**Figure 1. Female CD4-PPAR $\gamma^{KO}$  but not male mice have higher proportions of T<sub>H</sub> cells and GC B cells.** (A) T<sub>H</sub> cells were induced by immunization of six- to eight-week-old male and female littermate control and CD4-PPAR $\gamma^{KO}$  mice with NP-OVA. Seven days after immunization, the proportion of T<sub>H</sub> cells was analyzed by flow cytometry. (B,C) NP-OVA-immunized male and female littermate control and CD4-PPAR $\gamma^{KO}$  mice were analyzed by staining with anti-Bcl-6 and anti-CXCR5 antibodies. Bcl-6 and CXCR5 double-positive cells gated on CD4<sup>+</sup>CD44<sup>high</sup> were identified as T<sub>H</sub> cells and the % of Bcl-6<sup>+</sup>CXCR5<sup>+</sup> T<sub>H</sub> cells was represented as a bar graph. (D,E) Germinal center (GC) B cells in male and female littermate control and CD4-PPAR $\gamma^{KO}$  mice were examined by staining with anti-GL-7 and anti-CD95 antibodies seven days after NP-OVA immunization and the % of GL-7<sup>+</sup>CD95<sup>+</sup> GC B cells gated on B220-positive cells was indicated as a bar graph. The data represent means  $\pm$  SEM (n = 4/group, three independent experiments). \**P* < 0.05, \*\**P* < 0.01 by a two-tailed, unpaired Student's *t*-test.

together, these results suggest that estrogen level has a positive correlation with PPAR $\gamma$  sensitivity to its ligand in females, thereby regulating T cell responses.

## Discussion

PPAR $\gamma$  is a master regulator in adipocyte differentiation, which has important roles in lipid metabolism. PPAR $\gamma$  has been recently studied in T cells where it was shown to regulate Th17 cells to prevent autoimmunity and was also found to be necessary for regulatory T cell functions. Here, we demonstrate gender-specific actions of PPAR $\gamma$  regarding effector T cell functions, such as T<sub>H</sub> responses, which are supported by estrogen.

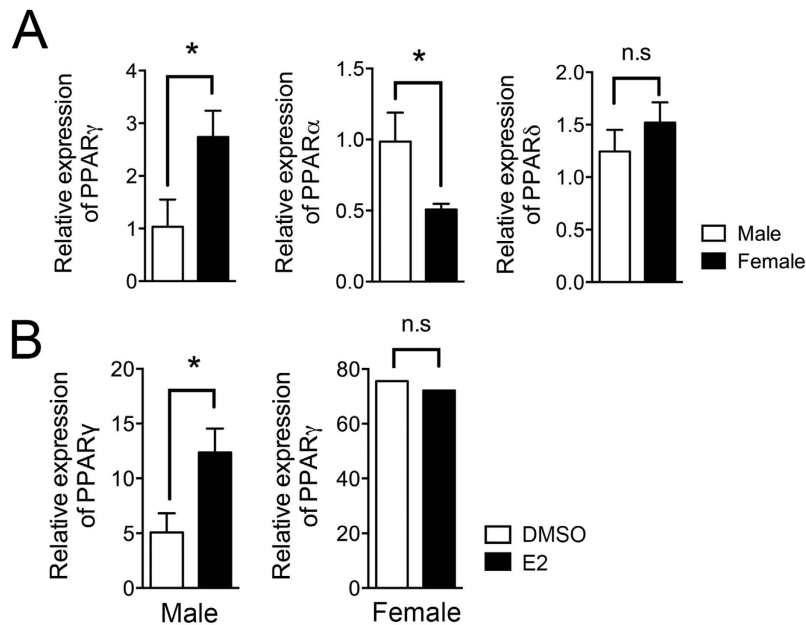
Previously, it was reported that treatment with the PPAR $\gamma$  agonist pioglitazone augmented the incidence of hypoglycemia in diabetic women<sup>24</sup> and that rosiglitazone showed a greater reduction of fasting plasma glucose



**Figure 2. PPAR $\gamma$  ligand pioglitazone treatment reduces T<sub>FH</sub> responses in female but not in male mice.** (A) Six- to eight-week-old male and female mice were immunized with SRBC or NP-OVA and were treated daily with pioglitazone (10 mg/kg) intra-peritoneally from day 1 to day 6. (B,C) The mice were sacrificed following SRBC immunization and the spleens were isolated and stained with anti-mouse CXCR5 and Bcl-6 antibodies to analyze the proportion of T<sub>FH</sub> cells and the % of CXCR5<sup>+</sup>Bcl-6<sup>+</sup>-positive T<sub>FH</sub> cells was represented as a bar graph. (D,E) GL-7 and CD95 double-positive cells gated on B220-positive cells from the spleens from male and female mice were analyzed and the % of GL-7<sup>+</sup>CD95<sup>+</sup> GC B cells was demonstrated as a bar graph (n = 3/group, three independent experiments). (F,G) Immunofluorescence analysis was performed to determine the number of GCs formed in spleens of SRBC-immunized male and female mice by staining with anti-PNA, -IgD, and -CD4 antibodies and the numbers of GCs were counted per spleen section. The average number of GCs per spleen section was determined (n = 9). (H,I) The inguinal lymph nodes were isolated from NP-OVA-immunized male and female mice. CXCR5<sup>+</sup>Bcl-6<sup>+</sup>-positive T<sub>FH</sub> cells were analyzed and displayed as a bar graph. (J,K) GL-7 and CD95 double-positive cells gated on B220-positive cells in the inguinal lymph nodes from male and female mice were analyzed and the % of GL-7<sup>+</sup>CD95<sup>+</sup> GC B cells was represented as a bar graph. The data shown represent means  $\pm$  SEM (n = 3 per group, two independent experiments). \**P* < 0.05, \*\**P* < 0.01 by a two-tailed, unpaired Student's *t*-test.

(FPG) levels in women than in men<sup>25</sup>, suggesting that women have a greater sensitivity to treatment with PPAR $\gamma$  ligands. This gender-based difference in sensitivity would be correlated to the sex-dimorphic expression of PPAR $\gamma$ . Previously, a sex-specific action of PPAR $\alpha$  was also reported when it was observed that male mice lacking PPAR $\alpha$  were more susceptible to EAE than were female mice and that PPAR $\alpha$  expression level was higher in male T cells than in female T cells<sup>26</sup>. In addition, PPAR $\alpha$  siRNA treatment affected only human male T cells by enhancing IFN- $\gamma$  production, while there was no difference in IFN- $\gamma$  expression in female T cells. PPAR $\alpha$  ligand



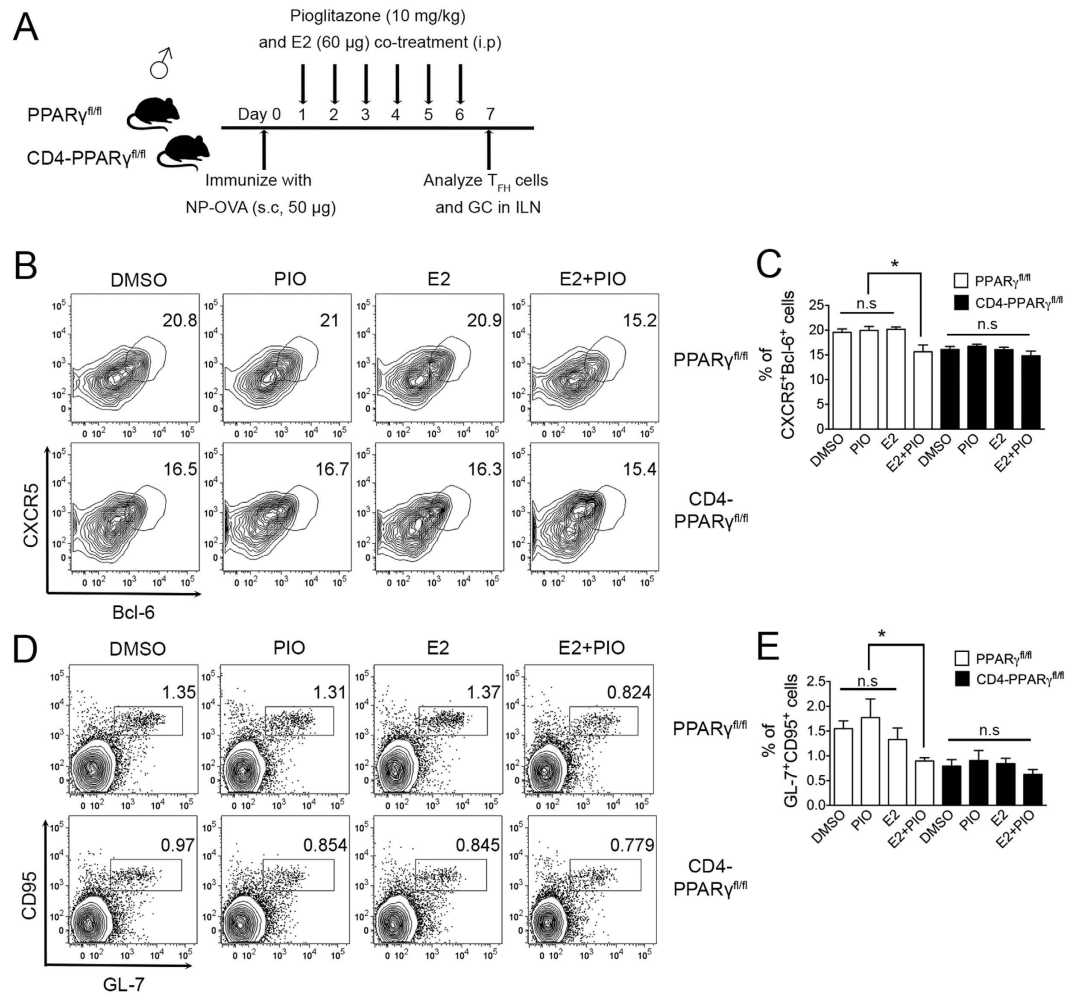


**Figure 3. Estradiol treatment enhances the PPAR $\gamma$  expression.** (A,B) Total RNA was isolated from male and female naïve T cells (CD4<sup>+</sup>CD62L<sup>high</sup>) to determine the PPAR expression levels. Basal expression of PPARs in male and female naïve T cells and PPAR $\gamma$  expression in 5 nM E2- or DMSO-treated male and female naïve T cells following TcR stimulation for 3 days were assessed using real-time PCR and were normalized to  $\beta$ -actin. \* $P < 0.05$  by two-tailed, unpaired Student's  $t$ -test. Values shown are means  $\pm$  SEM ( $n = 3$ ).

fenofibrate diminished IFN- $\gamma$  production in males, but not in females<sup>27</sup>. Fenofibrate was also reported to reduce body weight and white adipose tissue (WAT) mass in high-fat-diet-fed male mice, but not in females<sup>28</sup>, suggesting that PPAR $\alpha$  and PPAR $\gamma$  have gender-based specific sensitivities in their biological roles.

In general, women are more susceptible to autoimmune diseases compared to men<sup>29–31</sup>. Several factors including hormones and the X-chromosome have been suggested to affect the higher prevalence rates of autoimmune diseases in females. We showed here that PPAR $\gamma$  expression is higher in T cells from female mice than in cells from males and that pioglitazone effects have a positive correlation with estrogen levels. Here we suggest that PPAR $\gamma$  controls T<sub>FH</sub> responses more sensitively in females with sufficient estrogen levels. This finding suggests that an abnormality in PPAR $\gamma$  activity in T cells might cause a more critical problem for maintaining homeostasis in females than in males. T<sub>FH</sub> cells are found in B cell follicles and interact with cognate B cells to promote isotype class switching, affinity maturation, and plasma cell differentiation to produce antibodies<sup>32,33</sup>. Therefore, T<sub>FH</sub> cells and GC reactions are important drivers of autoimmune disease by supporting autoantibody production<sup>34</sup>. Regulation of T<sub>FH</sub> cells is now considered to be an important target for treatment of autoimmune diseases<sup>35</sup>. We demonstrate that PPAR $\gamma$  plays a significant role in the regulation of T<sub>FH</sub> responses, especially in females, which would be important to prevent autoimmunity.

Decreased numbers of T<sub>FH</sub> cells in male CD4-PPAR $\gamma$ <sup>KO</sup> mice following NP-OVA immunization were observed in our experiments, raising the possibility that the discrepancy in PPAR $\gamma$  function acts as a negative regulator of effector T cell functions, although pioglitazone treatment has no effect in males. One recent paper reported that PPAR $\gamma$  is required for the development of autoimmunity in lymphopenic conditions due to increased apoptosis with reduced IL-7R $\alpha$  expression of CD4-PPAR $\gamma$ <sup>KO</sup> T cells<sup>19</sup>, while there is a controversial result from previous studies showing that PPAR $\gamma$  is a negative regulator of T cell activation. It was reported that the transfer of PPAR $\gamma$ -deficient effector T cells into RAG-knockout mice showed a robust induction of colitis<sup>17</sup>. In addition, PPAR $\gamma$  deficiency in T cells showed increased Th17 and EAE disease pathogenesis and pioglitazone treatment selectively inhibited Th17 factors, suggesting a role for this drug in suppressing Th17 differentiation<sup>15</sup>. More recently, Treg-specific PPAR $\gamma$  deficiency showed an abnormality on Treg migration into adipose tissue in a high-fat diet animal model<sup>16</sup>. We hypothesize that this discrepancy could result from the different genders of the mice used. We determined that male PPAR $\gamma$ -deficient T cells have reduced levels of Bcl-2 and IL-7R $\alpha$  expression, which are critical for T cell survival, while there is no difference in female CD4-PPAR $\gamma$ <sup>KO</sup> mice compared to their controls (Supplementary Fig. S5a,b). We also found that male PPAR $\gamma$ -deficient T cells are more apoptotic than wild-type controls in serum starvation conditions (Supplementary Fig. S5c,d). Therefore, PPAR $\gamma$  in males contributes to the survival of T cells by maintaining Bcl-2 and IL-7R $\alpha$  expression while an estrogen-sufficient environment might compensate to sustain anti-apoptotic molecular expression in females<sup>36</sup>. Recently, role of IL-7R $\alpha$  signaling in the regulation of T<sub>FH</sub> cells has been suggested that IL-7 suppressed the expression of Bcl-6 and other T<sub>FH</sub> genes<sup>37</sup>. In addition, IL-7R $\alpha$ -STAT5 axis is suggested as a negative regulator of T<sub>FH</sub> responses like IL-2-STAT5<sup>38</sup>. However, another previous study regarding Foxo1 deletion in T cells with transgenic of IL-7R $\alpha$  expression demonstrated that alteration of T<sub>FH</sub> responses are dependent on Foxo1 but not IL-7R $\alpha$ <sup>39</sup>. Thus,

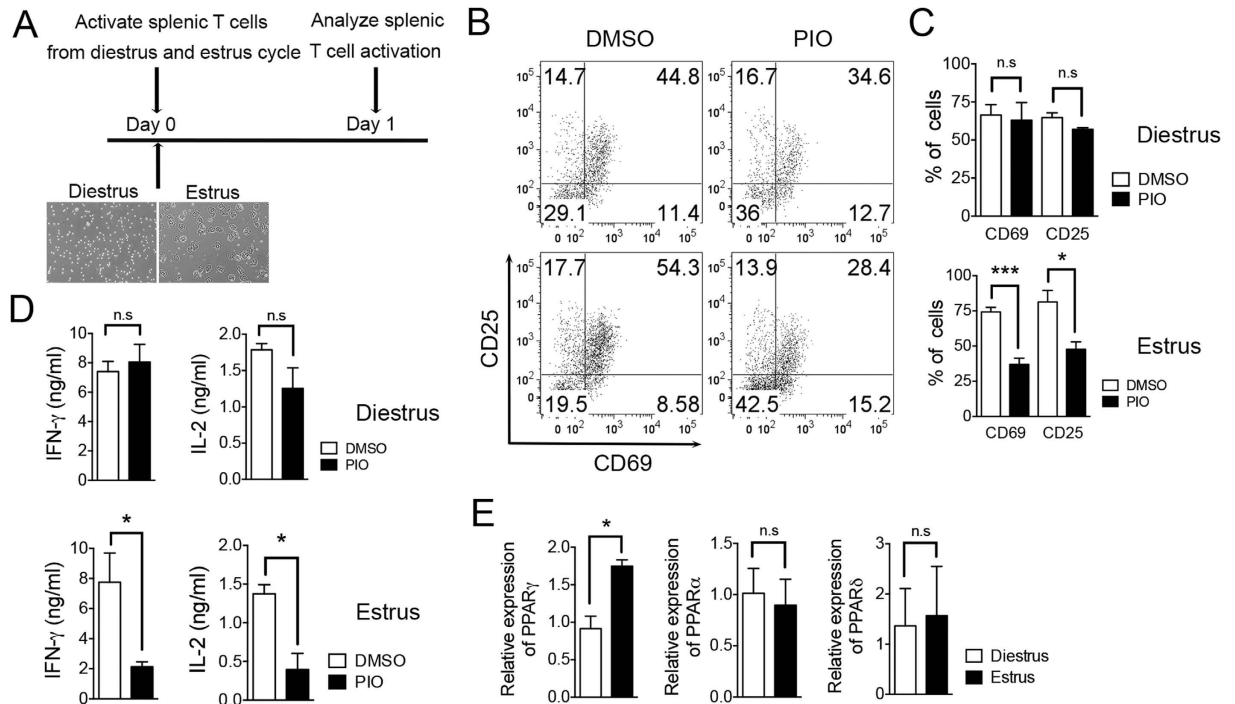


**Figure 4. Pioglitazone and estradiol co-treatment in males reduces T<sub>FH</sub> responses.** (A) Six- to eight-week-old male CD4-PPAR $\gamma$ <sup>KO</sup> mice and the littermate control mice were immunized with NP-OVA and were treated daily with DMSO, pioglitazone (10 mg/kg), E2 (60  $\mu$ g), or pioglitazone (10 mg/kg) + E2 (60  $\mu$ g) intraperitoneally once a day from day 1 to day 6. (B,C) The mice were sacrificed on day 7 and inguinal lymph nodes were isolated and the cells were stained with anti-mouse CXCR5 and Bcl-6 antibodies to analyze the proportion of T<sub>FH</sub> cells and the % of CXCR5<sup>+</sup>Bcl-6<sup>+</sup>-positive T<sub>FH</sub> cells was indicated as a bar graph. (D,E) GL-7 and CD95 double-positive cells gated on B220-positive cells from the lymph nodes were analyzed and the % of GL-7<sup>+</sup>CD95<sup>+</sup> GC B cells were represented as a bar graph. The data shown represent means  $\pm$  SEM (n = 5/group, two independent experiments). One-way ANOVA was used for statistical analysis. \**P* < 0.05.

possible reason for reduced induction of T<sub>FH</sub> cells without PPAR $\gamma$  in males need to be further investigated regarding decreased IL-7R $\alpha$  and Bcl-2 levels.

Synthetic ligands for PPAR $\gamma$  are used as anti-diabetes drugs for the treatment of type II diabetes mellitus<sup>40–44</sup>. Those ligands are also effective in the regulation of autoimmune diseases including colitis and EAE. Troglitazone and rosiglitazone treatment remarkably reduced disease severity in a mouse colitis model by inhibiting activation of NF- $\kappa$ B<sup>45</sup>, while pioglitazone selectively inhibited Th17 cells to ameliorate the clinical features of EAE<sup>15</sup>. In addition to the synthetic ligands, various types of polyunsaturated fatty acid (PUFA) metabolites can serve as endogenous ligands for PPAR $\gamma$  to ameliorate inflammatory responses and autoimmune diseases. Previous studies have reported that PUFA metabolites, including eicosanoids and linoleic acid, modulated upregulation of PPAR $\gamma$ , resulting in reduced inflammation and IFN- $\gamma$  production<sup>46,47</sup>. Animal models of inflammatory bowel diseases displayed significantly reduced colonic inflammation with PUFA feeding<sup>48</sup> and reduced severity of EAE after PUFA supplementation<sup>49</sup>, suggesting that PPAR $\gamma$  has a potential role in the treatment of autoimmune diseases. Dietary intake of these metabolites could be an advantageous strategy, especially for females, to prevent autoimmune diseases by stimulating PPAR $\gamma$  to regulate sensitive T<sub>FH</sub> responses.

Estrogen is the predominant sexual hormone in females and several studies have demonstrated the anti-inflammatory<sup>50</sup> and protective role of E2 in an EAE model<sup>51,52</sup>. Due to the protective role of estrogen in autoimmune diseases, the incidence and severity of autoimmune diseases are worse in postmenopausal patients<sup>53</sup>, suggesting that the estrogen signal is essential to preventing autoimmunity. In our study, pioglitazone treatment inhibits activation of splenic T cells only when cells were collected during the estrus cycle and E2 treatment



**Figure 5. Pioglitazone inhibits T cell activation in the estrus but not in the diestrus stage of the menstrual cycle in females.** (A) Menstrual cycle was monitored in six- to eight-week-old female C57BL/6 mice and splenocytes were isolated from the mice at estrus and diestrus stages. The cells were activated with anti-CD3 and anti-CD28 antibodies for 24 h in the presence of DMSO or pioglitazone. (B,C) CD69 and CD25 expression gated on CD4-positive cells were analyzed with flow cytometry and the % of CD69- and CD25-positive cells were represented. (D) IFN- $\gamma$  and IL-2 cytokine production levels following TcR stimulation were analyzed by ELISA using cultured supernatant. (E) Total RNA was isolated from female splenocytes from diestrus and estrus stages and the expression level of PPARs was analyzed by real-time PCR and normalized to  $\beta$ -actin. The data shown represent means  $\pm$  SEM ( $n = 5/\text{group}$ ). \* $P < 0.05$ , \*\*\* $P < 0.001$  by a two-tailed, unpaired Student's  $t$ -test.

increased the PPAR $\gamma$  expression level in male T cells, suggesting that estrogen and PPAR $\gamma$  have a positive correlation in the regulation of T cell response. We did not observe any effect of E2 treatment alone on the regulation of T<sub>FH</sub> cells or GC responses, suggesting that E2 enhances PPAR $\gamma$  level to increase the sensitivity to its ligand. Therefore, estrogen hormonal imbalance in females might result in abnormal control of PPAR $\gamma$  action to regulate T cells, which could contribute to sensitive auto-antibody production via T<sub>FH</sub> responses. Molecular mechanisms of estrogen on PPAR $\gamma$  regulation and estrogen receptor deficiency in T cells will be further investigated regarding gender-specific regulatory mechanisms for T<sub>FH</sub> responses.

In conclusion, our findings suggest that there is gender-specific sensitivity of PPAR $\gamma$  in the regulation of T<sub>FH</sub> responses and that PPAR $\gamma$ -mediated regulation requires the estrogen signal in mice. Gender-based differences in therapeutic strategies using PPAR $\gamma$  agonists and combination treatments with estrogen should be considered for the treatment of autoimmune diseases.

## Materials and Methods

**Mice.** B6.129-Pparg<sup>tm2Rev/J</sup> (PPAR $\gamma^{\text{fl/fl}}$ ) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). CD4-Cre<sup>+/-</sup> transgenic mice were crossed with PPAR $\gamma^{\text{fl/fl}}$  mice to generate CD4-specific PPAR $\gamma$ -knockout mice (CD4-PPAR $\gamma^{\text{KO}}$ ). Mice were maintained at the Hanyang University mouse facilities under pathogen-free conditions with *ad libitum* feeding. All animal protocols in this study were approved by the Animal Experimentation Ethics Committee of Hanyang University and experiments were performed according to the guidelines of the Institutional Animal Care and Use Committees (IACUC) of Hanyang University.

**SRBC and NP-OVA immunization.** Mice were immunized intra-peritoneally (i.p.) with sheep red blood cells (Innovative Research, Novi, MI, USA) diluted with DPBS at a 1:1 ratio and subcutaneously with 100  $\mu\text{g}$  of NP-OVA (Bioresearch Technologies, Novato, CA, USA). Seven days after immunization, mice were sacrificed and spleens and inguinal lymph nodes were isolated and analyzed by flow cytometry and confocal microscopy. For PPAR $\gamma$  agonist treatment, pioglitazone was purchased from Sigma and dissolved in DMSO. To assess the regulatory effect of pioglitazone on T<sub>FH</sub> cell differentiation *in vivo*, 10 mg/kg of pioglitazone was injected i.p. daily from day 1 to day 6 and the lymph nodes were isolated from the mice for further analysis.

**Flow cytometry.** Splenocytes, mesenteric, and inguinal lymph node cells were isolated and then stained with anti-mouse CD4-APC, CD8-PerCP-Cy5.5, CD44-PE, CD62L-FITC, GL-7-FITC, CD95-PE, and

B220-PerCP-Cy5.5 antibodies (eBioscience, San Diego, CA) for 15 min at 4 °C. For T<sub>FH</sub> differentiation analysis, the cells were stained with anti-mouse CXCR5-biotin for 30 min at 4 °C followed by anti-mouse CD44-FITC, CD4-PerCP-Cy5.5, and streptavidin-APC staining. After fixation and permeabilization using the Foxp3 Staining Kit (eBioscience), anti-mouse Bcl-6-PE was stained for 1 h at room temperature. Cells were examined using the FACSCanto II system (BD Bioscience, San Jose, CA, USA) and data were analyzed using Flow Jo software (Treestar, Ashland, OR, USA). In all cases, doublets (FSC-area versus FSC-height gating) were excluded.

**RNA isolation and real-time PCR.** RNA was isolated by a RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA yield and purity were determined by NanoDrop. Total RNA (500 ng) was used for cDNA synthesis in a 20- $\mu$ l reaction volume using qPCR RT Master Mix (Toyobo, Japan). Real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). Actin was used as a control housekeeping gene. The following primer sequences were used (forward/reverse): PPAR $\gamma$ , 5'-CTCCAAGAATACCAAGTGC GA-3' and 5'-GCCTGATGCTTTATCCCCACA-3'; Actin, 5'-TGTCCCTGTATGCCTCTGGT-3' and 5'-CACGCACGATTTCCCTCTC-3'.

**Immunofluorescence.** For GC formation analysis, the spleens from six- to eight-week-old sheep red blood cell (SRBC)-immunized mice were isolated 7 days after immunization and frozen in OCT compound. Tissues were sectioned to a 7- $\mu$ m thickness, fixed in acetone at -20 °C, washed with PBS, and blocked with 0.1% BSA containing PBS for 30 min at room temperature. Tissues were stained with anti-PNA-FITC (Sigma-Aldrich, St. Louis, MO, USA), IgD-PE, and CD4-APC (eBioscience) antibodies diluted in blocking solution overnight at 4 °C. After three washes, tissues were incubated with ProLong Gold anti-fade reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) for 30 min at 4 °C and images were obtained using a Leica DM IRE2 confocal microscope.

**ELISA.** Cytokine production in activated T cells and Th1, Th2, and Th17 cells was measured by ELISA using mouse IL-4, IL-13, and IL-17 Ready-SET-Go kits (eBioscience) and IFN- $\gamma$  and IL-2 ELISA Deluxe sets (BioLegend, San Diego, CA, USA) according to the manufacturers' instructions. Anti-dsDNA antibody in mouse serum was quantified by ELISA (Alpha Diagnostic International Inc, San Antonio, TX, USA).

**Statistical analysis.** Data were analyzed statistically with the Student's t-test and multiple comparisons were analyzed with one-way ANOVA using Prism5 (GraphPad, San Diego, CA, USA). P-values (P) less than 0.05 were considered statistically significant.

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## Author Contributions

H.-J.P. and J.-M.C. conceived and designed the experiments. H.-J.P. performed most of the experiments. H.-S.P. and J.-U.L. technically supported immunization, flow cytometry, real-time PCR and ELISA assays. H.-J.P., A.-B. and J.-M.C. analyzed the data and wrote the paper.

## Additional Information

**Supplementary information** accompanies this paper at <http://www.nature.com/srep>

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