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RESEARCH ARTICLE

Estrogen enhanced the expression of IL-17 by tissue-resident memory γδT cells from uterus via interferon regulatory factor 4

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Abstract

Tissue-resident memory γδT cells at mucosal and epithelial sites play an important role for pathogen clearance, immunosurveillance, and participating in physiological processes. Different from other barrier sites, the immune cells in uterus face the protection against infections and tolerate an allogeneic fetus during a successful pregnancy. In the previous study, we found that tissue-resident memory γδT cells were enriched both in human and murine uterus and highly expressed IL-17 that promoted the invasion of trophocytes in vitro. In the current study, we found that γδT cells in uterus but not in blood or spleens expressed higher levels of estrogen receptors. The injection of estrogen into mice increased the proportion of γδT cells in uterus but not in spleens in vivo via CXCR3-CXCL10 chemokine axis. In addition, we found that estrogen enhanced the production of IL-17 but not IFN-γ in vivo and in vitro via interferon regulatory factor 4 but not RORγt and pSTAT3 at mRNA and protein levels. The analysis of cell transcriptome sequence further identified multiple differentially expressed genes between estrogen and control γδT cells. Our study demonstrated that estrogen directly act on γδT cells in uterus to enhance the production of IL-17 that might promote the invasion of trophocytes. Furthermore, our study might provide a new idea that estrogen increased the prevalence of autoimmune diseases in women by enhancing γδT cell-derived IL-17 production in uterus and uncover the critical pathological roles for estrogen in the development of autoimmune diseases.

KEYWORDS
CXCR3-CXCL10 chemokine axis, estrogen, IL-17, IRF-4, γδT cells

Abbreviations: ER, estrogen receptor; IRF-4, interferon regulatory factor 4; OVX, ovariectomy; TCR, T-cell receptor.

1 | INTRODUCTION

γδT cells produced IFN-γ or IL-17 depending on the TCR signal strength they encountered during embryonic development and mainly distributed in mucosal and epithelial sites greatly exposed to potential infectious agents.[1–4] Heterogeneous distribution in the normal and infectious tissues and non-MHC-restricted antigenic specificity of γδT cells make it possible to respond quickly to invading pathogens.[5] Recently, tissue-resident γδT cells have gained more and more attention, which were reported to participating in many beneficial processes such as pathogen clearance and immunosurveillance but also exacerbated some autoimmune diseases and cancers.[6–9]

Over the past decades, IL-17 has attracted much attention for its versatile functions, including triggering rapid immunity to infection antigens, generating immunopathology autoimmunity, involving in divergent physiological processes.[10] Th17 cells are considered the major source of IL-17. However, mounting evidence showed that γδT cell-derived IL-17 is one of the earliest sources of IL-17 in autoimmune diseases which is crucial for breaking immune tolerance.[11–13] RORγt and STAT3 are the key transcription factors regulating the production of IL-17 both in Th17 cells and γδT cells.[13–16]

Estrogen, in addition to regulating reproductive functions, also regulates various physiological processes such as immune, central nervous, and integumentary systems. It was reported that estrogen could not only regulate chemotaxis, infiltration, and phagocytic activity of neutrophils and macrophages, but also modulated all subsets of T cells including $CD4^+$T cells and $CD8^+$T cells.[17] It is now well established that there is a sex bias exists in many autoimmune diseases and women tend to respond more aggressively compared to their male counterparts. Accumulating studies ascribed the sex bias to the effect of estrogen. The effect of estrogen on Th17 cells has mostly been studied where the beneficial or pathogenic role varies in different diseases.[17–19] Surprisingly, the effect of estrogen on γδT cells has received little attention and few literature on the topic has been published.

Our previous study demonstrated that γδT cells were enriched both in human and murine uterus and characterized as tissue-resident memory γδT cells. In addition, uterine γδT cells produced high levels of IL-17 that were the main source of IL-17 in uterus to promote the invasion of trophocytes in vitro.[20] Our current study showed that subcutaneous injection of mice with estrogen could recruit γδT cells to uterus by CXCR3-CXCL10 chemokine axis. In addition, we found that estrogen enhanced the production of IL-17 by promoting the expression of transcription factor IRF-4 at mRNA and protein levels but not RORγt and STAT3. Our study might provide a new idea for probing the sex bias in autoimmune diseases that estrogen increased the prevalence of autoimmune diseases in women by enhancing γδT cell-derived IL-17 production in uterus.

2 | MATERIALS AND METHODS

2.1 | Animals and injection of estrogen

Female C57BL/6 mice aged 6–8 weeks were purchased from the Laboratory Animal Center of Sun Yat-sen University (S.C.XK 2016-0029) and maintained under pathogen-free conditions. Mice were age- and weight-matched in each experiment. All animal studies were approved by the Zhongshan School of Experimental Animal Ethics Committee, Sun Yat-sen University, Guangzhou, China. β-Estradiol (Sigma-Aldrich, USA) suspended in olive oil was subcutaneously injected into the back of mice with 5 mg/kg each time, once a week for 2 consecutive weeks. Mice were sacrificed 1 week after the second injection of estrogen.

2.2 | Cell isolation

Mice were sacrificed in sterile environment and blood in uterus was effectively removed by cardiac perfusion with 50 ml PBS. Uterus from control and estrogen groups were collected after opening the abdominal cavity. Uterus were cut into small pieces and digested with 5–10 ml digestion medium which contained 2 mg/ml collagenase I and 10 mg/ml DNase I (Sigma-Aldrich, USA) in RPMI 1640 medium for 1 h at 37°C with regular gentle shaking. Single-cell suspensions were obtained after filtering through a 100-μm nylon cell strainer (BD Bioscience Pharmingen, USA). The cell suspension was carefully layered over a discontinuous (40%/70%) Percoll (GE healthcare, USA) density gradients and centrifuged at speed 2500 rpm/min for 20 min. The mononuclear cells in the middle layer were collected and washed twice with complete RPMI 1640. The cells were counted and adjusted to a density of 2×10^6 cells/ml in complete RPMI 1640 medium. Spleen tissues were mechanically disrupted and filtered through a 40-μm cell strainer (Falcon, USA). The splenocytes were treated with red blood cell (RBC) lysis buffer, washed twice in complete RPMI 1640 medium, and loaded onto Ficoll-Hypaque (Tianjin HaoYang Biological

Manufacture, China) density gradient for isolation of mononuclear cells. The cells were counted and adjusted to a density of 2×10^6 cells/ml in RPMI 1640 medium.

2.3 | Flow cytometry and mAbs

The procedures for studying cell phenotype, intracellular cytokines, and transcriptional factor expression had been previously described.[20] Generally, the cells were washed and suspended in 100 μl of PBS containing 0.1% BSA and 0.05% sodium azide. For surface staining, the cells were incubated with the respective mAbs at 4°C in the dark for 30 min. For the detection of intracellular cytokines, the cells were fixed with 4% paraformaldehyde and permeabilized in PBS buffer containing 0.1% saponin (Sigma-Aldrich, USA), 0.1% BSA, and 0.05% NaN_3 for at least 2 h or overnight at 4°C and stained with conjugated mAbs for intracellular cytokines. For the detection of intracellular transcription factors, the cells were stained for surface antigens, followed by fixation, permeabilization with Permeabilization/Fixation buffer (BD Bioscience) and stained with conjugated mAbs for transcription factors. The data from flow cytometry were acquired with FACS Arial II (BD Bioscience, USA) and analyzed with FlowJo software (Tree Star, USA). The following mAbs were used for cell surface or intracellular staining: PE-CF594-labeled anti-CD3, FITC-labeled anti-γδT, anti-CD4, APC-labeled anti-CCR8, anti-CXCR6, anti-IFN-γ, PE-labeled anti-γδT, anti-IL-17, anti-CXCR3, anti-CX3CR1, anti-CCR5, anti-RORγt, anti-pSTAT3, anti-IRF-4, PE-cy7-labeled anti-CCR4, anti-CCR6, Apc-cy7-labeled anti-IL-17, and isotype-matched control antibodies were all purchased from BD Bioscience PharMingen (San Jose, USA). Rabbit anti-estrogen receptor alpha, Alexa Fluor® 488 goat anti-rabbit IgG, and control antibodies were purchased from Abcam.

2.4 | OVX model

All mice were anesthetized with injection of chloral hydrate (i.p.). The sham surgery for mice from control and estrogen groups involved the exposure of the ovaries with extraction of the surrounding fatty tissue, leaving the ovaries intact, whereas bilateral ovariectomy for the mice from OVX and OVX+estrogen groups involved the full removal of the left and right ovaries. After the surgery, the wound was cleaned with iodine to prevent infection twice daily for 2 days. After 3 weeks, the mice from estrogen and OVX+estrogen groups were injected with estrogen 5 mg/kg each time, once a week for 2 consecutive weeks. Mice were sacrificed 1 week after the second injection of estrogen.

2.5 | Enzyme-linked immuno sorbent assay

For the quantitative enzyme-linked immuno sorbent assay (ELISA) assay of cytokines, uterine cells were suspended in complete RPMI 1640 medium at a density of 2×10^6 cells/ml, and stimulated with or without anti-CD3 in the presence or absence of estrogen for 96 h, in a round-bottomed 96-well plate, 200 μl/well, at 37°C and 5% CO_2. In addition, sorted uterine γδT cells from control and estrogen-treated murine uterus were cultured with PMA and ionomycin for 48 h, 100 μl/well, at 37°C and 5% CO_2. The culture supernatants were collected for the detection of IFN-γ (BD Biosciences, USA) and IL-17 (Thermofish Scientific, USA) by ELISA according to the manufacturer's instructions.

2.6 | Reverse transcription-polymerase chain reaction

For the gene expression of chemokine CXCL10, control and estrogen-treated murine uterus were collected after subcutaneously injected with estrogen for 3 days in vivo. Uterine mononuclear cells were treated by different concentration of estrogen for 24 h in vitro. Total RNA was extracted by Trizol reagent (Invitrogen, USA) and then reverse-transcribed with a cDNA Synthesis Supermix (novoprotein scientific Inc, China) according to the manufacturer's instruction. The amplification of cDNA was conducted in a DNA thermal cycler (Biometra, Germany). The following sense and antisense primers for each molecule were used: CXCL10, Forward: 5′-AATCATCCCTGCGAGCCTATCC-3′, Reverse: 5′-TGTGCGTGGCTTCACTCCAGTT-3′; and GAPDH, Forward: 5′-ATGACCACAGTCCATGCCATCAC-3′, Reverse: 5′-ATGCCTGCTTCACCACCTTCTTG-3′. The ratio of CXCL10 to GAPDH was calculated according to the relative intensities of the bands revealed under UV illumination with Bio-1D software (Vilber Lourmat, Marne la Vallee France).

2.7 | Transwell migration technology

Five-micrometer pore size Transwell was purchased from Corning (USA). The procedures for Transwell migration technology were according to the manufacturer's instructions. Generally, mouse splenocytes were suspended in RPMI 1640 medium with no fetal calf serum at a density of 1×10^6 cells/ml and 200 μl cell suspension was added to the upper chamber. The lower chamber was added with or without uterus homogenate supernatant diluent in the presence or absence of anti-CXCL10 antibody, and incubated for 3 h at 37°C in a 5% CO_2 incubator. The recruited cells of lower chamber were counted.

2.8 | Purification of uterine γδT cells

The cells isolated from control and estrogen uterus were stained with PE-CF594-labeled anti-CD3 and FITC-labeled anti-γδT antibody. γδT cells were further sorted by using FACS Arial II (BD Bioscience, USA). γδT cells of >98% purity were used for subsequent functional experiments.

2.9 | Analysis of cell transcriptome sequence

The samples of γδT cells from control and estrogen uterus were prepared according to the previous description, which were added to lysis buffer containing ribonuclease inhibitor to amplify by the Smart-Seq2 method. The amplification product was purified and further constructed the library. Qualified libraries were loaded on Illumina Hiseq platform.

The amount of each gene was calculated by Fragments per Kilobase per Million Mapped Fragments (FPKM = 103*F/NL/106). Differentially expressed genes were performed with DEGseq package. The differentially expressed genes were picked out whose *p*-value was less than .05 and the difference multiple was greater than 2.

R package was used for hierarchical cluster analysis and the differentially expressed genes were shown in the heat map. Gene enrichment analysis of GO and KEGG were performed to determine the biological functions or pathways mainly affected by differentially expressed genes.

2.10 | Carboxyfluorescein succinimidyl ester labeling

Carboxyfluorescein succinimidyl ester (CFSE) staining was performed by CFSE Cell Proliferation Kit (Invitrogen, USA). The mononuclear cells isolated from uterus were resuspended in prewarmed PBS containing 0.1% BSA. CFSE was added at a final concentration of 5 μM, and the cells were incubated for 10 min at 37℃ in 5% CO_2. The staining was quenched using cold complete RPMI 1640 medium for 5 min.

2.11 | Statistical analysis

Data were presented as the mean ± standard error of mean (SEM). Statistical significance was analyzed by Mann–Whitney test using Prism6 (GraphPad, USA). Significant *p*-values are indicated in figures for the following ranges: NS, no significance; $^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$; and $^{****}p < .0001$.

3 | RESULTS

3.1 | Subcutaneous injection of mice with estrogen increased the proportion of γδT and αβT cells in uterus

To investigate the influence of estrogen on uterus, estrogen suspended in olive oil was subcutaneously injected into the back of mice with 5 mg/kg each time, once a week for 2 consecutive weeks, and olive oil was used as controls. Mice were sacrificed 1 week after the second injection. The mononuclear cells from control and estrogen groups were prepared and stained with anti-CD3, anti-γδT, anti-CD4, and anti-CD8 mAbs. The results indicated that there was a significant increase in the number of mononuclear cells and proportion of γδT cells in uterus from estrogen group compared to that of control group ($p < .0001$, Figure 1A,B). In addition, estrogen could also increase the proportion of $CD4^+$ and $CD8^+$T cells in uterus ($p < .01$, Figure 1C,D). Interestingly, there was no difference in the proportion of γδT cells in spleens from control and estrogen groups (Figure 1E). To further confirm the influence of estrogen on uterus, mice of OVX and OVX+estrogen groups were performed with a bilateral ovariectomy, while control and estrogen group mice were performed a sham surgery. Three weeks after the surgery, the estrogen and OVX+estrogen group mice were injected with estrogen 5 mg/kg each time, once a week for 2 consecutive weeks. Consistently, compared to control group, the proportion of γδT cells fell to 1.25% in OVX group mice, and exogenous estrogen supplement could recover the decrease of γδT cells proportion (Figure 1F). In addition, our study demonstrated that the recruited γδT could persist in uterus more than 8 weeks after removing the estrogen treatment, but there was no significant difference in the proportion of γδT cells at the 11th week (Figure 1G). Furthermore, our study found that estrogen could not affect the proportion of γδT cells in lungs, bladder, and vagina (Figure 1H). Taken together, these data suggested that subcutaneous injection of mice with estrogen could increase the proportion of γδT and αβT cells in uterus.

3.2 | Estrogen recruited γδT cells to the uterus by CXCR3-CXCL10 chemokine axis

In order to gain insight into the mechanisms of γδT cells recruitment into uterus, we analyzed the expression of CXCR3, CXCR6, CX3CR1, CCR4, CCR5, CCR6, and CCR8 on uterine γδT cells by FACS. The results showed that the expression of CXCR3 in estrogen group was significantly higher than that of control group ($p < .0001$).

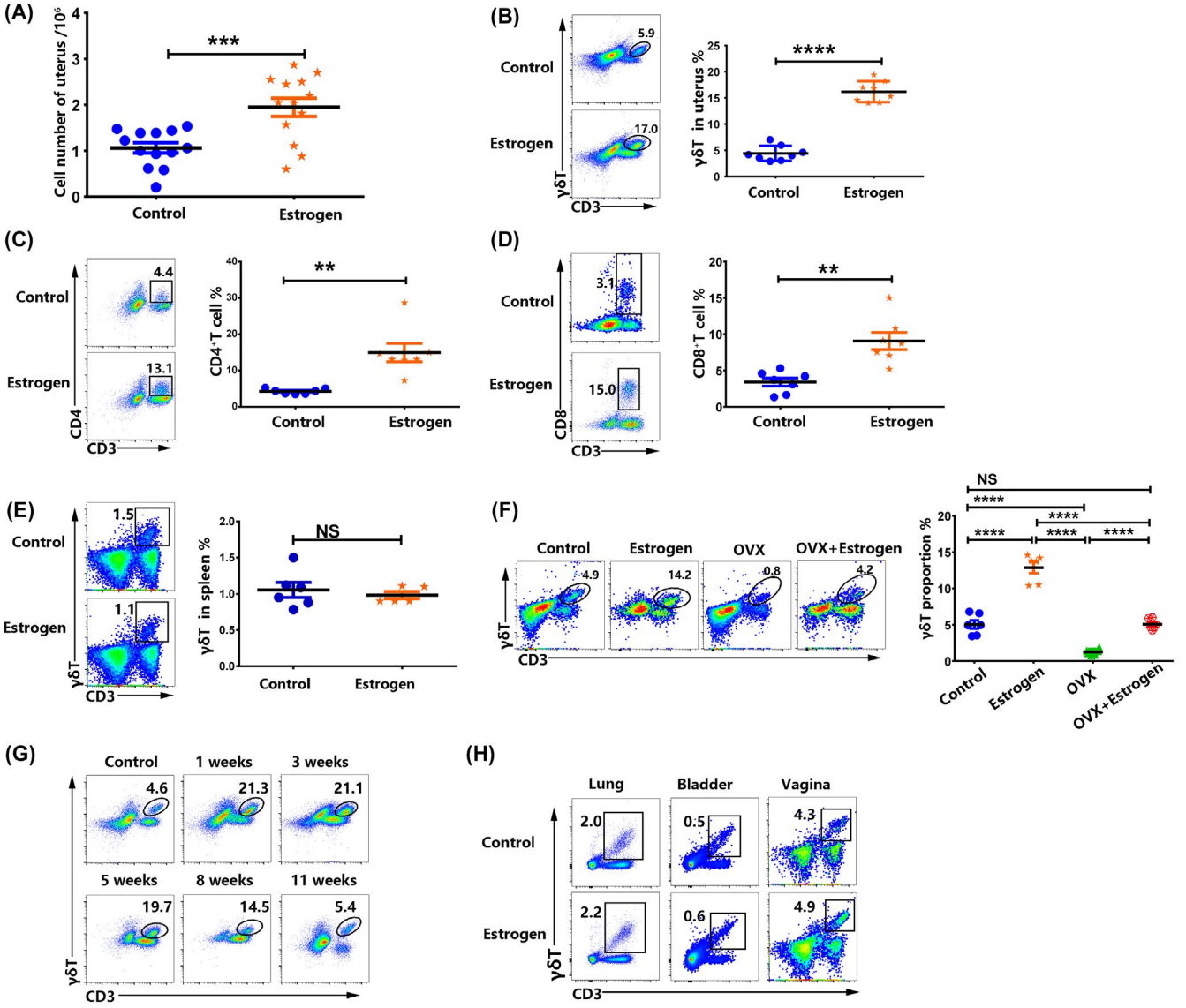
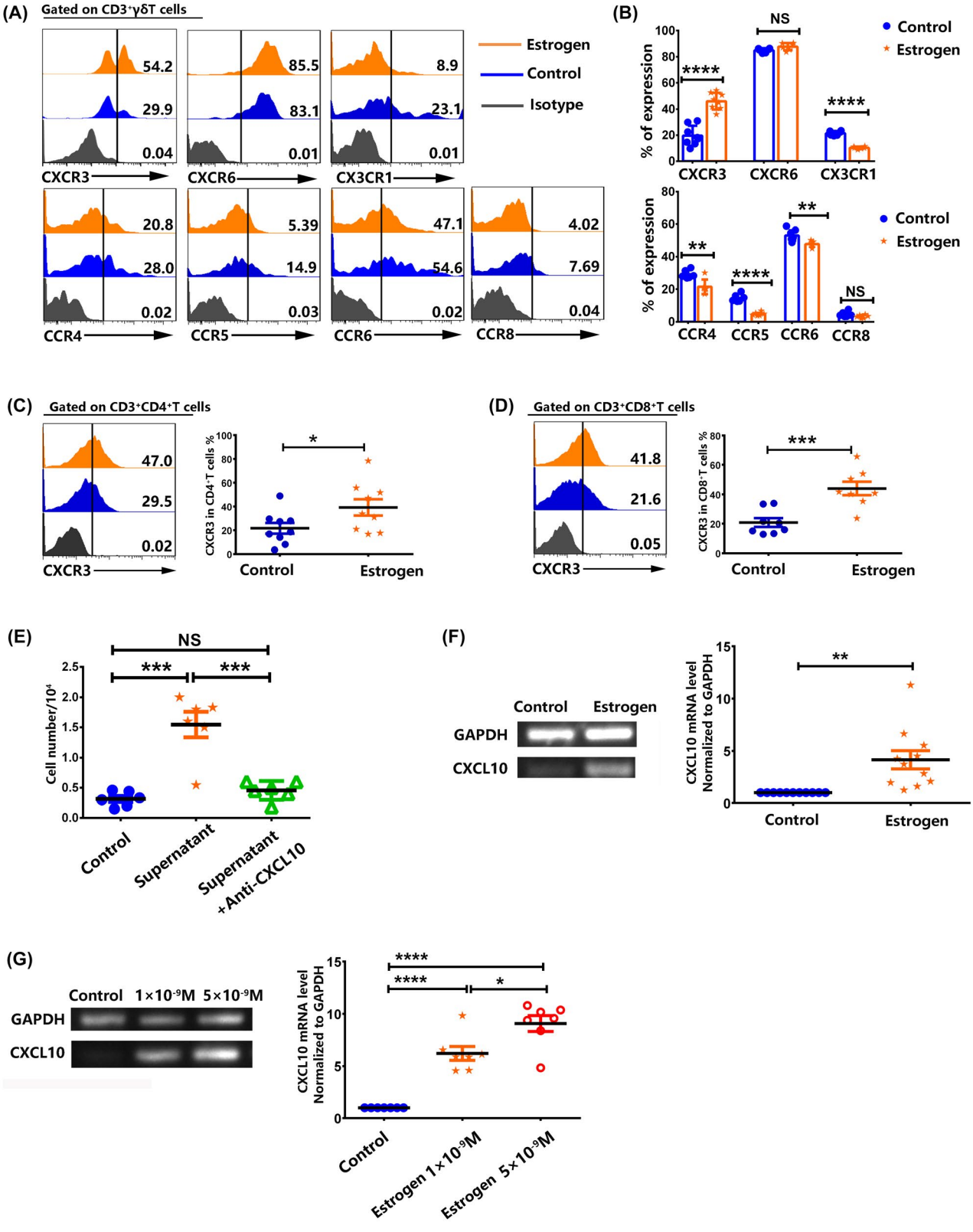


FIGURE 1 Subcutaneous injection of mice with estrogen increased the proportion of T cells in uterus but not in spleens. Mice were subcutaneously injected with estrogen 5 mg/kg each time, once a week for 2 consecutive weeks, and sacrificed 1 week after the second injection. The mononuclear cells from control and estrogen groups were prepared and stained with anti-CD3, anti-γδT, anti-CD4, and anti-CD8 mAbs, and assessed by FACS after stainings. The statistical results of mononuclear cell number in uterus from control and estrogen groups were shown (A). The representative graphs and statistical results for the proportion of γδT (B), $CD4^+$ (C), and $CD8^+$T cells (D) in control and estrogen uterus were shown. The representative graphs and statistical results for the proportion of γδT (E) in control and estrogen spleens were shown. All mice were processed a sham surgery or bilateral ovariectomy. After 3 weeks, the mice from estrogen and OVX+estrogen groups were subcutaneously injected with estrogen 5 mg/kg each time, once a week for 2 consecutive weeks. All mice were sacrificed 1 week after the second injection. The representative graphs and statistical results for the proportion of γδT cells in control, estrogen, OVX, OVX+estrogen groups were shown (F). The representative graphs for the proportion of γδT cells in uterus after removing the estrogen treatment were shown (G). The representative graphs for the proportion of γδT cells in lungs, bladder, and vagina from control and estrogen groups were shown (H). The graphs were representative of three independent experiments with six mice in each experiments. Each point represented an independent experiment with six mice and expressed as the mean ± SEM. The statistical significance was determined with the Mann–Whitney *U* test. NS, no significance; $^{**}p < .01$, $^{***}p < .001$, and $^{****}p < .0001$. OVX, ovariectomy

However, the expression of CX3CR1, CCR4, CCR5, and CCR6 in estrogen group decreased than that of control group, and there was no difference in the expression of CXCR6 and CCR8 (Figure 2A,B). In addition to γδT cells, $CD4^+$T cells and $CD8^+$T cells from estrogen groups also expressed higher percentages of CXCR3 than that of control groups (Figure 2C,D). The upregulated expression of CXCR3 in estrogen group reminded us to further



confirm whether estrogen recruited γδT cells to uterus by CXCR3-CXCL10 chemokine axis. Transwell migration technology was used to prove the hypothesis. The upper chamber was added with cell suspension and the lower chamber with or without uterus homogenate supernatant diluent in the presence or absence of anti-CXCL10 antibody. The recruited cells to lower chamber were counted. The result showed that a significant increase in recruited cells was observed in homogenate supernatant diluent group. More importantly, the addition of neutralizing

Abs to CXCL10 could effectually inhibit the recruitment effect of the uterus homogenate supernatant diluent (Figure 2E). Further study also demonstrated that estrogen could increase the expression of mRNA encoding chemokine CXCL10 in vivo (Figure 2F) and in vitro (Figure 2G). Overall, those data demonstrated that estrogen could recruit γδT cells to uterus by CXCR3-CXCL10 chemokine axis.

3.3 | Estrogen enhanced the production of IL-17 but not IFN-γ by γδT cells and $CD4^+$T cells in uterus

The mononuclear cells from uterus were prepared and stimulated with or without anti-CD3 in the presence or absence of estrogen (5×10^{-9}M) for 96 h. The levels of IL-17 and IFN-γ in culture supernatants were assessed by ELISA. Uterine cells produced high levels of IL-17 and IFN-γ following stimulation with anti-CD3. The addition of estrogen in vitro enhanced the production of IL-17 but not IFN-γ (Figure 3A,B). To clarify the effect of estrogen on different subsets of T cells, the mononuclear cells were stimulated with or without PMA plus ionomycin in the presence of Brefeldin A (BFA) for 6 h and analyzed by FACS for the expression of IL-17 and IFN-γ. The results showed that uterine γδT cells from control group stimulated with PMA plus ionomycin expressed IL-17 at the mean of 30.5% and subcutaneous injection of mice with estrogen could promote the expression of IL-17 up to 48.1%. Furthermore, uterine γδT cells from control and estrogen groups both expressed low levels of IFN-γ and there was no difference between control and estrogen groups (Figure 3C,D). Consistently, estrogen also could enhance the expression of IL-17 but not IFN-γ in $CD4^+$T cells (Figure 3E,F). In order to exclude the influence of other cells in uterus, we sorted uterine γδT cells from control and estrogen groups by FACS. The purified cells (purity > 98%, Figure 4A) were stimulated with PMA and ionomycin for 48 h. The cell culture supernatants were harvested and measured for the levels of IL-17 and IFN-γ by ELISA. The results showed that subcutaneous injection of mice with estrogen enhanced the production of IL-17 by γδT cells (Figure 4B) but had no effect on the production of IFN-γ (Figure 4C). Those results demonstrated that estrogen directly enhanced the production of IL-17 by uterine γδT cells but not splenic γδT cells. After stimulation with anti-CD3 plus anti-CD28, 58.8% of γδT cells were divided. However, the addition of estrogen (5×10^{-9}M) into cultures did not affect the percentages of cell division (Figure 4D). In order to further explore the reason why estrogen could selectively promote the production of IL-17 by uterine γδT cells, but not spleen γδT cells, flow cytometry was used to detect the estrogen receptor of spleen, peripheral blood, and uterine γδT cells. The results showed that uterine γδT cells expressed higher levels of estrogen receptors than spleen and peripheral blood γδT cells (Figure 4E,F).

3.4 | The enhancement effect of estrogen on the production of IL-17 by γδT cells was related to its promotion of IRF-4 expression

To investigate the possible mechanism of the effect of estrogen on IL-17 production, we evaluated the expression of transcription factors, RORγt, pSTAT3, and IRF-4, on γδT cells from mice of control and estrogen groups. The mononuclear cells from control and estrogen uterus were stimulated with or without PMA plus ionomycin for 6 h in the presence of BFA and stained with anti-γδT, anti-IL-17, anti-RORγt, anti-pSTAT3, and anti-IRF-4 mAbs. The results showed that the levels of RORγt and pSTAT3 were not significantly changed between control and estrogen groups (Figure 5A,B). However, the expression of IRF-4 on γδT cells from mice of estrogen group was significantly higher than that of control uterus (Figure 5C). In addition, the results from cell transcriptome sequence confirmed that the expression of IRF-4 in γδT cells from estrogen group was around three times more than that of control group at mRNA levels (data not shown). In addition, our study showed that γδT cells in spleen expressed higher levels of IRF-4 than that in uterus (Figure 5D), and estrogen

FIGURE 2 Estrogen recruited γδT cells to the uterus by CXCR3-CXCL10 chemokine axis. The mononuclear cells from control and estrogen uterus were prepared and stained with anti-CD3, γδT, and chemokine receptor mAbs for FACS analysis. Gated on $CD3^+$γδT cells from estrogen (orange) and control (blue) uterus, the representative graphs for the expression of CXCR3, CXCR6, CX3CR1, CCR4, CCR5, CCR6, and CCR8 were shown (A). Statistical results in each experiment were shown (B). Gated on $CD3^+CD4^+$T cells (C) and $CD3^+CD8^+$T cells (D) from estrogen (orange) and control (blue) uterus, the representative graphs and statistical results for the expression of CXCR3 were shown. The cell suspension was added to the upper chamber. The lower chamber was added with or without uterus homogenate supernatant diluent in the presence or absence of anti-CXCL10 antibody. The recruited cells of lower chamber were counted (E). The levels of CXCL10 and GAPDH mRNA in control and estrogen uterus were determined by RT-PCR in vivo (F) and after cultured with or without estrogen for 24 h in vitro (G). Each point represented an independent experiment with six mice and expressed as mean ± SEM. The statistical significance was determined with the Mann–Whitney *U* test. NS, no significance; *$p < .05$, **$p < .01$, ***$p < .001$, and ****$p < .0001$

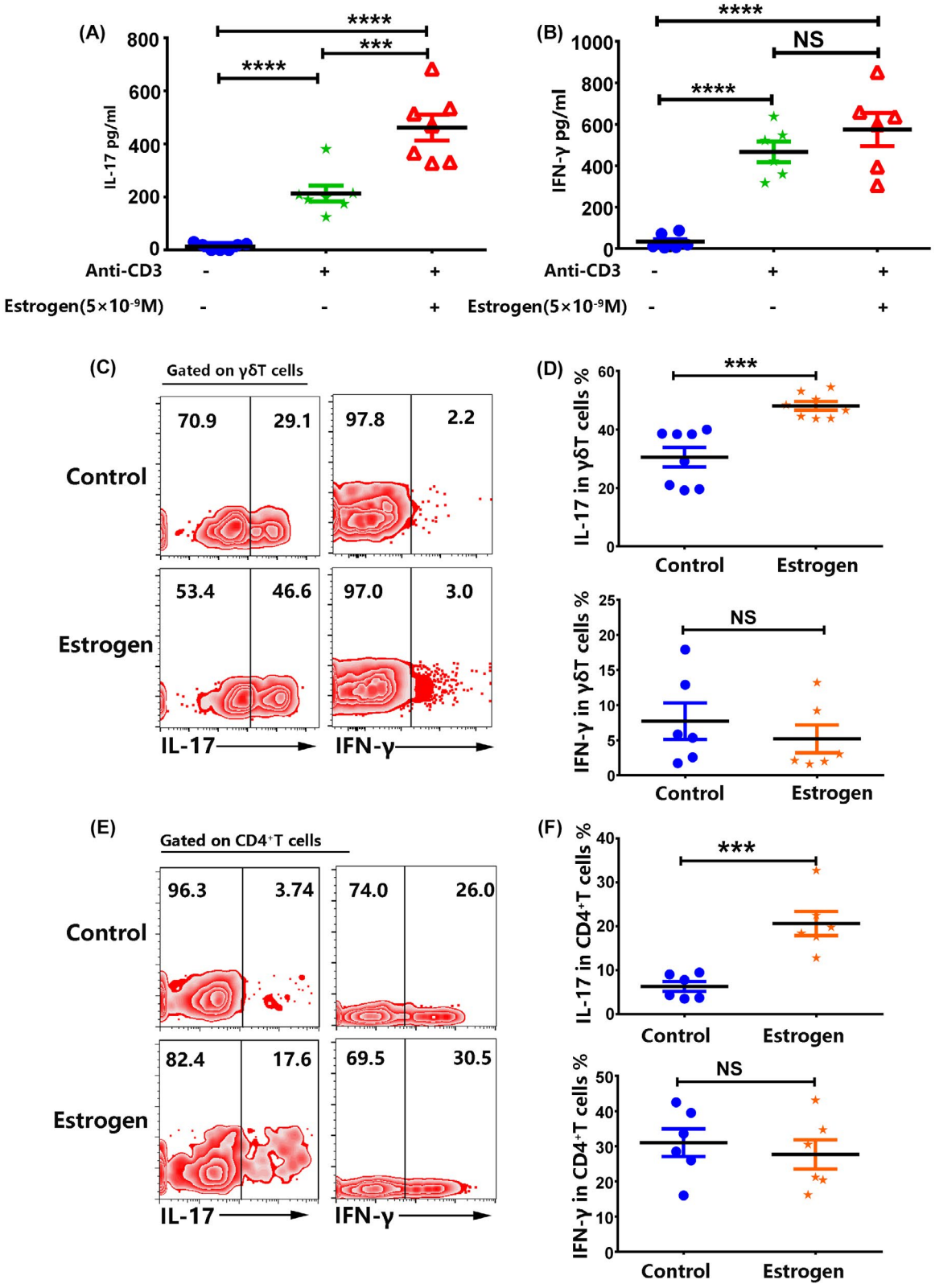


FIGURE 3 Estrogen increased the production of IL-17 but not IFN-γ by γδT and $CD4^+$T cells in uterus. The mononuclear cells from uterus were prepared and suspended in complete RPMI 1640 medium at a density of 2×10^6/ml. The cells were stimulated with or without anti-CD3 in the presence or absence of estrogen (5×10^{-9}M) for 96 h. The levels of IL-17 ($n = 7$) (A) and IFN-γ ($n = 6$) (B) were measured by ELISA. The mononuclear cells from control and estrogen uterus were stimulated with or without PMA plus ionomycin in the presence of BFA for 6 h and analyzed by FACS for the expression of IL-17 and IFN-γ. Gated on $CD3^+$γδT cells, the representative graphs (C) and statistical results (D) for the expression of IL-17 and IFN-γ were shown. Gated on $CD3^+CD4^+$T cells, the representative graphs (E) and statistical results (F) for the expression of IL-17 and IFN-γ were shown. Each point represented an independent experiment with six mice. Data were shown as mean ± SEM. The statistical significance was determined with the Mann–Whitney *U* test. NS, no significance; ***$p < .001$ and ****$p < .0001$

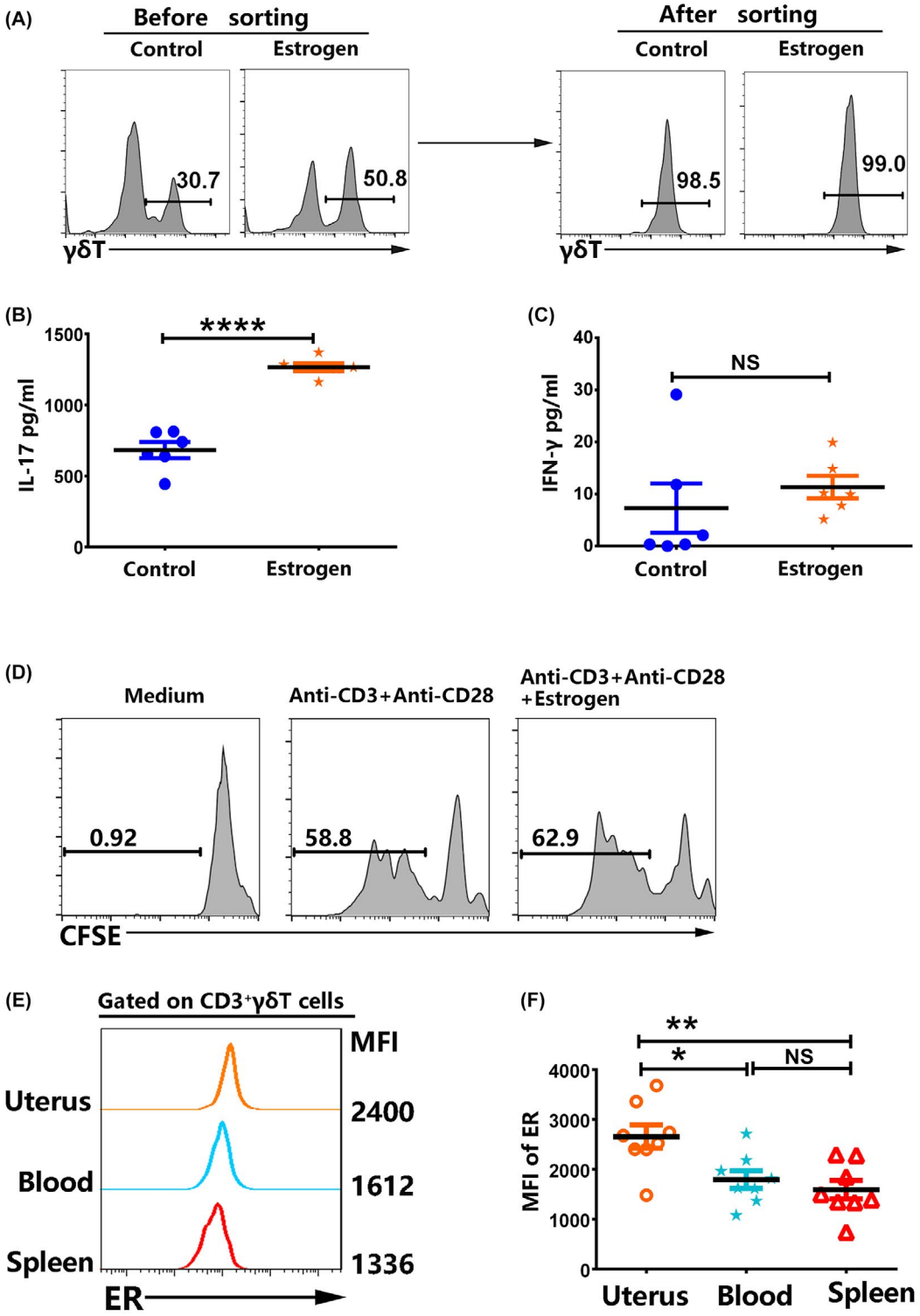


FIGURE 4 Sorted γδT cells from uterus treated in vivo with estrogen produced higher levels of IL-17 but not IFN-γ than γδT cells from control uterus. γδT cells from control and uterus treated in vivo with estrogen were sorted and the purity of sorted γδT cells were detected by FACS. The graphs were representative of six independent experiments (A). The sorted γδT cells were stimulated with PMA and ionomycin for 48 h. The cell culture supernatants were harvested and measured the levels of IL-17 (B) and IFN-γ (C) by ELISA ($n = 6$). Each point represented an independent experiment with 20 mice in control group and 10 mice in estrogen group. The uterine mononuclear cells were stimulated for 4 days with anti-CD3 plus anti-CD28 in the presence or absence of estrogen (5×10^{-9}M). The proliferative response was determined by CFSE Cell Proliferation Kits (D). The graphs were representative of three independent experiments. The mononuclear cells from uterus, blood, and spleen were prepared and stained with anti-CD3, anti-γδT, and anti-ER. The representative histogram graphs (E) and statistical data of MFI (F) for the expression of ER on γδT cells from uterus, blood, and spleen were shown ($n = 8$). Data were shown as mean ± SEM. The statistical significance was determined with the Mann–Whitney *U* test. NS, no significance; *$p < .05$, **$p < .01$, and ****$p < .0001$. ER, estrogen receptor

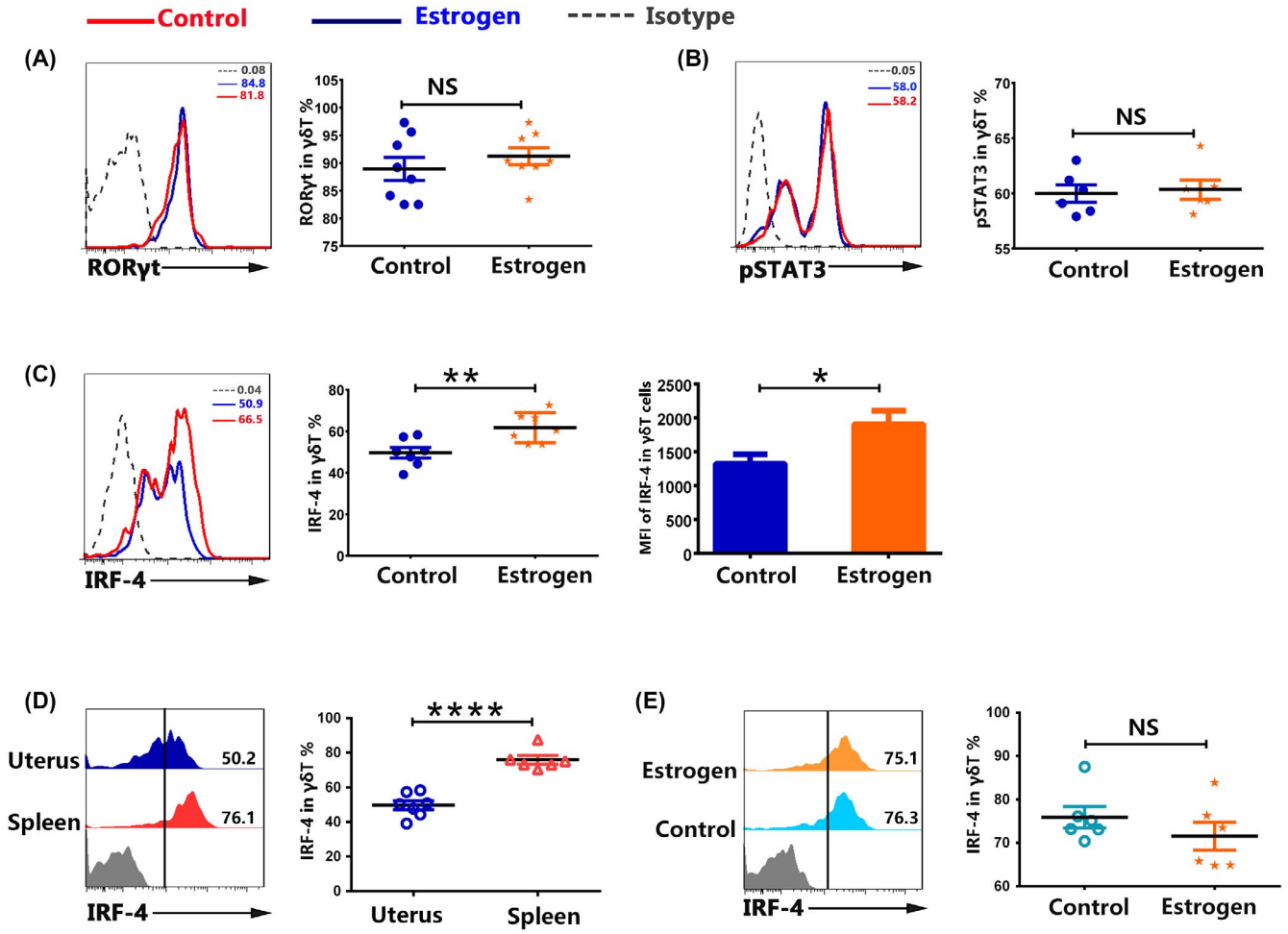


FIGURE 5 γδT cells from uterus treated in vivo with estrogen expressed higher levels of transcription factor IRF-4 than γδT cells from control uterus. The mononuclear cells from control and uterus treated in vivo with estrogen were stimulated with or without PMA plus ionomycin in the presence of BFA for 6 h. The expression of RORγt (A), pSTAT3 (B), and IRF-4 (C) on γδT cells were shown ($n = 6$–8). The representative graphs and statistical results for the expression of IRF-4 on γδT cells from uterus and spleen were shown (D). The representative graphs and statistical results for the expression of IRF-4 on γδT cells from control and estrogen group spleen were shown (E). Each point represented an independent experiment with six mice and expressed as the mean ± SEM. The statistical significance was determined with the Mann–Whitney U test. NS, no significance; $^{*}p < .05$, $^{**}p < .01$, and $^{****}p < .0001$

could not enhance the expression of IRF-4 in splenic γδT cells (Figure 5E).

3.5 | Estrogen induced different mRNA expression compared to control γδT cells

The difference in phenotypes and functions between estrogen and control γδT cells prompted us to clarify the heterogeneity of estrogen and control γδT cells at mRNA levels. γδT cells from uterus of estrogen and control groups were sorted by FACS and added to lysis buffer. Cell transcriptome sequence was used for the analysis of their gene expression profiles. The results showed that compared to control γδT cells, there were 382 upregulated and 162 downregulated genes in estrogen γδT cells, based upon the standard of fold change ≥2 for upregulation and ≤0.5 for downregulation and $p < .05$ (Figure 6A). Moreover, heat map of the top 50 genes differentially expressed is shown in Figure 6B.

3.6 | The potential biological function of differentially expressed genes between estrogen and control γδT cells

To further study the function of identified 544 differentially expressed genes, we used the KEGG database to perform pathway analysis on differential protein-coding genes. The results showed that a total of 61 differentially expressed genes were enriched in signal transduction pathways, followed by 51 differentially expressed genes in the immune system, and 45 differentially expressed genes

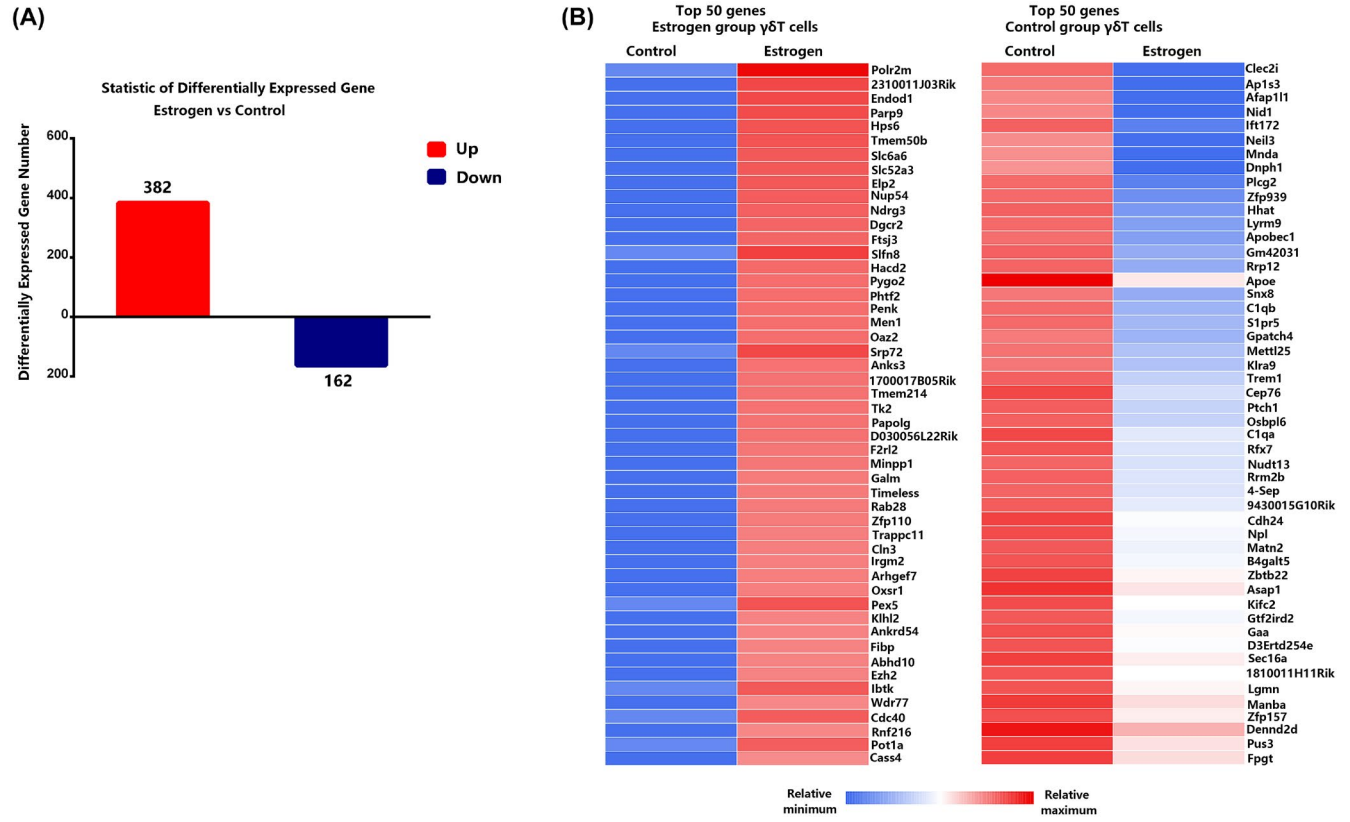


FIGURE 6 Identification of differentially expressed genes (DEGs) between estrogen and control γδT cells. γδT cells from control uterus and uterus treated in vivo with estrogen were sorted and added to lysis buffer. The RNA sequencing process was accomplished using Illumina Hiseq platform. The statistical data of upregulated and downregulated genes between estrogen and control γδT cells were shown (A). Heat map of the top 50 genes differentially expressed (p value $< .01$) between control group (left) and estrogen group (right) γδT cells was shown (B)

involved in cancers (Figure 7A). These were the most abundant genes involved in KEGG pathways. In addition, we analyzed the top of 20 significant enrichment pathways. In accordance with previous study, differentially expressed genes between estrogen and control γδT cells were enriched in the IL-17 signaling pathway and Th17 cell differentiation pathway. At the same time, other immunological pathways such as TNF signaling pathway, Th1 and Th2 cell differentiation, and chemokine signaling pathway were enriched with differentially expressed genes (Figure 7B). Taken together, we further verified the effect of estrogen on the IL-17 signaling pathway and explored other potential function of estrogen at the mRNA level using cell transcriptome sequence.

4 | DISCUSSION

Different from αβT cells, γδT cells acquire their effector functions during embryonic development and quickly seed in epithelial and mucosal barriers. γδT cells are shaped by tissue environmental which in turn shaped the immune response of that environmental.[7,13] In the model of experimental arthritis, Annica et al, showed that estrogen treatment could reduce the severity of arthritis by preventing the migration of Th17 cells and IL-17$^+$γδT cells from lymph nodes to joints.[21,22] Given the abundance of γδT cells in uterus and the localization of γδT cells close to ovary, a better understanding of the mechanism how estrogen regulated the immune function of uterine γδT cells may be crucial for the development of novel therapeutic targets. However, there was no literature on estrogen effect on uterine γδT cells published yet.

Subcutaneous injection of mice with estrogen could increase the proportion of γδT cells in uterus as well as CD4$^+$T cells and CD8$^+$T cells. Consistently, ovariectomized mice suffered a big loss in the proportion of γδT cells, and exogenous estrogen supplement could recover the losses. However, there was no difference in the percentage of γδT cells in spleens. Those data indicated that estrogen could recruit γδT cells to uterus. It has been shown that estrogen induced the expression of chemokines CXCL10 and CXCL11 within human endometrium which activated NK cell migration and accounted for the

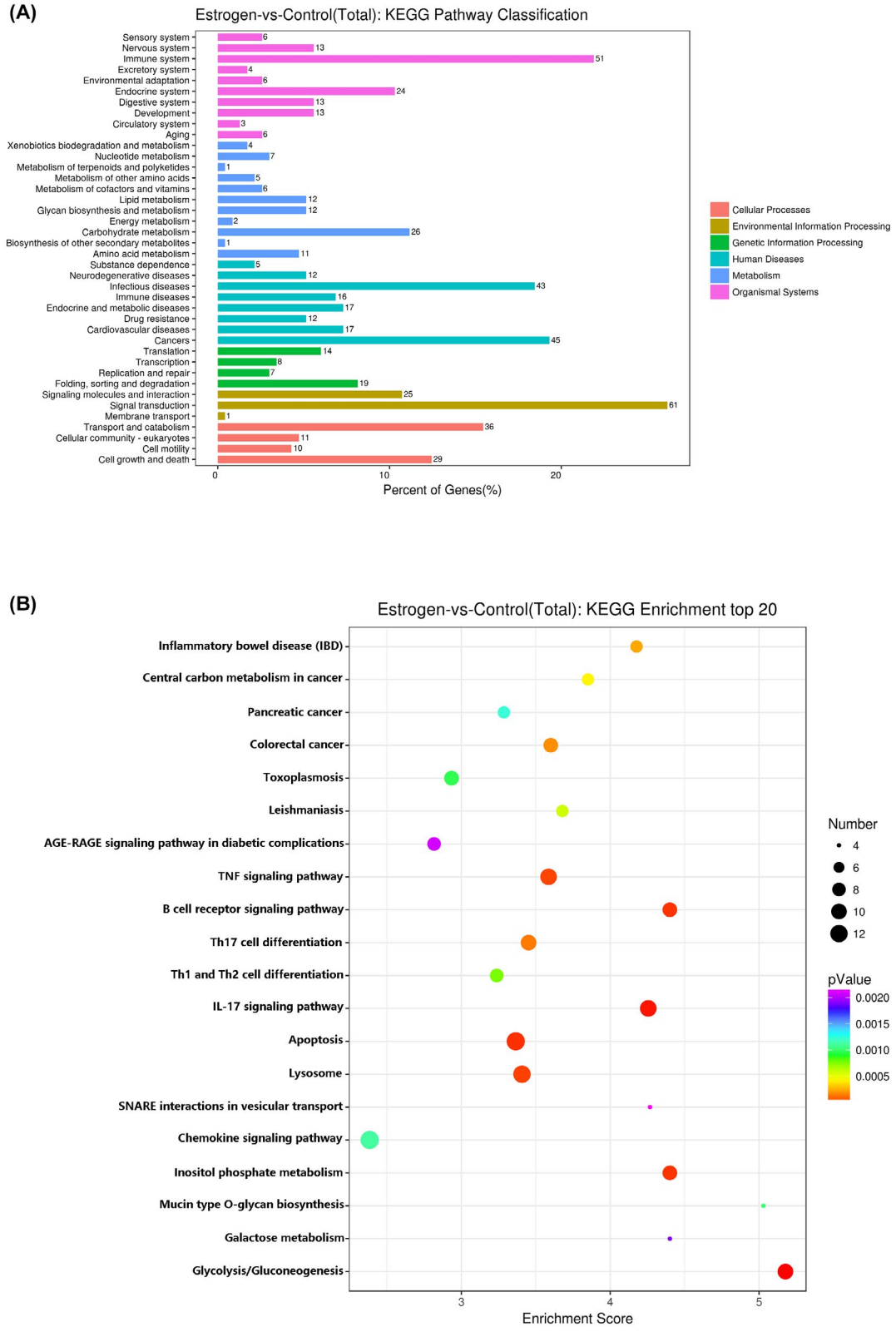


FIGURE 7 KEGG pathway classification and enrichment analysis of differentially expressed genes between estrogen and control γδT cells. KEGG analysis classified the differentially expressed genes between estrogen and control γδT cells for cellular processes, environmental information processing, genetic information processing, human diseases, metabolism, and organismal systems (A). The bubble diagram displayed the top 20 pathways using KEGG enrichment analysis. KEGG enrichment analysis was corresponding to the number of differential genes greater than 2 and sorting them according to the −log10*p*Value corresponding to each entry from large to small. The horizontal axis in the figure was the enrichment score. The larger the bubble, the more the number of differentially expressed encoding genes. The bubble color changes from purple to blue to green to red, and the smaller the enrichment *p*-value, the greater the significance (B)

accumulation of NK cells in endometrium during the menstrual cycle.[23] In line with the findings of NK cells in human endometrium, our results demonstrated that estrogen could increase the expression of CXCR3 on γδT cells in uterus and recruited γδT cells to uterus by CXCR3-CXCL10 chemokine axis.

Aside from the protective effect to eliminate extracellular bacteria and fungi, IL-17 strongly contributes to pathogenic inflammation of various autoimmune diseases, particularly critical in the early stages of autoimmune disease.[10] IL-17 could recruit different inflammatory myeloid population accumulation which drive tissue damage.[10,24] Antibodies targeting IL-17 or IL-23, the upstream driver of IL-17, were reported to have a remarkable efficacy in the treatments of psoriasis, and ankylosing spondylitis (AS).[25–27] Although Th17 cells are considered to be the major source of IL-17 in many autoimmune diseases, accumulating evidence from murine and human studies showed that γδT cell-derived IL-17 is one of the earliest sources of cytokines and promotes the development of colitis, rheumatoid arthritis, psoriasis, and type 1 diabetes.[9,13,28–30] Persistent unopposed estrogen stimulation is a central oncogenic mechanism driving the formation of some female cancer, such as breast cancer and type I endometrial cancer. IL-17 produced by infiltrating macrophages in endometrial microenvironment could induce the upregulation of ERα expression, which in turn sensitized endometrial cells to estrogen stimulation.[31,32] However, it remains unknown how γδT cell-derived IL-17 influence endometrial microenvironment. Our previous work showed that γδT cells were enriched in uterus and highly expressed IL-17.[20] In the present study, we found that estrogen could increase the production of IL-17 in uterus after stimulation with anti-CD3 in vitro. During polyclonal activation, γδT cells in uterus expressed high levels of IL-17 but the expression of IFN-γ was much scarcer. In contrast, $CD4^+$T cells in uterus expressed low levels of IL-17 but expressed high percentages of IFN-γ. Furthermore, estrogen could increase the expression of IL-17 not only in γδT cells but also in $CD4^+$T cells, but there was no difference in the expression of IFN-γ. The enhancement effect of estrogen on IL-17 production was confirmed when the production of IL-17 increased in purified uterine γδT cells where we eliminated the interruption of other cells in uterus. In addition, our study found that γδT cells from uterus expressed significantly higher level of estrogen receptor than that of blood and spleen, which might be the reason why estrogen could selectively promote the production of IL-17 in uterus but not in spleen. Estrogen could promote the expression of estrogen receptor in HepG2 cells, the upregulated estrogen receptor could reduce HBV transcription and inhibit cell proliferation.[33,34] However, whether estrogen could promote the expression of estrogen receptors on γδT cells has not been reported.

Aside from transcription factors RORγt and STAT3, IRF-4 is absolutely required for the production of IL-17.[35,36] Our results showed that there was no significant difference in the expression of RORγt and STAT3 between uterine γδT cells from control and estrogen groups. However, the expression of IRF-4 in γδT cells from estrogen group was much higher than that of control group. Estrogen/ERα signaling could increase the expression of cellular IRF-4, which was required for $CD11b^+$ and $Langerin^+$DC differentiation mediated by GM-CSF.[37] Algorithms within the ECR Browser at the National Center for Biotechnology Information identified four conserved estrogen response elements (EREs) proximal to the murine *Irf4* gene.[38] ERα also may complex with other factors such as Sp-1, NF-Y, FoxA1/HNF3α, AP-1, or NF-κB and binding sites for these factors present proximal to the *Irf4* gene.[39,40] Our study provided evidence that the enhancement effect of estrogen on the production of IL-17 by γδT cells was related to its promotion of IRF-4 expression. However, the detailed mechanism from estrogen receptor to IRF-4 remains unknown.

In summary, our study for the first of time, to our best knowledge, found that estrogen could recruit γδT cells to uterus via CXCR3-CXCL10 chemokine axis and promote the production of IL-17 from uterine γδT cells but not splenic. More importantly, our study demonstrated that transcription factor IRF-4 was related to the enhancement effect of estrogen on the production of IL-17 by γδT cells. Our study provide a new idea for probing the sex bias in autoimmune diseases that estrogen increased the prevalence of autoimmune diseases in women by enhancing γδT cell-derived IL-17 production in uterus and might contribute to the understanding of the estrogen in the development of autoimmune diseases.

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DISCLOSURES

The authors declare that they have no conflict of interest to this work. They declare that they do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

AUTHOR CONTRIBUTIONS

Shuangpeng Kang performed research and wrote the paper. Qiongli Wu analyzed the data. Binyan Yang

contributed new reagents and analytic tools. Changyou Wu designed research.

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