

Article

Open Access

Single-cell sequencing reveals alterations in the ovarian immune microenvironment regulated by 17 β -estradiol in neonatal mice

Yu-Tong Yan^{1,2}, Yan-Xue Li^{1,2}, Yi-Ting Meng^{1,2}, Qian Li^{1,2}, Xiao-E Zhao^{1,2}, Qiang Wei^{1,2}, Meng-Hao Pan^{1,2,*}, Sha Peng^{1,2,*}, Bao-Hua Ma^{1,2,*}

¹ College of Veterinary Medicine, Northwest A & F University, Yangling, Shaanxi 712100, China

² Key Laboratory of Animal Biotechnology of the Ministry of Agriculture and Rural Affairs, Northwest A & F University, Yangling, Shaanxi 712100, China

ABSTRACT

The immunomodulatory function of estrogen within the ovary remains a subject of ongoing debate, and the neonatal ovarian immune microenvironment, particularly its modulation by estrogen, has not been comprehensively characterized. In this study, the effects of 17 β -estradiol (E₂), a key regulator of immune function, were investigated using single-cell transcriptomic profiling of C57BL/6J neonatal mouse ovaries after E₂ treatment. Results revealed dynamic alterations in the proportion of immune cell types after E₂ treatment, accompanied by changes in cytokine and chemokine expression. Detailed analyses of gene expression, cell states, and developmental trajectories across distinct cell types indicated that E₂ treatment influenced cell differentiation and development. Notably, E₂ treatment reduced the abundance of macrophages and promoted a phenotypic transition from M1 to M2 macrophages. These findings demonstrate that the neonatal mouse ovarian immune microenvironment is sensitive to estrogenic modulation, which governs both the distribution and functional specialization of resident immune cells, offering novel mechanistic insights into the immunomodulatory roles of estrogen across various immune cell types.

Keywords: Ovary; Immune microenvironment; Immune cells; 17 β -estradiol; Transcriptional alterations

INTRODUCTION

Immune effectors play a vital role in regulating female reproductive physiology (Yang et al., 2019), influencing key processes such as folliculogenesis, menstruation, ovulation,

implantation, and ovarian aging (Ben Yaakov et al., 2023; Isola et al., 2024). Age-related alterations in the composition of ovarian immune cells have been documented, suggesting a dynamic immune landscape throughout reproductive life (Ben Yaakov et al., 2023; Isola et al., 2024). Immune cells, including macrophages, dendritic cells, granulocytes, T and B lymphocytes, and others, are distributed throughout the entire female reproductive tract (Givan et al., 1997; Lee et al., 2015), and are predominantly present within the ovaries (Ben Yaakov et al., 2023; Best et al., 1996; Bukulmez & Arici, 2000; Carlock et al., 2013; Isola et al., 2024; Yang et al., 2019). Within this context, ovarian immune cells contribute to essential reproductive events, including follicle development, ovulation, and the formation and regression of corpus luteum (Cohen-Fredarow et al., 2014; Fair, 2015; Oakley et al., 2010; Wu et al., 2004; Yang et al., 2019).

Ovulation itself is widely recognized as an inflammation-like process (Duffy et al., 2019; Richards et al., 2008), with changes in immune cell composition, especially in macrophage and dendritic cell populations, linked to reduce ovulation rates, decrease endothelial cell abundance, increase follicular atresia, and delay estrus cycle progression (Cohen-Fredarow et al., 2014; Turner et al., 2011; Wu et al., 2004). Co-culture studies have further shown that M1 macrophages can activate primordial follicles, while M2 macrophages inhibit this process via PI3K/AKT/mTOR signaling pathway (Xiao et al., 2022). Moreover, the ovarian immune composition also shifts with age. In aged mice (9 months), immune cell abundance doubles compared to that of young mice (3 months), with lymphocyte expansion constituting the most prominent change (Isola et al., 2024). While the immunological alterations accompanying ovarian aging have been characterized in adult and aged murine models (Ben Yaakov et al., 2023; Isola et al., 2024), the immune microenvironment of the neonatal mouse ovary remains largely unknown.

Estrogen, a steroid hormone, the main active form is E₂,

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0/>), which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Copyright ©2025 Editorial Office of Zoological Research, Kunming Institute of Zoology, Chinese Academy of Sciences

Received: 16 December 2024; Accepted: 14 January 2025; Online: 15 January 2025

Foundation items: This work was supported by the National Natural Science Foundation of China (32072941)

*Corresponding authors, E-mail: panmenghao@nwfau.edu.cn; pengshacxh@nwfau.edu.cn; malab@nwfau.edu.cn

which regulates multiple biological processes in mammals. During primordial follicle formation, estrogen plays a positive role in hamsters (Wang & Roy, 2007) and baboons (Pepe et al., 2006) but exerts inhibitory effects in fetal bovine (Yang & Fortune, 2008) and mice (Chen et al., 2007). Disruptions in E_2 -regulated follicle assembly have been shown to impair subsequent follicular development (Yan et al., 2024). In addition, estrogen functions as a key immunomodulator, acting through both innate and adaptive immune pathways (Grossman, 1985). Estrogen modulates immune cell development, differentiation, and effector functions via direct interactions with various immune cell types (Asaba et al., 2015; Kovats, 2015; Panchanathan & Choubey, 2013). In macrophages, E_2 regulates chemotactic behavior, phagocytic activity, and the production of cytokines, inducible nitric oxide synthase (iNOS), and nitric oxide (Dai et al., 2008; Hsieh et al., 2009; Karpuzoglu & Ahmed, 2006; Karpuzoglu et al., 2009). It also facilitates the maturation of dendritic cells and regulates cytokine and chemokine expression (Bachy et al., 2008; Liu et al., 2002), including interleukin-6 (IL-6), IL-10, C-X-C motif chemokine ligand 8 (CXCL8), and C-C motif chemokine ligand 2 (CCL2).

Despite growing evidence linking estrogen to immune regulation, its functional complexity and effects require further clarification. Most prior studies have focused on bulk ovarian tissues, often neglecting cellular heterogeneity, which limits the understanding of how estrogen acts on immune microenvironment within the ovary. Although single-cell transcriptomic approaches have delineated the principal ovarian cell types, including oocytes, pre-granulosa cells, stromal cells, immune cells and others, the study has yet to provide a detailed characterization of immune cell diversity in the neonatal ovary of mice (Yan et al., 2024). A systematic analysis of the neonatal mouse ovarian immune landscape, particularly its modulation by estrogen, is therefore essential for advancing mechanistic understanding of early ovarian development and immune-endocrine interactions.

Several recent studies have explored the influence of estrogen on the immune system at the single-cell level (Kimmel et al., 2019; The Tabula Muris Consortium, 2020). Notably, estrogen levels are reported to decline markedly during primordial follicle formation (Dutta et al., 2014). In this study, immune cell subpopulations were extracted from single-cell RNA sequencing (scRNA-seq) data obtained from control (CTRL) and E_2 -treated neonatal mouse ovaries. Comprehensive single-cell analyses were conducted to determine transcriptional and cellular alterations induced by E_2 exposure. This study represents the first detailed characterization of the immune system composition with the neonatal murine ovary, revealing that E_2 treatment promoted a shift toward innate immune dominance. Transcriptomic profiling uncovered changes in gene expression across multiple immune cell types, particularly involving inflammatory mediators, such as *IL-1B*, *IL-10*, *CCL2*, *CCL4*, and *CCR1*. Moreover, E_2 exposure was associated with a reduction in M1 macrophages and a concomitant increase in M2 macrophages, suggesting a transition toward an anti-inflammatory immune phenotype. Collectively, this study provides a systematic analysis of estrogen-induced modulation of ovarian immune cells, including transcriptional dynamics, cell states, and differentiation trajectories. These findings offer a foundational framework for understanding the interactions between estrogen and the immune system in

reproductive-age female mammals.

MATERIALS AND METHODS

ScRNA-seq data acquisition and processing

The scRNA-seq datasets were obtained from the NCBI Sequence Read Archive (SRA) under accession number PRJNA1056475. Neonatal mice at 1 day post-partum (1 dpp) received daily subcutaneous injections of either 20 μ L corn oil or vehicle containing 20 μ g E_2 for 3 days. Ovaries were then collected at 4 dpp for scRNA-seq and analyzed followed previously reported protocols (Yan et al., 2024). Briefly, FASTQ files were processed using Cell Ranger (v.5.0.0) to generate gene expression matrices and barcode information. STAR (v.2.7.2a) was used to align reads to the mouse reference genome (GRCm39, NCBI) and to produce output files for downstream analyses using the R package Seurat (v.3.1.1), including gene and cell filtration, normalization, principal component analysis (PCA), variable gene identification, and clustering analysis. DoubletFinder (v.2.0.3) was utilized to mitigate the impact of double or multiple cells in the dataset. Quality control criteria included retention of cells with “200<gene counts<4 900, unique molecular identifier (UMI) counts<38 000, and percentage of mitochondrial genes<25%”. Gene expression normalization was performed using the LogNormalize method, with the formula “Log (1+(UMI A/UMI total) $\times 10^4$)”. Data integration and scaling were carried out using Harmony, followed by PCA for dimensionality reduction, with selected principal components used for subsequent analyses.

ScRNA-seq data analyses

Cell clustering and visualization were performed using the Uniform Manifold Approximation and Projection (UMAP) algorithm. Genes were defined as up-regulated if they were expressed in more than 25% of the target cell population, with a $\log_2$ fold-change (FC) ≥ 0.36 and $P \leq 0.01$. Differentially expressed genes (DEGs) were defined based on $|\log_2 FC| \geq 0.36$, $P < 0.05$, and detection in at least 10% of cells within a specific cluster. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using the identified DEGs. Pseudotime trajectory analyses were performed using Monocle 2 (v.2.10.1). Cell cycle status was determined, and cells with maximal cell cycle scores below zero were classified as non-cycling. The above analysis methods were described in the report (Yan et al., 2024).

Mice

All study protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the College of Veterinary Medicine, Northwest A&F University (No. 2023010708). C57BL/6J mice were obtained from the Experimental Animal Center of Xi'an Jiaotong University (Xi'an, Shaanxi, China) and housed under a controlled constant-temperature (22–25°C) and fixed-light environment (12 h light/dark cycle), with adequate feed and water provided. Female mice with a vaginal plug the morning after mating were designated as embryonic day 0.5 (E0.5), while pups delivered at E19.5 were designated as 1 dpp.

Mouse treatment

Mouse treatment protocols were conducted as previously described (Yan et al., 2024). Briefly, 17 β -estradiol (E2758,

Sigma Aldrich, USA) was dissolved in dimethyl sulfoxide (DMSO, D4540, Sigma Aldrich, USA) and stored at -20°C . Prior to subcutaneous administration, $20\text{ }\mu\text{g}$ E_2 was dissolved in $20\text{ }\mu\text{L}$ corn oil (C7030, Solarbio, China). Female pups (1 dpp) received daily subcutaneous injections of either $20\text{ }\mu\text{L}$ corn oil or vehicle consisting of $20\text{ }\mu\text{g}$ E_2 for 3 days. Ovaries were collected at 4 dpp for subsequent analyses.

RNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted utilizing a MiniBEST Universal RNA Extraction Kit (9767, TaKaRa, China); cDNA synthesis was performed using a PrimeScript RT Master Mix reverse transcription kit (RR036, TaKaRa, China); qPCR was performed using a SYBR Green Premix Pro Taq HS qPCR Kit (AG11718, Accurate Biotechnology, China), with the following qPCR parameters: 95°C for 30 s, followed by 40 cycles each at 95°C for 5 s and 60°C for 30 s. The primers used are listed in Supplementary Table S1. Gene expression was normalized to *ACTIN*, and relative mRNA levels were estimated using the $2^{-\Delta\Delta\text{ct}}$ method.

Immunostaining of ovarian sections

Freshly isolated ovaries were fixed in 4% paraformaldehyde (P1110, Solarbio, China) at 4°C overnight, embedded in paraffin, and serially sectioned at a thickness of $5\text{ }\mu\text{m}$. Sections were taken from the region surrounding the maximal ovarian cross-sectional area. The sections were deparaffinized with xylene and hydrated with different concentrations of ethanol. For immunohistochemistry, staining was performed according to the manufacturer's instructions (SP0041, Solarbio, China). For immunofluorescence staining, antigen retrieval was conducted in $1\times$ antigen retrieval solution (C1032, Solarbio, China) at 96°C for 15 min. Sections were then blocked with 10% donkey serum for 1 h and incubated with primary antibodies overnight at 4°C , followed by incubation with secondary antibodies for 2 h. Nuclear counterstaining was performed with 4',6-diamidino-2-phenylindole (DAPI, C1002, Beyotime, China). The antibodies used were as follows: F4/80 (30325T, Cell Signaling Technology, USA), IL-1 β (TA5103S, Abmart, China), CD163 (ab182422, Abcam, UK), iNOS (18985-1-AP, Proteintech, China), and Alexa Fluor[®] 488 donkey anti-rabbit IgG H&L (ab150073, Abcam, UK).

Western blotting (WB)

Ovaries ($n=6$) were extracted using RIPA lysis solution (R0010, Solarbio, China) supplemented with protease and phosphatase inhibitors. Protein samples were denatured in boiling water for 10 min and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer onto polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat milk and incubated with primary antibodies at 4°C overnight. After washing with TBST three times, the membranes were incubated with secondary antibodies for 2 h at room temperature. Detection was performed using enhanced chemiluminescence (ECL) reagents. Primary antibodies included F4/80 (30325T, Cell Signaling Technology, USA) and ACTIN (K200058M, Solarbio, China).

Statistical analysis

Results are presented as mean $\pm$ standard error of the mean (SEM) from at least three independent experiments. Statistical analyses were performed using GraphPad Prism (v.8.0), with

unpaired Student's *t*-test applied for experiments with two groups. Significance thresholds were denoted as follows: $^{\circ}$: $P<0.05$ and $^{\circ\circ}$: $P<0.01$, with $P>0.05$ defined as not significant (ns).

RESULTS

E_2 treatment alters the neonatal mouse ovarian immune microenvironment

To characterize the cell landscape of neonatal mouse ovaries and assess its response to exogenous estrogen exposure, scRNA-seq was performed on ovaries collected from C57BL/6J mice at 4 dpp, following daily subcutaneous injections of either corn oil or vehicle containing $20\text{ }\mu\text{g}$ E_2 starting at 1 dpp for three consecutive days (Yan et al., 2024). After quality control, 10 899 and 10 292 high-quality cells were retained from the CTRL and E_2 -treated groups, respectively (Figure 1A). Using established cell type-specific markers (Yan et al., 2024), seven major ovarian cell populations were identified, including oocytes (*Dppa3*, *Sycp3*, *Figla*, and *Dazl*), pre-granulosa cells (*Fst*, *Kitl*, *Amhr2*, and *Wnt6*), stromal cells (*Nr2f2*, *Tcf21*, *Mfap4*, *Col1a1*, and *Dcn*), erythrocytes (*Alas2*, *Rhd*, and *Car2*), immune cells (*Lyz2*, *Tyrbp*, *Fcer1g*, *Cd52*, *Plac8*, and *Ptpcr*), endothelial cells (*Aplnr*, *Egfl7*, *Cldn5*, *Flt1*, *Cdh5*, and *Kdr*), and smooth muscle cells (*Des*, *Abcc9*, *Rgs5*, *Gm13889*, and *Itga1*) (Figure 1B). These populations exhibited distinct differences after E_2 treatment: stromal cells (CTRL vs. E_2 : 25.57% vs. 28.19%), erythrocytes (55.12% vs. 37.48%), pre-granulosa cells (11.64% vs. 19.44%), oocytes (1.93% vs. 4.71%), endothelial cells (1.85% vs. 4.27%), immune cells (3.16% vs. 5.22%), and smooth muscle cells (0.73% vs. 0.70%).

Immune cells were identified by high expression of *Cd52*, *Tyrbp*, *Lyz2*, *Fcer1g*, *Ptpcr*, and *Plac8* (Supplementary Figure S1A–F). In neonatal mouse ovaries, immune cells comprised approximately 4.16% of total cells ((344+537)/(10 899+10 292)). This proportion was lower than levels reported in adult (3 months, 9.15%) and aged (9 months, 20.99%) ovaries (Isola et al., 2024), indicating a developmental increase in immune cell representation (Figure 1C). From the total immune cell subpopulation ($n=881$), eight transcriptionally distinct immune clusters were identified (Figure 1D–F; Supplementary Figure S1G, H). The number of up-regulated genes within each cluster is shown in Supplementary Figure S1I and Table S2, with the top five up-regulated genes shown in Supplementary Figure S1J. Estrogen receptor gene expression was examined across immune clusters, revealing *Esr1* expression in clusters 3, 5, and 7, *Esr2* expression in cluster 1, and low *Gper1* expression across clusters (Supplementary Figure S1K).

Based on canonical gene signatures (Ben Yaakov et al., 2023; Isola et al., 2024; Winkels et al., 2018; Winkler et al., 2024), clusters were annotated as macrophages (clusters 0, 1, and 3: *Itgam*, *Cd44*, *Cd14*, *Il1b*, *Lyz2*, *Acod1*, *S100a9*, *Ly6c2*, *Ccr2*, *Cd68*, *Apoe*, *Mafb*, *Csf1r*, *Cd86*, and *Adgre1*), B cells (cluster 4: *Ighm*, *Cd37*, *Igkc*, *Cd79a*, *Cd79b*, *Ebf1*, and *Cd74*), innate lymphoid cells (cluster 5: *Tmem176b*, *Auts2*, *Nfib*, *Tcf4*, and *Sfrp1*), dendritic cells (cluster 7: *Il13*, *Il1rl1*, *Cx3cr1*, and *Il6*), and mitotic immune cells (clusters 2 and 6: *Top2a*, *Mki67*, *Cenpe*, *Ccna2*, and *PCNA*) (Supplementary Figure S2A). Marker gene expression for each immune subtype is presented in Supplementary Figure S2B–F. Cell type distributions are depicted in Figure 1G and Supplementary

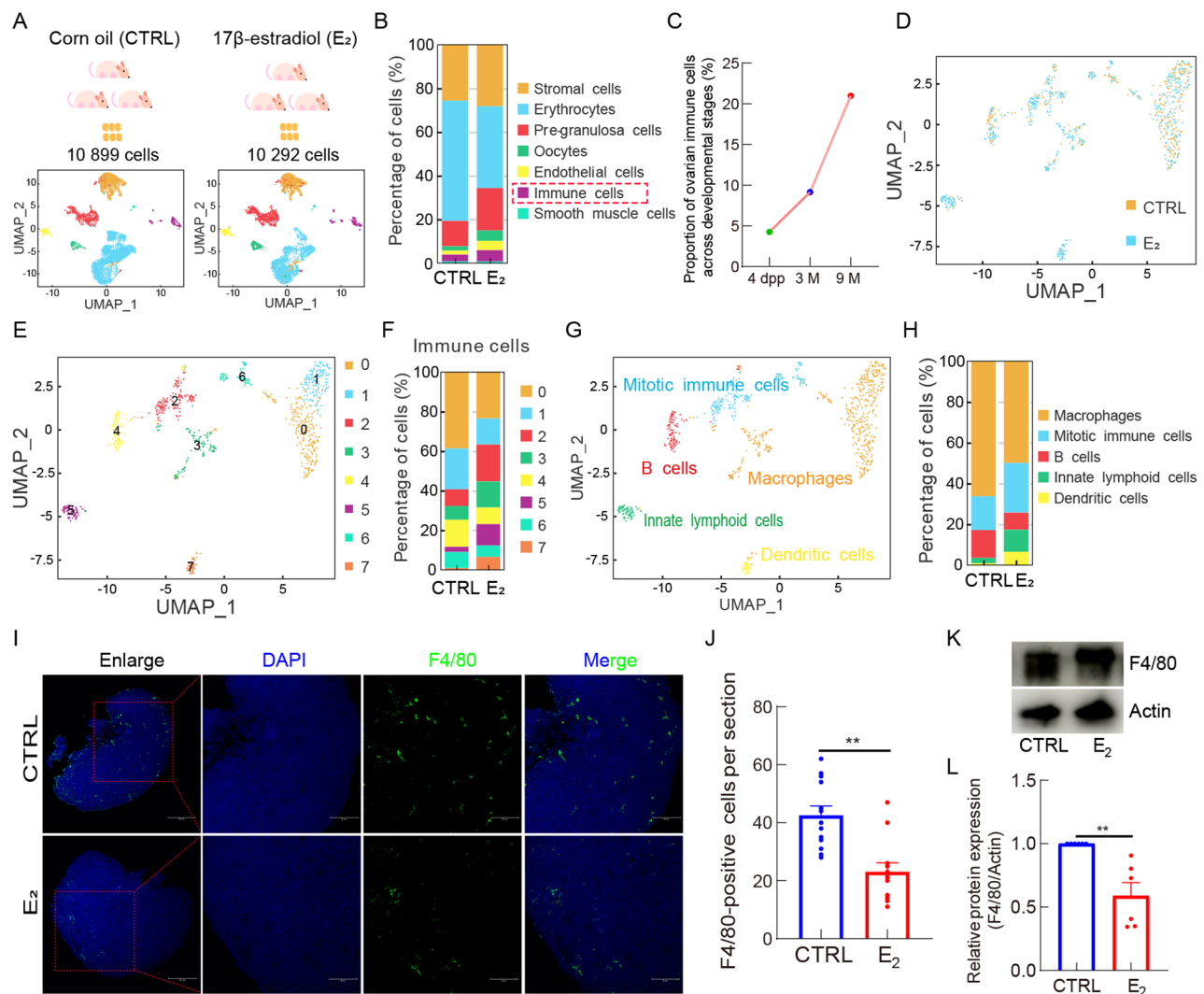


Figure 1 E₂ treatment alters the ovarian immune cell landscape in neonatal mice

A: UMAP plot of single-cell transcriptome from 4 day post-partum (dpp) mouse ovaries, colored by cell cluster. B: Proportions of major ovarian cell types between CTRL and E₂-treated groups. C: Proportions of ovarian immune cells in 4 dpp, 3-month-old (3 M), and 9-month-old (9 M) mice. D: UMAP plot of immune cell subpopulations between CTRL and E₂-treated groups. E: UMAP plot showing eight immune cell clusters. F: Distribution of immune cell clusters between CTRL and E₂-treated groups. G: UMAP plot of five annotated immune cell subtypes between CTRL and E₂-treated groups. H: Proportion of five immune cell subtypes between CTRL and E₂-treated groups. I: Representative immunofluorescence staining of macrophages (F4/80) in ovarian sections from CTRL and E₂-treated groups. Scale bar: 100 μ m (left), 50 μ m (others). J: Analysis of F4/80-positive cells in ovarian sections from CTRL and E₂-treated groups. K, L: Detection and analysis of F4/80 protein expression in ovaries. CTRL: Control group. E₂: 17 β -estradiol group. Data are shown as mean $\pm$ SEM. All experiments were repeated at least three times (**: $P < 0.01$).

Figure S2G, H. Quantitative comparisons revealed that E₂ exposure altered the proportions of five immune subtypes (Figure 1H). Specifically, macrophages (66.28% vs. 49.90%) and B cells (13.66% vs. 8.38%) were reduced, while mitotic immune cells (16.57% vs. 24.39%), innate lymphoid cells (2.62% vs. 10.8%), and dendritic cells (0.87% vs. 6.53%) were increased (Figure 1H). The number of up-regulated genes per subtype varied, with B cells showing the lowest and innate lymphoid cells the highest (Supplementary Figure S2I and Table S3). The top 10 up-regulated genes for each subtype are presented in Supplementary Figure S2J. F4/80, a classical macrophage marker (Sambamurthy et al., 2018; van den Berg & Kraal, 2005), was used to detect the number of macrophages by immunostaining (Figure 1I). Results showed that E₂ treatment significantly reduced F4/80-positive cells in the ovary (Figure 1J) and down-regulated F4/80 protein expression (Figure 1K, L). Collectively, these findings

demonstrate that exogenous E₂ treatment during early postnatal development substantially remodels the ovarian immune microenvironment.

E₂ treatment inhibits immune cell proliferation and disrupts differentiation trajectories

To investigate the transcriptomic impact of E₂ on immune cell dynamics, DEGs were identified between the CTRL and E₂-treated groups within the immune cell subpopulation. In total, 1 750 DEGs were detected between groups (Figure 2A; Supplementary Table S4), including 1 006 down-regulated genes and 744 up-regulated genes (Figure 2A; Supplementary Figure S3A–D). Based on DEG analysis of immune cells (Supplementary Table S4), E₂ treatment up-regulated the expression of *Ccl2*, *Ccl4* and *Cd68*, and down-regulated the expression of *Il1b* and *Il10rb*, while *Il6* expression remained unchanged (Figure 2B, C). GO

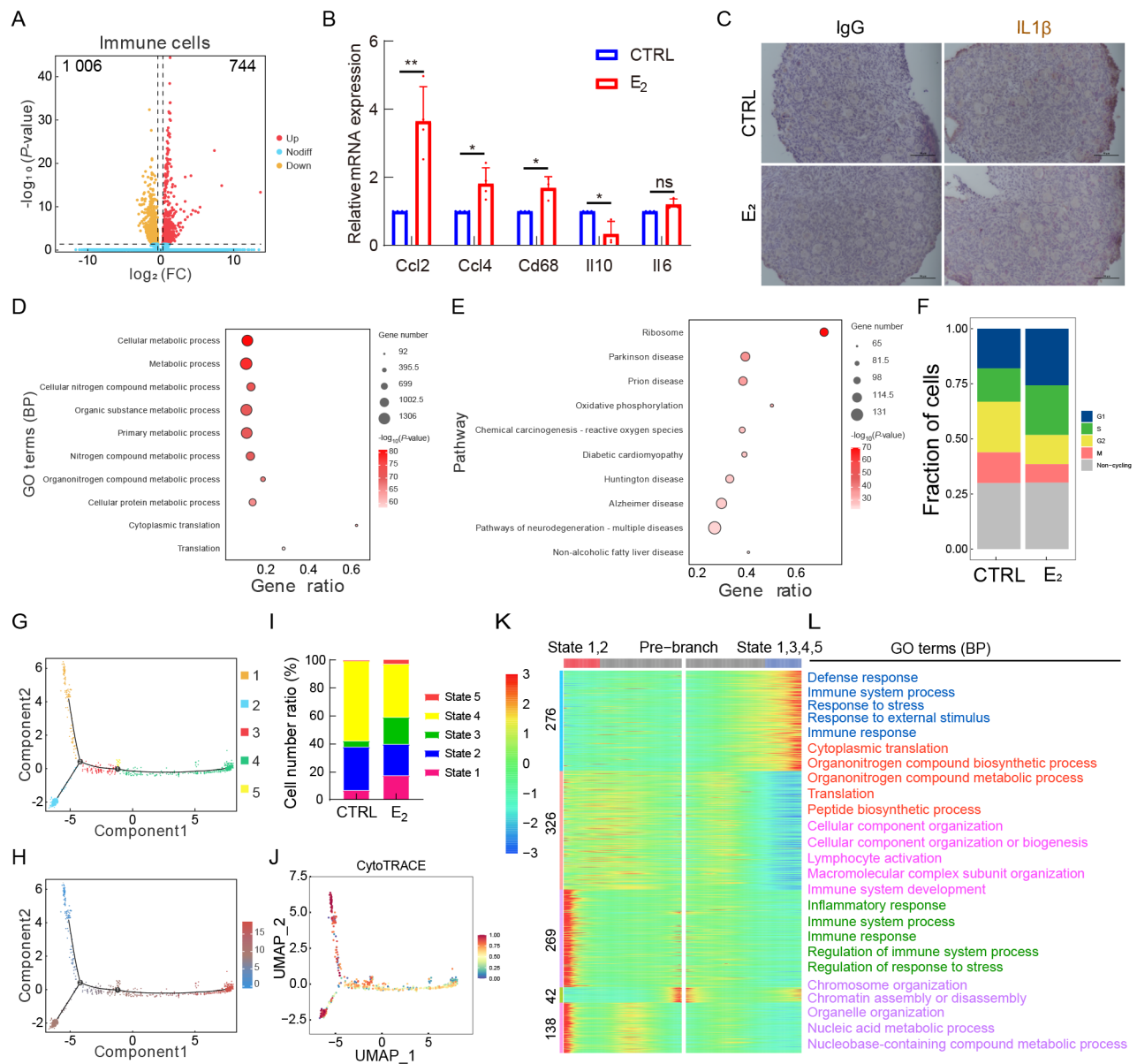


Figure 2 E₂ treatment suppresses immune cell proliferation and disrupts their cell fate dynamics

A: Volcano plot showing DEGs in immune cell subpopulations between CTRL and E₂-treated groups. B: mRNA expression levels of *Ccl2*, *Ccl4*, *Cd68*, *Il10*, and *Il6* in 4 dpp ovaries from CTRL and E₂-treated groups. C: Immunohistochemical staining of IgG and IL-1 β in ovarian sections from CTRL and E₂-treated groups. Scale bar: 50 μ m. D: Top 10 GO (BP) terms enriched in immune cell DEGs between CTRL and E₂-treated groups. E: Top 10 KEGG pathways enriched in immune cell DEGs. F: Cell cycle phase distribution in immune cells from CTRL and E₂-treated groups. G: Distribution of five cell states across pseudotime between CTRL and E₂-treated groups. H: Pseudotime trajectory of immune cells in CTRL and E₂-treated groups. I: Proportion of cells in each state between CTRL and E₂-treated groups. J: CytoTRACE analysis of differentiation potential in immune cells. K: Heatmap of DEGs distinguishing two cell fates at branch point 2. L: Top five GO terms (BP) in each gene set. CTRL: Control group. E₂: 17 β -estradiol group. Data are shown as means $\pm$ SEM. All experiments were repeated at least three times (ns: Not significant; *: $P < 0.05$; **: $P < 0.01$).

enrichment analysis revealed significant enrichment in biological processes (BP) related to “cellular metabolic process”, “metabolic process”, and “cellular nitrogen compound metabolic process” (Figure 2D). KEGG pathway analysis identified “ribosome” and “oxidative phosphorylation” as the top enriched pathways (Figure 2E).

A substantial down-regulation of genes associated with cell proliferation was observed after E₂ treatment (Supplementary Table S4), including *Mki67*, *Top2a*, *Cdkn1b*, and *Cdkn2d* (Supplementary Figure S3E–H), prompting further analysis of immune cell cycle dynamics (Figure 2F). Heatmap

visualization of different cell cycle-related genes is shown in Supplementary Figure S3I, with up-regulated genes presented with a bubble map (Supplementary Figure S3J). Results showed that E₂ treatment led to a marked decrease in the proportion of cells in the G2 (CTRL vs. E₂: 22.97% vs. 13.22%) and M phases (13.95% vs. 8.38%), accompanied by increased proportions in the non-cycling (29.94% vs. 30.17%), G1 (18.02% vs. 25.70%), and S phases (15.12% vs. 22.53%).

To evaluate how E₂ affects immune cell fate trajectories, pseudotime analysis was performed on the immune cell subpopulation (Supplementary Figure S3K), which resolved

into five cell states (Figure 2G). The distribution of pseudotime values is shown in Figure 2H. E₂ treatment altered the frequency of these states (Figure 2I): state 1 (CTRL vs. E₂: 6.69% vs. 17.32%), state 2 (31.10% vs. 22.35%), state 3 (4.36% vs. 19.37%), state 4 (57.27% vs. 38.18%), and state 5 (0.58% vs. 2.79%). CytoTRACE analysis indicated that state 1 exhibited the lowest degree of differentiation (Figure 2J), supporting the starting point of differentiation. Marker genes defining pseudotime transitions are shown in Supplementary Figure S3L–P. Gene expression analysis across pseudotime identified 1 051 DEGs in branch 2, which were categorized into five gene modules (Figure 2K; Supplementary Table S5). The top five GO biological processes associated with these genes are shown in Figure 2L. Collectively, these findings demonstrate that E₂ treatment suppresses immune cell

proliferation and disrupts differentiation trajectories in neonatal mouse ovaries.

E₂ treatment alters B cell transcription and disrupts their cell state dynamics

Following the global assessment of E₂-induced changes in the ovarian immune microenvironment, transcriptional and state-specific alterations were examined in the B cell subpopulation. Analysis identified 416 DEGs between the CTRL and E₂-treated groups (Figure 3A; Supplementary Table S6), including 158 down-regulated genes and 258 up-regulated genes (Figure 3A). GO enrichment revealed that the DEGs were predominantly associated with biological processes such as “cytoplasmic translation”, “peptide metabolic process”, and “translation” (Figure 3B). KEGG analysis further identified

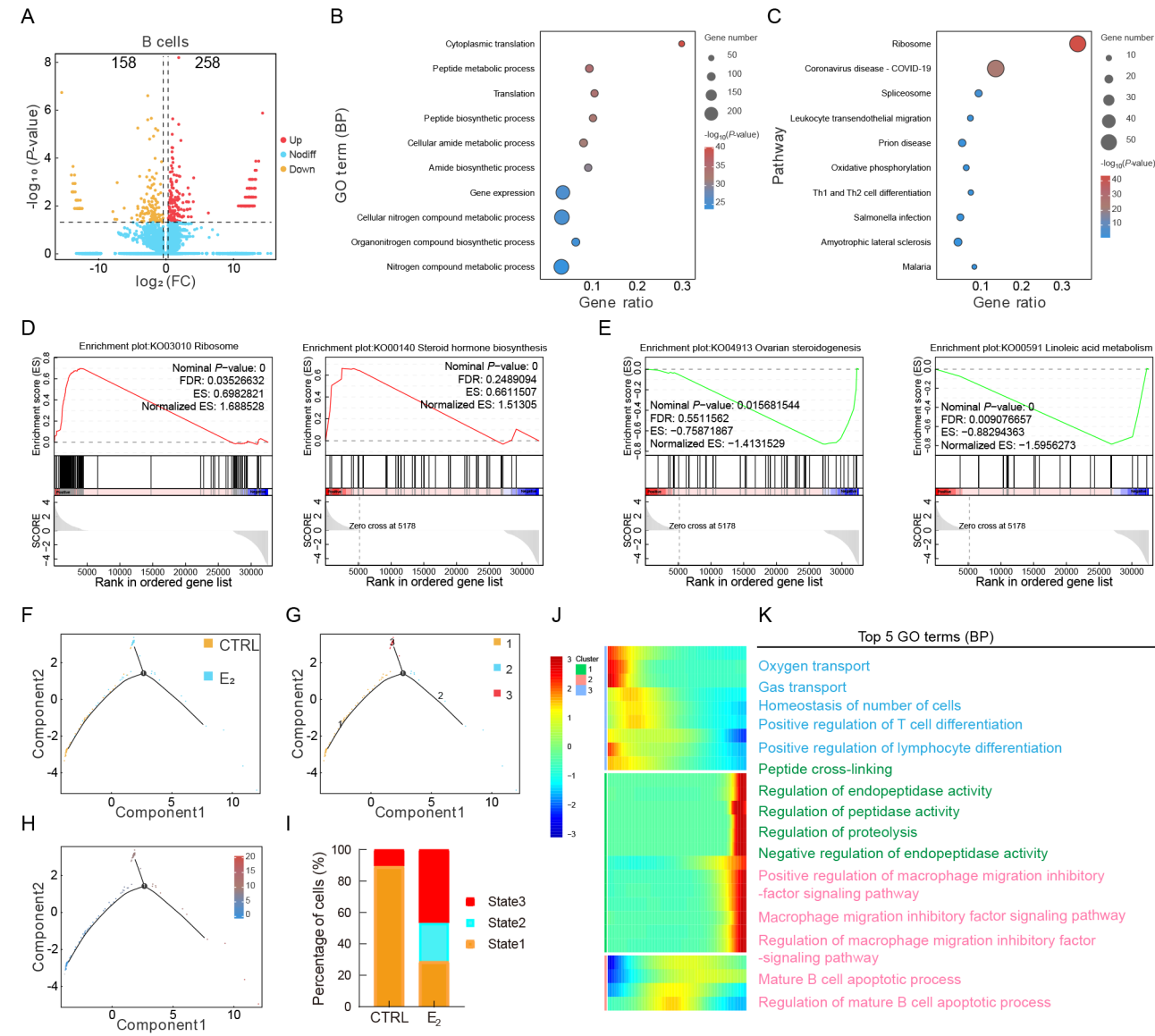


Figure 3 E₂ treatment alters B cell transcription and disrupts their cell state dynamics

A: Volcano plot showing DEGs in B cell subpopulations between CTRL and E₂-treated groups. B: Top 10 GO (BP) terms enriched in B cell DEGs between CTRL and E₂-treated group. C: Top 10 KEGG pathways enriched in B cell DEGs. D, E: GSEA of representative pathways (ribosome, steroid hormone biosynthesis, ovarian steroidogenesis, and linoleic acid metabolism) in B cells between CTRL and E₂-treated groups. F: Developmental trajectory of B cells between CTRL and E₂-treated groups. G: Distribution of three B cell states between CTRL and E₂-treated groups. H: Pseudotime trajectory of B cells between CTRL and E₂-treated groups. I: Proportion of B cells in each state. J: Heatmap of DEG expression in B cells across pseudotime. K: Top five GO terms (BP) enriched in DEGs of each cluster. CTRL: Control group. E₂: 17 β -estradiol group.

enrichment in “ribosome”, “oxidative phosphorylation”, and “Th1 and Th2 cell differentiation” pathways (Figure 3C). Gene set enrichment analysis (GSEA) supported these findings, indicating that E₂ treatment significantly enhanced ribosome (Normal $P=0$, enrichment score (ES)=0.6983) and steroid hormone biosynthesis (Normal $P=0$, ES=0.6612), but down-regulated ovarian steroidogenesis (Normal $P=0.01568$, ES=-0.7587) and linoleic acid metabolism (Normal $P=0$, ES=-0.8829) (Figure 3D, E).

To assess potential shifts in B cell differentiation trajectories, pseudotime analysis was performed using Monocle2 (Figure 3F). This analysis resolved the B cell subpopulation into three cell states (Figure 3G), with pseudotime values indicating that state 1 represented the earliest stage (Figure 3H). E₂ treatment dramatically altered the distribution of cell states. Notably, the proportion of cells in state 1 decreased after E₂ treatment (89.36% vs. 28.89%), while the proportion of cells in state 2 (0 vs. 24.44%) and state 3 (10.64% vs. 46.67%) increased (Figure 3I). Further analysis of pseudotime-dependent gene expression identified 26 DEGs along trajectories (Figure 3J; Supplementary Table S7), which were divided into three clusters based on expression dynamics. Functional annotation of cluster 1 (13 genes) revealed enrichment in “peptide cross-linking”, “regulation of endopeptidase activity”, “regulation of peptidase activity”, “regulation of proteolysis”, and “negative regulation of endopeptidase activity” (Figure 3K). Cluster 2 (four genes) was associated with “positive regulation of macrophage migration inhibitory factor signaling pathway”, “macrophage migration inhibitory factor signaling pathway”, “regulation of macrophage migration inhibitory factor signaling pathway”, “mature B cell apoptotic process”, and “regulation of mature B cell apoptotic process”. Cluster 3 (nine genes) was predominantly enriched in “oxygen transport”, “gas transport”, “homeostasis of number of cells”, “positive regulation of T cell differentiation”, and “positive regulation of lymphocyte differentiation”. Collectively, these results demonstrate that E₂ treatment significantly reprograms the transcriptional landscape of B cells and perturbs their differentiation trajectories.

E₂ treatment alters innate lymphoid cell transcription and disrupts their cell state dynamics

To evaluate the impact of E₂ on innate lymphoid cells, transcriptomic profiling was performed at single-cell resolution. Differential expression analysis revealed 3 340 DEGs between the CTRL and E₂-treated groups (Figure 4A; Supplementary Table S8), including 3 020 down-regulated genes and 320 up-regulated genes (Figure 4A). GO enrichment analysis of biological processes highlighted terms related to “cellular metabolic process”, “metabolic process”, and “cellular macromolecule metabolic process” (Figure 4B). KEGG pathway analysis identified significant associations with “cell cycle”, “pathways of neurodegeneration-multiple diseases”, and “ubiquitin mediated proteolysis” (Figure 4C). Furthermore, GSEA demonstrated that E₂ treatment was associated with increased enrichment of oxidative phosphorylation (Normal $P=0$, ES=0.5196) and nitrogen metabolism (Normal $P=0.2209$, ES=0.6895), while suppressing key pathways such as cell cycle (Normal $P=0.2962$, ES=-0.604) and linoleic acid metabolism (Normal $P=0.004158$, ES=-0.8081) (Figure 4D, E).

To explore how E₂ influences innate lymphoid cell

differentiation trajectories, pseudotime reconstruction was conducted using Monocle2 (Figure 4F), which divided the cell subpopulation into three cell states (Figure 4G). Pseudotime values revealed that state 1 represented the initiation phase of cell differentiation (Figure 4H), with E₂ treatment shown to significantly alter the distribution of the three states. Notably, cells in state 1 decreased (100% vs. 29.31%), while those in state 2 (0 vs. 53.45%) and state 3 (0 vs. 17.24%) increased (Figure 4I). A total of 22 pseudotime-associated DEGs were identified (Supplementary Table S9), which were grouped into three clusters. Key representative genes included *Hbb-bs*, *S100a8*, *S100a9*, and *Igkc* (Figure 4J–M), which associated with innate lymphoid cell development and differentiation. Collectively, these results demonstrate that E₂ treatment extensively reprograms the transcriptome of innate lymphoid cells and disrupts their cell states.

E₂ treatment alters mitotic immune cell transcription and disrupts their cell state dynamics

To evaluate the effects of E₂ on proliferating immune cells, transcriptomic and cell state analyses were conducted on the mitotic immune cell subpopulation. A total of 1 288 DEGs were identified between the CTRL and E₂-treated groups (Figure 5A; Supplementary Table S10), including 627 down-regulated genes and 661 up-regulated genes (Figure 5A). GO enrichment analysis of the DEGs revealed significant associations with “cytoplasmic translation”, “organonitrogen compound metabolic process”, and “peptide metabolic process” (Figure 5B), while KEGG pathway analysis highlighted enrichment in “ribosome” and “oxidative phosphorylation” pathways (Figure 5C). Cell cycle phase distribution analysis revealed that E₂ treatment suppressed the proliferative capacity of mitotic immune cells. Specifically, the proportion of cells in the G2 phase (CTRL vs. E₂: 35.09% vs. 14.50%) and M phase (12.28% vs. 5.34%) was reduced, while an increase was observed in the proportion of cells in the non-cycling (26.32% vs. 29.77%), G1 (12.28% vs. 27.48%), and S phases (14.04% vs. 22.90%) (Figure 5D). GSEA further confirmed that E₂ treatment enhanced the oxidative phosphorylation (Normal $P=0$, ES=0.6900) and ribosome pathways (Normal $P=0$, ES=0.82), while suppressing the primary immunodeficiency (Normal $P=0$, ES=-0.7604) and B cell receptor signaling pathways (Normal $P=0.007634$, ES=-0.6296) (Figure 5E, F).

Pseudotime trajectory reconstruction using Monocle2 further revealed E₂-driven alterations in the mitotic immune cell subpopulation (Figure 5G), with three cell states identified (Figure 5H). Pseudotime mapping indicated that state 1 represented the initiation point of differentiation (Figure 5I). Notably, E₂ treatment increased the proportion of cells in state 1 (3.51% vs. 78.63%), while reducing the proportion of cells in state 2 (47.37% vs. 6.87%) and state 3 (49.12% vs. 14.50%) (Figure 5J). A total of 233 pseudotime-associated DEGs were identified (Supplementary Table S11) and classified into three clusters (cluster 1: 105; cluster 2: 48; cluster 3: 80) (Figure 5K), the top 5 GO terms (BP) were shown in Figure 5L, which were associated with mitotic immune cell development and differentiation (Figure 5L). Overall, these findings indicate that E₂ treatment alters the transcriptomic landscape and impairs the developmental trajectory of mitotic immune cells.

E₂ treatment alters dendritic cell transcription and disrupts their cell state dynamics

To assess the impact of E₂ treatment on dendritic cell subpopulations within the neonatal mouse ovary, transcriptional profiling and pseudotime trajectory analyses were performed. A total of 309 DEGs were identified between the CTRL and E₂-treated groups (Figure 6A; Supplementary Table S12), including 132 down-regulated genes and 177 up-regulated genes (Figure 6A). GO enrichment analysis revealed that DEGs were predominantly involved in “cytoplasmic translation”, “translation”, “peptide metabolic process”, and “peptide biosynthetic process” (Figure 6B). KEGG pathway analysis highlighted enrichment in pathways such as “ribosome”, “oxidative phosphorylation”, “diabetic cardiomyopathy”, “amyotrophic lateral sclerosis”, “thermogenesis”, and “non-alcoholic fatty liver disease” (Figure 6C). GSEA indicated that E₂ treatment enhanced the ribosome (Normal $P=0.056$, ES=0.7250) and nitrogen

metabolism pathways (Normal $P=0.13788$, ES=0.87724), while inhibiting the intestinal immune network for IgA production (Normal $P=0.05797$, ES=-0.4867) and fat digestion and absorption pathways (Normal $P=0$, ES=-0.79095) (Figure 6D, E).

Pseudotime trajectory reconstruction using Monocle2 (Figure 6F) resolved the dendritic cell population into three cell states (Figure 6G). Pseudotime mapping indicated that state 1 represented the earliest point in the differentiation (Figure 6H). Notably, E₂ treatment markedly altered cell state distribution, increasing the proportion of cells in state 1 (0 vs. 54.29%) and state 2 (0 vs. 28.57%), while decreasing the proportion of cells in state 3 (100% vs. 17.14%) (Figure 6I). Pseudotime-associated gene expression analysis identified only three DEGs, including *Hbb-bs*, *H19*, and *Hsp90aa1*, as significantly altered along the differentiation trajectory, associated with

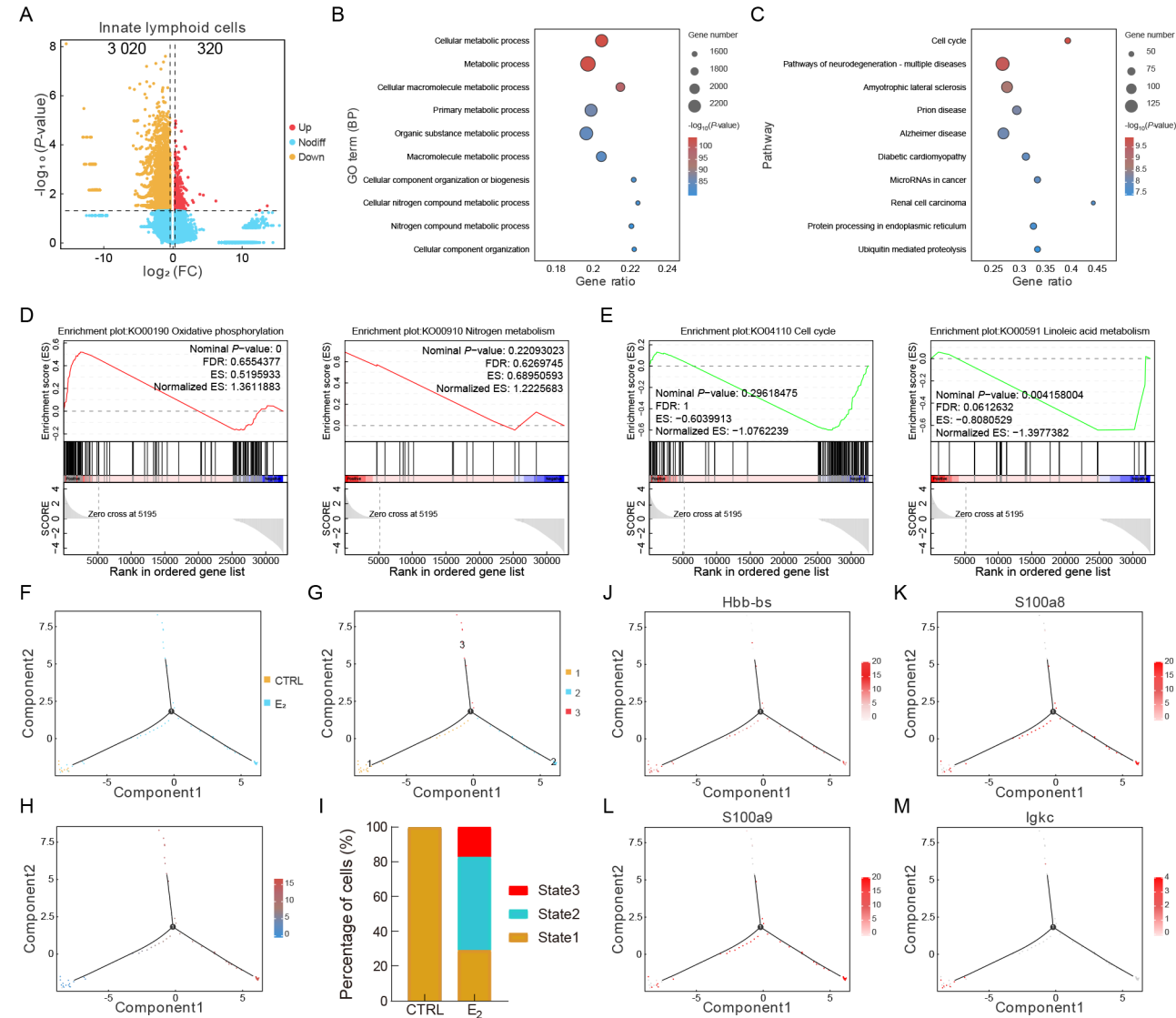


Figure 4 E₂ treatment alters innate lymphoid cell transcription and disrupts their cell fate dynamics

A: Volcano plot showing DEGs in innate lymphoid cell subpopulations between CTRL and E₂-treated groups. B: Top 10 GO (BP) terms enriched in innate lymphoid cell DEGs between CTRL and E₂-treated groups. C: Top 10 KEGG pathways enriched in innate lymphoid cell DEGs. D, E: GSEA of representative pathways (oxidative phosphorylation, nitrogen metabolism, cell cycle, and linoleic acid metabolism) in innate lymphoid cells between CTRL and E₂-treated groups. F: Developmental trajectory of innate lymphoid cells between CTRL and E₂-treated groups. G: Distribution of three innate lymphoid cell states between CTRL and E₂-treated groups. H: Pseudotime trajectory of innate lymphoid cells between CTRL and E₂-treated groups. I: Proportion of innate lymphoid cells in each state. J–M: Expression of representative DEGs (*Hbb-bs*, *S100a8*, *S100a9*, and *Igkc*) in innate lymphoid cells across pseudotime. CTRL: Control group. E₂: 17 β -estradiol group.

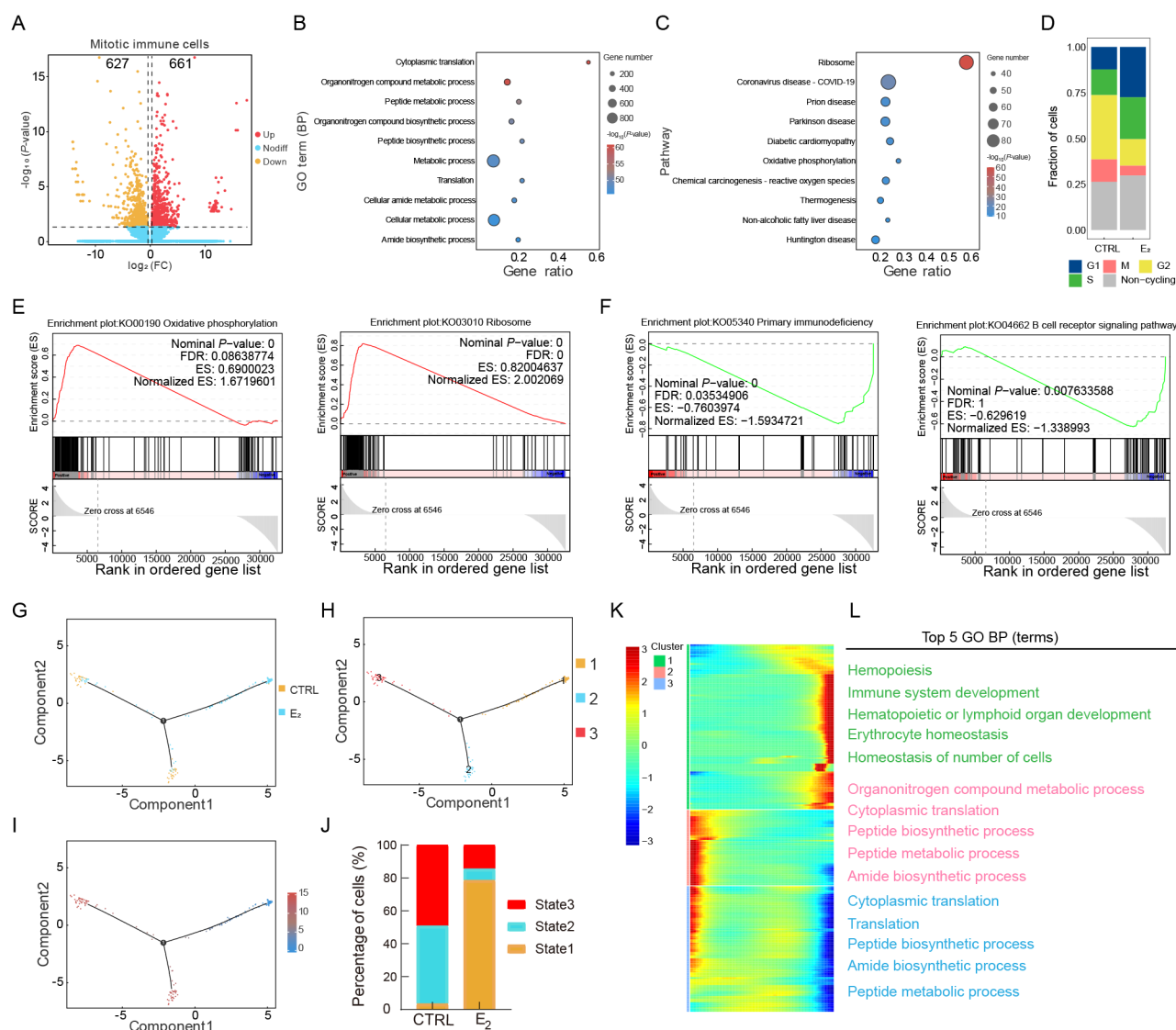


Figure 5 E_2 treatment alters mitotic immune cell transcription and disrupts their cell state dynamics

A: Volcano plot showing DEGs in mitotic immune cell subpopulations between CTRL and E_2 -treated groups. B: Top 10 GO (BP) terms enriched in mitotic immune cell DEGs between CTRL and E_2 -treated group. C: Top 10 KEGG pathways enriched in mitotic immune cell DEGs. D: Cell cycle analysis of mitotic immune cells between CTRL and E_2 -treated groups. E, F: GSEA of representative pathways (oxidative phosphorylation, ribosome, primary immunodeficiency, and B cell receptor signaling pathway) in mitotic immune cells between CTRL and E_2 -treated groups. G: Developmental trajectory of mitotic immune cells between CTRL and E_2 -treated groups. H: Distribution of three cell states between CTRL and E_2 -treated groups. I: Pseudotime trajectory of mitotic immune cells between CTRL and E_2 -treated groups. J: Proportion of mitotic immune cells in each state. K: Heatmap of DEG expression in mitotic immune cells across pseudotime. L: Top five GO terms (BP) enriched in DEGs in each cluster. CTRL: Control group. E_2 : 17 β -estradiol group.

dendritic cell development and differentiation. Collectively, these findings indicate that E_2 treatment alters the transcriptome and disrupts the progression of cell states in neonatal mouse ovarian dendritic cells.

E_2 treatment alters macrophage transcription and inhibits M1 to M2 macrophage transformation

To investigate the impact of E_2 on ovarian macrophage subpopulations, differential gene expression analysis was performed between CTRL and E_2 -treated groups. A total of 1 261 DEGs were identified, including 764 down-regulated genes and 497 up-regulated genes (Figure 7A; Supplementary Table S13). GO enrichment analysis indicated that these DEGs were mainly involved in “cellular metabolic process”, “metabolic process”, and “cytoplasmic translation”

(Figure 7B). KEGG pathway analysis further revealed significant enrichment in pathways related to “ribosome” and “oxidative phosphorylation” (Figure 7C). GSEA corroborated these findings, showing increased enrichment of ribosome (Normal $P=0$, ES=0.8265), oxidative phosphorylation (Normal $P=0$, ES=0.7729), and DNA replication pathways (Normal $P=0.01252$, ES=0.7945), while suppressing B cell receptor signaling (Normal $P=0.01319$, ES=-0.5146), FoxO signaling (Normal $P=0.0369$, ES=-0.5289), and starch and sucrose metabolism pathways following E_2 treatment (Normal $P=0.01754$, ES=-0.7733) (Supplementary Figure S4A–F).

Clustering analysis of the macrophage subpopulation revealed four transcriptionally distinct clusters (Figure 7D, E), with their respective proportions shown in Figure 7F. Notably, E_2 treatment led to a marked expansion of cluster 0 (CTRL vs.

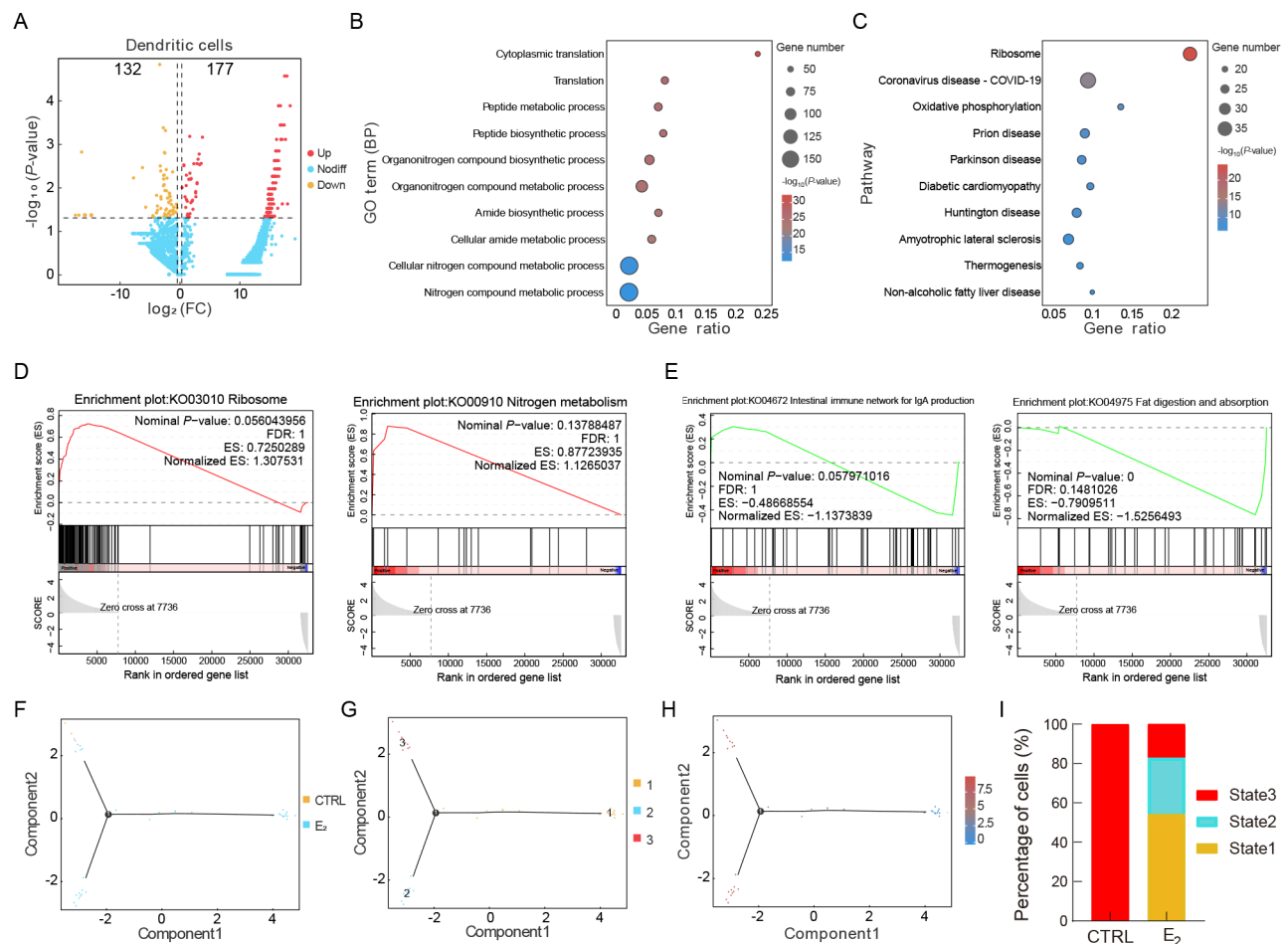


Figure 6 E_2 treatment alters dendritic cell transcription and disrupts their cell state dynamics

A: Volcano plot showing DEGs in dendritic cell subpopulations between CTRL and E_2 -treated groups. B: Top 10 GO (BP) terms enriched in dendritic cell DEGs between CTRL and E_2 -treated groups. C: Top 10 KEGG pathways enriched in dendritic cell DEGs. D, E: GSEA of representative pathways (ribosome, nitrogen metabolism, intestinal immune network for IgA production, and fat digestion and absorption) in dendritic cells between CTRL and E_2 -treated groups. F: Developmental trajectory of dendritic cells between CTRL and E_2 -treated groups. G: Distribution of three states in dendritic cells between CTRL and E_2 -treated groups. H: Pseudotime trajectory of dendritic cells. I: Proportion of dendritic cells in each state. CTRL: Control group. E_2 : 17 β -estradiol group.

E_2 : 23.25% vs. 47.39%) and a corresponding reduction in cluster 1 (39.47% vs. 30.97%), cluster 2 (26.32% vs. 20.52%) and cluster 3 (10.96% vs. 1.22%). Based on established markers described in previous research (Dai et al., 2023; Winkler et al., 2024), *S100a9* and *Acod1* were used to identify M1 macrophages, while *Tgfb1*, *Arg1*, *Mrc1*, and *Ccr2* were used to identify M2 macrophages. Analysis of gene expression profiles and cluster-specific distributions (Supplementary Figure S4G–K) indicated that cells in cluster 0 exhibited high expression of M2 markers, while clusters 1–3 exhibited high expression of M1 markers (Figure 7G; Supplementary Figure S4L, M). DEG analysis identified 489 up-regulated genes in M1 macrophages and 315 up-regulated genes in M2 macrophages following E_2 treatment (Supplementary Figure S4N and Table S14). The top 10 up-regulated genes in each subtype are shown in Supplementary Figure S4O. Quantitative assessment of cell subtype composition demonstrated a marked shift in macrophage polarization, with the proportion of M1 macrophages declining (76.75% vs. 52.61%) and the proportion of M2 macrophages increasing (23.25% vs. 47.39%) in response to E_2 (Figure 7H). To validate these findings at the protein level, immunostaining of ovarian sections was performed using iNOS and CD163 as

markers for M1 and M2 macrophages, respectively (Sambamurthy et al., 2018; Yan et al., 2021) (Figure 7I, K). Results showed that E_2 treatment significantly decreased iNOS-positive cells (Figure 7J) and increased CD163-positive cells (Figure 7L). In summary, these findings suggest that E_2 treatment alters the transcription and inhibits the transformation of M1 macrophages into M2 macrophages.

E_2 treatment alters macrophage differentiation trajectories and alters cell fate in M1 and M2 subtypes

To investigate the changes in macrophage cell fate after E_2 treatment, pseudotime trajectory analysis was conducted using Monocle2, with macrophages resolved into three cell states (Figure 8A–C). Notably, E_2 treatment significantly altered their distribution: the proportion of cells in state 1 decreased (91.23% vs. 68.66%), while state 2 (6.14% vs. 18.28%) and state 3 (2.63% vs. 13.06%) increased following E_2 exposure (Figure 8D). Further analysis revealed two major cell fate branches and 39 pseudotime-associated DEGs, organized into five clusters (cluster 1: 22; cluster 2: three; cluster 3: five; cluster 4: four; cluster 5: five) (Figure 8E; Supplementary Table S15). Functional enrichment of these gene clusters indicated involvement in pathways related to

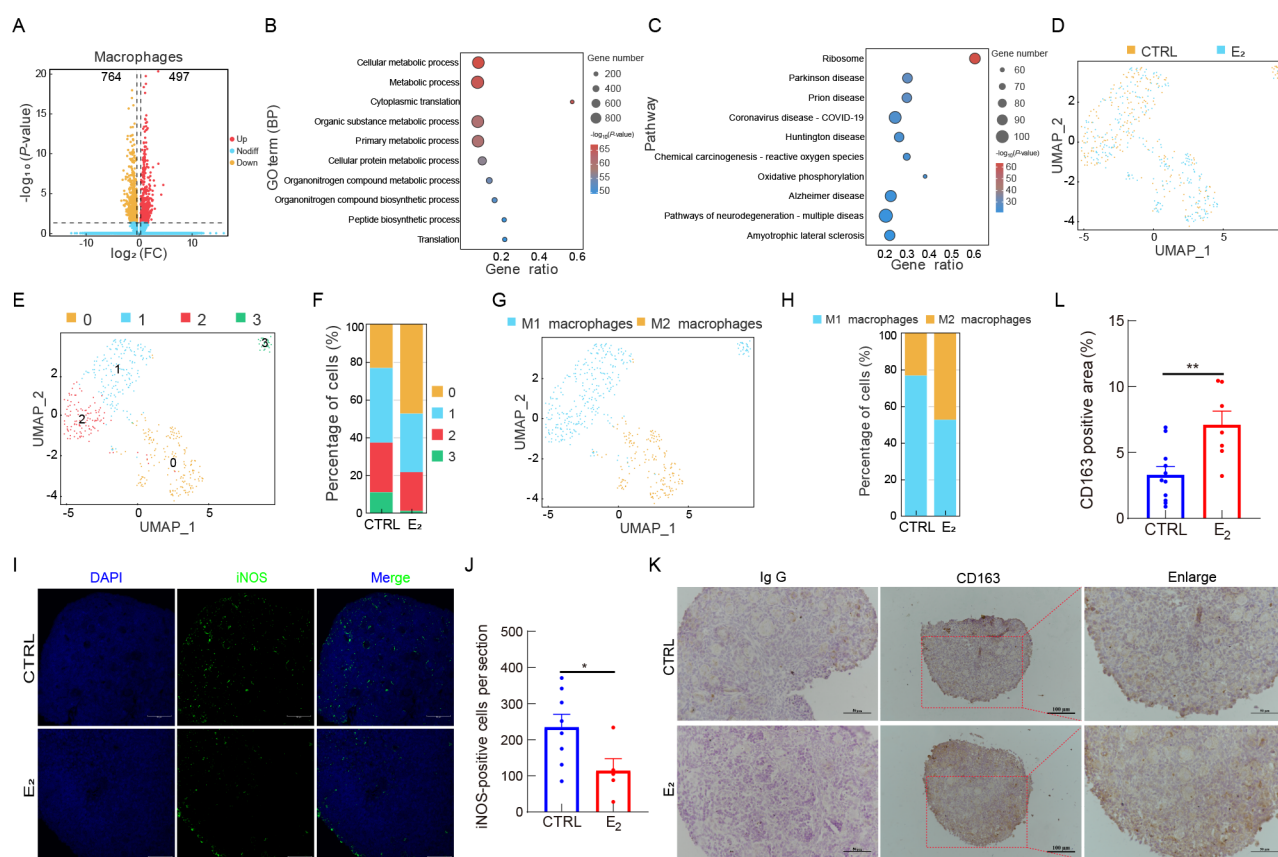


Figure 7 E₂ treatment alters macrophage transcription and inhibits M1-to-M2 transition

A: Volcano plot showing DEGs in macrophage subpopulations between CTRL and E₂-treated groups. B: Top 10 GO (BP) terms enriched in macrophage DEGs between CTRL and E₂-treated groups. C: Top 10 KEGG pathways enriched in macrophage DEGs. D: UMAP plot of macrophages between CTRL and E₂-treated groups. E: UMAP plot of four clusters in macrophages between CTRL and E₂-treated groups. F: Proportion of each macrophage cluster between CTRL and E₂-treated groups. G: UMAP plot of M1 and M2 macrophages between CTRL and E₂-treated groups. H: Proportion of M1 and M2 macrophages. I: Representative immunofluorescence of M1 macrophage staining (iNOS) in ovarian sections between CTRL and E₂-treated groups. Scale bar: 50 μ m. J: Number of iNOS-positive cells per section between CTRL and E₂-treated groups. K: IgG and CD163 staining of ovarian sections between CTRL and E₂-treated groups. Scale bar: 100 μ m (Middle); 50 μ m (others). L: Analysis of CD163-positive area in each section between CTRL and E₂-treated groups. CTRL: Control group. E₂: 17 β -estradiol group. Data are shown as mean $\pm$ SEM. All experiments were repeated at least three times (*: $P < 0.05$; **: $P < 0.01$).

macrophage development (Figure 8F).

Further subpopulation analysis identified 547 DEGs in M1 macrophages between the CTRL and E₂-treated groups (Supplementary Figure S5A; Supplementary Table S16), including 449 down-regulated genes and 98 up-regulated genes. GO term analysis showed enrichment in "cellular metabolic process", "metabolic process", and "cytoplasmic translation" (Supplementary Figure S5B), while KEGG pathway analysis revealed enrichment in "ribosome", "ferroptosis", "oxidative phosphorylation", "autophagy-animal", and "Foxo signaling pathway", (Supplementary Figure S5C).

In contrast, M2 macrophages exhibited 223 DEGs between the CTRL and E₂-treated groups (Supplementary Figure S5D and Table S17), including 19 down-regulated genes and 204 up-regulated genes. These genes were enriched in GO terms related to "cytoplasmic translation", "translation", "peptide metabolic process", and "peptide biosynthetic process" (Supplementary Figure S5E), with KEGG analysis indicating enrichment in "ribosome", "proteasome", and "thermogenesis" pathways (Supplementary Figure S5F). Comparative analysis revealed 40 DEGs shared between M1 and M2 macrophages (Supplementary Figure S5G), with enrichment in GO terms related to "cytoplasmic translation", "translation", and "peptide

biosynthetic process" (Supplementary Figure S5H) and enrichment in KEGG pathways related to "ribosome", "leukocyte transendothelial migration", "necroptosis", "ferroptosis", and "cholesterol metabolism" (Supplementary Figure S5I). GSEA further revealed that the PI3K-Akt signaling pathway was suppressed in M1 macrophages but enhanced in M2 macrophages (Supplementary Figure S6A), while the MAPK signaling pathway was suppressed in both subtypes (Supplementary Figure S6B).

Monocle2 analysis of M1 macrophages revealed five differentiation states (Figure 8G, H), with pseudotime mapping showing that state 1 was the initiation point of differentiation (Figure 8I). E₂ treatment increased the proportion of cells in state 2 (2.29% vs. 18.44%) and state 3 (20.57% vs. 24.11%), while state 1 (34.86% vs. 34.75%), state 4 (10.86% vs. 4.26%), and state 5 (31.43% vs. 18.44%) showed a decrease (Figure 8J). Pseudotime-associated analysis identified 164 DEGs, and clustered into five groups (cluster 1: 81; cluster 2: 41; cluster 3: 28; cluster 4: eight; cluster 5: six) (Supplementary Figure S6C and Table S18). Functional enrichment analysis revealed key terms associated with the development and differentiation of M1 macrophages (Supplementary Figure S6D).

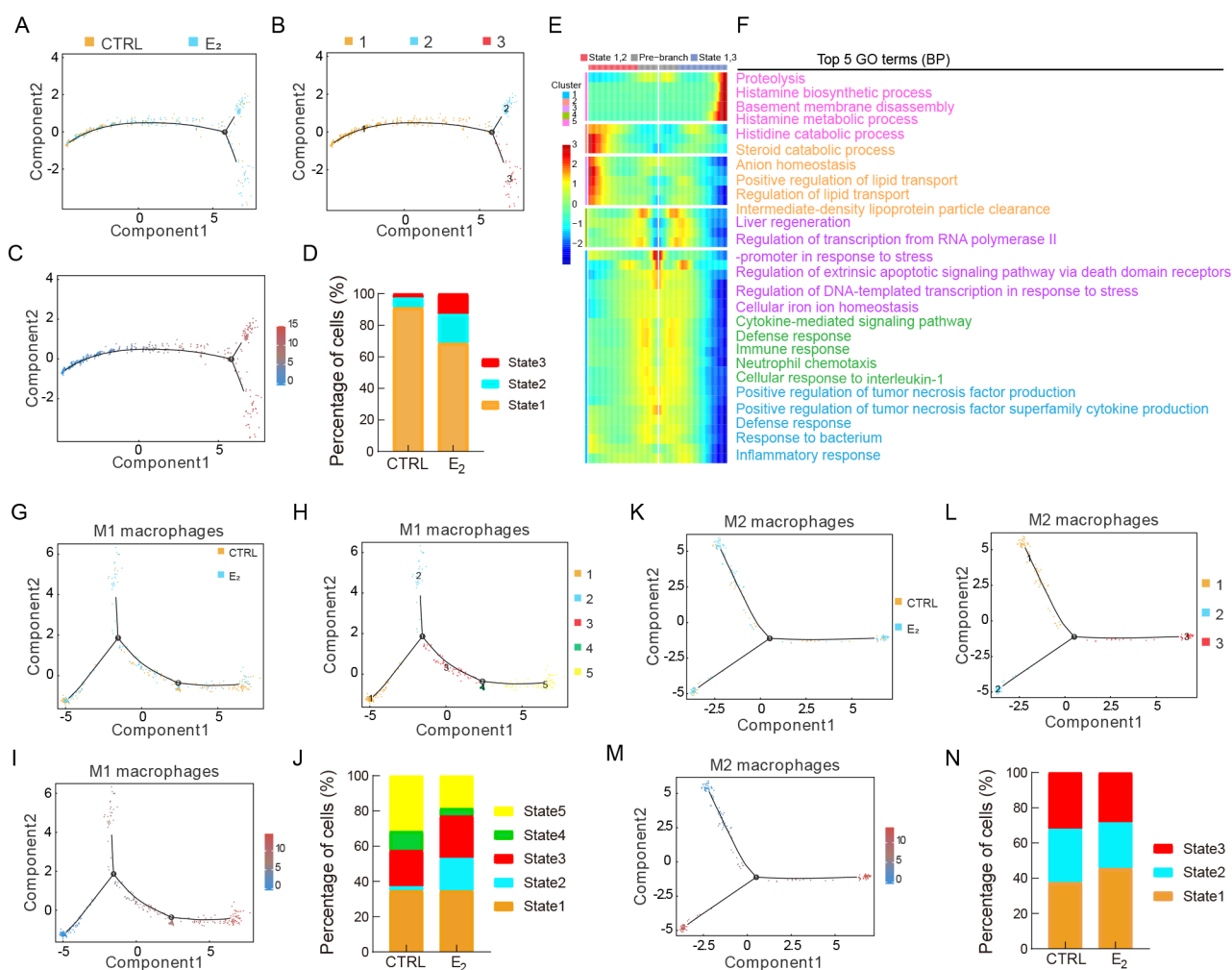


Figure 8 E₂ treatment alters macrophage differentiation trajectories and alters cell fate in M1 and M2 subtypes

A: Developmental trajectory of macrophage subpopulations between CTRL and E₂-treated groups across pseudotime. B: Three cell states of macrophages across pseudotime. C: Pseudotime trajectory of macrophage subpopulations. D: Proportion of macrophages in each state between CTRL and E₂-treated groups. E: Heatmap of DEG expression in macrophages from two cell fates. F: Top five GO terms (BP) enriched in DEGs in each gene set. G: Developmental trajectory of M1 macrophages between CTRL and E₂-treated groups. H: Distribution of five states in M1 macrophage subpopulation. I: Pseudotime trajectory of M1 macrophages. J: Proportion of M1 macrophages in each state. K: Developmental trajectory of M2 macrophages between CTRL and E₂-treated groups. L: Three cell states of M2 macrophages between CTRL and E₂-treated groups. M: Pseudotime trajectory of M2 macrophages. N: Proportion of M2 macrophages in each state. CTRL: Control group. E₂: 17β-estradiol group.

Monocle2 analysis of M2 macrophages revealed three differentiation states (Figure 8K, L), with the pseudotime mapping showing that state 1 was the initiation point of differentiation (Figure 8M). E₂ treatment increased the proportion of cells in state 1 (37.74% vs. 45.67%), while state 2 (30.19% vs. 25.98%) and state 3 (32.08% vs. 28.35%) showed a decrease (Figure 8N). Pseudotime-associated analysis identified 99 DEGs, and clustered into three groups (cluster 1: 26; cluster 2: 15; cluster 3: 58) (Supplementary Figure S6E and Table S19). Functional enrichment analysis revealed key terms associated with the development and differentiation of M2 macrophages (Supplementary Figure S6F). Collectively, these results indicate that E₂ treatment alters the differentiation trajectories of both M1 and M2 macrophages.

DISCUSSION

This study conducted the first comprehensive characterization

of the neonatal mouse ovarian immune microenvironment during the primordial follicle formation stage and analyzed its transcriptional and compositional alterations in response to exogenous E₂ treatment. Single-cell transcriptomic profiling revealed that the neonatal mouse ovarian immune cell compartment consisted of macrophages, B cells, dendritic cells, mitotic immune cells, and innate lymphoid cells, among which macrophages represented the most abundant subtype (Figure 9). Following E₂ treatment, marked shifts in immune cell composition were observed, including increased proportion of mitotic immune cells, innate lymphoid cells, and dendritic cells, accompanied by a concomitant reduction in the proportion of macrophages and B cells (Figure 9), indicating that E₂ exposure significantly reshapes the cellular landscape of the neonatal mouse ovarian immune milieu. While the current analysis was based on scRNA-seq of dissociated cells, additional studies are needed to validate these findings.

The proportion and composition of ovarian immune cells are known to vary with age. In comparison to adult (3 months) and

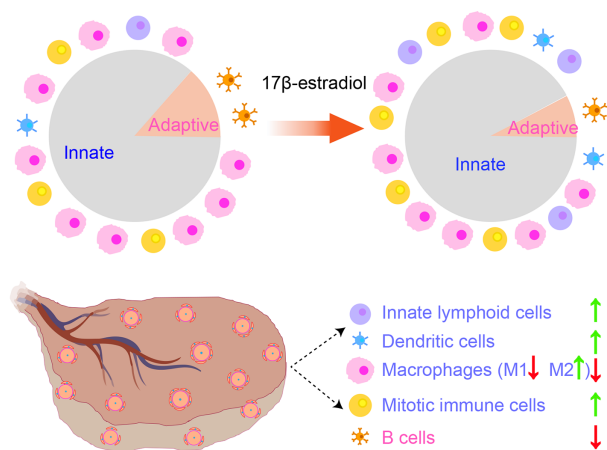


Figure 9 Effects of 17 β -estradiol on ovarian immune system in neonatal mouse ovaries

E_2 treatment led to increase proportions of several innate immune cells, including innate lymphoid cells, dendritic cells, and mitotic immune cells, within the total immune cell population. Conversely, proportions of B cells and macrophages decreased, with a reduction in M1 macrophages and an increase in M2 macrophages. Additionally, E_2 exposure induced marked cellular heterogeneity and altered the expression profiles of multiple cytokines and chemokines, reflecting broad immunomodulatory effects on the neonatal mouse ovarian immune microenvironment.

aged (9 months) mice (Isola et al., 2024), neonatal mouse ovaries harbored the lowest proportion of immune cells, suggesting a progressive immune cell expansion during ovarian aging. Furthermore, the relative composition of immune cells differed substantially among neonatal, adult, and aged ovaries, underscoring dynamic immunological remodeling across the reproductive lifespan. Prior work has shown that ovarian aging in fertile females is associated with a transition from innate immune dominance (macrophages, neutrophils, dendritic cells, natural killer (NK) cells, innate lymphoid cells, and NKT cells) to adaptive immune dominance (T and B cells) (Ben Yaakov et al., 2023). In this context, our findings indicated that E_2 inhibited this immunological trajectory by suppressing the innate-to-adaptive transition in the neonatal mouse ovary, as evidenced by reduced proportions of macrophages, dendritic cells, and innate lymphoid cells and increased proportions of B cells. Collectively, these results suggest that E_2 acts as a regulator of the ovarian immune microenvironment during development.

Estrogen primarily functions through its interactions with estrogen receptors (ERs), including nuclear receptors *Esr1* and *Esr2* (Heldring et al., 2007) and membrane-bound G protein-coupled estrogen receptor 1 (*Gper1*). Expression of these ERs varies across immune cell types. In humans, *Esr1* and *Esr2* are expressed in peripheral blood mononuclear cell (PBMC) subpopulations (Laffont et al., 2014; Phiel et al., 2005), and monocytes exhibit functional responses to estrogen stimulation (Escribese et al., 2008; Mor et al., 2003; Seillet et al., 2012). Consistent with these findings, our results showed that *Esr2* was expressed in cluster 1 (macrophages) and *Esr1* was expressed in clusters 3, 5, and 7 (macrophages, innate lymphoid cells, and dendritic cells), while *Gper1* showed negligible expression across all immune clusters.

The expression patterns and distribution of ERs likely contribute to their divergent functional roles across immune

cell types. Nuclear ERs can bind directly to estrogen response elements or indirectly associate with DNA through transcription factor complexes involving NF- κ B, Sp1, AP1, and C/EBP β (O'Lone et al., 2004), which are essential for immune cell function (Leitman et al., 2010). In this study, Sp1 was significantly down-regulated, and the subunit composition of AP-1 was altered in immune cells following E_2 treatment, suggesting regulatory involvement in transcription responses. Estrogen also initiates rapid non-nuclear signaling transduction (seconds to minutes), which affects various physiological processes, including intercellular calcium mobilization, cAMP generation, potassium current modulation, phospholipase C activation, nitric oxide production, and kinase pathway activation, such as PI3K/AKT and ERK signaling (Ueda & Karas, 2013). However, the mechanisms underlying nuclear and membrane ER signaling remain incompletely defined, particularly with respect to subtype-specific immune cell responses.

Given the differential responses of immune cell types to E_2 treatment, further analysis was performed on individual subpopulations. B cells, components of the adaptive immune system, contribute to antigen presentation, antibody production, and cytokine-mediated immunomodulation. In murine ovaries, the proportion of B cells increases with age (Ben Yaakov et al., 2023; Isola et al., 2024). Compared to young and adult ovaries (Isola et al., 2024), our study showed a higher proportion of B cells in neonatal samples, indicating variation in immune composition across reproductive stages. Moreover, E_2 treatment led to a reduction in the proportion of B cells, suggesting that E_2 regulates B cell development and differentiation. ER signaling has been implicated in the regulation of B cell activation, with relevance to both immune defense and pathophysiological processes (Asaba et al., 2015).

DEGs identified in B cells were enriched in pathways related to ribosome, oxidative phosphorylation, Th1 and Th2 cell differentiation, RNA degradation, Th17 cell differentiation, regulation of active cytoskeleton, and ribosome biogenesis in eukaryotes. GSEA showed that E_2 treatment enhanced ribosome and oxidative phosphorylation pathways, while suppressing ovarian steroidogenesis and linoleic acid metabolism. Th1 and Th2 cells are CD4⁺ T helper cells that enhance the activity of other leukocytes (Hao & Whitelaw, 2013). Herein, the differentiation of Th1 and Th2 cells was suppressed following E_2 treatment, indicating that estrogen can directly or indirectly affect cell functions. IL-10-producing B cells can inhibit Th1 differentiation (Mauri et al., 2003), further implicating B cells in the regulation of T cell function. Monocle 2 analysis demonstrated that E_2 altered both the cell states and differentiation trajectories of B cells. These findings indicate that E_2 modulates B cell transcriptional programs and developmental dynamics.

Dendritic cells, key components of the innate immune system (Ben Yaakov et al., 2023), initiate and coordinate both innate and adaptive immune responses (Banchereau & Steinman, 1998). In murine ovaries, the abundance of dendritic cells increases with reproductive aging (Ben Yaakov et al., 2023; Isola et al., 2024). In this study, E_2 exposure markedly elevated the proportion of dendritic cells from 0.87% in the CTRL group to 6.53% in the E_2 -treated group, far exceeding the less than 2% typically reported in adult and aged ovaries (Isola et al., 2024). These findings suggest a prominent role for estrogen in promoting dendritic cell

differentiation during early ovarian development. Under physiological conditions, dendritic cells generally exist in an immature state (Banchereau & Steinman, 1998) and serve as efficient stimulators of B and T lymphocytes. Estrogen signaling has been shown to regulate the development and function of both conventional and plasmacytoid dendritic cells in mice and humans (Laffont et al., 2017; Zhang et al., 2018). Notably, E₂ treatment enhanced oxidative phosphorylation in dendritic cells, implicating estrogen in the metabolic programming underlying dendritic cell maturation.

ER-induced epigenetic modifications in dendritic cell progenitors may influence the gene expression programs that govern the functional responses of mature dendritic cells (Kovats, 2015). Compared to male mice, elevated systemic estradiol in female mice is associated with increased chromatin accessibility at genes involved in type 1 interferon signaling (Kovats, 2015), potentially elucidating the enhanced production of type 1 interferon in females. In mature dendritic cells, ERs may exert acute regulatory effects by modulating gene expression upon activation via pattern recognition receptors or cytokines. For instance, ER recruitment to immune regulatory elements may promote or inhibit the expression of genes involved in innate immunity, including members of the NF- κ B pathway that regulate cytokine responses. Consistently, this study observed increased activity of the NF- κ B signaling pathway following E₂ treatment in dendritic cells, supporting a functional role for estrogen in modulating innate immune signaling and cell fate.

Although E₂ treatment increased the proportion of mitotic immune cells, expression of key proliferation-related genes (*Mki67* and *Top2a*) was significantly decreased, consistent with earlier observations in whole ovarian tissue (Yan et al., 2024). The higher baseline abundance of mitotic cells in neonatal mouse ovaries compared with adult and aged mice (Isola et al., 2024) further highlights developmental stage-dependent complexity of the ovarian immune microenvironment.

Innate lymphoid cells are critical components of the innate immune system (Ben Yaakov et al., 2023). In this study, their proportion in mouse ovaries declined with age. However, previous work has reported no significant difference in the abundance of such cells between adult and aged ovaries, with levels consistently below 2% (Isola et al., 2024). These discrepancies likely reflect methodological differences in cell isolation strategies. In the present study, E₂ treatment markedly increased the proportion of innate lymphoid cells in neonatal ovaries from 2.62% to 10.8%. Compared to adult and aged ovaries, neonatal ovaries exhibited the lowest baseline innate lymphoid cell abundance, suggesting that cell expansion is developmentally regulated. Given the high plasticity of innate lymphoid cells and their potential to differentiate into various effector cell types, the observed increase in their proportion after E₂ exposure suggests that estrogen may constrain differentiation, thereby altering immune cell fate trajectories.

Macrophages represent the predominant immune population in ovaries and play indispensable roles in folliculogenesis, ovulation, luteal formation and regression, and follicle atresia (Tang et al., 2023). In other contexts, such as bone marrow of ovariectomized osteoporotic mice, an elevated M1/M2 macrophage ratio has been reported, which can be reversed by estrogen treatment (Dou et al., 2018). In this study, the abundance of ovarian macrophages decreased

following E₂ treatment. While this reduction may suggest E₂-mediated modulation of macrophage differentiation or survival, it remains unclear whether this effect is directly linked to the inhibitory role of E₂ in primordial follicle formation, as previously reported (Yan et al. 2024). Further investigation is required to delineate the mechanistic relationship between estrogen exposure, macrophage dynamics, and follicular development in the neonatal ovary (Yan et al., 2024).

In adult mouse ovaries, macrophages can be categorized into five distinct subtypes (Jokela et al., 2020). Within ovarian immunology, particular emphasis has been placed on characterizing M1 and M2 macrophages due to their opposing roles in regulating primordial follicle activation in newborn mice (Xiao et al., 2022). In the present study, macrophages were subdivided into M1 and M2 subtypes. E₂ treatment led to a reduction in M1 macrophages and a concurrent increase in M2 macrophages. Given the contrasting immunological roles of these subtypes, these results indicate that estrogen exerts anti-inflammatory effects in the neonatal mouse ovary. This anti-inflammatory shift may partially explain the increase in ovarian inflammation with age as estrogen levels decline. Previous studies have shown that ER α signaling suppresses pro-inflammatory cytokine production following Toll-like receptor (TLR) stimulation in both dendritic cells and macrophages (Kovats, 2015). Herein, E₂ treatment modulated the expression of multiple cytokines and chemokines, including *Il1r2*, *Tnfrsf1a*, *Tnfaip2*, *Il1b*, *Il7r*, *Il10rb*, *Il36g*, *Ccl2*, *Ccl4*, *Cxcr1*, and *Cxcr2*.

M1 and M2 macrophages differentially regulate primordial follicle activation through the PI3K/AKT/mTOR signaling pathway (Xiao et al., 2022). Our GSEA results showed that E₂ treatment suppressed PI3K/AKT/mTOR signaling in M1 macrophages but enhanced it in M2 macrophages, suggesting a shift in signaling dynamics that may influence follicle formation and development. Experimental evidence further supports the functional necessity of M1 macrophages for folliculogenesis, whereas M2 depletion does not impair this process (Ono et al., 2018). Thus, the decrease in M1 macrophage abundance may contribute to the negative effect of estrogen on primordial follicle formation in neonatal mice. In pathological ovarian conditions, such as polycystic ovary syndrome, an increased M1/M2 ratio has been observed within antral and preovulatory follicles (Lima et al., 2018), highlighting the importance of the immune microenvironment in the reproductive system. Collectively, these findings underscore the complex regulatory roles of macrophage subtypes in female reproductive health and suggest potential immunomodulatory targets for the treatment of ovarian disorders.

Energy metabolism is a critical aspect of immune cell development, activation, and differentiation. As early as the 1950s, neutrophils were found to rely on aerobic glycolysis—a phenomenon known as the “Warburg effect”. Previous research has shown that the metabolic preferences for glycolysis and oxidative phosphorylation differ between M1 and M2 macrophages. M1 macrophages, which mediate pro-inflammatory responses, rely predominantly on glycolysis and the pentose phosphate pathway for ATP production, characterized by reduced oxygen consumption and disrupted tricarboxylic acid (TCA) cycle function at two specific points. This metabolic configuration is also accompanied by suppression of oxidative phosphorylation and fatty acid oxidation (Feingold et al., 2012; Freemerman et al., 2014;

Fukuzumi et al., 1996; Funk et al., 1993; Jha et al., 2015; Meiser et al., 2016; Tannahill et al., 2013). In contrast, M2 macrophages —associated with anti-inflammatory functions —maintain an intact TCA cycle and demonstrate enhanced oxidative phosphorylation and fatty acid oxidation (Jha et al., 2015). Importantly, estrogen is known to influence cellular energy metabolism. In the present study, oxidative phosphorylation pathways were significantly up-regulated across immune cell populations following E₂ treatment, indicating that estrogen plays complex roles in immune cell differentiation and function.

Although the mouse model remains a cornerstone of reproductive immunology research, various aspects of their ovarian biology differ significantly from those of nonhuman primates and humans, such as poly-ovulation versus mono-ovulation as well as variation in follicular formation, recruitment, and selection. Thus, the extent to which estrogen-mediated remodeling of the mouse ovarian immune microenvironment translates to primate models remains to be determined. Furthermore, the bidirectional interactions between follicles and immune cells remain incompletely understood, particularly during primordial follicle formation—a process that establishes the foundational ovarian reserve and thereby determines reproductive lifespan. Whether estrogen-induced immune changes are beneficial or harmful to the body remain unclear. Given that estrogen can induce changes in the immune microenvironment, future studies are needed to determine whether estrogen or its analogs could be therapeutically applied to modulate ovarian function.

CONCLUSIONS

This study presents a comprehensive single-cell analysis of E₂-induced alterations in the ovarian immune microenvironment of neonatal mice. Exposure to E₂ elicited pronounced changes in immune cell composition and the immune microenvironment, characterized by increased proportions of innate lymphoid cells, dendritic cells, and mitotic immune cells, alongside reduced proportions of macrophages and B cells. Transcriptional profiling and pseudotime trajectory analysis further revealed shifts in gene expression, cell state, and differentiation trajectories across immune subtypes. Notably, E₂ treatment promoted the transition from M1 to M2 macrophages, indicating a potential immunomodulatory role for estrogen. Collectively, these findings provide mechanistic insights into how estrogen regulates the ovarian immune microenvironment during early female reproductive development.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Y.T.Y. conceived the study, performed major experiments, analyzed the data, and wrote the manuscript. Y.T.Y., Y.X.L., Y.T.M., Q.L., X.E.Z., Q.W., M.H.P., and S.P. participated in experimental design. B.H.M. led the experimental design and was responsible for overall direction and planning. All authors read and approved the final version of the manuscript.

REFERENCES

Asaba J, Bandyopadhyay M, Kindy M, et al. 2015. Estrogen receptor signal in regulation of B cell activation during diverse immune responses. *The*

International Journal of Biochemistry & Cell Biology, **68**: 42–47.

Bachy V, Williams DJ, Ibrahim MAA. 2008. Altered dendritic cell function in normal pregnancy. *Journal of Reproductive Immunology*, **78**(1): 11–21.

Banchereau J, Steinman RM. 1998. Dendritic cells and the control of immunity. *Nature*, **392**(6673): 245–252.

Ben Yaakov T, Wasserman T, Akin E, et al. 2023. Single-cell analysis of the aged ovarian immune system reveals a shift towards adaptive immunity and attenuated cell function. *Elife*, **12**: e74915.

Best CL, Pudney J, Welch WR, et al. 1996. Localization and characterization of white blood cell populations within the human ovary throughout the menstrual cycle and menopause. *Human Reproduction*, **11**(4): 790–797.

Bukulmez O, Arici A. 2000. Leukocytes in ovarian function. *Human Reproduction Update*, **6**(1): 1–15.

Carlock C, Wu J, Zhou C, et al. 2013. Ovarian phagocyte subsets and their distinct tissue distribution patterns. *Reproduction*, **146**(5): 491–500.

Chen Y, Jefferson WN, Newbold RR, et al. 2007. Estradiol, progesterone, and genistein inhibit oocyte nest breakdown and primordial follicle assembly in the neonatal mouse ovary *in vitro* and *in vivo*. *Endocrinology*, **148**(8): 3580–3590.

Cohen-Fredarow A, Tadmor A, Raz T, et al. 2014. Ovarian dendritic cells act as a double-edged pro-ovulatory and anti-inflammatory sword. *Molecular Endocrinology*, **28**(7): 1039–1054.

Dai MY, Xu Y, Gong GD, et al. 2023. Roles of immune microenvironment in the female reproductive maintenance and regulation: novel insights into the crosstalk of immune cells. *Frontiers in Immunology*, **14**: 1109122.

Dai RJ, Phillips RA, Zhang Y, et al. 2008. Suppression of LPS-induced Interferon- γ and nitric oxide in splenic lymphocytes by select estrogen-regulated microRNAs: a novel mechanism of immune modulation. *Blood*, **112**(12): 4591–4597.

Dou C, Ding N, Zhao CR, et al. 2018. Estrogen deficiency-mediated M2 macrophage osteoclastogenesis contributes to M1/M2 ratio alteration in ovariectomized osteoporotic mice. *Journal of Bone and Mineral Research*, **33**(5): 899–908.

Duffy DM, Ko C, Jo M, et al. 2019. Ovulation: parallels with inflammatory processes. *Endocrine Reviews*, **40**(2): 369–416.

Dutta S, Mark-Kappeler CJ, Hoyer PB, et al. 2014. The steroid hormone environment during primordial follicle formation in perinatal mouse ovaries. *Biology of Reproduction*, **91**(3): 68.

Escribese MM, Kraus T, Rhee E, et al. 2008. Estrogen inhibits dendritic cell maturation to RNA viruses. *Blood*, **112**(12): 4574–4584.

Fair T. 2015. The contribution of the maternal immune system to the establishment of pregnancy in cattle. *Frontiers in Immunology*, **6**: 7.

Feingold KR, Shigenaga JK, Kazemi MR, et al. 2012. Mechanisms of triglyceride accumulation in activated macrophages. *Journal of Leukocyte Biology*, **92**(4): 829–839.

Freemerman AJ, Johnson AR, Sacks GN, et al. 2014. Metabolic reprogramming of macrophages: glucose transporter 1 (GLUT1)-mediated glucose metabolism drives a proinflammatory phenotype. *The Journal of Biological Chemistry*, **289**(11): 7884–7896.

Fukuzumi M, Shinomiya H, Shimizu Y, et al. 1996. Endotoxin-induced enhancement of glucose influx into murine peritoneal macrophages via GLUT1. *Infection and Immunity*, **64**(1): 108–112.

Funk JL, Feingold KR, Moser AH, et al. 1993. Lipopolysaccharide stimulation of RAW 264.7 macrophages induces lipid accumulation and foam cell formation. *Atherosclerosis*, **98**(1): 67–82.

Givan AL, White HD, Stern JE, et al. 1997. Flow cytometric analysis of leukocytes in the human female reproductive tract: comparison of fallopian tube, uterus, cervix, and vagina. *American Journal of Reproductive Immunology*, **38**(5): 350–359.

Grossman CJ. 1985. Interactions between the gonadal steroids and the immune system. *Science*, **227**(4684): 257–261.

Hao N, Whitelaw ML. 2013. The emerging roles of AhR in physiology and immunity. *Biochemical Pharmacology*, **86**(5): 561–570.

Heldring N, Pike A, Andersson S, et al. 2007. Estrogen receptors: how do

- they signal and what are their targets. *Physiological Reviews*, **87**(3): 905–931.
- Hsieh CH, Nickel EA, Chen JG, et al. 2009. Mechanism of the salutary effects of estrogen on kupffer cell phagocytic capacity following trauma-hemorrhage: pivotal role of Akt activation. *The Journal of Immunology*, **182**(7): 4406–4414.
- Isola JVV, Ocañas SR, Hubbart CR, et al. 2024. A single-cell atlas of the aging mouse ovary. *Nature Aging*, **4**(1): 145–162.
- Jha AK, Huang SCC, Sergushichev A, et al. 2015. Network integration of parallel metabolic and transcriptional data reveals metabolic modules that regulate macrophage polarization. *Immunity*, **42**(3): 419–430.
- Jokela H, Lokka E, Kiviranta M, et al. 2020. Fetal-derived macrophages persist and sequentially mature in ovaries after birth in mice. *European Journal of Immunology*, **50**(10): 1500–1514.
- Karpuzoglu E, Ahmed SA. 2006. Estrogen regulation of nitric oxide and inducible nitric oxide synthase (iNOS) in immune cells: implications for immunity, autoimmune diseases, and apoptosis. *Nitric Oxide*, **15**(3): 177–186.
- Karpuzoglu E, Phillips RA, Dai RJ, et al. 2009. Signal transducer and activation of transcription (STAT) 4 β , a shorter isoform of interleukin-12-induced STAT4, is preferentially activated by estrogen. *Endocrinology*, **150**(3): 1310–1320.
- Kimmel JC, Penland L, Rubinstein ND, et al. 2019. Murine single-cell RNA-seq reveals cell-identity- and tissue-specific trajectories of aging. *Genome Research*, **29**(12): 2088–2103.
- Kovats S. 2015. Estrogen receptors regulate innate immune cells and signaling pathways. *Cellular Immunology*, **294**(2): 63–69.
- Laffont S, Rouquié N, Azar P, et al. 2014. X-chromosome complement and estrogen receptor signaling independently contribute to the enhanced TLR7-mediated IFN- α production of plasmacytoid dendritic cells from women. *The Journal of Immunology*, **193**(11): 5444–5452.
- Laffont S, Seillet C, Guéry JC. 2017. Estrogen receptor-dependent regulation of dendritic cell development and function. *Frontiers in Immunology*, **8**: 108.
- Lee SK, Kim CJ, Kim DJ, et al. 2015. Immune cells in the female reproductive tract. *Immune Network*, **15**(1): 16–26.
- Leitman DC, Paruthiyil S, Vivar OI, et al. 2010. Regulation of specific target genes and biological responses by estrogen receptor subtype agonists. *Current Opinion in Pharmacology*, **10**(6): 629–636.
- Lima PDA, Nivet AL, Wang Q, et al. 2018. Polycystic ovary syndrome: possible involvement of androgen-induced, chemerin-mediated ovarian recruitment of monocytes/macrophages. *Biology of Reproduction*, **99**(4): 838–852.
- Liu HY, Buenafe AC, Matejuk A, et al. 2002. Estrogen inhibition of EAE involves effects on dendritic cell function. *Journal of Neuroscience Research*, **70**(2): 238–248.
- Mauri C, Gray D, Mushtaq N, et al. 2003. Prevention of arthritis by interleukin 10-producing B cells. *The Journal of Experimental Medicine*, **197**(4): 489–501.
- Meiser J, Krämer L, Sapcaru SC, et al. 2016. Pro-inflammatory macrophages sustain pyruvate oxidation through pyruvate dehydrogenase for the synthesis of itaconate and to enable cytokine expression. *The Journal of Biological Chemistry*, **291**(8): 3932–3946.
- Mor G, Sapi E, Abrahams VM, et al. 2003. Interaction of the estrogen receptors with the Fas ligand promoter in human monocytes. *The Journal of Immunology*, **170**(1): 114–122.
- Oakley OR, Kim H, El-Amouri I, et al. 2010. Periovarian leukocyte infiltration in the rat ovary. *Endocrinology*, **151**(9): 4551–4559.
- O'Lone R, Frith MC, Karlsson EK, et al. 2004. Genomic targets of nuclear estrogen receptors. *Molecular Endocrinology*, **18**(8): 1859–1875.
- Ono Y, Nagai M, Yoshino O, et al. 2018. CD11c $^{+}$ M1-like macrophages (M Φ s) but not CD206 $^{+}$ M2-like M Φ are involved in folliculogenesis in mice ovary. *Scientific Reports*, **8**(1): 8171.
- Panchanathan R, Choubey D. 2013. Murine BAFF expression is up-regulated by estrogen and interferons: implications for sex bias in the development of autoimmunity. *Molecular Immunology*, **53**(1-2): 15–23.
- Pepe GJ, Billiar RB, Albrecht ED. 2006. Regulation of baboon fetal ovarian folliculogenesis by estrogen. *Molecular and Cellular Endocrinology*, **247**(1-2): 41–46.
- Phiel KL, Henderson RA, Adelman SJ, et al. 2005. Differential estrogen receptor gene expression in human peripheral blood mononuclear cell populations. *Immunology Letters*, **97**(1): 107–113.
- Richards JS, Liu ZL, Shimada M. 2008. Immune-like mechanisms in ovulation. *Trends in Endocrinology & Metabolism*, **19**(6): 191–196.
- Sambamurthy N, Zhou C, Nguyen V, et al. 2018. Deficiency of the pattern-recognition receptor CD14 protects against joint pathology and functional decline in a murine model of osteoarthritis. *PLoS One*, **13**(11): e0206217.
- Seillet C, Laffont S, Trémollières F, et al. 2012. The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol in vivo through cell-intrinsic estrogen receptor α signaling. *Blood*, **119**(2): 454–464.
- Tang MX, Zhao MZ, Shi YH. 2023. New insight into the role of macrophages in ovarian function and ovarian aging. *Frontiers in Endocrinology*, **14**: 1282658.
- Tannahill GM, Curtis AM, Adamik J, et al. 2013. Succinate is an inflammatory signal that induces IL-1 β through HIF-1 α . *Nature*, **496**(7444): 238–242.
- The Tabula Muris Consortium. 2020. A single-cell transcriptomic atlas characterizes ageing tissues in the mouse. *Nature*, **583**(7817): 590–595.
- Turner EC, Hughes J, Wilson H, et al. 2011. Conditional ablation of macrophages disrupts ovarian vasculature. *Reproduction*, **141**(6): 821–831.
- Ueda K, Karas RH. 2013. Emerging evidence of the importance of rapid, non-nuclear estrogen receptor signaling in the cardiovascular system. *Steroids*, **78**(6): 589–596.
- van den Berg TK, Kraal G. 2005. A function for the macrophage F4/80 molecule in tolerance induction. *Trends in Immunology*, **26**(10): 506–509.
- Wang C, Roy SK. 2007. Development of primordial follicles in the hamster: role of estradiol-17 β . *Endocrinology*, **148**(4): 1707–1716.
- Winkels H, Ehinger E, Vassallo M, et al. 2018. Atlas of the immune cell repertoire in mouse atherosclerosis defined by single-cell RNA-sequencing and mass cytometry. *Circulation Research*, **122**(12): 1675–1688.
- Winkler I, Tolkachov A, Lammers F, et al. 2024. The cycling and aging mouse female reproductive tract at single-cell resolution. *Cell*, **187**(4): 981–998. e25.
- Wu RJ, Van der Hoek KH, Ryan NK, et al. 2004. Macrophage contributions to ovarian function. *Human Reproduction Update*, **10**(2): 119–133.
- Xiao Y, Peng XX, Peng Y, et al. 2022. Macrophage-derived extracellular vesicles regulate follicular activation and improve ovarian function in old mice by modulating local environment. *Clinical and Translational Medicine*, **12**(10): e1071.
- Yan SX, Zhou M, Zheng XY, et al. 2021. Anti-inflammatory effect of curcumin on the mouse model of myocardial infarction through regulating macrophage polarization. *Mediators of Inflammation*, **2021**: 9976912.
- Yan YT, Zhang H, Xu R, et al. 2024. Single-cell sequencing reveals the transcriptional alternations of 17 β -estradiol suppressing primordial follicle formation in neonatal mouse ovaries. *Cell Proliferation*, **57**(9): e13713.
- Yang MY, Fortune JE. 2008. The capacity of primordial follicles in fetal bovine ovaries to initiate growth in vitro develops during mid-gestation and is associated with meiotic arrest of oocytes. *Biology of Reproduction*, **78**(6): 1153–1161.
- Yang XH, Gilman-Sachs A, Kwak-Kim J. 2019. Ovarian and endometrial immunity during the ovarian cycle. *Journal of Reproductive Immunology*, **133**: 7–14.
- Zhang J, Ye ZW, Chen W, et al. 2018. S-glutathionylation of estrogen receptor α affects dendritic cell function. *The Journal of Biological Chemistry*, **293**(12): 4366–4380.