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ORIGINAL RESEARCH

Increased CD163+ Macrophage Activation and High Expression of CD163 Promote Granulosa Cell Apoptosis in Polycystic Ovary Syndrome

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Background: The inflammatory microenvironment disrupts the ovarian niche, impairing granulosa cell function and leading to aberrant follicular development, a key pathological feature of polycystic ovary syndrome (PCOS). However, the precise mechanisms by which inflammation influences granulosa cell function remain poorly understood.

Methods: Differentially expressed genes (DEGs) were identified from the GSE34526 dataset, with the inflammatory marker CD163 selected for further investigation due to its upregulation in ovarian granulosa cells in PCOS. Serum levels of soluble CD163 (sCD163) were measured in patients with PCOS, and a dehydroepiandrosterone (DHEA)-induced PCOS mouse model was utilized to examine the relationship between CD163 expression, inflammatory mediators, and macrophage activity in the ovaries and uterus. Granulosa cell apoptosis, inflammatory cytokine secretion, and sCD163 release from conditioned media (CM) of differently polarized macrophages co-cultured with COV434 cells were assessed.

Results: Elevated serum sCD163 levels were observed in patients with PCOS. The DHEA-induced PCOS mice exhibited characteristic oestrous cycle abnormalities, as well as morphological and pathological alterations in the ovaries and uterus. Increased CD163 expression was detected in ovarian and uterine macrophages of PCOS mice, alongside elevated inflammatory cytokines. Conditioned media from M1-polarized macrophages induced apoptosis in COV434 granulosa cells, with concomitant increases in pro-inflammatory cytokines (IL-1β and IL-6) and sCD163 secretion. Furthermore, $CD163^{+}$ cell apoptosis was heightened in the ovaries of PCOS mice.

Conclusion: These findings suggest that ovarian macrophages, through elevated CD163 expression, contribute to granulosa cell apoptosis and the secretion of sCD163, which may play a critical role in the pathogenesis of PCOS.

Keywords: CD163, high expression, macrophage activation, granulosa cell apoptosis, polycystic ovary syndrome

Introduction

Polycystic ovary syndrome (PCOS) affects 5–20% of women of reproductive age globally and is recognized as a major contributor to infertility.[1] It is characterized by hyperandrogenism, chronic oligoovulation or anovulation, and polycystic ovaries. Long-term, PCOS also increases the risk of dyslipidemia, metabolic syndrome, coronary artery disease, and cerebrovascular events.[2] While the exact pathogenesis of PCOS remains unclear, current research suggests that metabolic disturbances associated with chronic low-grade inflammation play a central role in ovarian dysfunction.[3,4] Under normal conditions, paracrine mediators, produced through the collaboration between ovarian granulosa cells (GCs) and surrounding immune cells, facilitate ovulation.[5] However, the inflammatory cytokines released by immune cells impair

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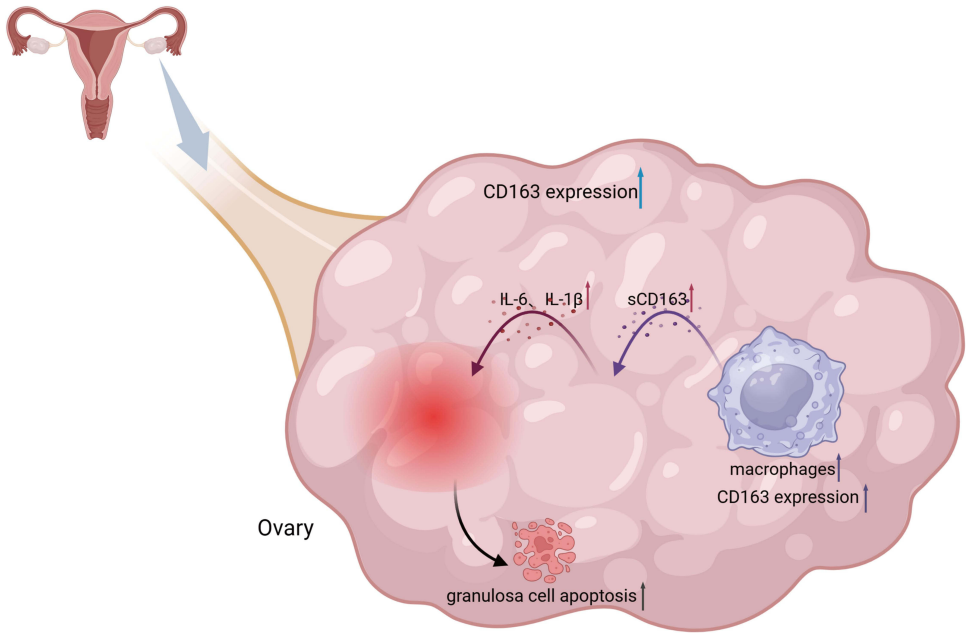
Graphical Abstract



the regulatory role of GCs on oocytes, thereby promoting anovulation in PCOS.[6,7] Despite these findings, the regulatory interactions between GCs and immune cells in PCOS remain poorly understood.

Macrophages, the predominant immune cells in ovarian tissue, are critically involved in the chronic inflammation observed in PCOS.[8] The number of inflammatory M1 macrophages is increased, accompanied by elevated levels of C-reactive protein, IL-6, TNF-α, and other inflammatory cytokines in the peripheral blood and ovarian tissues of patients with PCOS.[7–9] However, the precise role of macrophages in the pathogenesis of PCOS has yet to be fully elucidated.

The scavenger receptor CD163, expressed by monocytes and macrophages, plays a key role in the clearance of hemoglobin-globin complexes and the regulation of inflammation.[10] Upon cleavage by TNF-α, CD163 is converted into soluble CD163 (sCD163), which is considered a marker of macrophage and monocyte activation.[10,11] Elevated levels of sCD163 have been reported in various acute and chronic inflammatory diseases,[12,13] and its serum levels are also elevated in patients with PCOS.[14] Interestingly, in a study on atherosclerosis, CD163-positive macrophages may have actually been converted from pre-existing M1 proinflammatory macrophages.[15] Furthermore, these newly identified CD163-positive macrophages exhibited anti-atherosclerotic and anti-inflammatory effects within atherosclerotic lesions.[15] However, the expression of CD163 in macrophages and its role in the inflammation associated with PCOS remains unclear.

In the present study, analysis of the GSE34526 dataset from the NCBI GEO database revealed increased expression of CD163 in the serum of patients with PCOS. Additionally, $CD163^+$ macrophages were found to be elevated in the ovaries and uterus of PCOS mice, alongside an increase in inflammatory cytokines. In vitro, conditioned medium (CM) derived from M1-polarized macrophages co-cultured with GCs promoted apoptosis in the GCs, accompanied by increased secretion of inflammatory cytokines and sCD163. Increased apoptosis of $CD163^+$ ovarian cells was also observed in PCOS mice. These findings suggest that the activation of $CD163^+$ macrophages in the ovary may promote GC apoptosis and contribute to the pathogenesis of PCOS.

Material and Methods

Materials

Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute 1640 (RPMI-1640) were procured from GIBCO (Grand Island, NY, USA). Phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS) were

obtained from Sigma-Aldrich (Saint Louis, MO, USA). Recombinant human interferon-γ (IFN-γ) and recombinant human interleukin-13 (IL-13) were sourced from Peprotech (Cranbury, NJ, USA). Dehydroepiandrosterone (DHEA) was purchased from APExBIO (Houston, TX, USA). Anti-mouse FITC-CD11b, APC-Arg1, and PE-iNOS were obtained from BioLegend (San Diego, CA, USA). CD163 protein was acquired from MedChemExpress (Monmouth Junction, NJ, USA).

Methods

Identification of Differentially Expressed Genes (DEGs)

The GSE34526 dataset, retrieved from the NCBI GEO public database (http://www.ncbi.nlm.nih.gov/geo), comprised 10 GC samples, including 7 from the PCOS group and 3 from the control group. The dataset underwent standardized preprocessing. DEGs were identified using the "limma" package for variance analysis, with filtering criteria of an adjusted P-value < 0.05, an average expression level > 5, and a log fold change > 1. A volcano plot of the DEGs was generated using the "ggplot2" package, sorting P-values from smallest to largest to highlight the top ten upregulated and downregulated genes based on the lowest P-values.

Patients

Thirty patients aged 20–40 years with PCOS, fulfilling the criteria of the ESHRE/ASRM-sponsored PCOS Consensus Workshop in Rotterdam,[16] and 20 age- and sex-matched healthy controls were enrolled in the study. Patients with PCOS were required to have irregular menstruation (cycle length outside 21–35 days or <8 cycles per year) and biochemical hyperandrogenemia (total testosterone levels >50 ng/dL or free androgen index > 3.87), with the exclusion of known disorders of irregular bleeding or hyperandrogenism.[17] Human blood sample procedure was in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and all subsequent revision. The study was approved by the Affiliated Hospital of Jiangsu University (KY2023K1001), and informed consent was obtained from all participants.

Establishment of the PCOS Mice Model

Sixteen female C57/BL6 mice (4 weeks old) were purchased from Yangzhou University. Eight mice were induced with the PCOS model by daily subcutaneous injections of DHEA (6 mg/100 g body weight, dissolved in 0.1 mL sesame oil) for 21 days, as previously described.[18] Eight control mice received sesame oil injections for the same duration. All animals were provided humane care, and were conducted in accordance with the Guidelines for the Protection and Use of Experimental Animals and the Measures for the Administration of Animal Use at Jiangsu University. Experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Jiangsu University (Permit Number: UJS-IACUC-2023061602).

Identification of Oestrous Cycle in PCOS Mice

The oestrous cycle of the mice was assessed through vaginal cytology over ten consecutive days, starting on the 21st day after DHEA injections and continuing until the day of sacrifice. To prevent ovarian self-recovery, DHEA was continuously administered subcutaneously during the vaginal cytology assessments. Samples were then stained with Wright-Giemsa stain (Baso, Zhuhai, China) for 10 minutes, and oestrous cycles were examined using an optical microscope as described previously.[19]

Histological Analysis

Ovarian and uterine tissues were fixed in formalin overnight and subsequently embedded in paraffin. Sections of 5 μm thickness were stained with hematoxylin and eosin (HE), followed by imaging with a microscope (Leica, Wetzlar, Germany).

THP-1 Cell Culture and Induced M1 and M2 Macrophages

THP-1 cells were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology (Shanghai, China), and were cultured in RPMI 1640 medium supplemented with 10% FBS at 37 °C in a 5% CO_2 atmosphere. Cells were seeded in 6-well culture plates at a density of 2.0×10^6 cells/well and stimulated with PMA (100 ng/mL) for 24 hours to induce macrophage differentiation (M0). Following this, cells were polarized into M1 and M2 macrophages by the addition

of IFN-γ (20 ng/mL) + LPS (20 pg/mL) and IL-13 (20 ng/mL), respectively, for 48 hours. After polarization, cells were washed with PBS and incubated in a fresh medium for an additional 72 hours. The culture supernatants from these macrophage cultures were then collected as CM.

Conditioned Media (CM) from Macrophages Co-Culture with COV434 Cells

COV434 cells were obtained from Zhenjiang Weigen Biotechnology Co., Ltd. (Zhenjiang, China), and were seeded in 24-well plates (3.0×10^5 cells/well) and cultured for 24 hours to allow cell adhesion. The culture medium was then replaced, and the conditioned media from M0, M1, and M2 macrophages were added to the plates (400 μL each), forming the COV434+M0, COV434+M1, and COV434+M2 groups, respectively. The COV434 group was treated with 400 μL of culture medium (DMEM + 10% FBS) as a control. Based on the previous study, cells were cultured at 37 °C and 5% CO_2 for 72 hours.[20] After 72 hours, the supernatants were collected for the detection of cytokines and sCD163.

Flow Cytometry

Apoptosis of COV434 cells after co-culture with conditioned media from M0, M1, and M2 macrophages was assessed using the ANNEXIN V-FITC/PI apoptosis detection kit (Solarbio, Beijing, China), following the manufacturer's instructions. In brief, after COV434 cells were collected and washed, 10 μL of Annexin V-FITC and 10 μL of Propidium Iodide (PI) were added for 15 min at room temperature in the dark, and then the cells were washed and detected by flow cytometry (BD Biosciences).

ELISA

The levels of sCD163 in serum and cell culture supernatants were measured using the human sCD163 ELISA kit (Peprotech, Cranbury, USA). In brief, 100 μL of sample per well was added to the ELISA plate and incubated at 37°C for 2h. The plates were washed and incubated at 37°C for 1h with 100 μL detection antibody per well. After washing. 100 μL HRP-conjugated secondary antibody was added and incubated for 40 min at 37°C. Following that, 100 μ of TMB substrate was added to each well and incubated at 37°C for 15 min. The reaction was stopped by adding 50ul of 2M sulfuric acid per well, and the results were read at 450 nm using in an ELISA reader from Bio-Rad.

Cytokine Measurement

Cytokine levels in both serum and cell culture supernatants were quantified using a twelve-cytokine detection kit (Biopredia, Taizhou, China) via multiplex flow cytometry luminescence, in accordance with the manufacturer's guidelines. In brief, 96-well Magpix plates were added 50 μL of labeled cytokine antibodies, 25 μL coupled microbeads, and 25 μL of samples in each well, then incubated at 25 °C for 2 hours. After washing, 70 μL SA-PE were added into each well, then incubated for 30 minutes. After washing, the reaction was terminated and a Luminex Magpix plate reader was used to detect the concentrations of multiple cytokines in the samples.

Quantitative Real-Time PCR Analysis

Total RNA was extracted from cells using RNA isolater total RNA extraction reagent (Vazyme, Nanjing, China), and cDNA was synthesized with a reverse transcription kit (Vazyme, Nanjing, China). The cDNA and specific gene primers were analyzed on the QuantStudio™5 system (Thermo Fisher, Waltham, USA). Relative gene expression was normalized to GAPDH levels. Primer sequences for each gene are provided in Table 1.

Immunofluorescence

Mouse ovarian and uterine tissues were subjected to dehydration, paraffin embedding, sectioning, and antigen retrieval. Endogenous peroxidase activity was blocked, followed by serum blocking. Sections were then incubated overnight with a mixture of primary antibodies against CD163 (Servicebio, Wuhan, China; diluted 1:3000), F4/80 (Servicebio; diluted 1:3000), iNOS (Servicebio, diluted 1:1000), and Arg1 (Servicebio; diluted 1:5000). After washing with PBS, tissue sections were incubated with corresponding fluorescent secondary antibodies (Servicebio; diluted 1:2000). Nuclei were stained with DAPI, and images were acquired using a confocal laser scanning microscope (CLSM, LMS-800, Carl Zeiss).

Table 1 Primers for Each Gene

Gene	Forward Primer	Reverse Primer
GAPDH	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA
TNF-α	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
IL-6	CATCCTCGACGGCATCTCAG	CCAGGCAAGTCTCCTCATTGAA
CCL-22	AGGGCCAGGGGACATCTAAT	GAGATCTGTGCCGATCCCAG
CCL-18	TGCATTGCAGCGTCATCTTG	GAGTCCCATCTGCTATGCCC
IL-10	GACTTTAAGGGTTACCTGGGTTG	TCACATGCGCCTTGATGTCTG

Immunohistochemistry

For additional immunohistochemical analysis, ovarian and uterine tissues were processed in the same manner as in *immunofluorescence* analysis, including dehydration, embedding, sectioning, antigen retrieval, and blocking of endogenous peroxidase and serum. The tissues were then incubated with primary antibodies against IL-6 (Servicebio; diluted 1:800) and IL-10 (Servicebio; diluted 1:200). Following incubation with secondary antibodies, DAB staining was performed, followed by hematoxylin counterstaining to visualize cell nuclei. The sections were examined under a microscope.

TUNEL Assay

Apoptosis in ovarian cells was assessed using the TUNEL assay, following the manufacturer's instructions (Servicebio, Wuhan, China). Paraffin-embedded mouse ovarian tissue sections were prepared for TUNEL staining after dewaxing and rehydration. Antigen retrieval was performed using EDTA solution (pH 8.0) at 92 °C for 48 minutes. After washing, the sections were blocked with 3% BSA for 30 minutes. The TUNEL reaction mixture was applied to cover the tissue and incubated at 37 °C for 1 hour. The sections were washed and incubated with DAPI solution for 10 minutes. After another wash with PBS, the sections were sealed with anti-fluorescence quenching tablets and observed under a fluorescence microscope.

Statistical Analysis

Data are presented as mean ± standard error of mean (SEM). Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software Inc., USA). Comparisons between two groups were made using Student's *t*-test. To compare differences among multiple groups, one-way analysis of variance (ANOVA) was applied, followed by a post-hoc Tukey multiple comparison test. (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

Results

Increased Serum Levels of sCD163 in Patients with PCOS

The GSE34526 database, encompassing three normal and seven PCOS GC datasets from ovarian tissues, identified the top 10 upregulated genes in patients with PCOS as PIK3CG, CD163, EPB41L3, MRC1, PECAM1, KCNE3, HMOX1, TGFB1, PTPRJ, and FCGR1B. Conversely, the top 10 downregulated genes included FRMPD2, OXTR, SLITRK5, DYNLRB1, MAFG-AS1, HOXD13, HAS2, LRRC16A, FAM229B, and EGFLAM. A volcano plot and heatmap of all DEGs are presented in Figure 1a and b. Given the low-grade inflammatory nature of PCOS and the elevated expression of CD163, a marker of monocytes and macrophages, CD163 was selected for subsequent focus. Serum levels of sCD163 in patients with PCOS were quantified, with results demonstrating a significant increase in sCD163 levels, as shown in Figure 1c. Serum levels of total testosterone (T) and luteinizing hormone (LH) were significantly higher in patients with PCOS compared to healthy controls ($p < 0.05$). The biological characteristics of the study population are presented in Table 2.

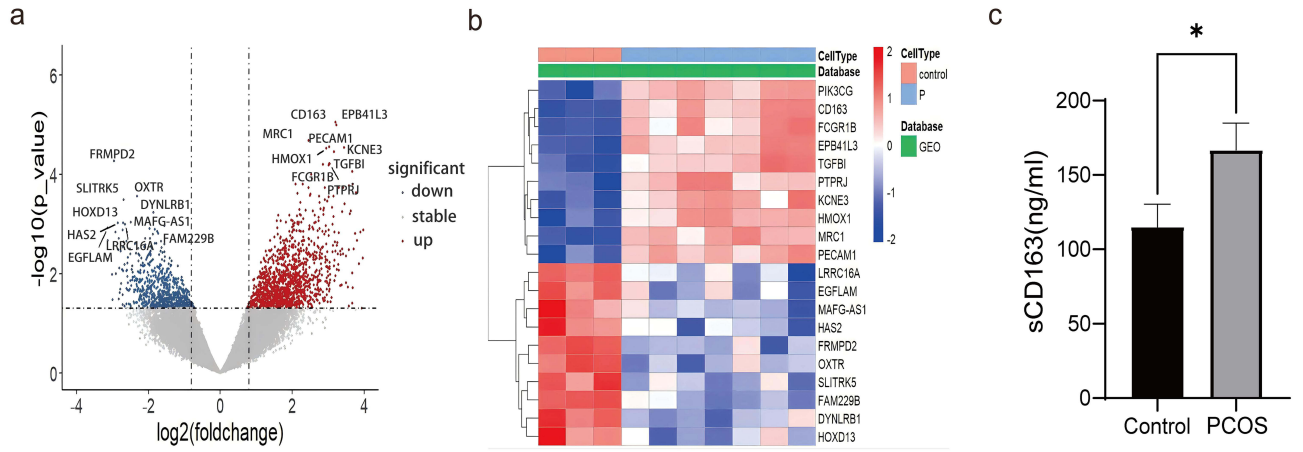


Figure 1 Serum sCD163 levels are upregulated in patients with PCOS. (**a**) Volcano plot depicting DEGs in GSE34526. Red dots represent upregulated genes, blue dots indicate downregulated genes, and grey dots denote genes without significant changes. Genes with logFC greater than 2 are labeled with their corresponding gene names. (**b**) Heatmap showing the top 20 DEGs between the PCOS and control groups. Red indicates high expression, and blue signifies low expression. (**c**) Serum levels of sCD163 in patients with PCOS and healthy controls. Data are presented as means ± SEM. *$p < 0.05$.

DHEA Induces Estrous Cycle Disorder, and Ovarian and Uterine Pathological Changes in PCOS Mice

PCOS was induced in mice via DHEA, resulting in oestrous cycle disruption (Figure 2a). In addition to oestrous cycle disturbances, PCOS mice exhibited pathological alterations and dysfunction in both the ovaries and uterus.[21,22] HE staining revealed normal ovarian structures in control mice, whereas in PCOS mice, aggregation of red blood cells was observed in the ovaries, with no cumulus cells present and dilated cystic atresia follicles identified (Figure 2b). Furthermore, the endometrial epithelium in PCOS mice was abnormally thickened, and uterine cavity diameter was increased, while the uterine morphology in control mice remained normal (Figure 2c). These results indicate successful induction of the PCOS mouse model.

Table 2 Characteristics of Patients and Healthy Controls

Clinical Parameters	PCOS (n=20)	Control (n=20)	P-Value
Age	28.90±5.088	31.30±3.389	0.0688
T (ng/dL)	43.90±16.92	32.65±13.86	0.0171*
E2 (pg/mL)	45.27±33.72	35.55±21.77	0.2607
FSH (mIU/mL)	6.086±1.764	6.624±1.976	0.3191
LH (mIU/mL)	11.05±12.62	5.185±3.368	0.0485*
PRL (ng/mL)	13.41±8.887	16.13±13.74	0.3992
P (ng/mL)	0.7723±0.8034	0.6525±0.5982	0.5718
GLU (mmol/L)	4.815±0.7241	4.521±0.7553	0.1732
INS (uIU/mL)	16.50±25.27	8.632±8.140	0.1869
HOMA-IR	4.170±7.959	1.863±2.035	0.2127
CHOL (mmol/L)	4.441±0.8942	4.120±0.4829	0.149
TG (mmol/L)	1.325±1.329	1.123±0.8284	0.5471
LDL (mmol/L)	2.554±0.7573	2.222±0.3490	0.073
HDL (mmol/L)	1.343±0.3196	1.407±0.2425	0.4507

Abbreviations: T, testosterone; E2, estradiol; FSH, follicle stimulating hormone; LH, luteinizing hormone; PRL, prolactin; P, progesterone; GLU, glucose; INS, insulin; HOMA-IR, homeostasis model assessment of insulin resistance; CHOL, cholesterol; TG, triglyceride; HDL, high density lipoprotein; LDL, low density lipoprotein.

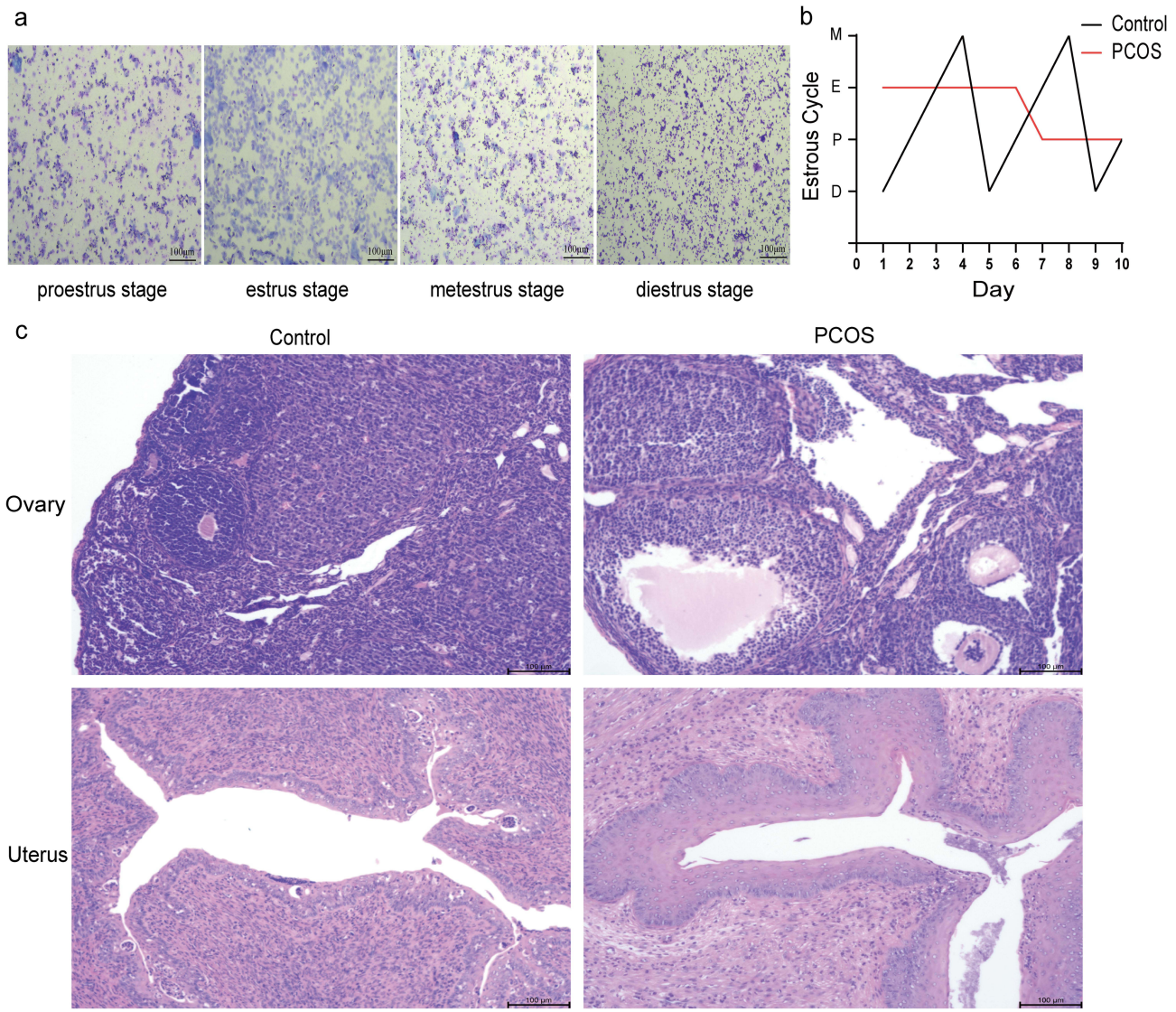


Figure 2 DHEA induces pathological changes in the ovary and uterus of mice. (**a**) Cell composition of different stages of the mouse oestrous cycle (Wright-Giemsa staining). P: proestrus stage with abundant nucleated epithelial cells; E: estrus stage with predominance of keratinizing epithelial cells; M: metestrus stage with a mixture of keratinized epithelial cells and leukocytes; D: diestrus stage with a predominance of white blood cells. (**b**) Representative oestrous cycle phases. (**c**) Representative hematoxylin and eosin (H&E) staining of uterus and ovary sections. Scale bars: 100 μm.
Abbreviations: P, proestrus; E, estrus; M, metestrus; D, diestrus.

Increased Macrophages and CD163 Expression in the Ovaries and Uterus of PCOS Mice

Given the pivotal role of macrophages in PCOS pathogenesis, the expression of F4/80$^+$ macrophages and CD163 was evaluated in the ovaries and uterus of PCOS mice. As shown in Figure 3a and b, both F4/80$^+$ macrophages and CD163 expression were significantly elevated, with increased CD163$^+$ expression in F4/80$^+$ macrophages (F4/80$^+$CD163$^+$ cells) in the ovaries of PCOS mice. Similar increases were observed in the uterus, where both F4/80$^+$ macrophages and CD163 expression in F4/80$^+$ macrophages were elevated. These results suggest an expansion of macrophages and an upregulation of CD163 expression in macrophages in the ovaries and uterus of PCOS mice.

The Expression of CD163 in M1 and M2 Macrophages Increased in the Ovaries and Uterus of PCOS Mice

Macrophages differentiate into pro-inflammatory M1 and anti-inflammatory M2 subsets, and an imbalance between M1 and M2 macrophages is implicated in PCOS pathogenesis.[8] Further investigation of macrophage populations from the

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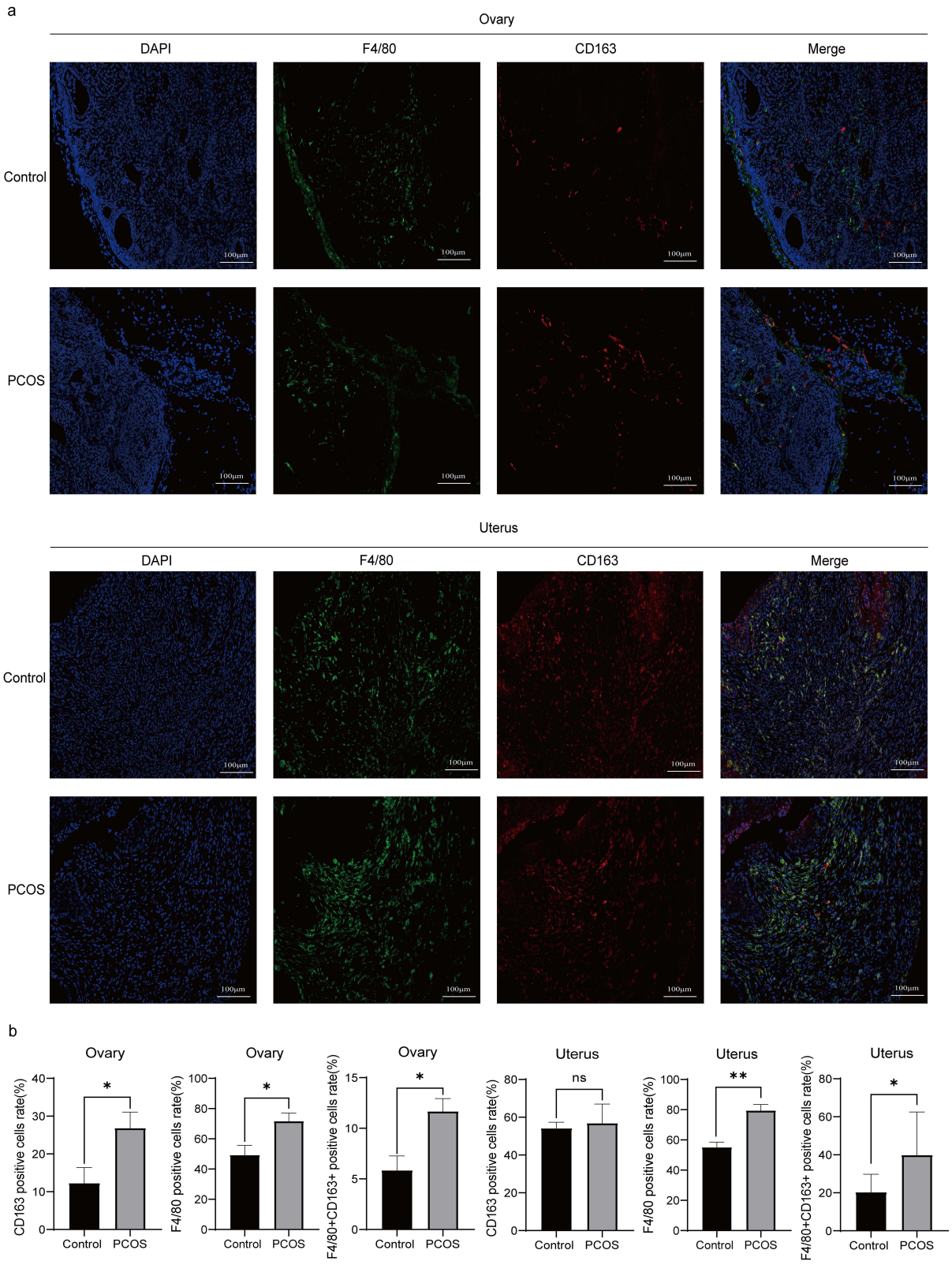

Figure 3 Increased macrophages and elevated CD163 expression in macrophages in the ovary and uterus of PCOS mice. (**a**) Representative immunofluorescence images of CD163 and F4/80 in ovaries and uteri of control and PCOS mice (scale bar = 100 μm). (**b**) Quantification of $CD163^+$, $F4/80^+$, and $F4/80^+CD163^+$ cell proportions in the ovaries and uteri of control and PCOS mice. *$p < 0.05$, **$p < 0.01$, ns: $p > 0.05$.

ovaries and uteri of PCOS mice revealed increased expression of iNOS in M1 macrophages and Arg1 in M2 macrophages, alongside elevated CD163 expression in both F4/80$^+$iNOS$^+$ M1 (F4/80$^+$iNOS$^+$CD163$^+$) and F4/80$^+$Arg1$^+$ M2 (F4/80$^+$Arg1$^+$CD163$^+$) macrophages (Figure 4a–d). These results indicate an increase in both M1 and M2 macrophages, with concurrent upregulation of CD163 expression in these subsets within the ovaries and uterus of PCOS mice.

Increased Expression of Local Inflammatory Factors in the Ovary and Uterus of PCOS Mice

Macrophage infiltration and chronic inflammation are critical factors in the pathogenesis of PCOS.[22,23] To assess the involvement of inflammatory factors, the expression of key cytokines in the ovaries and uterus of PCOS mice was measured. As shown in Figure 5a and b, IL-6 expression was significantly higher in both the ovaries and uterus of PCOS mice. However, IL-10 expression exhibited a divergent pattern: it was reduced in the ovaries but increased in the uterus of PCOS mice compared to the control group. These results suggest a lack of uniformity in the inflammatory response across the ovaries and uterus in PCOS mice.

Promotion of Granulocyte Apoptosis by Conditioned Medium (CM) from M1 Macrophage Stimulation

GCs are essential for oocyte maturation, which is critical for folliculogenesis.[24] Aberrant proliferation or apoptosis of GCs disrupts follicular development and ovulation, contributing to PCOS pathogenesis.[25] To investigate the impact of different macrophage subpopulations on GCs, COV434 cell apoptosis was evaluated following exposure to CM from M1 and M2 macrophages. As shown in Figure 6a, THP-1 cells were successfully differentiated into M1 cells with elevated expression of TNF-α, IL-1β, and IL-6 mRNA, and into M2 cells with increased expression of IL-10, CCL-18, and CCL-22 mRNA, confirming the successful polarization of THP-1 cells. CM from M1 macrophages induced significantly more apoptosis in COV434 cells compared to the unstimulated (M0) group, whereas CM from M2 macrophages induced significantly less apoptosis than M1-derived CM (Figure 6b and c). These results suggest that M1 macrophages promote GC apoptosis, while M2 macrophages exert a protective effect against apoptosis.

Upregulated IL-1β, IL-6, and sCD163 in Supernatants from CM from M1 Macrophages-Stimulated Granulocytes

To identify the factors in M1 macrophage CM responsible for increasing GC apoptosis, the secretion of key cytokines and sCD163 in the supernatants was measured. As expected, CM from M1 macrophages showed significantly higher levels of IL-1β, IL-6, and sCD163 compared to the M0 group (Figure 7a–c). These results suggest that elevated IL-1β, IL-6, and sCD163 levels in M1 macrophage CM contribute to the increased apoptosis observed in GCs.

Increased Apoptosis in CD163+ Ovarian Cells of PCOS Mice

To further examine the in vivo consequences of the increased CD163$^+$ macrophages in PCOS, the apoptosis of CD163$^+$ ovarian cells in PCOS mice was assessed. Consistent with the in vitro findings, PCOS mice exhibited a significant increase in TUNEL$^+$ cells, particularly CD163$^+$TUNEL$^+$ cells in the ovaries, compared to controls (Figure 8a and b). These results indicate an elevated rate of apoptosis in CD163$^+$ ovarian cells in PCOS mice.

Discussion

PCOS represents a heterogeneous disorder characterized by macrophage infiltration and activation, which contribute to the low-grade inflammation involved in its pathogenesis and progression.[4,23] Recent studies have shown that CD163, a marker of monocyte and macrophage activation, can be cleaved to release sCD163, which serves as an indicator of macrophage activation and is implicated in a variety of inflammatory conditions, including asthma,[26] atherosclerosis,[27] cirrhosis,[28] non-alcoholic fatty liver disease (NAFLD),[29] type 2 diabetes mellitus (T2DM),[30,31] obesity in chronic kidney disease (CKD),[32] lupus nephritis,[33,34] autoimmune hepatitis,[35] systemic sclerosis,[36,37] and systemic lupus erythematosus.[38] In this study, bioinformatics algorithms were employed to identify CD163 as a DEG in PCOS using data from the public GSE34526 database. This finding aligns with

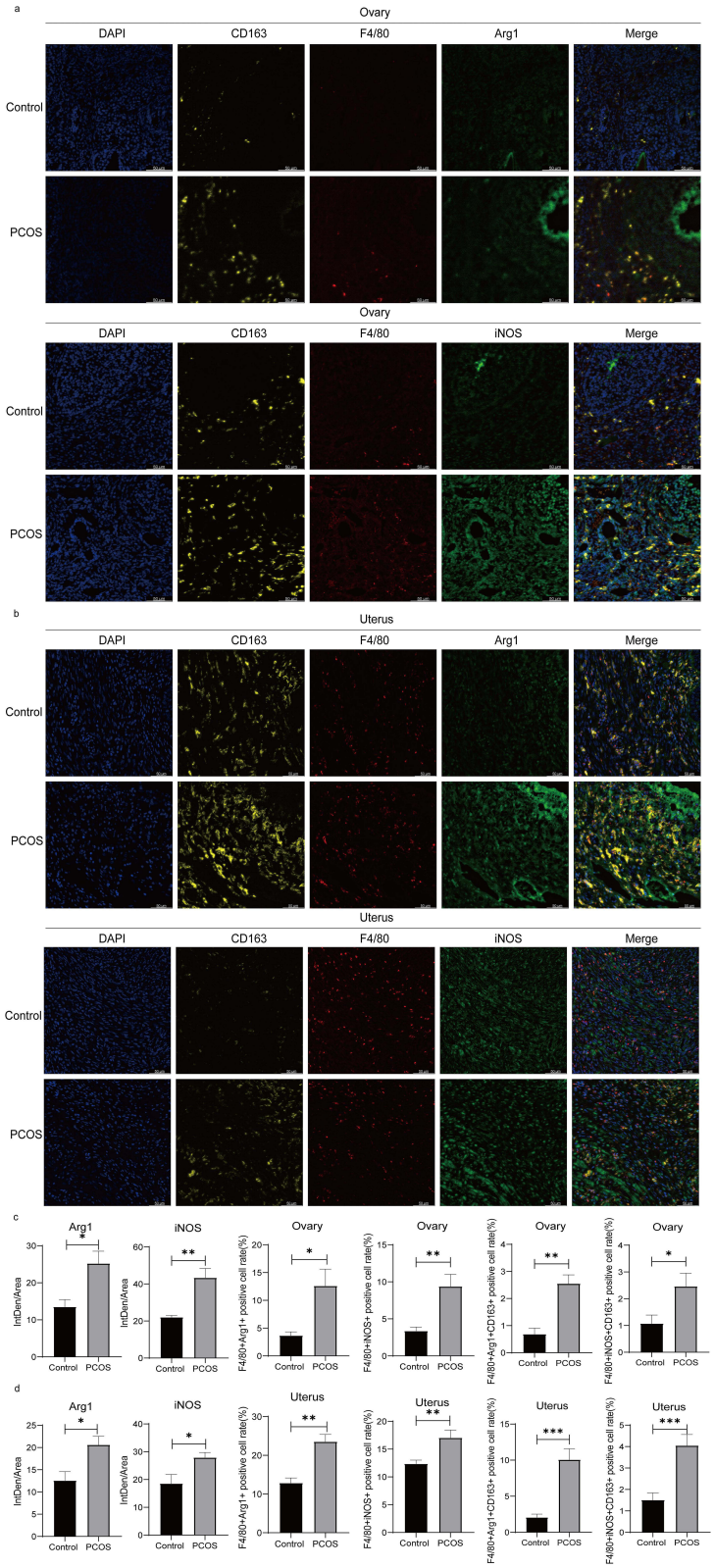

Figure 4 Increased M1 and M2 macrophages and elevated CD163 expression in M1 and M2 macrophages in the ovary and uterus of PCOS mice. (**a**) Representative immunofluorescence images of CD163, F4/80, iNOS, and Arg1 in the ovaries of control and PCOS mice (scale bar = 50 μm). (**b**) Representative immunofluorescence images of CD163, F4/80, iNOS, and Arg1 in the uterus of control and PCOS mice (scale bar = 50 μm). (**c**) Quantification of $F4/80^{+}iNOS^{+}CD163^{+}$ and $F4/80^{+}Arg1^{+}CD163^{+}$ cell proportions in the ovaries. (**d**) Quantification of $F4/80^{+}iNOS^{+}CD163^{+}$ and $F4/80^{+}Arg1^{+}CD163^{+}$ cell proportions in the uterus. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

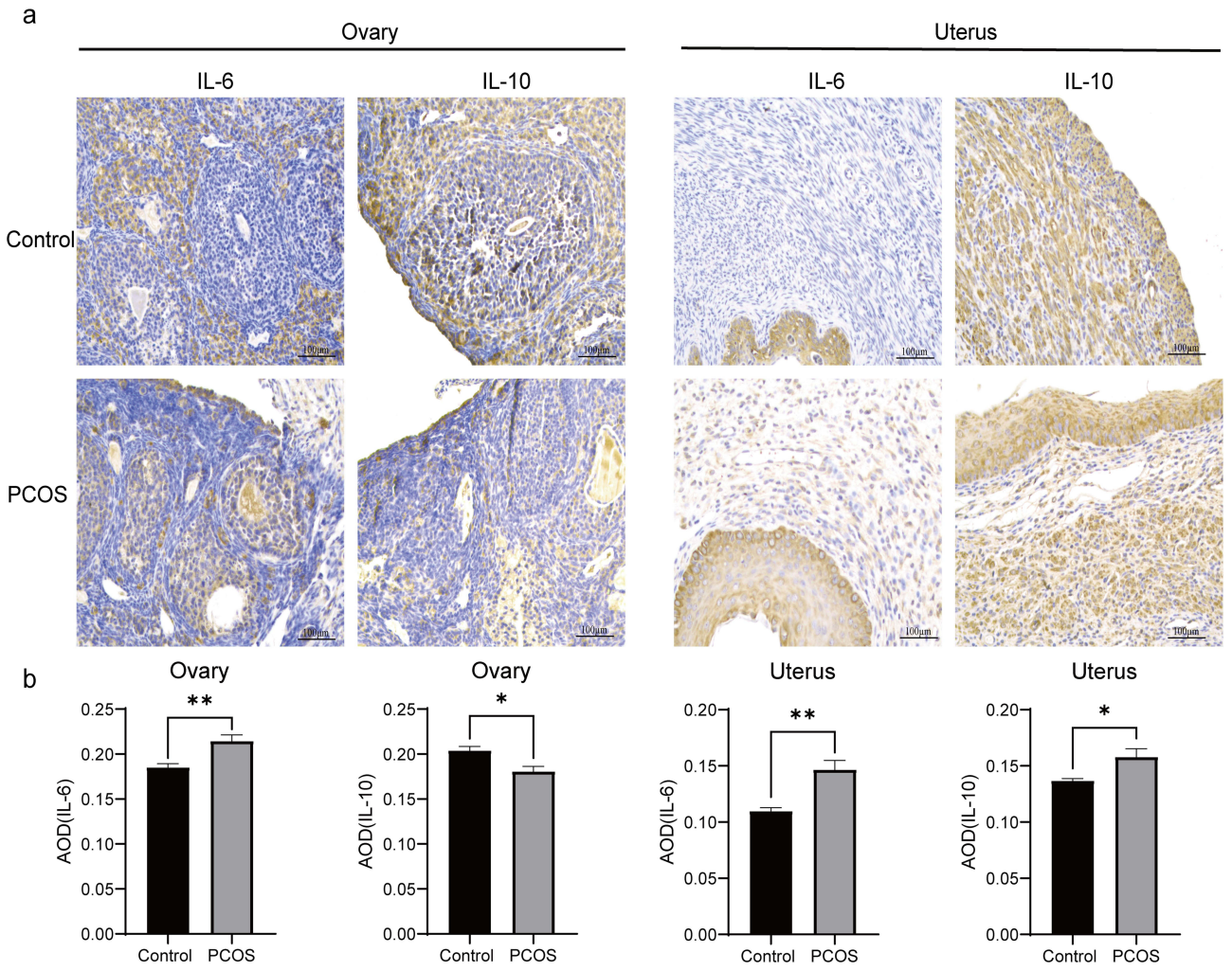

Figure 5 Expression of inflammatory factors in the ovary and uterus of PCOS mice. (**a**) Representative images showing IL-6 and IL-10 expression in the ovaries and uterus of control and PCOS mice via immunohistochemistry analysis (scale bar = 100 μm). (**b**) Quantification of IL-6 and IL-10 expression. *$p < 0.05$, **$p < 0.01$.

the work of Yuan et al, who also identified CD163 as a DEG in patients with PCOS, with a reported area under the curve (AUC) of 0.778 for PCOS prediction based on ROC analysis.[39] Furthermore, in line with previous research,[14] elevated serum levels of sCD163 were observed in patients with PCOS. As a biomarker of macrophage activation, sCD163 levels increase in response to inflammatory diseases, enabling the monitoring of disease progression and therapeutic response.[40] However, the precise role of elevated sCD163 in the pathogenesis of PCOS warrants further investigation.

CD163, an endocytosis receptor, is predominantly expressed on M2 macrophages, which are commonly recognized as a marker of these cells.[41,42] To further elucidate the role of CD163 and macrophages in PCOS, a DHEA-induced PCOS mouse model was utilized. Consistent with previous studies,[18,19] DHEA exposure induced ovarian and uterine pathological changes and disrupted the oestrous cycle in these mice. Macrophages, the most abundant immune cells in the ovary, are essential for maintaining ovarian function and stability. However, macrophage infiltration, along with systemic low-grade and chronic inflammation in ovarian tissue, constitutes a key pathogenic mechanism in PCOS.[8] In agreement with these observations,[18] an increase in $F4/80^+$ macrophages was observed in the ovaries and uteri of PCOS mice. Nevertheless, Torstensson et al reported a reduction in ovarian macrophage populations, alongside an increase in uterine macrophages, in dihydrotestosterone (DHT)-induced PCOS mice.[43] This discrepancy may be attributed to differences in host models, disease induction methods, and macrophage detection or labelling techniques. Moreover, in line with the elevated serum levels of sCD163, CD163 expression was also found to be upregulated in the ovaries of PCOS mice. While CD163 expression was not significantly altered in the uterine tissue of PCOS mice, macrophage CD163 expression

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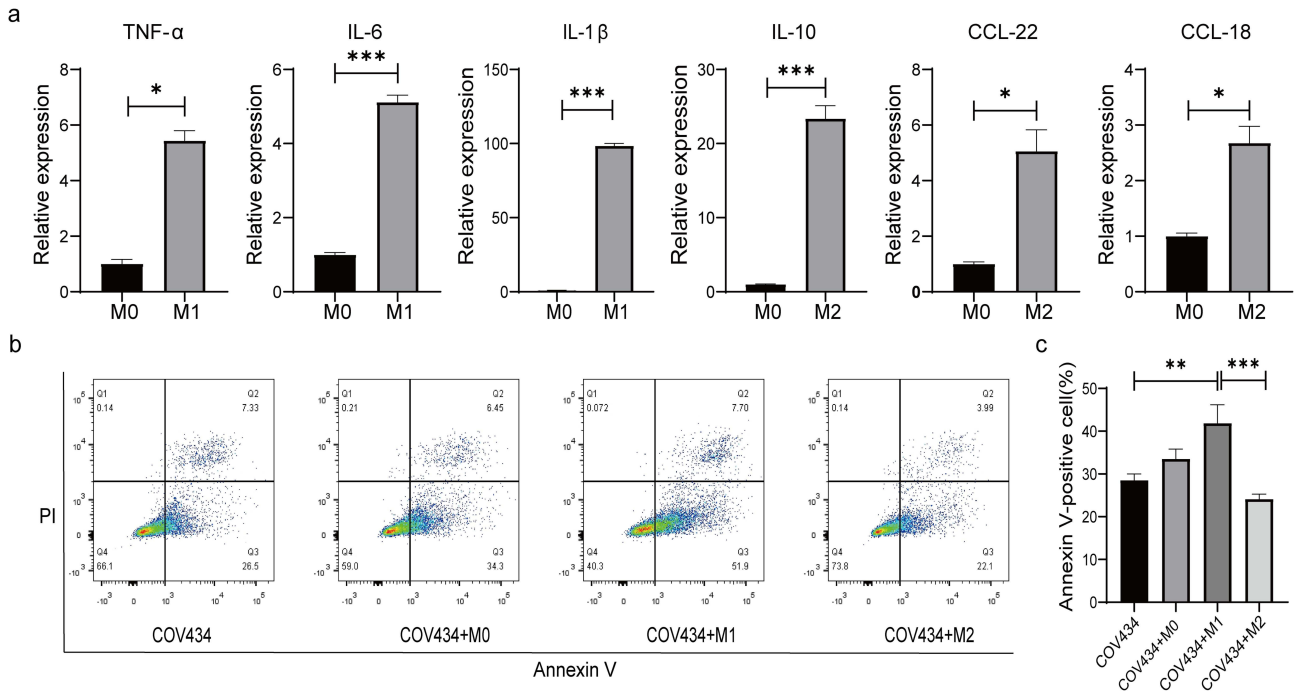

Figure 6 CM from M1 macrophages increases COV434 cell apoptosis. (**a**) mRNA expression levels of M1 markers (TNF-α, IL-1β, IL-6) and M2 markers (IL-10, CCL-18, CCL-22). Relative fold changes in gene expression were calculated using the 2-ΔΔCt method and normalized to GAPDH. (**b**) Representative FACS plots of COV434 cell apoptosis after treatment with CMs from M1 and M2 macrophages. (**c**) Quantification of COV434 cell apoptosis following CM treatment. The data are expressed as the mean ± SEM of three experiments performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

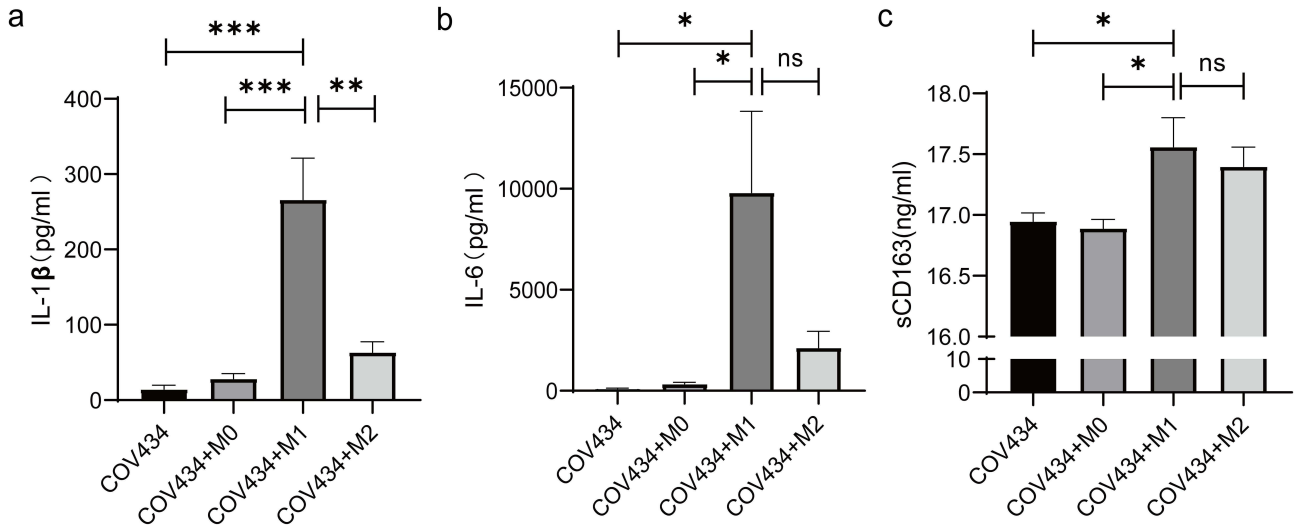

Figure 7 IL-1β, IL-6, and sCD163 were upregulated in COV434 cell supernatants stimulated by CM from M1 macrophages. (**a**) Secretion levels of IL-1β, (**b**) IL-6, and (**c**) sCD163 in COV434 cell supernatants stimulated by CM from macrophages. The data are expressed as the mean ± SEM of three experiments performed in triplicate. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns: $p > 0.05$.

was notably increased in both the ovaries and uteri. This observation aligns with the findings of Yuan et al, who reported upregulated ovarian CD163 mRNA expression in PCOS mice.[39] The increase in $CD163^+$ macrophages in PCOS mice may contribute to pathological changes in the ovaries and uterus, mediated by DHEA-induced inflammation.

Macrophages represent a heterogeneous cell population with diverse origins and functions. While numerous studies have reported an increase in M1 macrophages in the peripheral blood of DHEA-induced PCOS mice[18,44] and in the ovaries of 5α-DHT-induced PCOS rats,[45] our findings indicate an increase in both $F4/80^+iNOS^+$ M1 and $F4/80^+Arg1^+$ M2 macrophages in

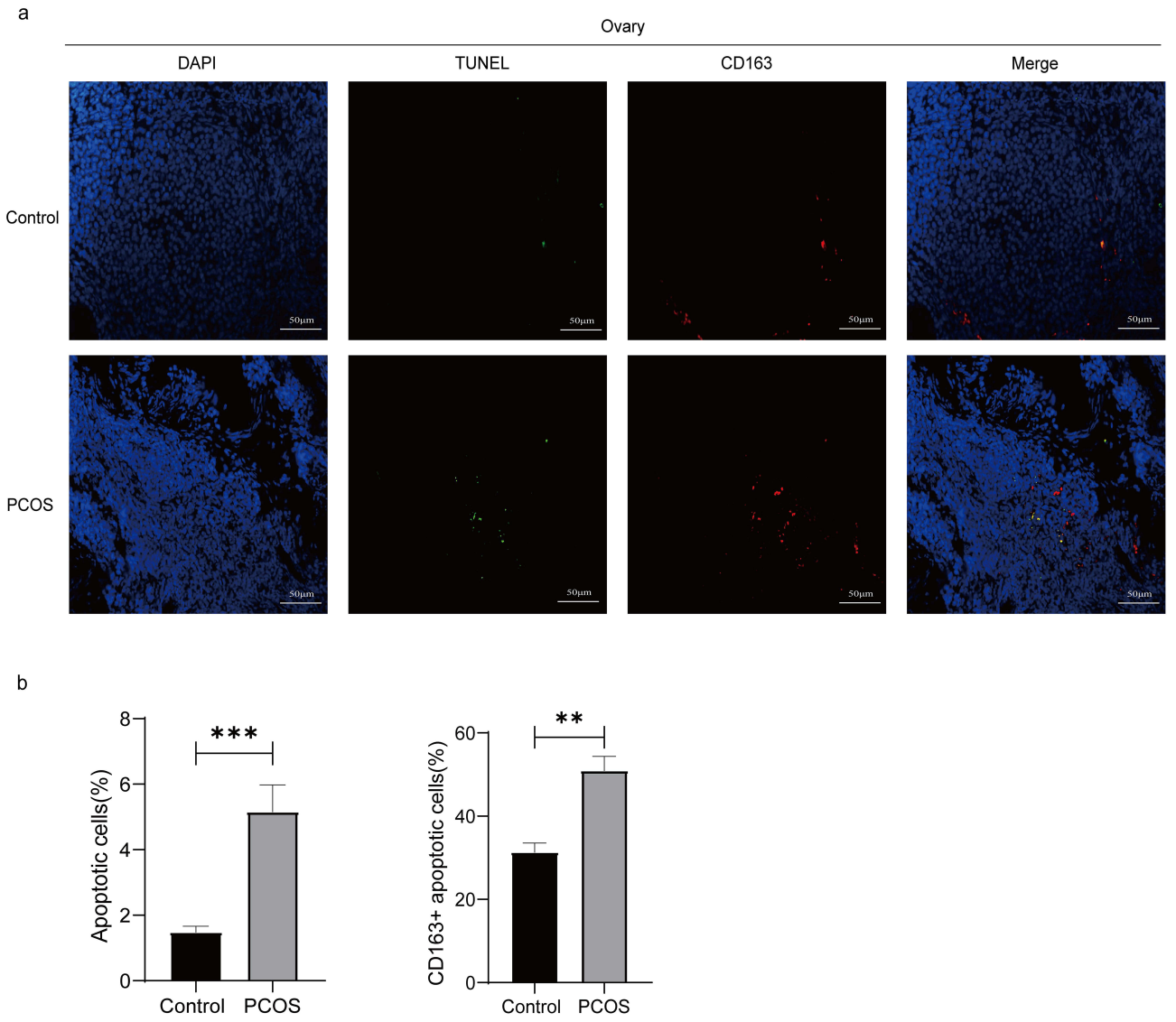

Figure 8 Increased apoptosis in CD163$^+$ ovarian cells from PCOS mice. (**a**) Representative images of TUNEL and CD163 in the ovaries of control and PCOS mice via immunofluorescence analysis (scale bar = 50 μm). (**b**) Quantification of TUNEL$^+$ and CD163$^+$TUNEL$^+$ cell proportions in the ovaries. $^{**}p < 0.01$, $^{***}p < 0.001$.

the ovaries and uteri of PCOS mice. Macrophages in the adult ovary are derived from both embryonic and bone marrow sources,[46] and their subpopulations exhibit varying distributions depending on ovarian development. Lima et al demonstrated that the numbers of M1 and M2 macrophages in DHT-induced rat ovaries are regulated by DHT and vary depending on the stage of follicular development.[45] Specifically, DHT treatment led to a significant increase in M1 macrophages in preovulatory follicles on day 7, while the frequency of M2 macrophages in sinus follicles and preovulatory follicles was slightly, though not significantly, reduced on days 7 and 15.[45] Interestingly, no significant increase in M1 expression was observed in the ovaries of patients with PCOS, although M2 expression was reduced.[45] Thus, discrepancies in M1 and M2 macrophage populations in the ovaries of PCOS mice are evident, underscoring the complexity of the inflammatory environment in this condition. Moreover, macrophages can adopt intermediate phenotypes, exhibiting characteristics of both M1 and M2 macrophages,[47,48] particularly under chronic inflammatory conditions.[49] Consequently, dysregulated macrophage activity in the ovary plays a critical role in sustaining the chronic low-grade inflammation observed in PCOS.

Although CD163 is widely considered a marker of M2 macrophages, an increase in CD163$^+$ expression was observed on both M1 and M2 cells in the ovaries and uteri of PCOS mice. CD163$^+$ macrophages are commonly elevated in both inflammatory and tumour microenvironments.[50] Notably, only a subset of M2 macrophages expresses CD163.[50] M1 macrophages are

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regulated by the transcription factors pSTAT1 and RBP-J, whereas M2 macrophages are modulated by CMAF.[51] Barros et al further challenged the M2-specificity of CD163 by demonstrating the presence of large populations of $CD163^+pSTAT1^+$ and $CD163^+RBP\text{-}J^+$ macrophages in Crohn's disease, suggesting that CD163 is not restricted to M2 macrophages and may be expressed by both M1 and M2 populations in Th1- or Th2-dominated immune responses.[51] This highlights the need for a more nuanced classification of $CD163^+$ macrophages in inflammatory diseases.[51] Previous research has also established that the number of $CD163^+$ macrophages increases as inflammation progresses, potentially due to phenotypic shifts in macrophages within the inflamed tissue and the transformation of recruited monocytes. For instance, Kobayashi et al observed a gradual increase in $CD163^+$ macrophages during the progression of cutaneous arteritis, with the highest numbers found in the subacute phase.[52] Similarly, Guo et al demonstrated that $CD163^+$ macrophages promote angiogenesis, vascular permeability, inflammation, and disease progression in atherosclerosis.[53] Thus, the accumulation of $CD163^+$ macrophages likely contributes to the inflammatory processes in the ovaries and uteri of PCOS mice. In parallel with the increase in macrophage and CD163 expression, elevated levels of IL-6 were observed in the ovaries and uteri of PCOS mice. Conversely, IL-10 expression was decreased in the ovaries but increased in the uterus of PCOS mice. These changes in IL-10 expression align with previous studies showing elevated IL-10 mRNA levels in the uteri of DHEA-treated mice.[18]

The inflammatory microenvironment plays a pivotal role in the pathogenesis of PCOS, contributing to impaired steroid hormone production and enhanced apoptosis in GCs.[54] To replicate the effects of an inflammatory environment on ovarian GCs in vitro, COV434 cells were treated with CM from M1 and M2 macrophages. In agreement with previous findings,[20] M1 macrophages showed high expression of TNF-α and IL-1β mRNA, while M2 macrophages exhibited high expression of CCL-18 and CCL-22 mRNA, confirming their successful induction. Consistent with earlier reports indicating that CM from M1 macrophages decreases GC survival and CM from M2 macrophages enhances GC survival,[20] CM from M1 macrophages significantly increased GC apoptosis, while CM from M2 macrophages reduced GC apoptosis relative to M1 macrophages in this study. Additionally, CM from M1 macrophages led to elevated secretion of IL-6 and IL-1β in the supernatants, consistent with the increased apoptosis of GCs. Notably, sCD163 secretion was also augmented in the supernatants after stimulation with CM from M1 macrophages. These results suggest that CM from M1 macrophages promotes GC apoptosis through the secretion of inflammatory factors (IL-1β and IL-6) and sCD163. Furthermore, aligning with the in vitro findings, PCOS mice exhibited increased apoptosis in ovarian cells, with a higher incidence of apoptosis observed specifically in $CD163^+$ cells. These results collectively suggest that $CD163^+$ macrophages, by virtue of their heightened expression, contribute to ovarian cell apoptosis and play a pivotal role in the pathogenesis of PCOS.

Of course, this study has some limitations. For example, there's the small sample size of PCOS patients, and the lack of functional validation for CD163. Interestingly, sCD163 levels are elevated in many diseases, particularly inflammatory conditions, which correlate with increased macrophage activity.[40] Large cohort studies have shown that sCD163 is associated with insulin resistance[31] and serves as a risk marker for the development of type 2 diabetes mellitus.[55] In the future, the sample size could be expanded to assess the diagnostic value of sCD163 and macrophage activation in patients with PCOS. Additionally, CD163+ macrophages at sites of inflammation produce inflammatory factors, and targeting CD163-expressing macrophages represents an intriguing therapeutic strategy.[40] Moving forward, using anti-inflammatory drugs conjugated to a CD163 antibody could target and inhibit macrophage activation and suppress the inflammatory response in PCOS.

Conclusions

In conclusion, our study demonstrates that elevated sCD163 levels in the serum of patients with PCOS, coupled with increased CD163 expression in the ovaries of PCOS mice, highlight the role of macrophages in driving inflammation in PCOS. These macrophages promote the secretion of sCD163 and inflammatory cytokines, which in turn exacerbate GC apoptosis. As an inflammatory marker, CD163 plays a pivotal role in the development and progression of PCOS by facilitating GC apoptosis.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Affiliated Hospital of Jiangsu University (KY2023K1001) and the Institutional Animal Care and Use Committee (IACUC) of Jiangsu University (Permit Number: UJS-IACUC-2023061602). Informed consent was obtained from all subjects in this study.

Consent for Publication

All authors consent to publication.

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Disclosure

The authors declare that they have no conflicts interests.

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