

Engineering structure-controlled aging ovarian organoid to explore immune modulation

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Competing interests

The authors declare no conflicts of interest.

Acknowledgments

This work was supported by a start-up package funding from The Hong Kong Polytechnic University (Y. C.), the Shenzhen Science and Technology Program (No. JCYJ20241202130520027), and the National Natural Science Foundation of China (32501277).

Abbreviations

FSH, follicle-stimulating hormone; ELISA, enzyme-linked immunosorbent assay; SASP, senescence-associated secretory phenotype; CXCL9, Chemokine ligand 9; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β

Citation

Yu PJ, Liu B, Li XC, et al. Engineering Structure-Controlled Aging Ovarian Organoid to Explore Immune Modulation. *Biomed Eng Commun*. 2026;5(6):33. doi: 10.53388/BMEC2026033.

Peer review information

Biomedical Engineering Communications thanks all anonymous reviewers for their contribution to the peer review of this paper.

Editorial Advisory Board: Dao-Ming Zhu.

Production editor: Jing-Wei Ma.

Received: 20 November 2025; **Revised:** 06 January 2026;

Accepted: 11 February 2026; **Available online:** 24 March 2026.

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Abstract

Background: Ovarian aging impairs fertility and endocrine homeostasis, a process accelerated by chemotherapy. However, mechanistic studies have been limited by the absence of physiologically relevant ovarian organoid models that incorporate immune components. **Methods:** We developed a long-lived, bilayer alginate ovarian organoid system from primary mouse granulosa and stromal cells, maintaining viability and functional markers for 45 days. By integrating splenic T cells, we further established an immune-competent ovarian organoid platform. **Results:** Organoids derived from senescent cells recapitulated key aging phenotypes, including upregulation of the senescence-associated secretory phenotype (SASP), enhanced p53/p21 signaling, and reduced follicle-stimulating hormone (FSH)-induced estradiol production. In immune-competent organoids, aging models exhibited increased T cell infiltration, which was further amplified upon chemical exposure. Notably, the functional consequences of T cell infiltration were age-dependent: in young organoids, it conferred chemical stress resistance and preserved estradiol secretion, whereas in aging organoids, it exacerbated estradiol decline and correlated with a pro-inflammatory ovarian immune microenvironment. **Conclusion:** This versatile platform accurately models ovarian aging and reveals an age-dependent sensitization of the ovarian immune niche to chemotherapy, leading to divergent steroidogenic outcomes. It thus provides a powerful tool for mechanistic discovery and therapeutic screening aimed at preserving ovarian function under aging and chemical stress.

Keywords: ovarian aging, ovarian organoid, immune modulation, chemotherapy, T cell infiltration

Introduction

Ovarian aging represents one of the earliest organ-specific declines in the female body, leading to reduced fertility, endocrine dysfunction, and a shortened healthy lifespan [1, 2]. Notably, ovarian aging exerts systemic effects throughout the aging process, subsequently impacting other organ functions [3, 4]. A key hallmark of ovarian aging is the persistent, progressive increase in systemic pro-inflammatory stress, which drives ovarian tissue remodeling and functional decline [5, 6]. Immune cells within the ovary maintain normal physiological function through dynamic immune remodeling, encompassing shifts in immune cell composition, chronic low-grade inflammation, and altered signaling within the aging ovarian microenvironment [7]. For example, research indicates that aging induces ovarian macrophages to transition towards a replacement activation phenotype, potentially driven by persistent tissue remodelling [8, 9]. Beyond macrophages, single-cell RNA sequencing (scRNA-seq) analysis further reveals profound age-related alterations within ovarian T lymphocyte populations [10]. Notably, prior to reproductive function decline, naivety-like T cells appear to undergo the most pronounced transformation, differentiating into multiple effector subsets [10]. These subsets secrete IL-17 and IFN- γ , thereby promoting chronic inflammatory responses within the aging ovarian microenvironment. Moreover, therapeutic drugs used to treat age-related diseases like cancer may further perturb the immune milieu, synergistically accelerating ovarian aging [11–14]. Current models for studying ovarian aging rely predominantly on endpoint analyses, such as scRNA-seq and histopathological examination, which provide only static snapshots and fail to capture the dynamic immune-ovarian interactions over time. Therefore, developing physiologically relevant ovarian organoid models is essential to enable longitudinal analysis of immune modulation during aging and to support therapeutic screening for interventions targeting ovarian aging.

Traditional ovarian organoid construction primarily follows a “top-down” strategy. Early efforts involved mechanically fragmenting ovarian tumor tissues to form organoids that retained multiple cell types for drug screening applications [15, 16]. This approach was later adapted using healthy ovarian tissues to study normal development and regeneration [17, 18]. However, these top-down organoids lack control over cellular composition and fail to model immune-ovarian crosstalk during aging. To overcome these limitations, a “bottom-up” approach, assembling key effector cell types to mimic physiological architecture, offers a promising strategy for modeling ovarian aging. In this context, granulosa cells and stromal cells are indispensable components for functional organoid construction. Granulosa cells form the core of the follicular unit, mediating endocrine function, while stromal cells provide essential three-dimensional structural support and hormonal precursors [19, 20]. Their integration enables the creation of a biomimetic, multicellular, and hormonally responsive organoid that more accurately recapitulates ovarian tissue function and immune interactions. Recently, the model of ovarian somatic aging developed by Shweta S. et al. has partially filled the gap in organoid models of ovarian aging [21]. However, organoids of the ovary for studying immunomodulation changes in ovarian aging have yet to emerge.

Here, we presented a modular, immune-integrated ovarian organoid platform designed to interrogate immunomodulatory processes during ovarian aging. Using primary mouse granulosa and stromal cells, we engineered bilayer alginate hydrogel organoids and generate young and aging constructs, the latter achieved through *in vitro* senescence induction. By incorporating spleen T cells, we reconstructed immune-competent ovarian microenvironment to create ovarian-immune organoids. This system was further challenged with clinically relevant chemotherapeutics to evaluate age- and drug-dependent effects on immune cell infiltration and endocrine output. Together, this approach establishes a tractable and physiologically relevant model that captures key hallmarks of ovarian aging, enabling mechanistic dissection of T cell-mediated modulation of the ovarian

niche and providing a foundation for therapeutic discovery aimed at preserving ovarian function under aging and cytotoxic stress.

Methods

Animals

Mice were obtained from Centralized Animal Facilities Shenzhen (CAF SZ) at Hong Kong Polytechnic University. All animals were housed at CAF SZ and experimental procedures were approved by the Animal Subjects Ethics Sub-committee at Hong Kong Polytechnic University (24-25/1176-BME-R-STUDENT). 21-day old female C57BL/6 mice were used as donors for the isolation of granulosa and stroma cells. Animals that exhibited any unrelated pathological conditions that would have affected the study outcome were excluded from the study based on pre-established conditions included in the original animal protocol.

Cells isolation, purification and culture

Follicular granulosa cells and stromal cells were isolated from female mouse ovaries. The ovaries collected in ice-cold Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12, Gibco, NY, USA) (containing HEPES (25 mM), 2% Fetal bovine serum (FBS), L-glutamine (2 mM), penicillin (10,000 IU/mL), streptomycin (10,000 μ g/mL)) were cleansed of extraneous tissues, washed twice with ice-cold DMEM/F12 and punctured gently with 27G syringe needles in order to release the loosely packed granulosa from the follicles; cells were centrifuged at 4 °C to obtain the granulosa cells. The remaining ovaries were chopped into fine pieces of ~ 0.25 mm² and the cells released during this process were also collected and kept separately on ice. The pieces of ovaries were then incubated with collagenase (1 mg/mL) and DNase (10 μ g/mL) in DMEM/F12 for 90 min with occasional mixing. The enzyme-digested pieces fragments were dispersed into a single-cell suspension using a pipette and stored on ice as separate components. Enzyme-digested fragments were dispersed into a single-cell suspension using a pipette to yield stromal cells. Cell type purity was assessed via immunofluorescence staining using cell-specific markers: AMH (14461-1-AP, Proteintech, Wuhan, China) for granulosa cells and STAR (2225-1-AP, Proteintech, Wuhan, China) for stromal cells, separately. Purified granulosa and stromal cells were cultured in DMEM/F12 containing 10% FBS. All cells were cultured at 37 °C under an atmosphere of 5% CO₂ in humidified air until they reached 80%–90% confluency. Upon reaching 80%–90% confluent stage (~ 96 h), digest with trypsin for passage or use in organoid construction for subsequent studies.

Organoids generation and culture

Briefly, for fabricating the organoid construct. Firstly, the granulosa cells were uniformly resuspended in a 0.5% (w/v) alginate solution. Subsequently, they were dispensed into a calcium chloride solution via a microfluidic device and allowed to stand for 2 min (for cross-linking), forming inner cell spheres with a diameter of approximately 500 to 600 μ m. The inner cell spheres were coated with 0.1% (w/v) poly-L-ornithine (PLO) for 5 min. Subsequently, the cell sphere was uniformly re-coated with a sodium alginate solution mixed with stromal cells. The cell sphere was gently resuspended in culture medium, and the organoids were harvested and transferred to a 24-well plate for continued culture in DMEM-F12 medium. After 7, 14, 28, and 45 days post-culture, organoids were harvested for flow cytometry assay of apoptosis (MA0220, MeilunBio, Dalian, China) via decrosslinking with sodium citrate dsolution. For chemotherapy drug treatment, on day 3 of culture, replace the medium surrounding the organoids with DMEM-F12 medium containing 10 ng/mL doxorubicin, 50 μ M cyclophosphamide, or 10 μ M cisplatin, and continue culturing the organoids for 48 h. To optimize detection of hormone secretion levels, all organoids were cultured with 10 ng/mL follicle-stimulating hormone (FSH). Following treatment, organoids were harvested from microtiter plates, transferred to centrifuge tubes, and placed on ice for subsequent experimental use.

Immunofluorescence staining

For whole-organoid staining, organoids harvested into centrifuge tubes were first fixed with 4% paraformaldehyde. Subsequently, they underwent overnight dehydration using a 10–30% sucrose solution. Following dehydration, the organoids were blocked with 10% serum, incubated overnight at 4 °C with primary antibody, then conjugated with secondary antibody. Fluorescence intensity was observed under confocal microscopy (Nikon AX R Confocal scope, Tokyo, Japan). For organoid frozen section staining, organoids harvested into centrifuge tubes were first fixed with 4% paraformaldehyde, followed by overnight dehydration using 10%–30% sucrose solutions. Dehydrated organoids were then OCT (optimal cutting temperature compound)-embedded via liquid nitrogen rapid freezing before sectioning. Following thawing at room temperature, sections were incubated overnight at 4 °C with primary antibody, followed by secondary antibody conjugation. Mounting was performed using Fluorescence-quenching mounting medium (DAPI-containing) (P0131, Beyotime, Shanghai, China), with fluorescence intensity assessed under confocal microscopy.

ELISA assay

Briefly, various concentrations of mouse estradiol proteins in the culture supernatant were determined using a sandwich enzyme-linked immunosorbent assay (ELISA) following the protocol provided by the manufacturer, the absorbance for each well was recorded at 450 nm using a Synergy H1 Hybrid Reader (BioTek, Winooski, VT, USA).

SA-β-gal staining.

The cultured cells were fixed with a fixative solution for 5 min, then incubated with SA-β-Gal staining solution (C0602, Beyotime, Shanghai, China) at 37 °C for 12 h. Images were subsequently captured using an inverted microscope (ECLIPSE Ts2, Nikon, Tokyo, Japan) and quantified using ImageJ software.

RNA extraction and real-time quantitative PCR (RT-qPCR)

The total RNA was extracted from mixed cells from uncrosslinked organoids using an RNeasy kit (R0027, Beyotime, Shanghai, China) in accordance with the manufacturer's instructions. Subsequently, 1 µg of total RNA was reverse transcribed using SuperScript II reverse transcriptase (RR047, TaKaRa, Kyoto, Japan). Quantitative PCR analysis was performed using SYBR qPCR Master Mix (Q712Vazyme, Nanjing, China) with a real-time PCR system (CFX960, BIO-RAD, Hercules, CA, USA). The primer sequences for RT-qPCR are listed in [Supplementary Table S1](#).

Western blot analysis

To begin with, cells were lysed using RIPA (Radio Immunoprecipitation Assay) buffer (P0013B, Beyotime, Shanghai, China) containing inhibitors for proteases and phosphatases, along with PMSF (Phenylmethylsulfonyl fluoride). Subsequently, the protein content was quantified using the BCA method. The samples were diluted 10 times with pure water and lysed with a 2 × SDS buffer containing 4% SDS and 100 mM Tris-HCl (pH 6.8). Afterward, 5 × SDS loading buffer was mixed with the lysate, and this mixture was subjected to a further 10-minute incubation at 95 °C. The protein samples were resolved by electrophoresis through an SDS-PAGE gel. Subsequently, the resolved proteins were transferred onto PVDF membranes, which were then blocked with a solution containing 5% BSA in 1 × TBST. The membranes were then incubated with primary antibodies at 4 °C overnight. Afterward, the membranes were treated with HRP (horseradish peroxidase)-linked secondary antibodies for a duration of 1 h at room temperature. Ultimately, the immunoreactive bands were visualized using the ChemiDoc imaging system (BIO-RAD). The antibodies for Western blot include anti-p21 (28248-1-AP, Proteintech, Wuhan, China) and anti-p53 (80077-1-RR, Proteintech, Wuhan, China).

Statistical Analysis

All experimental data were analyzed using Prism software (GraphPad version 9). One-way or two-way ANOVA, two-sided Student's *t* test was used. A *p*-value of less than 0.05 was regarded as statistically significant. The use of *, **, ***, **** footnotes was to mark significance levels (*p*-value < 0.05, *p*-value < 0.01, *p*-value < 0.001, *p*-value < 0.0001), and ns denotes "not significant".

Results

Establishment of a bilayer alginate ovarian organoid with sustained viability and function

To restore the fundamental structure of the ovaries/follicles, we first isolated granulosa cells and stromal cells from mouse ovaries, the two primary cell types that, aside from the sparsely distributed oocytes, most closely represent ovarian structure and function ([Figure 1A](#)), and analyzed their markers by immunofluorescence using the granulosa cell marker AMH and the stromal cell marker StAR, separately ([Figure 1B](#)). We then employed a sodium alginate hydrogel as a three-dimensional scaffold and encapsulated the two cell types at defined cell densities in a bilayer configuration to mimic the native follicular/ovarian microenvironment ([Figure 1C](#)). By optimizing the crosslinking conditions, we ensured both cellular viability and structural stability ([Supplementary Figure S1A](#)).

After a period of culture, the cells organized within the hydrogel into organoid-like structures, exhibiting robust viability and expression of functional markers ([Figure 1D–E](#)). Notably, ovarian cells within these organoid-like encapsulated maintained the viability of most cells for up to 45 days ([Supplementary Figure S1B–C](#)). Although we did not assess the extent of degradation of the organoid-like encapsulate structure, we consider these observations to be consistent with the construction's stability over the experimental period.

Generation of aging ovarian organoids exhibiting SASP activation and impaired steroidogenesis

Cellular senescence accumulates with age in human and mouse ovaries and is a major driver of ovarian aging [[11](#), [22](#)]. Accordingly, we encapsulated primary mouse granulosa cells and stromal cells that had been expanded *in vitro* to a senescent state (granulosa cells at passage 6 and stromal cells at passage 7) to generate organoid-like constructs ([Figure 2A](#) and [Supplementary Figure S2A–B](#)). After assembling the constructs, we cultured young and senescent ovarian organoid-like encapsulates *in vitro* for 7 days, then dissociated them by a decoupling agent to obtain mixed-cell populations for analysis of senescence markers. Cells derived from the senescent constructs exhibited significantly increased expression of senescence-associated secretory phenotype (SASP) genes ([Figure 2B](#)) and elevated levels of senescence-associated proteins p53 and p21 ([Figure 2C](#)). Moreover, as granulosa and stromal cells are the two key steroidogenic cell types in the ovary, the organoid-like encapsulates likewise displayed hormone secretory capacity. Following stimulation with FSH, the estradiol (E2) and progesterone levels in the culture supernatants from aging organoids cultures exhibited a tendency to be lower than those in the young control group, with this effect being more pronounced at 48 h post-stimulation ([Figure 2D](#) and [Supplementary Figure S2C](#)). Consistent with the reduced hormone output, the expression of key steroidogenic genes, including *Cyp11a1*, *Cyp19a1*, and *Hsd3b*, was significantly downregulated in the aging group compared to young organoids ([Figure 2E](#)). These findings collectively indicate that the senescence-induced organoids not only secrete less hormone but also exhibit attenuated transcription of essential enzymes in the steroidogenesis pathway.

To evaluate whether this replicative senescence model recapitulates key features of physiological ovarian aging, we isolated granulosa and stromal cells directly from reproductively aged mice (13 months old). Cells from aged ovaries displayed positive SA-βgal staining when

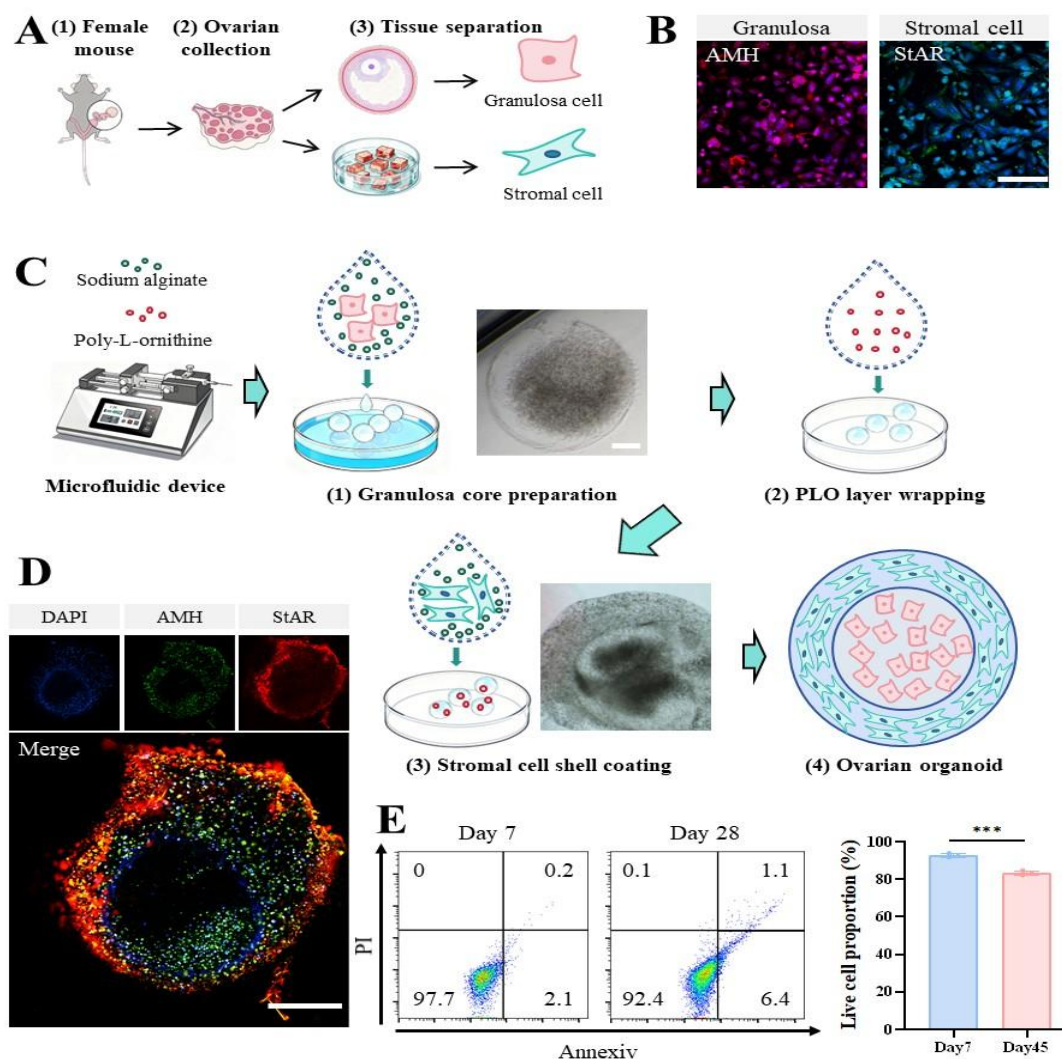


Figure 1 Ovarian organoid preparation. (A) Schematic diagram of extracting ovarian primary cells from mice for synthesizing organoids. (B) Detection of positive rates for primary mouse ovarian granulosa cells and stromal cells by immunofluorescence staining. Scale bar, 100 μ m. (C) Schematic diagram of synthetic hydrogel ovarian organoids. (D) Whole-organ immunofluorescence staining of organoids: Outer layer: stromal cells (StAR, red); Inner layer: granulosa cells (AMH, green). Scale bar, 250 μ m. (E) Flow cytometry analysis of cell viability within organoids. The left flow cytometry dot plots show representative images at Day 7 (leftmost) and Day 28 (second from left); statistical data are presented on the right, $n = 3$. The use of *, **, *** footnotes was to mark significance levels (p -value < 0.05, p -value < 0.01, p -value < 0.001). DAPI, 4',6-diamidino-2-phenylindole; AMH, Anti-Müllerian hormone; StAR, Steroidogenic acute regulatory protein; PLO, poly-L-ornithine.

cultured in monolayers (Supplementary Figure S2D). Moreover, compared to cells from young mice, they showed upregulated expression of senescence-associated genes (Supplementary Figure S2E), which was consistent with that observed in our aging organoids.

Reconstitution of the ovarian-immune interface reveals heightened T-cell infiltration in aging organoids

To recapitulate the ovarian immune microenvironment, we co-cultured the ovarian organoids with mouse spleen T cells (Figure 3A), thereby integrating them into an ovarian-immune organoid system. Through flow cytometry analysis of these spleen-derived T cells, we confirmed they primarily comprise $CD4^+CD44^{high}CD62L^{low}$ effector T cells or effector memory T cells, representing one of the principal T cell types responsible for senescence clearance (Supplementary Figure S3A). Immunofluorescence staining revealed markedly greater infiltration of T cells and other immune cells into aging organoid-like encapsulates compared with young counterparts after 48 h (Figure 3B–C and Supplementary Figure S3B–C). This phenomenon may be attributable to inflammatory recruitment factors expressed by senescent cells, thereby enabling senescent ovarian

organoid-like encapsulates to spontaneously integrate T cells. It aligns with the increased proportion of adaptive immune cells observed in aging mouse ovaries and suggests that T cells influence the immunoregulatory functions of the organoids.

To further investigate the molecular mechanisms underlying this recruitment, we examined the expression of major chemokines in aging ovarian organoid cells. Strikingly, among the chemokines evaluated, only CXCL9 was found to be markedly upregulated in aging compared to young organoids (Figure 3D–E). Consistent with this, elevated CXCL9 protein levels were detected in the supernatant of aging organoids (Figure 3F). These findings suggest that aging organoids may specifically recruit $CD4^+$ effector/effector memory T cells via CXCL9, thus facilitating the targeted migration of these T cells to inflamed sites, where they can exert cytotoxic or helper functions. This process likely contributes to immunosurveillance or clearance of senescent cells.

Collectively, these data further confirm that the immune microenvironment within senescent ovarian organoid-like encapsulates is remodeled toward a more pro-inflammatory state that favors the initiation and progression of inflammation, providing

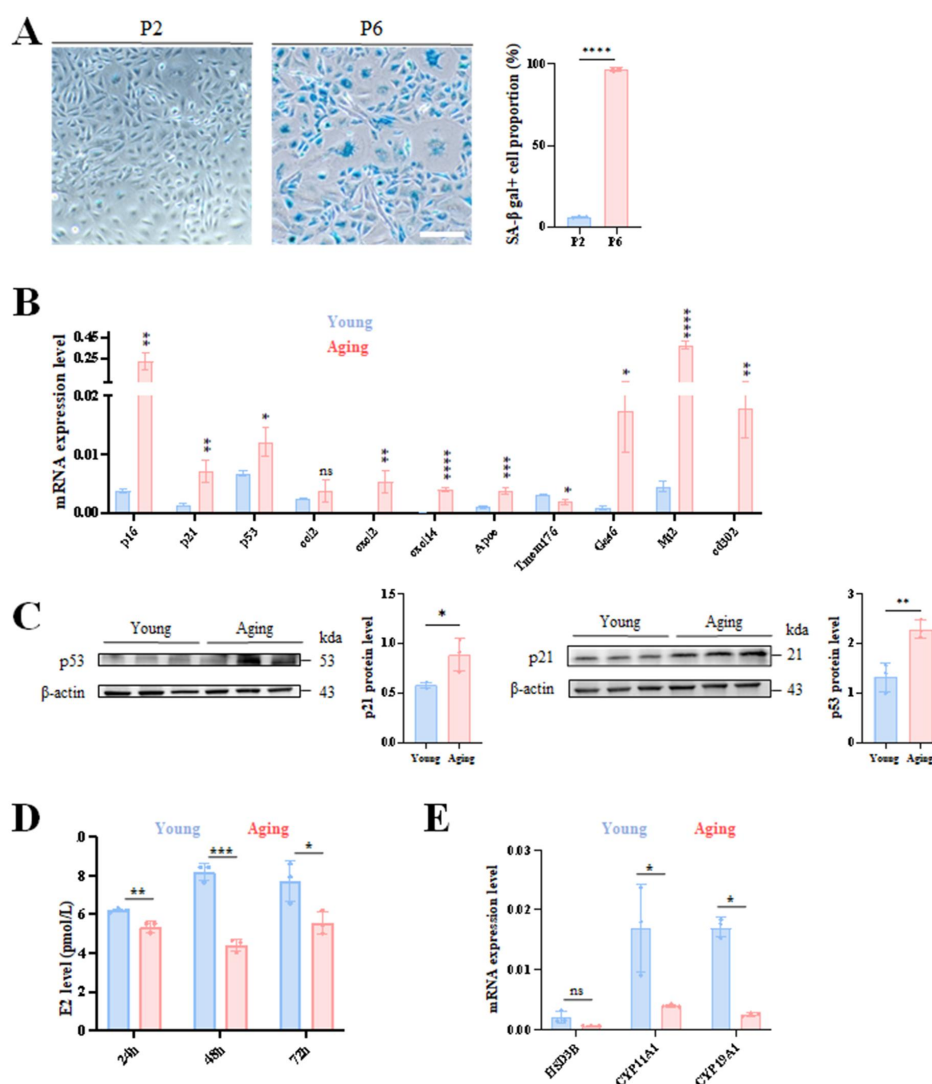


Figure 2 Aging ovarian organoid construction. (A) SA-β-gal staining of senescent ovarian cells. Representative images (left); statistical data (right), $n = 3$. Scale bar, 100 μm . (B) qPCR detection of SASP mRNA levels in young/aging organoid cells, $n = 3$. (C) Western blot analysis the levels of senescence marker proteins in young/aged organoid cells, $n = 3$. (D) Expression levels of estradiol (E2) in the supernatant of young/aged ovarian organoid culture medium by ELISA assay, $n = 3$. (E) qPCR detection of hormone secretion-related genes mRNA levels in young/aging organoid cells, $n = 3$. The use of *, **, ***, **** footnotes was to mark significance levels (p -value < 0.05 , p -value < 0.01 , p -value < 0.001 , p -value < 0.0001), and ns denotes “not significant”. p16, Cyclin-Dependent Kinase Inhibitor 2A; p21, cyclin dependent kinase inhibitor 1A; p53, tumor protein p53; ccl2, CC Motif Chemokine Ligand 2; cxcl2, C-X-C Motif Chemokine ligand 2; cxcl14, C-X-C Motif Chemokine Ligand 14; apoe, apolipoprotein E; Tmem176, transmembrane protein 176; gas6, growth arrest specific 6; mt2, metallothionein 2; cd302, CD302 molecule; e2, estradiol; cyp11a1, cytochrome P450 family 11 subfamily A member 1; Cyp19a1, cytochrome P450 family 19 subfamily A member 1.

robust experimental support for deeper insights into immune alterations during ovarian aging.

Chemotherapy selectively amplifies T-cell infiltration in aging ovarian-immune organoids and modulates estradiol output

Chemotherapy accelerates ovarian aging, heightens intraovarian inflammation, and consequently impairs ovarian function. Based on studies involving in vitro experiments on follicular or ovarian cells, we challenged the ovarian-immune organoids with commonly used chemotherapeutics (doxorubicin, cisplatin, and cyclophosphamide) and found that, relative to PBS-treated controls, T cell infiltration did not change significantly in young ovarian-immune organoids (Figure 4A). In contrast, T-cell infiltration became more pronounced in old ovarian immune organoids following exposure to doxorubicin or cisplatin (Figure 4B), suggesting that the aging ovarian immune microenvironment is more susceptible to chemotherapeutic perturbation.

To determine whether chemotherapy affected cellular localization within organoids, we performed immunofluorescence staining on frozen sections (Supplementary Figure S4A). The results showed that the overall cellular localization patterns remained largely unchanged after Dox treatment, indicating that increased T cell infiltration is not due to major structural alterations of the organoid architecture. We further examined whether hormone secretory function was affected. Compared with PBS controls, young ovarian immune organoids exposed to doxorubicin exhibited a modest but significant increase in E2 secretion (Figure 4C). We hypothesized that the incorporation of T cells may alter the effects of chemotherapy drugs on organoids. Consequently, we concurrently assessed T cell inflammatory factors in the supernatant, revealing significantly elevated expression levels of tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) in the aged ovarian immune organoids group compared to the younger control group (Figure 4D).

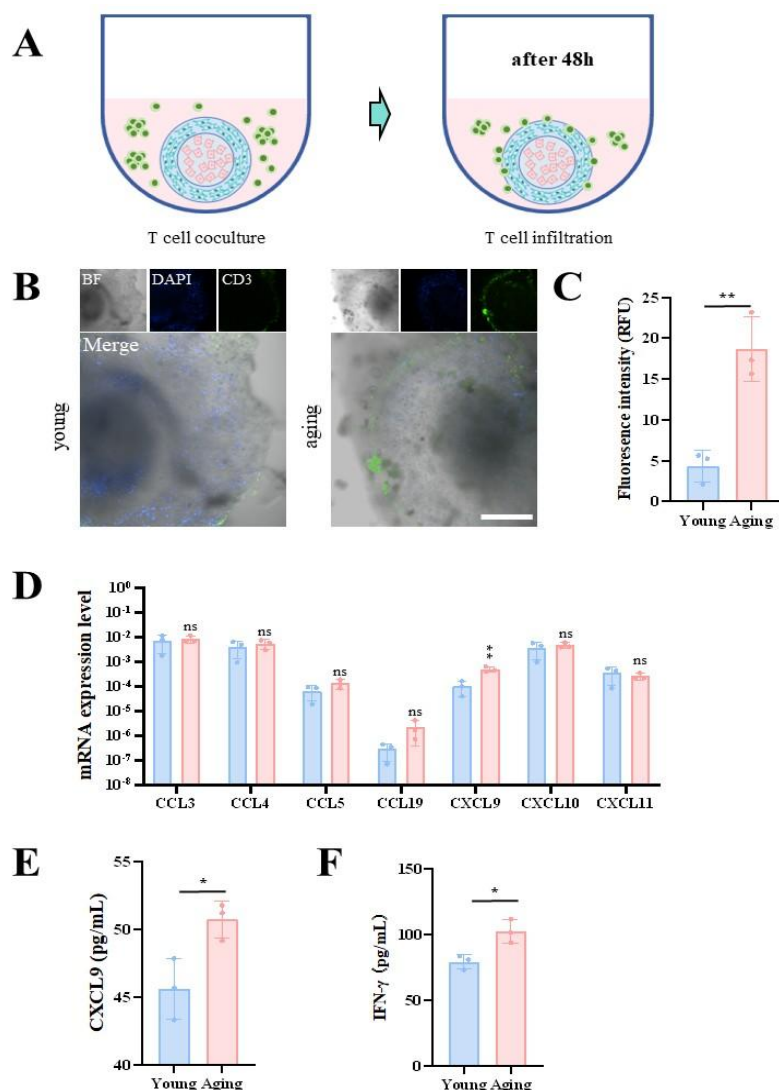


Figure 3 T cell infiltration in ovarian organoids. (A) Schematic diagram of co-culture between aged/young ovarian organoids and T cells. (B) Whole-organ representative images of organoid immunofluorescence staining to detect T-cell infiltration following 48 h of co-culture. Scale bar, 100 μ m. (C) Statistical analysis of immunofluorescence in the CD3 channel in (B) using ImageJ. (D) qPCR detection of chemokine mRNA levels in young/aging organoid cells, $n = 3$. (E-F) Expression levels of Chemokine ligand 9 (CXCL9) and Interferon gamma (IFN- γ) in the supernatant of young/aged ovarian organoid culture medium by ELISA assay, $n = 3$. The use of *, ** footnotes were to mark significance levels (p -value < 0.05, p -value < 0.01), and ns denotes "not significant". ccl3, C-C motif chemokine ligand 3; ccl4, C-C motif chemokine ligand 4; ccl5, C-C motif chemokine ligand 5; ccl19, C-C motif chemokine ligand 19; cxcl9, C-X-C motif chemokine ligand 9; cxcl10, C-X-C motif chemokine ligand 10; cxcl11, C-X-C motif chemokine ligand 11; ifn- γ , interferon gamma.

Moreover, following doxorubicin stimulation, IL-1 β levels markedly increased whilst TNF- α levels markedly decreased in the aged ovarian immune organoids group (Figure 4D). To further clarify the molecular basis for this observation, we measured the expression of cyp19a1 in the supernatant after chemotherapeutic stimulation and found that its levels paralleled the increase in E2 in the corresponding groups (Supplementary Figure S4B). This may indicate a regulatory role for T cell inflammatory factors in modulating ovarian cytokine secretion capacity. These findings indicate that the T cell containing ovarian immune microenvironment can modulate chemotherapy-induced alterations in ovarian function.

Discussion

Aging profoundly impacts female reproductive health, with ovarian function declining earlier than most organ systems, leading to reduced fertility and increased risks of chronic diseases. This decline is driven

by complex interactions between cellular senescence and immune remodeling, processes that are increasingly understood through advanced *in vivo* models and multi-omics approaches. However, traditional *in vivo* models face limitations in throughput and experimental control, prompting the development of innovative systems such as organoids and spatial transcriptomics to dissect these mechanisms with greater precision.

We established a bilayer alginate hydrogel system encapsulating primary mouse granulosa and stromal cells at defined densities, achieving structural stability and viability up to 45 days. Aging was modeled by using *in vitro*-expanded cells (granulosa P6, stromal P7) that exhibit robust senescence signatures (SASP upregulation; increased p53/p21) and reduced FSH-stimulated E2 and progesterone output. The immune niche was reconstituted via co-culture with spleen-derived T cells to generate ovarian-immune organoids. This modular workflow provides a reproducible route to aging organoids suitable for functional and pharmacological interrogation.

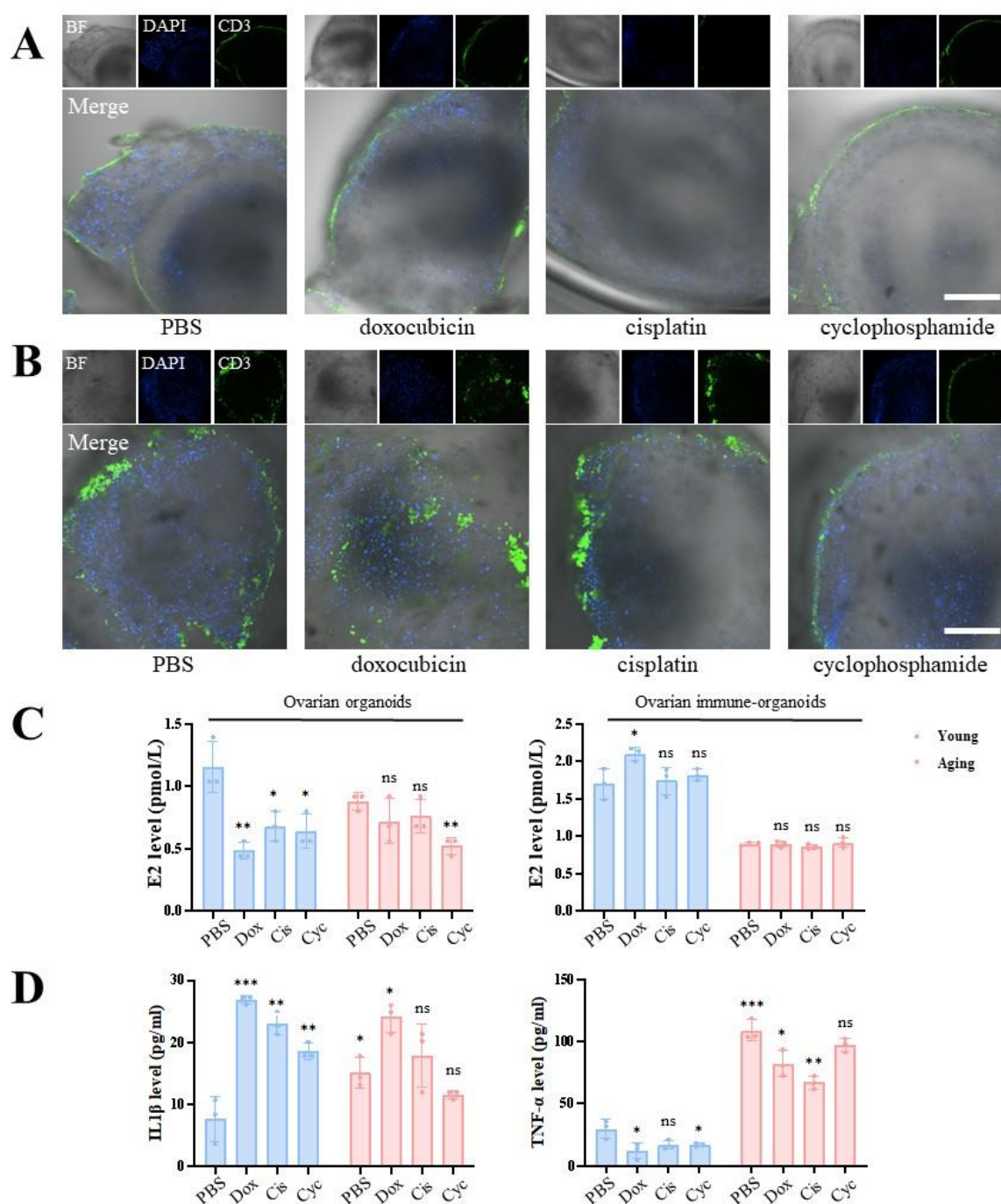


Figure 4 Chemotherapeutic agents affect the ovarian infiltrating capacity of T cells. Representative immunofluorescence images following 48-hour co-culture of young (A) or aged (B) ovarian organoids with T cells, followed by 24-hour stimulation with chemical reagents. Scale bar, 100 μ m. (C) E2, (D) IL-1 β and TNF- α expression levels in the supernatant following 48 h of co-culture between young or aged ovarian organoids and T cells, after 24 h of stimulation with chemical reagents. Scale bar, 100 μ m. The use of *, **, ***, **** footnotes were to mark significance levels (p -value < 0.05, p -value < 0.01, p -value < 0.001, p -value < 0.0001), and ns denotes “not significant”. BF, bright field; PBS, Phosphate buffered saline; Dox, doxorubicin; Cis, cisplatin; Cyc, cyclophosphamide; e2, estradiol; IL1- β , interleukin 1 beta; TNF- α , tumour necrosis factor alpha-like.

Our study also revealed that T cell infiltration is markedly increased in aging ovarian organoids, particularly following exposure to relevant chemotherapeutic agents. Mechanistically, this enhanced infiltration appears closely linked to upregulation of the chemokine CXCL9, which we found to be significantly elevated in aged organoids at both the mRNA and protein levels. Notably, CXCL9 is a well-established ligand for CXCR3, a receptor highly expressed on effector and effector memory T cells, especially in the context of IFN- γ -rich environments. Indeed, increased IFN- γ expression further

supports a microenvironment favoring T cell recruitment and activation in aging organoids. This cytokine milieu, in turn, could account for the preferential migration and retention of CD4⁺CD44^{high}CD62L^{low} T cells observed in our system.

Interestingly, upon doxorubicin exposure, culture supernatants from young ovarian immune-organoids showed a modest increase in E2, accompanied by elevated IL1 β and reduced TNF α . T cell-derived inflammatory mediators are known to regulate ovarian steroidogenesis. Specifically, the IL1 system is a key modulator in

preovulatory bovine follicles, and IL1 β has been shown to upregulate STAR and promote progesterone production in human granulosa–theca cells via ERK1/2 and p38-mediated CREB signaling [23–25]. By contrast, TNF α is widely reported to impair ovarian steroid hormone secretion [26]. The upregulation of cyp19a1 (aromatase) in aging organoids and its further elevation following doxorubicin treatment suggest that recruited T cells can modulate steroidogenic enzyme expression and subsequently estradiol production potentially via CXCL9/IFN- γ -mediated signaling. This points to a bidirectional dialogue between infiltrating T cells and ovarian parenchymal cells, wherein immune-derived signals reshape not only the local inflammatory landscape but also the endocrine activity of the aging ovary. The temporal sequence and causality along this ovarian–immune axis, especially how these T cell subsets and chemokine–cytokine networks regulate key steroidogenic pathways, will require prospective perturbations and multi-omics analyses for definitive validation.

However, several limitations in this study should be acknowledged. (1) The senescent cell model used in our organoids was established through in vitro expansion, inducing replicative and culture stress. While this approach reliably evokes senescence markers and phenotypes and partially recapitulates aspects of ovarian aging, it does not fully represent the complexity of cellular aging that occurs in vivo, where additional factors such as reactive oxygen species (ROS), genotoxic stress/DNA damage, metabolic shifts, and mitochondrial dysfunction all contribute to the aging process. This limitation could constrain the generalizability of the findings to "natural" ovarian aging. To address this, future studies should incorporate orthogonal senescence triggers, including oxidative stress (such as H₂O₂ treatment), irradiation, telomere dysfunction, or low-dose chemotherapeutic regimens, and compare immune and endocrine responses across models. Combining different stressors would more accurately model the multifaceted nature of tissue aging and may reveal unique or shared features of immune cell recruitment and organoid behavior in aging contexts. (2) We utilized spleen-derived T cells, which, though convenient for isolation in mice and amenable to flow cytometric subsetting, are not directly equivalent to the diverse tissue-resident T cell populations that inhabit the ovarian microenvironment in vivo. Our flow analysis validated that the majority of these T cells are of the CD4⁺CD44^{high}CD62L^{low} phenotype, tentatively classifying them as effector or effector memory T cells, which is a relevant subset for tissue infiltration and immune surveillance. However, the precise contribution of this specific T cell subset to the observed phenomena, as well as the potential roles of other tissue-resident subsets (such as tissue-resident memory T cells, Tregs, or cytotoxic CD8⁺ T cells), remains uncertain. Future studies should isolate and purify the subset for focused co-culture experiments and simultaneously explore the roles of alternative T cell populations to dissect their different effects on ovarian organoid function. (3) Matrix biology, the organoids were embedded in alginate hydrogels, which are inert and provide a tunable mechanical environment but lack inherent degradability and cell-adhesive motifs (e.g., RGD). The absence of degradability may impede authentic cell migration and tissue remodeling, underestimating immune cell infiltration and the physical interactions between immune and ovarian cells. To overcome this, future studies should employ bioengineered matrices with controlled degradability, such as partially oxidized alginate, peptide-cleavable hydrogels, or hybrids incorporating ECM components. Such material innovations will make it possible to study not only infiltration but also dynamic immune–parenchymal interactions and matrix remodeling, offering more physiologically and translationally relevant insights. (4) Readouts and kinetics, immune infiltration was primarily assessed by immunofluorescence; quantitative live imaging, transwell migration surrogates, or 3D segmentation would provide dynamic insight. Finally, dose–time matrices and recovery phases would improve translational relevance.

Conclusion

We established a modular ovarian–immune organoid platform that recapitulates key hallmarks of ovarian aging, including senescence activation, impaired hormone synthesis, and heightened immune infiltration. This tractable platform enabled direct testing of how senescence altered endocrine output and immune behavior, and how chemotherapeutics perturbed these axes. It fills a critical gap for hypothesis-driven discovery and preclinical screening aimed at preserving ovarian health during aging and cytotoxic stress.

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