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Immune imbalance in the pre-ovulatory follicular microenvironment of overweight and obese women during IVF

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Abstract

Background Overweight and obesity can induce an inflammatory milieu in the oocyte microenvironment and are closely associated with reduced assisted reproductive outcomes.

Objective How are immune cells, cytokines and lipid profiles altered in the pre-ovulatory microenvironment of overweight and obese women?

Methods 32 women undergoing in vitro fertilization (IVF) were included, with 14 overweight or obese (OW) and 18 normal weight (NW) participants. Serum was collected before ovulation induction, follicular fluid (FF) and aspirates were obtained during oocyte retrieval for flow cytometry, cytokines, hormone, and lipid profiles measurement. Clinical outcomes were recorded through a one-year follow-up.

Results The percentage of T cells in the pre-ovulatory follicular microenvironment, especially $CD4^+$ T cells, increased significantly in the OW group, which positively related with BMI. Notably, type 2 cytokine *IL4* and *IL13* transcription level in OW group had significantly increased, while the type 1 cytokine *IFNG* only showed a non-statistically significant upward trend. Lipid profiles were screened, revealing no difference between the two groups, however, levels were higher in serum compared to FF. Additionally, the concentration gradient of TG between serum and FF was 22-fold in OW group (2.92 ± 3.66 vs. 0.13 ± 0.03), which was significantly higher than the 12-fold gradient observed in NW group (1.72 ± 0.95 vs. 0.14 ± 0.08). Furthermore, day 3 high quality embryos rate is negatively associated with BMI and exhibits a decreasing trend in OW group.

Conclusion Overweight and obesity can disrupt immune hemostasis in the pre-ovulatory follicular microenvironment, potentially leading to adverse effects on assisted reproductive outcomes.

Keywords Obesity, Pre-ovulatory follicular microenvironment, T cells, Cholesterol, IVF

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Introduction

The escalating prevalence of obesity and overweight individuals poses a significant global public health challenge [1]. In China, the incidence rate of overweight and obese females approximates 37%, with a consistent upward trend [2, 3]. Apart from the rising incidence of metabolic syndrome, cardiovascular disease, type 2 diabetes and other diseases [4, 5], excess weight can adversely impact female fertility by compromising oocyte quality, early embryonic development and endometrial receptivity [6, 7]. The pathogenesis of these conditions has been linked to the chronic inflammatory state induced by overweight and obesity [8, 9].

Chronic inflammation in overweight and obese individuals is primarily characterized by an alteration in immune cells profile within adipose tissue towards a pro-inflammatory state, accompanied by changes in the distribution and abundance of circulating immune cells [10–12]. The follicular microenvironment, comprising cells in the follicular fluid and granulosa cells surrounding the oocyte, plays a crucial role in oocyte maturation, embryo fertilization, and early development [13–15]. The quantity and functionality of immune cells within the follicular microenvironment, including dendritic cells, macrophages, NK cells, B cells, and T cells are closely associated with follicular development, ovulation, and oocyte quality [16–18]. Prior research has confirmed that diet-induced obesity in mice led to a significant increase in immune cell presence in the ovaries and heightened expression of pro-inflammatory genes [19, 20].

Inflammation and pro-inflammatory cytokines, such as IL-6 and IL-8, can impair oocyte competence and reduce both day 3 embryo quality and blastocyst quality [21, 22]. Concurrently, a significant rise in inflammation levels in follicular fluid is correlated with an increase in body mass index (BMI) or obesity, primarily characterized by elevated levels of C-reactive protein (CRP), pro-inflammatory cytokines interleukin (IL)-18, IL-1β, and tumor necrosis factor-α (TNF-α) [23–26]. Obesity-induced changes in inflammatory proteins within the follicular microenvironment can upregulate pro-inflammatory transcripts in oocytes, potentially disrupting oocyte and embryo development [27]. During assisted reproduction, the clinical pregnancy rate, cumulative pregnancy rate, and cumulative live birth rate of overweight and obese women decline with increasing BMI [28, 29]. This diminished reproductive prognosis may be attributed to chronic inflammation of the ovaries [30, 31].

While inflammation is recognized to exist in the oocyte microenvironment of overweight and obese individuals, alterations in immune cells are not well comprehended. Follicular aspiration during IVF oocyte retrieval involves retrieving follicular fluid, numerous granulosa cells, non-granulosa cells, and erythrocytes. The primary non-granulosa cell type is immune cells, which have been demonstrated to differ from circulating immune cells and can reflect the unique characteristic of immune cells within the follicular microenvironment [32]. Hence, we opted to use follicular aspirates to examine changes in immune cells within the pre-ovulatory follicular microenvironment, aiming to investigate the impact of overweight and obesity on the oocyte microenvironment.

Methods

Patient selection

This study enrolled participants who underwent in vitro fertilization (IVF) at the Shanghai JiAi Genetics and IVF Institute from December 2022 to March 2023. This study received approval and oversight from the Ethics Committee of Shanghai JiAi Genetics and IVF Institute (JIAI E2021-27). All patients provided signed informed consent forms prior to the commencement of the study.

According to the demographic characteristics of China, the obesity diagnosis in this study adheres to the following criteria: normal range, $18.50 < \text{BMI} < 24.00$ kg/m^2; Overweight, $24.00 \leq \text{BMI} < 28.00$ kg/m^2; Obesity, $\text{BMI} \geq 28.00$ kg/m^2 [2, 33]. The inclusion criteria are as follows: [1] Normal menstrual cycle; [2] Accept IVF treatment for male factor infertility or tubal factor infertility. The exclusion criteria consist of: [1] Patients with diminished ovarian reserve; [2] Patients with endometriosis and adenomyosis; [3] Patients with polycystic ovary syndrome; [4] Women with genital abnormalities, chronic hypertension, diabetes, autoimmune diseases, infectious diseases or liver, kidney, cardiovascular or thyroid diseases. Participants will be categorized into normal weight (NW) group and overweight and obese (OW) group.

Controlled ovarian stimulation

All women selected the gonadotropin-releasing hormone antagonist (GnRH-ant) ovarian stimulation protocol. They were administered recombinant human follicle stimulating hormone (rFSH; Gonal-f; Merck Serono, Geneva, Switzerland) and human menopausal gonadotropin (HMG, Livzon, Zhuhai, China) starting on the 2nd or 3rd of menstrual cycle. When the dominant follicle reaches 14 mm or the serum estradiol (E_2) level reaches 350 pg/ml, the GnRH-ant (Cetrotide; Merck Serono, Geneva, Switzerland) is administered. This regimen is continued until the dominant follicle reaches 18 mm or until two follicles reach 16 mm. Subsequently, 6000 IU of recombinant human chorionic gonadotropin (rhCG; Lidebo, Zhuhai, China) was used as a trigger. Transvaginal oocyte retrieval was performed 36 h after trigger injection.

Patient and cycle variables collect

Demographic data, cycle characteristics and outcomes of participants were collected. Demographic information comprised age, basal hormone levels, and ovarian reserve indicators. Oocyte maturity was defined as the proportion of metaphase II (MII) oocytes to the total number of oocytes collected. Normal fertilization was confirmed by the presence of two pronuclei 16–18 h after insemination. Evaluation of day 3 embryo quality included assessing the number of blastomeres, the degree of fragmentation, and the uniformity of blastomeres. High quality day 3 embryos were defined as those with at least seven blastomeres and were morphologically evaluated as grade 1–2. Embryo morphology was scored based on Scott's criteria, grade 1 embryos had blastomeres of equal size with no cytoplasmic fragments, and grade 2 embryos had blastomeres of equal size with minor cytoplasmic fragments or blebs [34]. In case embryo transfer is conducted, document the cycle outcomes post-transfer. Positive pregnancy test is identified by β-hCG positivity. Clinical pregnancy is defined by the identification of a gestational sac during transvaginal ultrasound examination around 7 weeks of gestation.

Biological sample collection

Before controlled ovulation stimulation, peripheral blood was drawn via venipuncture, and the serum was obtained and store at -80 ℃ until required. Transvaginal oocyte retrieval was performed under ultrasound guidance. Follicular fluid was extracted from the most accessible single follicle to minimize blood contamination. After centrifugation, the supernatant was collected and stored at -80 ℃ for future use. Follicular aspirates were collected from the remaining follicles of each individual. Lymphocyte separation solution (Hao Yang Biological Manufacture Co., LTD; Tianjin, China) was utilized for performing differential centrifugation. Cells from the middle layer of the separation solution were harvested. Red blood cells were lysed using red blood cell lysis buffer (Invitrogen; Thermo Fisher Scientific; Massachusetts, USA). After stopping the lysis process, the cell sediment was collected for further experiments.

Flow cytometry

According to the manufacturer's protocol, incubate the harvested lymphocytes collected from follicular aspirates with the specified antibodies at 4 ℃, shielded from light for 30 min: CD45-APC/Cy7 (BioLegend Cat# 368515, RRID: AB_2566375), CD3-FITC (BioLegend Cat# 300305, RRID: AB_314041), CD4-APC (BioLegend Cat# 300514, RRID: AB_314082), CD8-PerCP/Cyanine5.5 (BioLegend Cat# 344709, RRID: AB_2044009), CD11c-APC (BioLegend Cat# 301613, RRID: AB_493024), CD14-FITC (Thermo Fisher Scientific Cat# 11-0149-41, RRID: AB_10597445), CD16-PE (BioLegend Cat# 302007, RRID: AB_314207), CD19-PE (BioLegend Cat# 302207, RRID: AB_314237), CD56-APC-R700 (BD Biosciences Cat# 565139, RRID: AB_2744429), CD66b-PerCP/Cyanine5.5 (BioLegend Cat# 305107, RRID: AB_2077856), HLA-DR-PECy7 (BD Biosciences Cat# 560651, RRID: AB_1727528). Flow cytometry data were acquired using BD FACS Celesta flow cytometer (BD Bioscience, California, USA) and analyzed with FlowJo software (version 10.8.1; TreeStar, California, USA).

RNA isolation and RT-qPCR

After isolating total RNA from lymphocytes collected from follicular aspirates, utilize cDNA reverse transcription kit (Takara Bio Inc., Shiga, Japan) to generate complementary DNA (cDNA). The primers for *IL4* (ID 3565), *IL5* (ID 3567), *IL6* (ID 3569), *IL10* (ID 3586), *IL13* (ID 3596), *IL17* (ID 3605), *TNFA* (ID 7124), and *IFNG* (ID3458) in humans were sourced from Primer Bank with Gene IDs retrieved from GenBank. Conduct quantitative PCR using the SYBR Premix Ex Taq II (Perfect Real Time) (Takara Bio Inc., Shiga, Japan) following the provided instructions. The expression level of each target gene was presented as a "fold change" relative to the control sample.

Follicular fluid and serum cytokine measurement

The follicular fluid and serum cytokines were evaluated using the Luminex platform following the guidelines provided in the ProcartaPlex Hu Th1/Th2/Th9/Th17/Th22/Treg plex kit (Thermo Fisher Scientific; Massachusetts, USA). The cytokines measured include granulocyte macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), TNF-α, IL-13, IL-12p70, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-18, IL-10, IL-17 A, IL-21, IL-23, IL-27, IL-9. The cytokine concentrations were quantified using Luminex 200 (Luminex Corporation, Texas, USA) following a standard curve.

Lipid profile measurement

Total cholesterol (TC), triglycerides (TG), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), and free fatty acid (FFA) levels in follicular fluid samples and serum were quantified utilizing an enzyme-linked immunosorbent assay (ELISA) reader (Tecan Sunrise; Männedorf, Switzerland) and commercially available kits (Nanjing Jiancheng Bioengineering Institute Co., Ltd., Jiangsu, China).

Statistical analysis

Continuous variables were compared using the Student's t-test or Mann-Whitney U test, while categorical variables were analyzed Fisher's exact test. ANOVA analysis was utilized for comparing multiple variables. The

Wilcoxon matched-pairs signed-rank test was applied for analyzing paired data analysis with non-normal distribution. Spearman and Pearson correlation analysis was used to investigate the associations between variables. Additionally, correlation and local estimation scatter plot smoothing (LOESS) analysis were employed to visually assess nonlinear relationships and trends. Statistical significance was determined based on the p value, with $p < 0.05$ considered indicative of statistical significance. Statistical analysis was carried out using GraphPad Prism 8 (version 9.5.1, GraphPad Software Inc., California, USA), SPSS (version 26.0, IBM, New York, USA) and R (version 4.4.1, R Foundation for Statistical Computing, Vienna, Austria).

Results

Clinical biochemical characteristics of recruited participants

The demographic and clinical biochemical characteristics of the participants are presented in Table 1. Only BMI was significantly higher in OW group compared to the NW group (20.96 ± 1.60 vs. 26.73 ± 2.03, $p < 0.001$). No significant differences were observed in age, anti-Müllerian hormone (AMH) levels, basal levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), E_2 or testosterone (T), as well as E_2 levels on rhCG trigger day between the two groups.

Alterations in the immune cell composition in the pre-ovulatory follicular microenvironment of overweight and obese women

Flow cytometry analysis revealed that the proportion of NK cells was comparable between the two groups (Fig. 1A). OW group exhibits a significantly higher proportion of B cells and T cells, in the follicular microenvironment before ovulation compared to NW group (Fig. 1B). Further analysis of T cell subsets revealed an increase in $CD4^+$ T cells, while there was no change in $CD8^+$ T cells. (Fig. 1C). In the main subsets of myeloid immune cells, the proportions of monocytes, dendritic cells and neutrophils did not differ significantly between the two groups (Fig. 1D-F). These findings suggest a more pronounced inflammatory response in the pre-ovulatory follicular microenvironment of OW women.

Cytokine levels in the follicular fluid and serum of the OW and NW group

Analysis of immune cell composition in the preovulatory follicular microenvironment suggests that OW group exhibit an increased $CD4^+$ T proportion. Therefore, we examined the related cytokines expression. The mRNA expression levels of *IL4* and *IL13* in the pre-ovulatory follicle microenvironment of OW women were significantly increased before ovulation (Fig. 2A). Compared with the NW group, the *IFNG* mRNA expression level of OW women tended to increase, but the difference was not significant ($p = 0.058$) (Fig. 2A). Additionally, we found that there are differences in the concentration of certain cytokines between blood and follicular fluid. The IFN-γ content in follicular fluid was approximately 2 times higher than its content in serum (2.02 ± 1.34 vs. 1.03 ± 0.72, $p < 0.05$) (Fig. 2B). The levels of IL-10 in serum were lower than those in follicular fluid (0.09 ± 0.44 vs. 0.41 ± 0.47, $p < 0.05$), while TNF-α exhibited a decreasing trend but not statistically significant (0.89 ± 0.81 vs. 1.08 ± 0.67, $p = 0.064$). However, there was no significant difference in the quantification of other cytokines in follicular fluid and serum, including IL-18, which previously been reported to be positively correlated with follicular maturation [24] (Fig. 2B). Collectively, our results suggested an increased transcription level of type 2 pro-inflammatory cytokines in the pre-ovulatory microenvironment of OW women.

Table 1 Baseline characteristics of the study groups

	Normal weight ($n = 18$)	Overweight and Obese ($n = 14$)	All ($n = 32$)	p value
	Mean ± SD	Mean ± SD	Mean ± SD	
Age (years)	33.89 ± 3.64	33.93 ± 4.03	33.91 ± 3.75	0.954
BMI (kg/m^2)	20.96 ± 1.60	26.73 ± 2.03	23.48 ± 3.40	< 0.001
AMH (ng/mL)	3.39 ± 1.33	3.07 ± 1.67	3.26 ± 1.46	0.563
Basal LH (mIU/mL)	5.39 ± 2.23	4.15 ± 2.73	4.85 ± 2.49	0.168
Basal FSH (mIU/mL)	7.74 ± 1.88	6.55 ± 1.83	7.22 ± 1.93	0.082
Basal E_2 (pg/L)	42.78 ± 14.94	47.51 ± 29.06	44.85 ± 21.96	0.586
Basal T (nmol/L)	0.90 ± 0.63	0.88 ± 0.75	0.89 ± 0.67	0.770
E_2 on hCG trigger day (pg/L)	3562.06 ± 1880.94	3407.21 ± 2098.00	3494.31 ± 1947.33	0.828

BMI, body mass index; AMH, anti-Müllerian hormone; LH, luteinizing hormone; FSH, follicle-stimulating hormone; E_2, estradiol; P, progesterone; T, testosterone; TC, total cholesterol; TG, triglycerides; LDL-C, low density lipoproteins cholesterol; HDL-C, high density lipoproteins cholesterol, hCG, human chorionic gonadotropin

The baseline characteristics were compared between the normal weight and overweight and obese women. Normal distributed data was compared using Student's t test and non-normal distributed data was analyzed using Mann-Whitney U Test

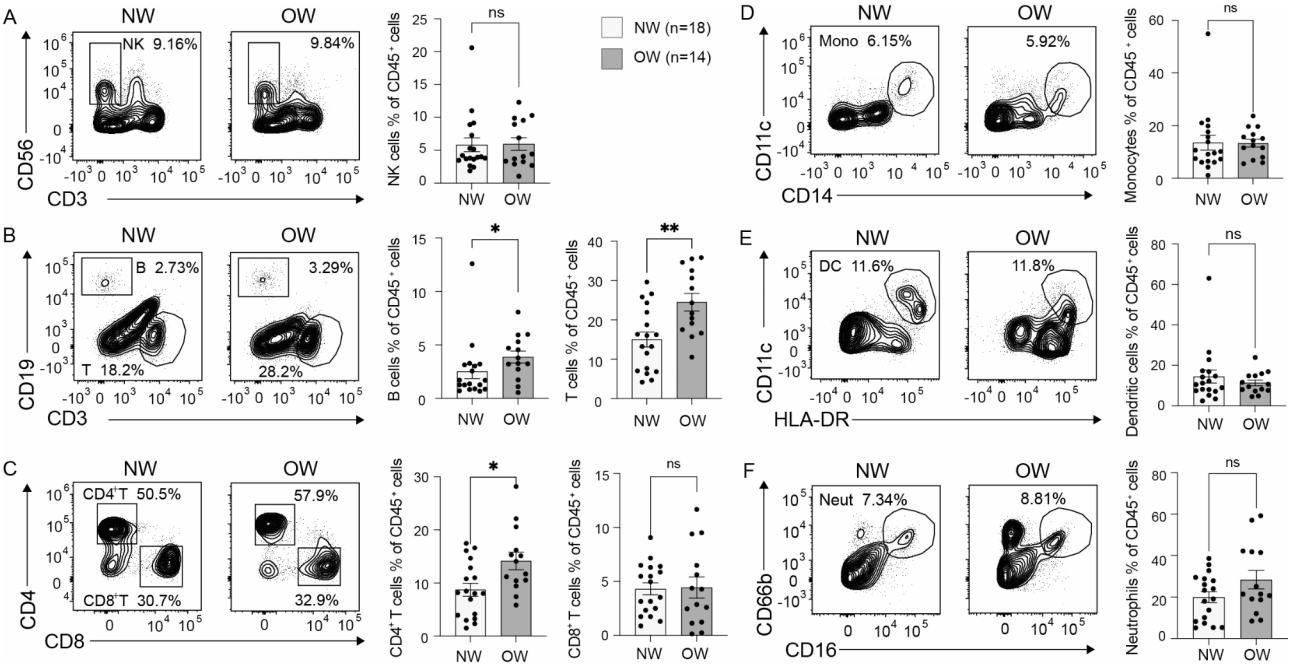

Fig. 1 The composition of immune cells in the pre-ovulatory microenvironment representative flow cytometry results, showing identification and comparing percentage of NK cells (**A**), B cells and T cells (**B**), $CD4^+$ T cells and $CD8^+$ T cells (**C**), monocytes (**D**), dendritic cells (**E**), neutrophils (**F**) between NW and OW patients. Each data point represents an individual subject, data are expressed as mean ± SEM, * $p < 0.05$, ** $p < 0.01$. ns, not significant. (Normal distributed data was compared using Student's t test and non-normal distributed data was analyzed using Mann-Whitney U Test.)

Lipid profiles in the follicular fluid and serum of OW and NW groups

As depicted in Table 2, TG, TC, LDL-C, and FFA exhibited no significant differences in follicular fluid and serum between the OW and NW groups. The serum HDL-C levels in the OW group showed a non-statistically significant downward trend ($p = 0.100$); however, this trend was not observed in the follicular fluid (Table 2). The lipid content in the serum is significantly higher than that in the follicular fluid. Additionally, the concentration gradient of TG between serum and FF was 22-fold in the OW group (2.92 ± 3.66 vs. 0.13 ± 0.03), which was markedly higher than that observed in the NW group, where the gradient was 12-fold (1.72 ± 0.95 vs. 0.14 ± 0.08) (Table 2). In the serum, TC is about 4 times higher, TG is about 17 times higher, LDL-C is about 15 times higher, HDL-C and FFA are about 2 times higher than that in the follicular fluid (Fig. 3). These findings suggest that the blood-follicle barrier plays a role in regulating the lipid composition in the follicular microenvironment.

Comparison of IVF outcomes between the OW and NW group

We follow-up on the IVF outcomes of two groups. No significant differences were found between the two groups regarding the number of retrieved oocytes, metaphase II (MII) rate, fertilization rate and normal cleavage rate (Fig. 4A-D). The day 3 high quality embryo rate in OW women was slightly lower than that in the NW group; however, the difference was not statistically significant. ($p = 0.072$) (Fig. 4E). Through comparison that the positive pregnancy test rate, clinical pregnancy rate, and cumulative pregnancy rate of the OW group showed a decreasing trend. However, this trend may not be statistically significant due to the limited sample size (Table 3). These findings suggest that overweight and obesity may affect embryo quality and assisted reproductive outcomes; however, studies with larger sample sizes are needed to confirm the above trends.

The correlation among BMI, embryo quality and immune cells in the follicular microenvironment before ovulation

T cells and $CD4^+$ T cells in the pre-ovulatory follicular microenvironment exhibit a positively correlated with BMI, and day 3 high quality embryos rate shows a negatively correlation with BMI (Table 4). LOESS correlation analysis revealed a consistent pattern, with T cells and $CD4^+$ T cells demonstrating an increasing trend at a BMI of around 23 kg/m^2 (Fig. 5A **and B**), while day 3 high quality embryos rate began to exhibit a decreasing trend at a BMI of approximately 22 kg/m^2 (Fig. 5C).

Discussion

The results of this study illustrate the unique follicular microenvironment in overweight and obese women, which appears to be unrelated to ovarian reserve

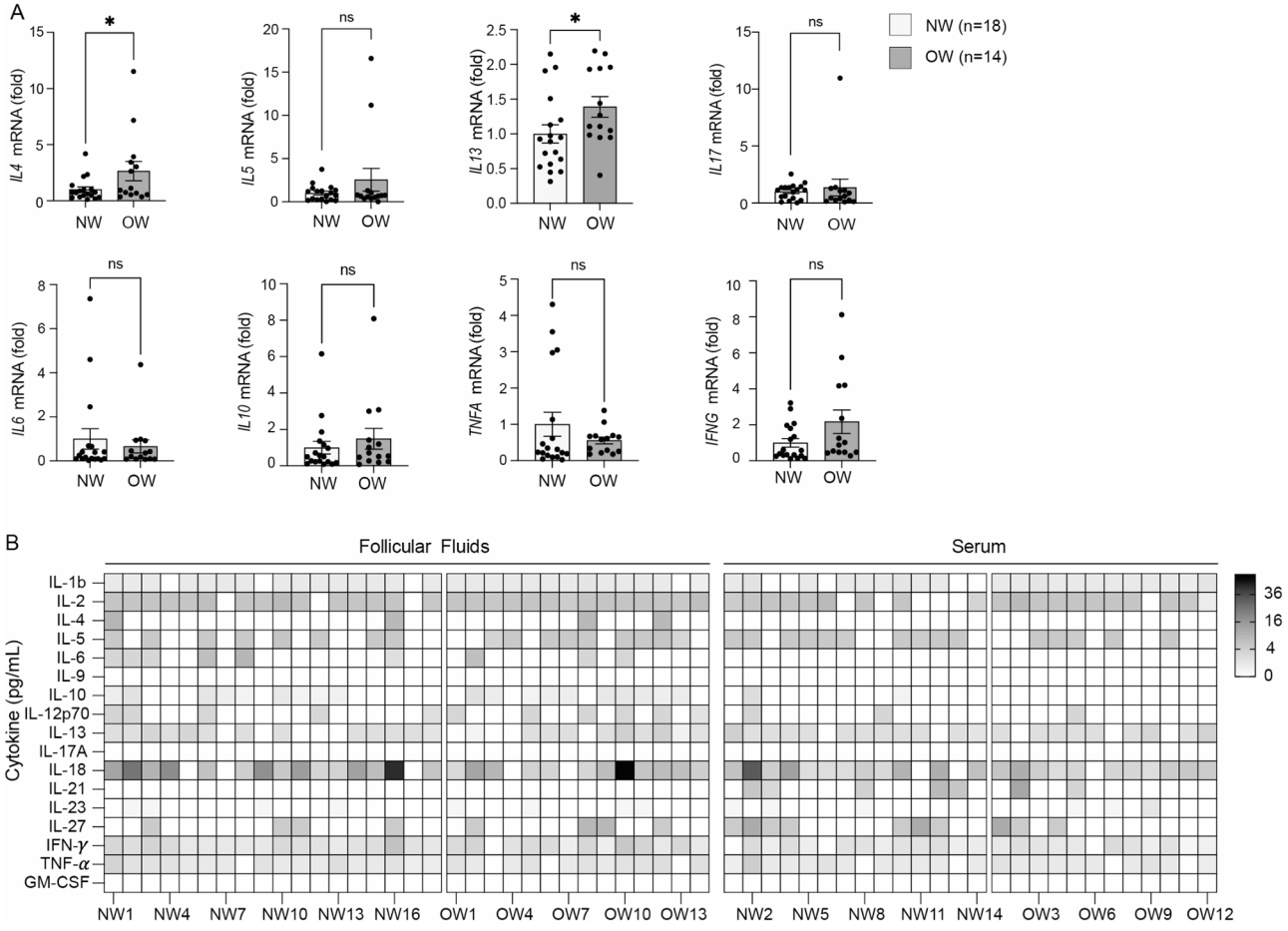

Fig. 2 Cytokines in the pre-ovulatory microenvironment and serum (**A**) The expression of *IL4*, *IL5*, *IL13*, *IL17*, *IL6*, *IL10*, *TNFA* and *IFNG* mRNA isolated from lymphocytes collected from follicular aspirates within the preovulatory microenvironment was assessed using RT-qPCR. Each data point represents an individual subject, data are expressed as mean ± SEM. * $p < 0.05$, ns, not significant. mRNA level from OW group compared to NW group (Mann-Whitney U Test). (**B**) The heatmap of cytokines detection in follicular fluids and serum. The levels of the cytokines were measured using Luminex 200 platform

Table 2 Lipid profiles in follicular fluid and serum

	Follicular fluid (Mean ± SD)			**Serum (Mean ± SD)**		
	Normal weight (*n* = 18)	**Overweight and obese (*n* = 14)**	***p* value**	**Normal weight (*n* = 14)**	**Overweight and obese (*n* = 12)**	***p* value**
TC, mmol/L	0.65 ± 0.71	0.46 ± 0.10	0.414	2.59 ± 0.34	2.34 ± 0.42	0.111
TG, mmol/L	0.14 ± 0.08	0.13 ± 0.03	0.392	1.72 ± 0.95	2.92 ± 3.66	0.504
LDL-C, mmol/L	0.14 ± 0.23	0.14 ± 0.17	0.879	2.04 ± 0.82	2.30 ± 1.30	0.758
HDL- C, mmol/L	1.96 ± 0.45	1.94 ± 0.46	0.890	3.48 ± 0.50	3.18 ± 0.43	0.100
FFA, mmol/L	0.30 ± 0.08	0.29 ± 0.16	0.725	0.45 ± 0.19	0.52 ± 0.31	0.508

TC, total cholesterol; TG, triglycerides; LDL-C, low density lipoproteins cholesterol; HDL-C, high density lipoproteins cholesterol; FFA, free fatty acids. Normal distributed data was compared using Student's t test and non-normal distributed data was analyzed using Mann-Whitney U Test

function and lipid content in serum and follicular fluid. We observed a significant increase in T cells, especially $CD4^+$ T cells in the pre-ovulatory follicular microenvironment of overweight and obese women, which exhibited a positive correlation with BMI. Additionally, B cells were also significantly elevated. The transcription levels of pro-inflammatory cytokines *IL4* and *IL13* were found to be significantly increased in overweight and obese women, and the level of *IFNG* only displayed an upward trend. The day 3 high quality embryo rate in overweight and obese women demonstrated a declining pattern and exhibited a negative correlated with BMI. In full cohort, the lipid contents in follicular fluid were all lower than that in serum.

B cells are increased in the peripheral blood of obese and overweight individuals, and they tend to accumulate

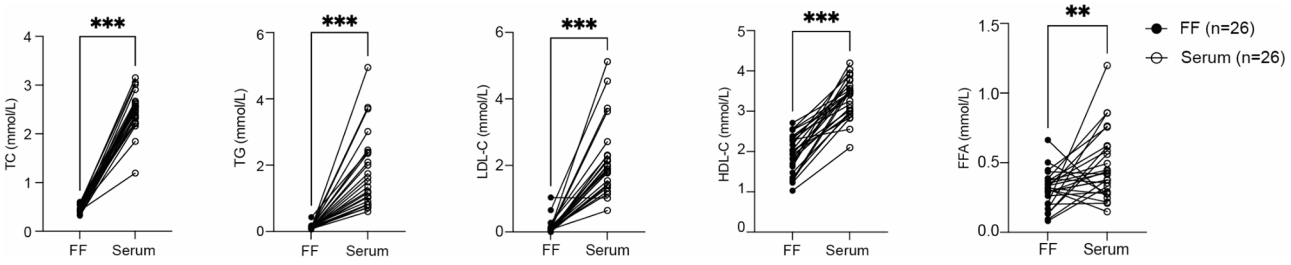

Fig. 3 Comparison of concentrations of lipid profiles between follicular fluids (FF) and serum within the same IVF cycle and individual patients. Data points of total cholesterol (TC), triglycerides (TG), low density lipoproteins cholesterol (LDL-C), high density lipoproteins cholesterol (HDL-C) and free fatty acids (FFA) in FF (●) and serum (○) obtained from the full cohort are shown for individual patients. The levels of the lipid profiles were measured using ELISA. ** $p < 0.01$, *** $p < 0.001$, lipid profile levels from serum compared to follicular fluids (Wilcoxon matched-pairs signed rank test)

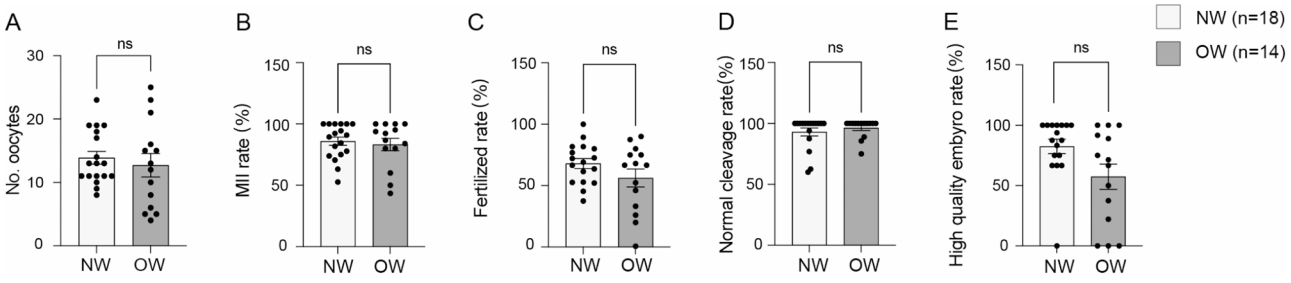

Fig. 4 Maturation of oocytes and formation of embryos. (**A**) The number of oocyte retrieval after 36 h of rhCG trigger. (**B**) The percentage of MII oocyte in the total number of retrieval oocytes. (**C**) The percentage of fertilized oocyte in the total number of retrieval oocytes. (**D**) The percentage of normal cleavage embryos in the total number of fertilized oocytes. (**E**) The percentage of day 3 high quality embryos in the total number of day 3 effective embryos. Each data point represents an individual subject, data are expressed as mean ± SEM, * $p < 0.05$, ns, not significant. (Normal distributed data was compared using Student's t test and non-normal distributed data was analyzed using Mann-Whitney U Test.)

Table 3 Comparison of patient's clinical reproductive outcomes

	Normal weight	Over-weight and obese	*p* value
Positive pregnancy test rate [a], n (%)	8/14 (57.14)	1/8 (12.5)	0.074
Clinical pregnancy rate [b], n (%)	5/14 (35.71)	1/8 (12.5)	0.351
Cumulative pregnancy rate [c], n (%)	8/11 (72.73)	4/8 (50.0)	0.377

Positive pregnancy test is identified by *β*-hCG positivity. Clinical pregnancy is defined by the identification of a gestational sac during transvaginal ultrasound examination around 7 weeks of gestation

[a] Calculated as follows: the number of positive pregnancy test originated from the first embryo transfer cycle divided by the number of women with transferred embryos

[b] Calculated as follows: the number of clinical pregnancies originated from the first embryo transfer cycle divided by the number of women with transferred embryos

[c] Calculated as follows: the number of clinical pregnancies originated from the index ART cycle divided by the number of women with transferred embryos

Comparison of IVF outcomes between normal weight and overweight and obese women using the Fisher's exact test method

Table 4 Correlation of T cells, $CD4^+$T cells, good quality embryo rate with BMI

	Overall BMI	
	R	*p* value
T cells % of $CD45^+$ cells	0.479	0.006*
$CD4^+$T cells % of $CD45^+$ cells	0.368	0.039*
Day 3 high quality embryo rate (%)	-0.358	0.048*

BMI, body mass index. The correlation analysis utilized Spearman and Pearson correlation analysis methods, and * $p < 0.05$

in visceral adipose tissue [35–37]. These B cells may play a role in the development of chronic inflammation by producing IgG and proinflammatory cytokines [37, 38]. Our study demonstrated for the first time that the levels of B cells in the pre-ovulatory follicular microenvironment were significantly elevated in overweight and obese women. In follicular fluid, leptin has been reported to positively correlate with BMI, and elevated leptin levels promoting the proliferation of pro-inflammatory B cells [39, 40]. This may account for the increased proportion of B cells.

Additionally, this is the first time that an increase in T cells and $CD4^+$ T cells has been identified in the pre-ovulatory follicular microenvironment of overweight and obese women. T cells are also markedly increased in both visceral and subcutaneous adipose tissue as well as in the peripheral blood of obese people [10, 41]. They contribute to the development of chronic inflammation by promoting the conversion of naïve $CD4^+$ T cells to T helper (Th) cells [11, 41]. Previous research has shown that the ratio of n-6 to n-3 free fatty acids can influence the proliferation and differentiation of $CD4^+$ T cells [42,

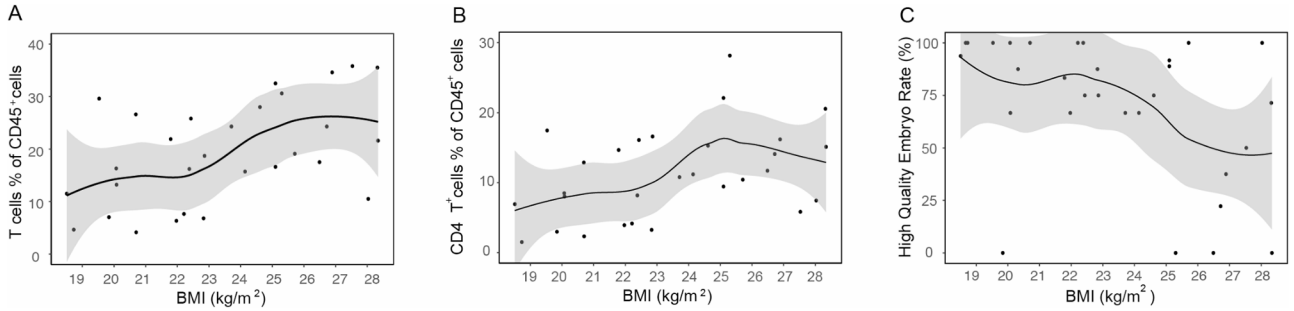

Fig. 5 Locally estimated scatterplot smoothing (LOESS) correlations for the corresponding immune cells, BMI or early in vitro embryonic development in the full cohort. (**A**) T cells % of $CD45^+$ cells and BMI. (**B**) $CD4^+$ T cells % of $CD45^+$ cells and BMI. (**C**) High quality embryo rate and BMI in the full cohort. The shade region represents the 95% confidence interval

43]. Although our study did not detect the specific types of free fatty acids, previous studies have confirmed an imbalance in the content of n-6 and n-3 free fatty acids in the follicular fluid of overweight and obese women, which may explain the increased proportion of $CD4^+$ T cells [44]. Due to the association between T cell dysregulation and various ovarian disease, such as ovarian dysfunction, premature ovarian failure, polycystic ovary syndrome (PCOS) and other ovulation disorders [45–47], we conducted an analysis of cytokines linked to $CD4^+$ T cell function in both follicular fluid and serum.

We found that a dysregulation of IFN-γ, IL-4, IL-13 levels may occur in the follicular microenvironment of overweight and obese women. The pro-inflammatory cytokine IFN-γ, primarily secreted by the $CD4^+$ T cell subtype Th1 cells, is believed to play a role in follicle maturation and fertilization. Existing studies have demonstrated that elevated levels of IFN-γ in peripheral blood are associated with poor IVF pregnancy outcomes [48, 49]. Elevated levels of IFN-γ in the follicular fluid disrupt the immune microenvironment balance within the follicle, potentially causing granulosa cell apoptosis and damage, impairing ovulation, and exerting toxic effects on early embryos, particularly at the 2-cell stage [47, 50]. IL-4 and IL-13 are primarily secreted by the $CD4^+$ T cell subtype Th2 cells. Increased IL-4 and IL-13 levels in follicular fluid negatively correlate with the number of mature oocytes, fertilized oocytes, and high quality embryos in the endometriosis population [49]. In follicular fluid IL-4 inhibits granulosa cell growth and induces apoptosis by activating the PI3K/AKT signaling pathway, thereby impairing oocyte development and potentially affecting IVF outcomes and early embryo development [51, 52]. While research on IL-13 is limited, it has been observed that IL-13 levels are elevated in the follicular fluid of patients with PCOS and are associated with T cell alterations [53]. The aforementioned findings suggest that the dysregulation of IFN-γ, IL-4, IL-13 levels may impact oocyte quality and early embryonic development.

In the follow-up of IVF pregnancy outcomes, we noted that overweight and obesity could potentially exert an adverse influence on IVF results, which is consistent with prior research [29]. Previous studies have reported that obese and overweight women tend to have fewer oocytes, lower fertilization rates, and zygotes that are less likely to complete cleavage after fertilization [44, 54]. However, our study observed no statistically significant differences in these parameters. This discrepancy may be attributed to the lack of significant differences in the lipid profiles between NW and OW women observed in our study. The rates of positive pregnancy tests, clinical pregnancies, and cumulative pregnancies demonstrated a declining trend. However, due to the limited sample size, these findings did not achieve statistical significance. Notably, prior studies have consistently reported that overweight and obese women experience reduced pregnancy and live birth rates during assisted reproductive treatments compared to women with a normal body weight [28, 29, 55].

In this study, we observed a partial imbalance in the proportion of immune cells and potential dysregulation of pro-inflammatory cytokines in the pre-ovulatory follicular microenvironment of overweight and obese women. However, several limitations should be acknowledged. First, the study cohort was limited in size and comprised exclusively of Chinese participants, which may have introduced geographical or cultural factors that influenced the results. Second, dietary and exercise habits were not standardized across participants. Although no differences in lipid profiles were observed, lipid metabolism is closely linked to the activation, differentiation, plasticity, and function of immune cells, including T cells [56, 57]. Therefore, future studies should explore the relationship between lipid metabolism and immune dysregulation within the follicular microenvironment. Moreover, further investigations are needed to explore the specific mechanisms by which immune cell composition imbalances in the pre-ovulatory follicular microenvironment of overweight and obese women impact assisted reproductive outcomes.

Conclusions

In conclusion, although no differences were observed in ovarian reserve function or lipid metabolism, imbalances in immune cell composition and cytokine exist in the pre-ovulatory follicular microenvironment of overweight and obesity women, potentially exerting a detrimental effect on assisted reproductive outcomes. We explained the mechanisms underlying poor pregnancy outcomes in obese patients from an immunological perspective and thereby indicating the necessity of weight management before assisted reproduction.

Abbreviations

IVF	In vitro fertilization
OW	Overweight or obese
NW	Normal weight
FF	Follicular fluid
BMI	Body mass index
CRP	C-reactive protein
IL	Interleukin
TNF-α	Tumor necrosis factor-α
IFN-γ	Interferon-γ
GM-CSF	Granulocyte macrophage colony-stimulating factor
GnRH-ant	Gonadotropin-releasing hormone antagonist
rFSH	Recombinant human follicle stimulating hormone
HMG	Human menopausal gonadotropin
E_2	Estradiol
rhCG	Recombinant human chorionic gonadotropin
AMH	Anti-Müllerian hormone
LH	Luteinizing hormone
FSH	Follicle-stimulating hormone
T	Testosterone
TC	Total cholesterol
TG	Triglycerides
LDL-C	Low-density lipoprotein cholesterol
HDL-C	High-density lipoprotein cholesterol
FFA	Free fatty acid
ELISA	Enzyme-linked immunosorbent assay
LOESS	Local estimation scatter plot smoothing
MII	Metaphase II
Th cells	T helper cells
PCOS	Polycystic ovary syndrome

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Author contributions

H.L. and L.J. conceived the idea, designed the study, and interpreted the results; L.J., H.Z., J.P. include patients performed or supervised the follicle punctures; Y.L., Y.Z performed the experiments, analyzed the data, Y.L. prepared the original manuscript; L.J., H.L., C.Z., H.H. revised the manuscript; H.H., H.L., L.J. was responsible for the financing of the study, helped in study coordination. All authors read and approved the final manuscript.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Human ethics and consent to participate declarations

This study was performed following the ethical guidelines of the Declaration of Helsinki and was approved by the Ethical Committee of Shanghai JiAi Genetics and IVF Institute (Approval number: JIAI E2021-27). All patients provided signed informed consent forms prior to the commencement the study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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