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Inflammatory Responses of Women with Polycystic Ovary Syndrome *in Vitro* Differ from Healthy Women

Fatemeh Rezayat[1,2], Mehri Hajiaghayi[1], Nazanin Ghasemi[1], Mehrnaz Mesdaghi[1], Fahimeh Ramezani Tehrani[3*], Nariman Mosaffa[1]

1. Department of Immunology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
2. Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.
3. Reproductive Endocrinology Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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ABSTRACT

Polycystic ovary syndrome is a low-grade inflammatory state with increased serum levels of TNF-α. The present study has compared the inflammatory responses to breast cancer cell lines in women with PCOS with healthy women. Peripheral blood mononuclear cells (PBMCs) isolated from 50 women with PCOS and 50 healthy controls were cultured in the trans-well co-culture system. These cells were stimulated with two distinct breast cancer cell lines. The proliferation of PBMCs, CD3+CD8+T cell percentages, and tumor necrosis factor-alpha (TNF-α) concentration were evaluated after 48 and 72 hours of incubation. TNF-α concentration and the proliferation rate of PBMCs after 48 hours of incubation significantly increased in the PCOS group. However, after 72 hours, TNF-α secretion significantly decreased in the PCOS group. The ability of PBMCs to produce TNF-α decreased gradually in women with PCOS. When the effects of low-grade inflammation and endocrine conditions on the cells decrease, the inability of PBMCs to create an inflammatory response will be altered.

Keywords: Inflammatory response, polycystic ovary syndrome, TNF-α, breast cancer

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***Corresponding** Fahimeh Ramezani Tehrani
Address: Reproductive Endocrinology Research Center, Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
E-mail: ramezani@endocrine.ac.ir

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Introduction

Polycystic ovary syndrome is one of the most common endocrinopathies among reproductive-aged women, with an estimated prevalence of about 11.1% according to Androgen Excess Society (1, 2) or 14.6% according to the Rotterdam consensus (2, 3). Clinically, PCOS is characterized by hyperandrogenism and chronic anovulation leading to menstrual irregularities and sub-fertility with polycystic morphology of ovaries (4-7). Although the etiology of PCOS is not fully understood, the role of obesity, hyperandrogenemia, and insulin resistance are known to be major factors involved in PCOS (8). Hyperandrogenism affects different immune cell subtypes and causes immune dysregulation in women with PCOS (9). M1 macrophages, neutrophils, and Th1 cells, all induced by hyperandrogenism in PCOS, produce high levels of tumor necrosis factor alpha (TNF-α). TNF-α is associated with increased insulin resistance, obesity, and hyperandrogenism, which is common in the pathogenesis of PCOS (10). The significant increase in TNF-α leads to insulin resistance by impairing the signal transduction pathway for insulin, increasing free fatty acids, inhibiting glucose transport, and reducing the expression of glucose-carrying protein (7, 11).

Obesity associated with hypertriglyceridemia in PCOS patients (12) is attributed to the obesity-induced change of adipokines, including TNFα, interleukin (IL)-6, and adiponectin (13). Also, it leads to a chronic inflammation state with increased levels of pro-inflammatory cytokines, chemokines, and oxidative stress marker levels, including C-reactive protein (CRP), IL-18, and ferritin (14-16). Chronic inflammatory markers are higher in PCOS women compared with age- and body mass index (BMI)-matched controls (13, 15, 16). This low-grade chronic inflammatory state is associated with inflammatory conditions such as metabolic syndrome, type 2 diabetes, and cardiovascular disease, which are common in PCOS (17). Also, chronic inflammation is associated with some types of cancer. As Virchow has defined for the first time, there is a positive association between cancer and chronic inflammation (18). Additional studies proved that chronic inflammation is associated with cancer risk (19).

In addition to chronic inflammation, there is an overlap between some other clinical manifestations of PCOS and risk factors for breast cancer, including nulliparity, obesity, hyperandrogenemia, and hyperinsulinemia (20-24). However, inflammatory responses and related disorders in women with PCOS are various depending on clinical manifestations, inflammatory factors, and immune cell function.

In the present study, some inflammatory factors of women with PCOS without obesity and insulin resistance have been investigated compared to age- and BMI-matched healthy controls. Therefore TNFα production, T CD8+ cell percentages, and cell proliferation response as three inflammatory parameters in PCOS women with hyperandrogenemia were studied in exposure to triple-negative and Estrogen receptor-positive breast cancer cell lines.

Materials and methods

This study was conducted at the Reproductive Endocrinology Research Center for over one year (2019-2020). Twenty-five women with PCOS who were referred to the Reproductive Endocrinology Research Center, based on the Rotterdam criteria (3, 25), participated in this study. The subjects were selected

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according to the following inclusion criteria: oligo ovulation, clinical symptoms of hyperandrogenism (26), polycystic ovaries with >12 follicles, and aged 18-45 years. Twenty-five non-hirsute eumenorrheic healthy age- and BMI-matched women were selected as controls.

The exclusion criteria were histories of cancers, primary or acquired immunodeficiency, suffering from infectious diseases, and systemic disorders. Also, women with a family history of cancer, those with early menopause (occurring at or before age 45), and substance dependents (addiction) were excluded. The medical Ethics Committee for the Research Institute for Endocrine Sciences (RIES) of Iran approved the study protocol. All of the participants completed an informed consent form.

Tumor Cell Line Culture

Two human breast cancer cell lines (MCF-7, MDA-MB-468) were purchased from the Cell Bank of Institute Pasteur Karaj, Iran. Cell lines were cultured in RPMI-1640 (Gibco, Invitrogen, USA) and DMEM (Gibco, Invitrogen, USA) media supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin/streptomycin (Atocel, Austria), and maintained in humidified 5% CO_2 incubator at 37°C. After reaching the log phase of growth at 80% confluency in the T25 tissue culture flask, growing cells were detached from the culture bed using the trypsin-EDTA solution 0.25% (Gibco, USA) and divided into 24-well culture plates or maintained at -80°C as a backup.

Isolation of Peripheral Blood Mononuclear Cells

Heparinized blood samples were obtained and diluted with RPMI medium at a ratio of 1:1. Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood using density gradient centrifugation (Ficoll-hypaque Sigma 1077, USA). Thus, mononuclear cells located in the second layer were collected. Then washed and suspended in Complete Tissue Medium (CTM). Cell viability was determined by trypan blue staining (>95%).

Co-culture System

Tumor cell lines (5×10^5cell/well) were pre-seeded in the lower chamber at Transwell 24-well plates with a pore size of 0.4 µm (SPL, Korea) in RPMI-1640/DMEM medium supplemented with 10% fetal bovine serum (FBS) and incubated overnight in humidified CO_2 incubator at 37°C. Subsequently, a total 2×10^6 PBMCs per well was added to the upper chamber at a ratio of 1:4 as the executive cells (PBMCs), and target cells (tumor cell lines). As the negative control, PBMCs were cultured in the upper chamber of the plates in the absence of tumor cell lines. The supernatants were elicited separately from each Trans-well chamber at two-time intervals of 48 and 72 h following co-culture.

Measurement Tumor Necrosis Factor alpha of Concentration

TNFα concentration in the supernatants collected from PBMCs cultured with and without tumor cell lines was determined and reported in pg/dL using the sandwich ELISA kit (Quantikine, R&D Systems, USA), for human TNFα, according to the manufacturer's instruction. Results were calculated using a standard curve.

Evaluation of Proliferative Response of PBMCs

PBMC proliferation rate was evaluated using the non-radioactive Cell Proliferation Assay ELISA kit, BrdU (Roche Diagnostic, Germany; Ref: 11647229001) during co-culture with tumor cell lines beside controls at two-time intervals of 48 and 72 h. The BrdU cell proliferation assay was

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performed according to kit's instructions. Absorbance was measured at 450 nm with a reference wavelength of 690 nm.

Determination of Changes in the Percentage of $CD3^+$ $CD8^+$ Lymphocytes

The percentages of total mononuclear cells and $CD3^+CD8^+$ T lymphocytes were evaluated by flow cytometry (FACS Calibur) at each time interval. For this purpose, cultured cells were stained by phycoerythrin (PE)-conjugated anti-human CD8 and fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 monoclonal antibodies (BD Biosciences, Cat No: 340044). The $CD3^+$ $CD8^+$ T lymphocytes ratio was analyzed using FlowJo software.

Statistical Analysis

In the present study, One-way Analysis of Variance (ANOVA) and Independent Samples t-test were conducted to compare the mean scores and evaluate the normal distribution of data, respectively. Also, nonparametric tests such as the Kruskal-Wallis test or Mann-Whitney U test were performed for variables with asymmetric distribution. Categorical variables, expressed as percentages, were compared using Pearson's test. Data analysis graph drawings were conducted using the software SPSS and GraphPad Prism. The results were expressed as Mean±SEM (Standard Error of Mean), the significance level was set at $P<0.05$, and the confidence interval (CI) was 95%.

Results

The demographic characteristics of participants are summarized in Table 1. There were no statistically significant differences in the demographic characteristics of the participants.

Table 1. The demographic characteristics of the study participants.

-	PCOS (n = 25)	Control (n = 25)	*P*- value
Age (years)	30.2 ± 6.1	30.8 ± 5.8	0.5
BMI (kg/m^2)	22.8 ± 6.4	23.09 ± 2.8	0.8
WC (cm)	79.8 ± 8.1	78.7 ± 8.5	0.4
HC (cm)	100.1 ± 6.8	99.1 ± 6.5	0.1
SBP (mmHg)	101.1 ± 10.7	101.1 ± 9.3	0.2
DBP (mmHg)	62.8 ± 11.7	65.4 ± 8.1	0.1
Parity	1.4 ± 0.6	1.5 ± 0.7	0.5
FBS (mg/dL**)**	91.2 ± 1.078	92.52 ± 1.04	0.4

PCOS, polycystic ovary syndrome; BMI, body mass index; WC, waist circumference; HC, hip circumference; SBP, systolic blood pressure; DBP, diastolic blood pressure; FBS, fasting blood sugar

The Proliferative Response in PBMCs

The co-culture of PBMCs with both tumor cell lines in all samples has stimulated their inflammatory response. The proliferative response has been measured using the BrdU cell proliferation assay and the determination of the optical density (OD) value.

For the cell proliferation assessment in the treated wells, the OD value of PBMC wells was subtracted from the total absorbance in these wells. Exposure to tumor cells stimulated the proliferative response in all treated wells (Figure 1 A, B). Compared to the healthy group, the PCOS group indicated a more increased mean cell proliferation rate ($P=0.04$; Figure 1 C). During the incubation time, the proliferation rate in the

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PCOS group increased to such an extent that after 72 hours, the cell proliferation rate in this group was significantly higher than at 48 hours (P=0.018).

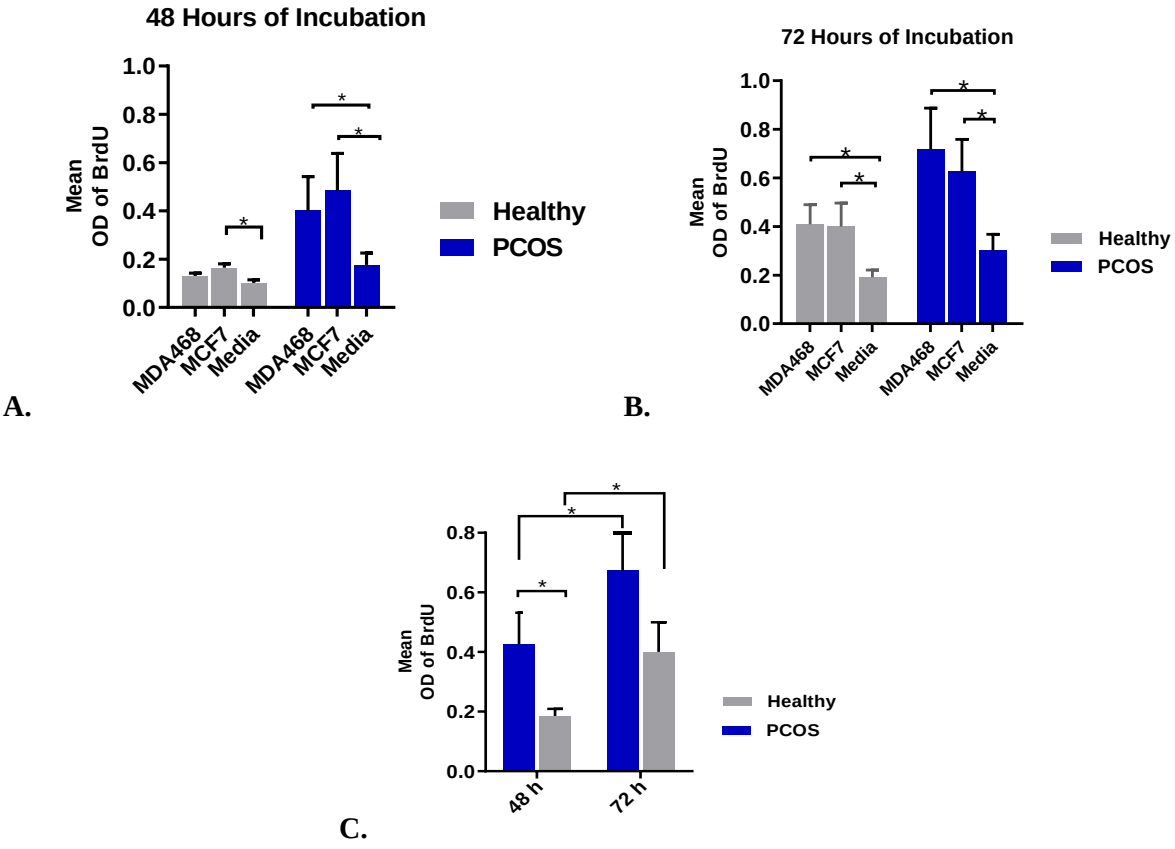

Fig.1. Comparison of the mean proliferation of PBMCs in the PCOS group and healthy controls. The mean lymphocyte proliferation of MD-468 and MCF-7 cell lines in co-culture with PBMCs culture is compared to negative controls in 48 h (A) and 72 h (B), respectively. Comparison of the mean proliferation at two-time intervals (48 and 72 h) during co-culture with the MDA-468 cell line (C) The proliferation rate in the PCOS group was significantly more.

The Concentration of TNFα

The mean TNFα concentration did not reveal any significant differences in the tumor cell lines, while its production significantly increased during co-culture compared to the negative control wells.

As presented in Figure 2 A, the mean TNFα concentration in the PCOS and healthy groups enhanced up to 48 hours of incubation (P=0.007). However, the trend of increasing TNFα concentration in the PCOS group decreased after 72 hours of incubation. At the end of 72 hours, the mean TNFα concentration in the healthy controls significantly increased compared to the PCOS group (Figure 2 B, $p<0.05$). Figure 3 compares the mean TNFα concentration in both study groups during incubation intervals. Accordingly, TNFα secreted by PBMCs isolated from women with PCOS decreased in vitro.

The percentages of $CD3^+CD8^+$ T lymphocytes

Figure 4 displays the flow cytometry plots associated with the percentages of cytotoxic T lymphocytes (CTLs) in the PCOS and healthy groups. $CD3^+CD8^+$ T cell population was determined at three stages: before

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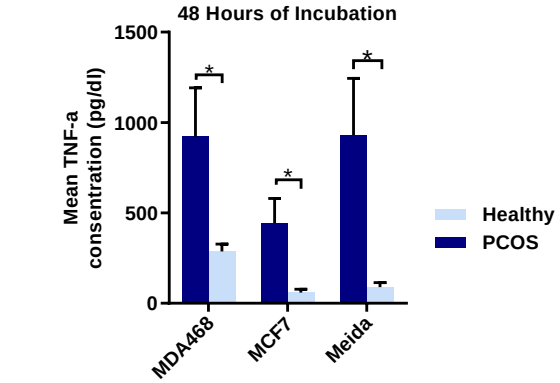
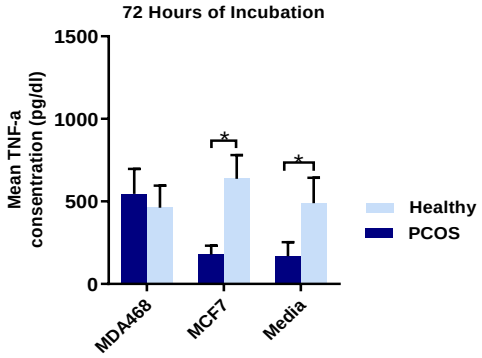
starting cell culture (a), 48 h (b), and 72 h of incubation (Figure 4). As presented in Figure 5, the percentages of $CD3^{+}CD8^{+}$ T cells increased after co-culture. But the mean percentage of cytotoxic cells exhibited no significant differences between the study groups (16.8 % ± 1.14 versus 13.33 % ± 1.84 in the PCOS and healthy groups, respectively, P>0.05). It should be noted that the initial percentage of peripheral $CD3^{+}CD8^{+}$ T cells in the women attending the studied groups did not differ significantly (data has not been supplied).



A.



B

Fig.2. Comparison of the mean TNFα concentration in the PCOS group and healthy control. The mean TNFα concentration in co-culture of MD-468 and MCF-7 cell lines with PBMCs is compared to negative controls in 48 h (A) and 72 h (B).

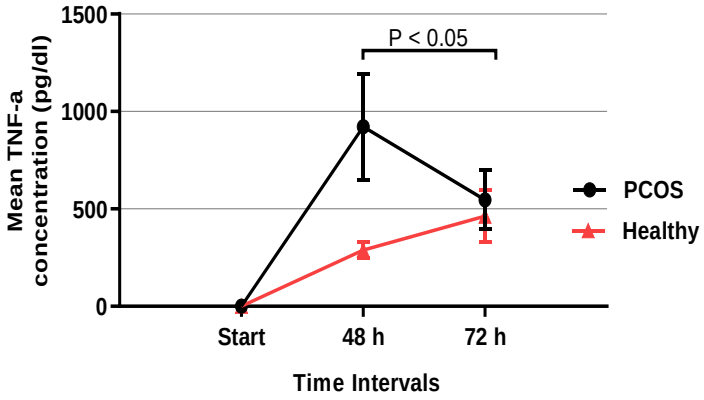


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Fig.3. The alterations in the concentration of TNFα at 48 and 72 hours after culture. The concentration of TNFα in the PCOS group increased up to 48 hours of incubation with tumor cell lines but then decreased. However, the process of TNFα production in healthy group co-cultures was increased by up to 72 h.

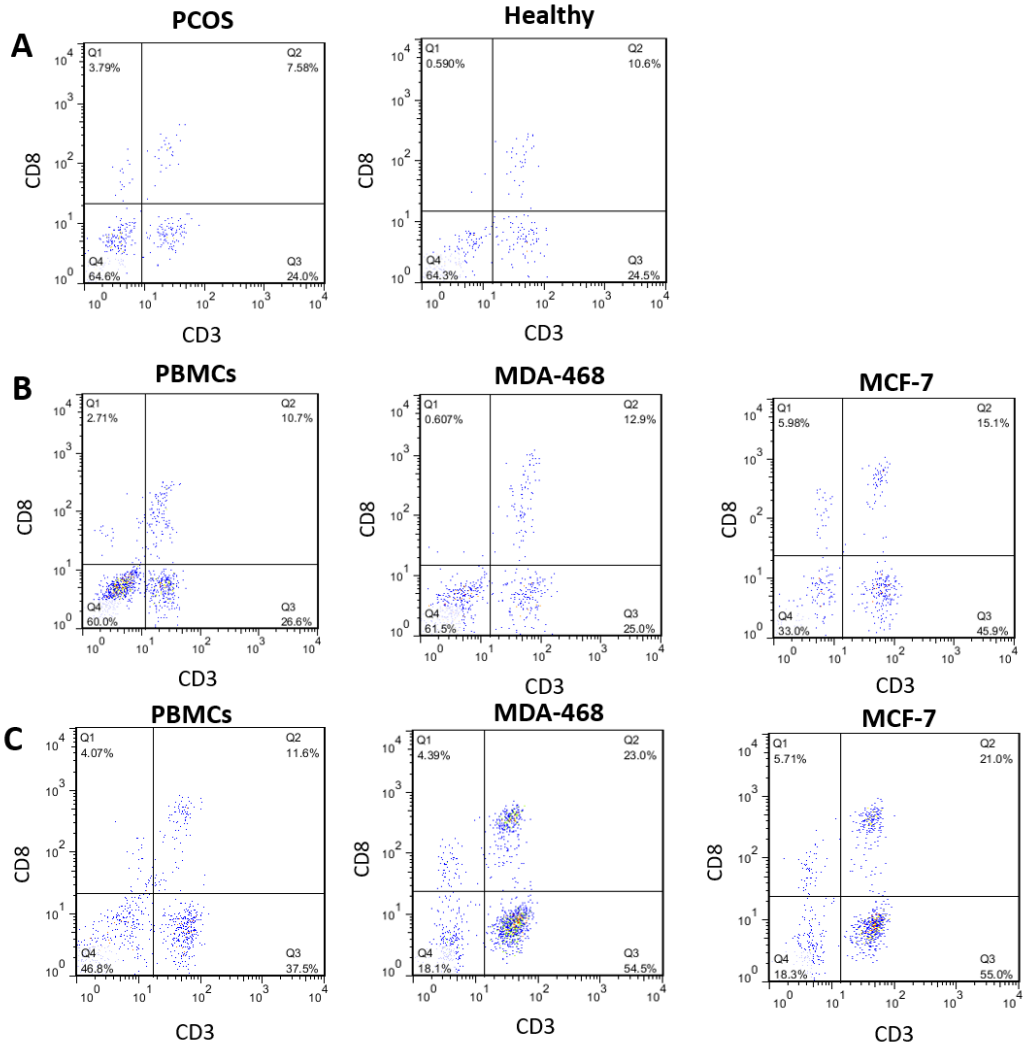

Fig.4. Flow cytometry results revealed the percentage of T CD3+ CD8+ lymphocytes in three intervals, the start of co-culture (A), 48 h (B), and 72 h (C) after incubation in the PCOS group.

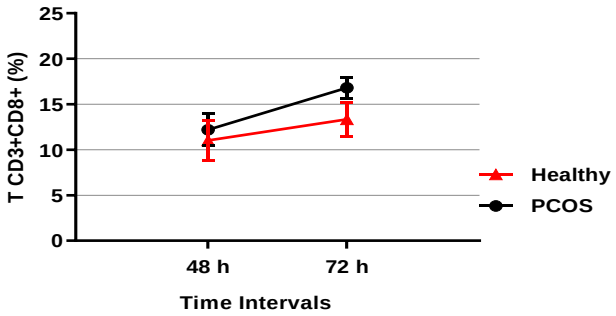

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Fig.5. The percentage of $CD3^+CD8^+$ T cells in two intervals following co-culture with the tumor cell lines. Following co-culture with both tumor cell lines and PBMCs culture, the ratio of these cytotoxic cells showed no statistically significant difference between the two healthy and PCOS groups in 48 h and 72 h intervals.

Discussion

PCOS is considered a low-grade inflammatory state with increased serum levels of TNFα.[8,18] This study has been aimed to investigate the inflammatory responses of women with PCOS. The indicators examined were the production of TNFα, the proliferative response of immune cells, and the population of cytotoxic T cells. These inflammatory responses are considered as indicators of immune system function. Tumor cells can stimulate the immune system by affecting mentioned indicators. Some clinical manifestations of PCOS, including obesity, hyperandrogenemia, hyperinsulinemia, high anti mullerian hormone (AMH) levels, and nulliparity, are considered common risk factors for breast cancer. However, no positive association between these two disorders has been proven (23, 27-31). Therefore, two of the most well-known breast cancer cell lines were examined to stimulate immune cells in vitro. To investigate the effect of hyperandrogenism in women with PCOS on inflammatory responses, MCF-7 and MDA-MB-468 cell lines were used, which are triple-negative and estrogen receptor-positive, respectively. Obesity is one of the factors involved in chronic inflammation. The obese women with PCOS, compared to their obese counterparts without PCOS, have higher serum levels of TNFα (32, 33). To minimize the effect of obesity on the inflammatory status of PCOS, PCOS women with normal BMI were studied. Inflammatory responses in these women were compared with the age- and BMI- matched control group.

Consequently, the common factors linking PCOS and breast cancer were excluded to minimize the impact of these factors on inflammatory responses in women with PCOS. Accordingly, the PCOS group and the healthy controls were not significantly different in BMI, taking medication, and fertility.

In this study, it has been found that the proliferation of PBMCs increased in women with PCOS compared to healthy controls. It means that the immune cells of women with PCOS exhibited a more significant proliferative response than healthy women in response to comparable and identical stimulation. Therefore both MDA-468 and MCF-7 cell lines could induce a proliferative response in PBMCs isolated from women with PCOS.

$CD8^+$ T cell percentages increased following co-culture with both breast tumor cell lines, with no significant differences between the study groups. So, according to these results, the observed proliferative response was not related so much to $CD8^+$ T cells. Previous studies on women with PCOS reported that the number of $CD8^+$ T cells decreased in the peripheral blood (34) while in the present study such a decrease was not observed.

Up to 48 hours of incubation, TNFα secretion increased following co-culture with breast cancer cell lines in the PCOS group, compared to healthy controls. Over time, TNFα concentration increased in the healthy group. However, TNFα secretion in the PCOS group decreased after 72 hours of incubation. Despite the significant initial inflammatory response in the first incubation interval, PBMCs of the PCOS group could not maintain their effectual response. Due to the high serum level of TNF-α in women with PCOS (32, 33, 35-38), the increased concentration of this cytokine up to 48 hours after co-culture can be attributed to their chronic inflammatory condition. It seems that by decreasing the impact of the low-grade inflammation and

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endocrine state on the immune cells, the inability of PBMCs to create an inflammatory response will be detectable. It should be noted that these observations were identical in both triple-negative (MCF-7) and Estrogen receptor-positive (MDA-468) cell lines.

No significant differences were observed in TNFα production, proliferative response, and increase of cytotoxic T cells in exposure to two cell lines. In the microscopic investigations of the 72-hour culture of the MCF-7 cell line with the serum of women with PCOS, apoptotic bodies and reduced cell proliferation were observed. These observations confirm the results of previous studies (data not supplied) (39, 40).

According to several studies, TNFα is crucial in linking inflammation with breast cancer development and evolution (41, 42). TNFα has a dual role in antitumor responses and cancer progression (43, 44). This cytokine increases many pro-apoptotic factors in triple-negative breast cancer cell lines like MCF-7 and leads to apoptosis (39, 40). Secreted TNFα in the tumor microenvironment is mainly produced by activated M1 macrophages, T lymphocytes, natural killer cells, stromal cells, and own tumor cells (32, 45, 46). Hajiaghayi et al. evaluated the inflammatory response of PBMCs in PCOS patients compared to healthy women by co-culture of these cells with ovarian cell lines. They reported the TNFα secretion as a pro-inflammatory cytokine in co-culture with the SKOV3 cell line was higher than that of co-culture with the A2780 cell line in the PCOS and healthy groups. The reported difference may be due to the SKOV3 cell line's ability to produce TNFα. Therefore, the observed decline in TNFα concentration in the present study may also be related to the decrease in these tumor cell lines' ability to produce TNFα (47).

Founded on the results, some factors involved in the inflammatory responses in women with PCOS are distinct compared to healthy women. Accordingly, analysis of other parameters related to inflammatory responses and immune system function in these women will be critical. $CD8^+$ T cell percentages and cell proliferation response, in addition to the production of the pro-inflammatory cytokines, are considered the principal indicators for the inflammatory and anti-tumor responses study in vitro (48).

The findings of this study confirmed previous reports, including high levels of TNFα and low-grade inflammation in women with PCOS. However, no decrease was observed in $CD8^+$ T cell percentages. This chronic inflammation is not caused by obesity and diabetes in these patients. The study's criteria attempted to decrease the heterogeneity in the study population. However, this study does have some limitations. More studies are required to comprehensively analyze the status of inflammatory responses in women with PCOS. Our study indicates that the inflammatory response of immune cells in women with PCOS because of stimulation with breast cancer cell lines is different compared to healthy women. The ability of PBMCs to produce TNFα in vitro decreased gradually in these women.

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