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Impact of IL-12 and IL-18 Combined Stimulation on INF-γ and TNF-α Production by PBMCs Derived from Egyptian Ovarian Cancer Patients

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Abstract

***Background*:** Cytokines play a crucial role in modulating the immune cells' response to tumors. Utilizing IL-18 and IL-12 as immunotherapeutic agents has gained much attention for cancer treatment. This study aimed to investigate the effect of combined stimulation with IL-12 and IL-18 on the ability of peripheral blood mononuclear cells (PBMCs) derived from Egyptian ovarian cancer (OC) patients to produce IFN-γ and TNF-α.

Methods and Results: This in vitro study included 8 patients with benign ovarian tumors, 8 patients with low-grade OC, and 8 patients with high-grade OC. PBMCs were stimulated with IL-12 (10 ng/mL) and IL-18 (50 ng/mL) for 7 days. The levels of IFN-γ and TNF-α were quantified before and after stimulation by quantitative sandwich ELISA. Stimulated PBMCs isolated from patients with low-grade OC showed good ability to produce high levels of IFN-γ, while stimulated PBMCs isolated from patients with high-grade OC patients showed good and high production of TNF-α.

Conclusion: We found that the combined stimulation with IL-12 and IL-18 significantly enhances the production of IFN-γ and TNF-α by PBMCs in OC patients, suggesting a potential avenue for cancer immunotherapy.(**International Journal of Biomedicine. 2024;14(3):435-440.)**

Keywords: ovarian cancer • IFN-γ • IL-12 • IL-18 • peripheral blood mononuclear cells • TNF-α

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Abbreviations

IL, interleukin; **IFN**, interferon; **NK**, natural killer; **NKT**, natural killer T cells; OC, ovarian cancer; **PBMCs,** peripheral blood mononuclear cells; **TNF-α**, tumor necrosis factor alpha.

Introduction

Ovarian cancer (OC) is one of the most common gynecologic cancers. It ranks third after cervical and uterine cancer [1] and has the worst prognosis and the highest mortality rate.[2] Although OC is less prevalent than breast cancer, it is three times more lethal.[3] Delayed onset of symptoms and lack of proper screening result in late diagnosis in the advanced stages, so "a silent killer" is one of the names given to OC.[3-5]

The epidemiological diversity of OC in different regions can be attributed to the risk factors that account for the occurrence of OC.[6] The highest prevalence of OC is seen in non-Hispanic white women, followed by Hispanic, non-Hispanic black, and Asian/Pacific Islander women.[7] The high

mortality rate of OC is seen in African populations.[8] OC is the 4th commonest cancer among Egyptian females, accounting for 4.5% of the population. It is estimated that the number of OC patients in Egypt will more than double by 2050.[9]

Interleukins play an important role in the biology of cancer. They can block or enhance the growth of tumors. For instance, IL-2 and IL-12 can inhibit tumor growth and strengthen the antitumor response.[10] IL-18, previously named IFN-γ inducing factor (IGIF), can facilitate the production of IFN-γ by Th1 cells, especially when combined with IL-12. In addition, IL-18 is a significant interleukin involved in differentiating naïve T-cells into Th1 cells, particularly in the presence of IL-12. Moreover, IL-18 plays a critical role in inducing Fas ligand (FasL) expression on Th1 cells and in the activation of natural killer (NK) cells.[11] On the other hand, IL-12 can boost the antitumor immune response and enhance the activity of T cells and NK cells. Indeed, IL-12 induces the differentiation and development of IFN-γ- producing Th1 cells.[12] Previous studies showed that IL-18, in synergy with IL-12, can be involved in the enhanced production of IFN-γ.[13]

IFN-γ is a pleiotropic cytokine that has antitumor activity and immunomodulatory function.[14] During the innate immune response, it is mainly produced by NK and NKT. In adaptive immunity, it is regulated by CD8+ and CD4+ T-cells.[15] These cells are mainly stimulated in situ by IL-12 and IL-18.[16] Another significant cytokine is TNF-α, which participates in the progression of different cancers and plays a role in malignant transformation.[17] It is also involved in the inflammatory process, proliferation, and differentiation of different cell types.[18]

Although many studies have examined IFN-γ production due to combined stimulation of IL-18 and IL-12, few studies have focused on other produced cytokines. In a study by Fehniger et al.,[19] TNF-α was differentially induced by various combinations of IL-18, IL-12, and IL-15 by NK cells. Stimulation of PBMCs may be beneficial in identifying other highly produced cytokines that would help further understand the immune mediators produced by activated PBMCs.

Materials and Methods

Reagents and Cytokines

Recombinant human IL-12 (Cat. No. PKSH033284) and recombinant human IL-18 (Cat. No. PKSH033626) were purchased from Elabscience (USA). RPMI 1640 culture media was purchased from Capricorn Scientific (Cat. No. RPMI-A). The media was supplemented with 10% fetal calf serum (Gibco by Life Technologies, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco by Life Technologies, USA).

ELISA kits for measuring IFN-γ (Cat. No. E0105Hu) and TNF-α (Cat. No. E0082Hu) were purchased from the Bioassay Technology Laboratory (BT-LAB, China).

Patient Information

All the enrolled patients attended the outpatient clinic at the Egyptian National Cancer Institute. In total, peripheral blood samples obtained before surgery from 16 OC patients (8 low-grade OC patients and 8 high-grade OC patients) and 8 patients with benign ovarian tumors were enrolled in this study. The mean age of the study participants was 47.1±16.4 years.

Cell Viability in MTT Assay

The altered cell viability was assessed by dye reduction assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). In 96-well plates, the PBMCs were cultured in the absence and presence of different concentrations of IL-12 and IL-18 combined. We used three different concentrations: 10 ng/mL of IL-12 and 50 ng/mL of IL-18; 5 ng/mL of IL-12 and 25 ng/ml of IL-18; 2.5 ng/mL of IL-12 and 50 ng/mL of IL-18. After 48 hours, 20 µL of MTT solution was added to each well, and then the cells were incubated for 4 h at 37°C; after that, DMSO was added (100 uL per well), and cells were incubated for 10 min. The absorbance was measured at 540 nm on a microplate reader, and the cell survival index was calculated. The best-combined concentration was that produced ≤50% loss of cell viability. IC50 values were calculated using GraphPad Prism.

PBMCs Isolation

We isolated PBMCs from the blood samples by centrifugation over a density gradient Lymphocyte Separation Medium (Cat. No.17-829E, Lonza BioWhittaker), washed twice with Hanks' Balanced Salt Solution (HBSS) (Cat No. 0313), and re-suspended in RPMI 1640 medium with L-glutamine, supplemented with 10% FBS, penicillin 100 U/mL, and streptomycin 100 µg/mL (Gibco by Life Technologies, USA).

Combined Stimulation with IL-12 and IL-18

PBMCs were cultured in RPMI complete medium in the absence (before stimulation) and the presence of a combined concentration of IL-12 (10 ng/mL) and IL-18 (50 ng/mL) for 7 days. All dilutions were prepared from a starting concentration solution (0.1µg/µL) of recombinant human IL-12 and recombinant IL-18. We tested these combined concentrations on the derived PBMCs from all 24 patients. After 3 and 7 days of culture, the medium was refreshed without discarding the cells. Supernatants were collected at three different times: (Time 1) before the beginning of stimulation, (Time 2) after 3 days of culture, and (Time 3) after 7 days of culture. The collected supernatants were used to measure the concentration of IFN-γ and TNF-α.

Determination of IFN-γ and TNF-α Levels

IFN-γ and TNF-α levels were measured by quantitative sandwich ELISA kits following the manufacturer's instructions. For IFN-γ, the standard curve range was from 1-400 ng/mL, and the analytical sensitivity was 0.49 ng/mL. For TNF-α, the standard curve range was from 3-900 ng/L, and the analytical sensitivity was 1.52 ng/L.

Statistical analysis was performed using the statistical software package SPSS version 21.0 (SPSS Inc, Armonk, NY: IBM Corp). Baseline characteristics were summarized as mean (M) ± standard deviation (SD). Multiple comparisons were performed with one-way ANOVA with adjusted *P*-value

for each comparison. A *P*-value of <0.05 was considered statistically significant.

Results

This in vitro study included 8 patients with benign ovarian tumors (BOT), 8 with low-grade OC, and 8 with high-grade OC.

Production of IFN-γ

We found that stimulated PBMCs isolated from patients with BOT and patients with OC produced different amounts of IFN-γ. No statistically significant difference in IFN-γ production was found before combined stimulation with IL-12 and IL-18. However, after three days of stimulation with IL-12 and IL-18, PBMCs isolated from low-grade OC patients produced significantly more IFN-γ than PBMCs isolated from high-grade OC patients (adjusted *P*-value=0.017 on pairwise comparison). Moreover, after seven days of stimulation with IL-12 and IL-18, PBMCs isolated from patients with BOT produced significantly less IFN-γ than PBMCs isolated from low-grade OC patients (adjusted *P*-value=0.003 on pairwise comparison) (Table 1).

Table 1.

IFN-γ production by PBMCs from patients with benign ovarian tumors (BOT) and patients with OC before and after 3 and 7 days of combined stimulation with IL-12 and IL-18.

	BOT (n=8)	Low-grade OC (n=8)	High-grade OC (n=8)	ANOVA
IFN-γ, ng/mL	Mean±SD	Mean±SD	Mean±SD	*P*-value
Before stimulation	72.0±13.9	82.4±17.8	79.2±23.5	0.534
After 3 days	83.5±12.9	142.2±28.0	97.8±37.2	0.001
After 7 days	109.7±30.9	184.4±32.9	137.1±50.8	0.004

Our findings also revealed that timing and grouping have a noticeable effect on IFN-γ production; there is a significant interaction effect of timing and grouping, so simple effects in each group were examined (Table 2).

Table 2.

Effect of timing and grouping on IFN-γ production by PBMCs before and after combined stimulation with IL-12 and IL-18

	BOT (n=8)	Low-grade OC (n=8)	High-grade OC (n=8)
IFN-γ, ng/mL	Mean±SD	Mean±SD	Mean±SD
Time 1	72.0±13.9	82.4±17.8	79.2±23.5
Time 2	83.5±12.9	142.2±28.0	97.8±37.2
Time 3	109.7±30.9	184.4±32.9	137.1±50.8
[a] *P*-value for timing	<0.001	*P*<0.001 for Time 1 vs. Time 2 *P*<0.001 for Time 2 vs. Time 3	
[a] *P*-value for group	0.005	*P*=0.004 for BOT vs. low grade *P*=0.668 for BOT vs. high grade *P*=0.071 for low grade vs. high grade	
[a] *P*-value for interaction	0.001		

[a] *Repeated measures ANOVA*

It was found that PBMCs isolated from patients with benign ovarian tumors produced significantly higher amounts of IFN-γ after 7 days of stimulation than after 3 days (*P*-value=0.010) and before stimulation (*P*-value=0.004). PBMCs isolated from low-grade OC patients produced significantly higher amounts of IFN-γ after 7 days of stimulation than after 3 days (*P*-value<0.001) and before stimulation (*P*-value<0.001), and they also produced significantly higher amounts of IFN-γ after 3 days of stimulation when compared to those before stimulation (*P*-value<0.001). Moreover, PBMCs isolated from high-grade OC patients produced significantly higher amounts of IFN-γ after 7 days of stimulation than after 3 days (*P*-value<0.001) and before stimulation (*P*-value=0.003).

Production of TNF-α

Un-stimulated PBMCs isolated from patients with benign ovarian tumors produce significantly lower amounts of TNF-α than PBMCs isolated from patients with high-grade OC (adjusted *P*-value=0.004 on pairwise comparison) (Table 3). In addition, PBMCs isolated from patients with benign ovarian tumors produced significantly less TNF-α than PBMCs isolated from patients with high-grade OC after 3 days of combined stimulation with IL-12 and IL-18 (adjusted *P*-value=0.007) and after 7 days of combined stimulation with IL-12 and IL-18 (adjusted *P*-value=0.002 on pairwise comparison). No significant interaction was found regarding the effect of timing and grouping on TNF-α production (*P*-value=0.273) (Table 4).

Table 3.

TNF-α production by PBMCs from patients with benign ovarian tumors (BOT) and patients with OC before and after 3 and 7 days of combined stimulation with IL-12 and IL-18.

	BOT (n=8)	Low-grade OC (n=8)	High-grade OC (n=8)	ANOVA
TNF-α, ng/L	Mean±SD	Mean±SD	Mean±SD	*P*-value
Before stimulation	126.6±49.5	171.4±56.3	219.7±43.1)	0.005
After 3 days	147.5±57.3	226.0±55.5	257.6±76.6)	0.007
After 7 days	168.6±83.5	219.5±48.4	304.8±69.6)	0.003

Table 4.

Effect of timing and grouping on TNF-α production by PBMCs before and after combined stimulation with IL-12 and IL-18.

	BOT (n=8)	Low-grade OC (n=8)	High-grade OC (n=8)
TNF-α, ng/L	Mean±SD	Mean±SD	Mean±SD
Time 1	126.6±49.5	171.4±56.3	219.7±43.1
Time 2	147.5±57.3	226.0±55.5	257.6±76.6
Time 3	168.6±83.5	219.5±48.4	304.8±69.6
[a] *P*-value for timing	<0.001	*P*<0.007 for Time 1 vs. Time 2 *P*<0.010 for Time 2 vs. Time 3	
[a] *P*-value for group	0.001	*P*=0.114 for BOT vs. low grade *P*=0.001 for BOT vs. high grade *P*=0.144 for low grade vs. high grade	
[a] *P*-value for interaction	0.273		

[a] *Repeated measures ANOVA*

Discussion

IFN-γ, one of the key mediators produced by Th1 cells, has macrophage antitumor effector function.[20] It has an antiproliferative activity against OC cell lines.[21] T cells and NK cells are the main IFN-γ producing cells in PBMC cultures and stimulation with IL-12 and IL-18 enhances IFN-γ production by these cells.[22] A positive correlation was described between the longer progression-free and the higher overall survival rate of women with ovarian carcinoma and the high level of IFN-γ in cancer tissue.[23]

IL-18 has a major effect in increasing the IFN-γ-dominant T-cell response induced by IL-12.[24] It was suggested that IL-18, in synergy with IL-12 and IL-15, triggers Th1 immune responses and may be involved in macrophage production of TNF-α inflammatory cytokines.[25] Employing IL-18 and IL-12 as immunotherapeutic agents has gained much attention in cancer treatment. For instance, in a hepatoma model, administration of IL-18 and IL-12 was found to reduce the tumor burden.[26] In addition, the adoptive transfer of preactivated NK cells with a combination of IL-18, IL-12, and IL-15 has been shown to induce a clinical response in myeloid leukemia patients[27] and reduce tumor burden in a murine lymphoma model.[28]

In the former years, many publications discussed the potential for using combined IL-18 and IL-12 treatment. Thus, we thought it would be beneficial to identify the cytokines produced from stimulated PBMCs derived from OC patients and patients with benign ovarian tumors. Our findings revealed that IFN-γ production by PBMCs from patients with low-grade OC, in response to IL-12 and IL-18 combined stimulation, was higher than that from patients with high-grade OC and benign ovarian tumors.

Nowak et al.[18] found that activated PBMC of patients at III-IV FIGO stages produced less IFN-γ than PBMC of non-cancer patients. The current study found that activated PBMCs from patients with low-grade OC produced high IFN-γ. We also revealed that PBMC isolated from low-grade OC patients produced significantly higher amounts of IFN-γ after 7 days of stimulation than after 3 days and before stimulation.

Kawashima & Miossec[29] reported that IFN-γ production by PBMCs isolated from patients with rheumatoid arthritis (RA) have a lower response to IL-12 and IL-18 than PBMCs isolated from healthy controls, even after phytohemagglutinin and phorbol 12-myristate 13-acetate activation. They also showed that synovium cells in RA patients present increased response to IL-12 and IL-18. However, in the present study, we found that stimulated PBMCs isolated from low-grade OC patients showed good ability to produce high levels of IFN-γ.

Our results revealed that a prolonged culture of stimulated PBMCs increased IFN-γ production. There was a significant difference in the level of IFN-γ production between stimulated PBMCs cultured for 7 and 3 days and before stimulation. We suggest that the time of culture is an important factor in increasing IFN-γ production. In addition, we found that the level of TNF-α production was increasing till day 3 of culture, and then it started to decrease. This result was only observed in the case of PBMCs isolated from low-grade OC patients; otherwise, there was an increase in TNF-α production along days of culture.

Our findings demonstrated that stimulated PBMCs from patients with benign ovarian tumors produced lower amounts of TNF-α than those produced from stimulated PBMCs from patients with high-grade OC. In line with our findings, Nowak et al.[18] reported that un-stimulated PBMC from OC patients produced more TNF-α than non-cancer patients. Another study showed that after stimulation with LPS, TNF-α was produced by cancerous ovarian tissue but not normal ovarian tissue.[30] Moreover, TNF-α cyst fluid level was high in women with malignant tumors when compared to those with benign ovarian tumors.[31] In addition, advanced stages of renal and prostate cancers were associated with high serum levels of TNF-α.[32] Here, we revealed that stimulated PBMCs isolated from high-grade OC patients showed good, constitutive, and high production of TNF-α.

In summary, IL-12 and IL-18 stimulation synergistically promote the production of IFN-γ and TNF-α by PBMCs isolated from Egyptian OC patients. Our results may be helpful in the therapeutic trials that study the cytokine profile produced by the immune cells in response to IL-12 and IL-18 combined stimulation.

Ethical Considerations

All protocols were approved by the Institutional Review Board (IRB approval number: CB2402-102-054) of the National Cancer Institute, Cairo University, Egypt.

Competing Interests

The authors declare that they have no competing interests.

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