



Original Research

Ovarian Cancer Immunotherapy en Route: IL9 Inhibits Growth of Ovarian Cancer and Upregulates its Expression of Ox40L and 4-1BBL

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Abstract

Objective: Ovarian cancer is the deadliest of all gynecologic tumors. Current treatment methods include debulking surgery with chemotherapy, however even with treatment, the five-year survival rate is below 45%. Cancer immunotherapy is an innovative treatment option being highly researched. Interleukins (ILs) are signaling molecules used by the human immune system to assist in detecting and destroying cancer cells. The ability of tumor cells to evade the immune system is a major challenge we face in fighting cancer. Ox40L/Ox40 and 4-1BBL/4-1BB are key immune costimulatory molecules that increase T cell activation to eliminate tumors. Past research has shown that IL9 has unique influences on various types of cancer, however, its role in ovarian cancer has not yet been assessed. In this study, ovarian cancer cells were treated with IL9 and the expression of Ox40L and 4-1BBL were measured. **Methods:** A2780 ovarian cancer cells were treated with IL9. Proliferation of ovarian cancer cells was measured by a Clonogenic Survival Assay and Quick Proliferation Assay. RT-PCR was conducted to determine whether IL9 upregulated the costimulatory molecules Ox40L and 4-1BBL. IHC was performed to further investigate IL9 upregulation of Ox40L and 4-1BBL. **Results:** Treatment of A2780 ovarian cancer cells with IL9 resulted in decreased proliferation of the ovarian cancer cells. By using RT-PCR, it was determined that IL9 treated ovarian cancer cells displayed upregulation of the costimulatory molecules Ox40L and 4-1BBL. Upregulation of Ox40L and 4-1BBL was further confirmed by IHC. **Conclusions:** IL9 inhibited growth of ovarian cancer cells, and IL9 upregulated the key immune costimulatory molecules Ox40L and 4-1BBL. This suggests that increased expression of Ox40L and 4-1BBL may be associated with the inhibitory effect of IL9 on proliferation of ovarian cancer. This study warrants further investigation of the role of Ox40L and 4-1BBL in ovarian cancer growth.

Keywords: ovarian cancer; interleukins; immunotherapy; costimulatory molecules

1. Introduction

Ovarian cancer is the most lethal gynecological cancer, and it is the fifth most common cause of overall cancer death in women [1,2]. Ovarian cancer is a dismal diagnosis, and it is unfortunately usually discovered at an advanced stage with metastasis [3]. Despite aggressive initial treatments with cytoreductive surgery and platinum-based chemotherapy, the five-year survival rates remain below 45% [4]. Although many ovarian cancer patients respond to initial treatment with chemotherapy and surgery, most patients will relapse within two years. Sadly, many patients develop chemotherapy-resistant disease due to molecular changes in ovarian cancer cells [5]. Improvements in chemotherapy is an indolent process, and chemotherapy has serious side effects that may lead to early termination of treatment [6]. The surgery required for treatment of ovarian cancer has many complications including infection and risk of damaging nearby organs.

For our immune system to effectively fight disease, immune cells must communicate with one another utilizing interleukins (ILs) [7]. ILs serve as messengers between cancer cells and immune cells, and thus hold potential as

novel immunotherapy agents [8]. ILs influence tumor development in two ways: ILs affect tumor cell proliferation/apoptosis and ILs drive anti-tumor immunity [9]. Our lab previously examined the role of interleukin 33 (IL33) and its inhibitory effect on ovarian cancer cell growth [10]. To further expand on the influence of interleukins on ovarian cancer, the highly influential interleukin 9 (IL9) and its role on ovarian cancer was investigated. IL9 can directly decrease the survival of tumor cells and participate in anti-tumor immunity by activating mast cells and recruiting dendritic cells into tumor sites [11,12]. Specifically, IL9 has been shown to demonstrate anti-tumor effects on melanoma, breast, lung, and colon cancer. However, IL9 was originally identified as a T cell growth factor, and in past research, IL9 was shown to have a proliferative effect on Hodgkin and diffuse large B cell lymphoma due to its lymphocyte growth factor abilities [9,11]. As discussed, IL9 plays a variety of roles in cancer immunity, and the question remains whether it can be used for immunotherapy. IL9 may be used to overcome the immunosuppressive obstacle of the cancer cell microenvironment and revolutionize the treatment of cancer [13,14].



T cells are one of our body's main defenses against cancer. T cell activation requires binding between the T cell receptor (TCR) and the antigen presenting cell (APC) MHC complex. In addition to the TCR-MHC binding, many other costimulatory signals are required to enhance the T cell response [15]. Two important costimulatory signals are Ox40/Ox40L and 4-1BB/4-1BBL, members of the tumor necrosis factor (TNF) superfamily [16,17]. Ox40 and 4-1BB are expressed on T cells, and Ox40L and 4-1BBL are expressed on many other immune system cells [18, 19]. Ox40/Ox40L and 4-1BB/4-1BBL are potent immune-stimulating agents that increases T cell survival to assist in tumor elimination [20].

Considering the devastating features and outcomes of ovarian cancer, the medical field is in desperate need of a powerful alternative to current chemotherapy and surgery regimens. The answer to improving cancer patient survival may lie in IL-based cancer immunotherapy. In this article, the effect of IL9 on ovarian cancer cell growth is investigated, and we will determine the effect it has on immune costimulatory molecules to help develop future treatments for ovarian cancer.

2. Materials and Methods

2.1 Tumor Cell Line

The human ovarian cancer cell line A2780 used in the study was cultured in DMEM medium supplemented with 10% heat-inactivated FBS and 1% penicillin-streptomycin (Invitrogen). The cultures were incubated in a humidified 5% CO₂ incubator at 37 °C. Cells were grown to 70% confluence and were then treated with IL9 or DMEM medium alone.

2.2 Treatment of Ovarian Cancer Cell Line

After reaching 70% confluence, the A2780 ovarian cancer cells were treated for 3 days with one of the following: IL9 (50 ng/mL) or DMEM medium alone. The concentration and incubation time for the treatments was established from our prior cytokine studies [21–25].

2.3 Clonogenic Survival Assay

After 3 days of treatment with IL9 or DMEM medium, A2780 ovarian cancer cells were detached and counted in a hemocytometer. Clonogenic survival assay was performed as previously described [21–25]. The number of treatment colonies were expressed as a percentage of total control colonies.

2.4 Determination of Proliferation with the Quick Cell Proliferation Assay Kit

A2780 ovarian cancer cell viability and proliferation was quantified using the Quick Cell Proliferation Assay Kit (BioVision) as described by the manufacture's protocol [21–25].

2.5 Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

A2780 ovarian cancer treatment and control cells were washed with PBS (Fisher Scientific, New Hampshire, USA) and homogenized in TRIzol (Invitrogen, Fisher Scientific, New Hampshire, USA). RNA was extracted, and NanoDrop was used to measure the concentration. RT-PCR was carried out using 1 µg of A2780 mRNA, and this yielded cDNA aliquots proportional to mRNA expression profiles of the original culture. Primer sequences specific for the costimulatory molecules (Ox40L and 4-1BBL) and GAPDH were amplified. GAPDH, a housekeeping gene, was used as an internal control for differences in amount of cDNA amplified. A more detailed description of the RT-PCR process can be found in our prior studies [21–25].

2.6 Immunohistochemistry (IHC)

IHC was conducted with the A2780 ovarian cancer cells treated with IL9. IHC was used to stain for the costimulatory molecules Ox40L and 4-1BBL by using primary antibodies purchased from Santa Cruz Biotechnology. IHC protocol was performed as described in our previous studies [21–25].

2.7 Statistics

All experiments were repeated three times. Statistical analysis was conducted using an unpaired two-tailed Student's *t* test. A *p*-value < 0.05 was considered significant.

3. Results

3.1 Treatment of Ovarian Cancer Cells with IL9 Demonstrated an Anti-proliferative Effect

A2780 ovarian cancer cells were grown to 70% confluence then treated with IL9 or DMEM medium, and ovarian cancer cell survival was monitored using the Clonogenic Survival and Quick Proliferation Assay. Treatment of ovarian cancer cells with IL9 demonstrated a statistically significant (*p* < 0.05) anti-proliferative effect when compared to the control (Fig. 1). In the presence of IL9, ovarian cancer cell colony numbers decreased from 100 ± 20% to 9 ± 3% (Fig. 1). These results emphasize the anti-growth role of IL9 on ovarian cancer cells.

3.2 IL9 Upregulates Expression of Ox40L and 4-1BBL on Ovarian Cancer Cells, Evaluated by RT-PCR

The direct effects of IL9 on the immune costimulatory molecules Ox40L and 4-1BBL on ovarian cancer cells was examined. Cell cultures of A2780 at 70% confluence were treated with IL9 for 3 days. RT-PCR was conducted to examine the mRNA expression alteration for key immune costimulatory molecules Ox40L and 4-1BBL. A2780 ovarian cancer cells treated with IL9 demonstrated a statistically significant increase in Ox40L and 4-1BBL expression levels (Fig. 2, *p* < 0.05).

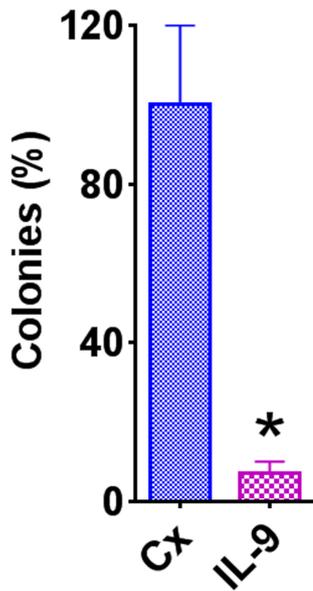


Fig. 1. Treatment of ovarian cancer cells with IL9 demonstrated an anti-proliferative effect. Shown is the clonogenic survival assay of A2780 ovarian cancer cells treated with and without IL9 (50 ng/mL). The number of colonies was counted and expressed as a percentage of the total colonies in medium alone (control). Error bars denote a mean \pm SD; Student's *t* test. Asterisk indicates significant difference ($p < 0.05$).

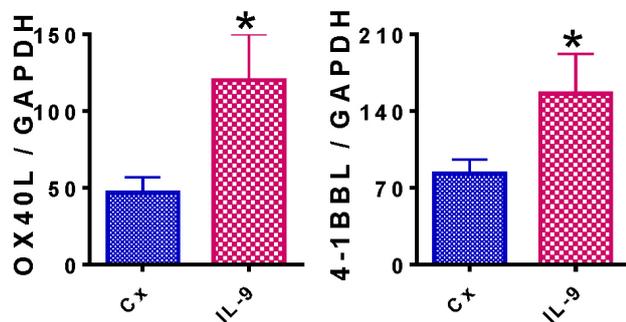


Fig. 2. IL9 upregulates expression of OX40L and 4-1BBL on ovarian cancer cells, evaluated by RT-PCR. Extraction of mRNA was performed as previously described in the methods. Results were performed in triplicate and expressed as the mean ratio of proliferative molecules densitometric units/GAPDH + SEM ($\times 100$). Significant difference in mRNA expression between cells treated with IL9 and those in control is indicated by the asterisk ($p < 0.05$).

3.3 IL9 Upregulates Expression of Ox40L and 4-1BBL on Ovarian Cancer Cells, Evaluated by IHC

RT-PCR results emphasized the critical role IL9 has on increasing Ox40L and 4-1BBL expression levels, which results in an anti-ovarian cancer cell environment. In order to further evaluate these results, immunohistochemistry (IHC) was conducted to examine whether IL9 up-

regulates Ox40L and 4-1BBL. As expected from the RT-PCR data, results from IHC staining further support increased Ox40L and 4-1BBL expression in IL9 treated ovarian cancer cells (Figs. 3, 4). The harmonious data from RT-PCR and IHC enhances our confidence that treating ovarian cancer cells with IL9 decreases growth of ovarian cancer cells by IL9's role in increasing the immune costimulatory molecules Ox40L and 4-1BBL expression.

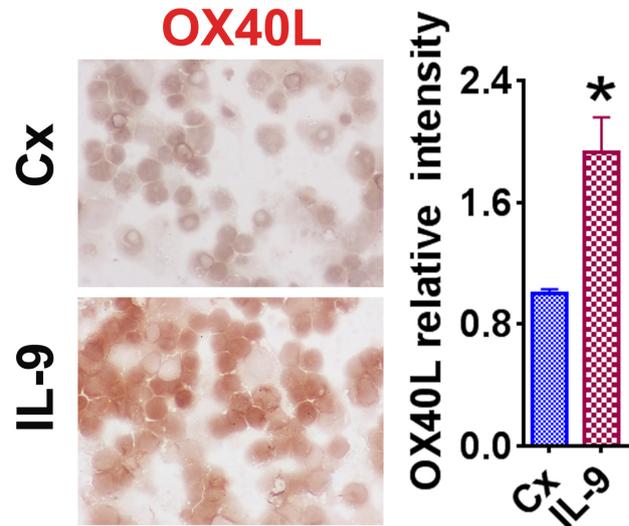


Fig. 3. IL9 upregulates expression of Ox40L on ovarian cancer cells, evaluated by IHC. Shown are representative pictures of IHC. Results are expressed as the average staining intensity relative to that in control group. A significant difference in staining intensity between both groups is indicated by the asterisk ($p < 0.05$). Original magnification: $\times 400$.

4. Discussion

The oncology field is in dire need of more effective treatment options for ovarian cancer [2]. In hopes of discovering a robust immunotherapy treatment, this research article examines how treatment of ovarian cancer cells with IL9 effect important costimulatory molecules that promote anti-tumor immunity. Treatment of A2780 ovarian cancer cells with IL9 demonstrated decreased proliferation. RT-PCR was conducted to examine IL9's influence on the immune costimulatory molecules Ox40L and 4-1BBL. IL9 was discovered to upregulate Ox40L and 4-1BBL, and the upregulation of Ox40L and 4-1BBL by IL9 was further evaluated with IHC.

A common approach to the treatment of ovarian cancer is combining debulking surgery and chemotherapy, however the relapse rate is high and survival rates are morosely low [4,6]. Treatment of ovarian cancer with immunotherapy is a budding area of research because it serves as an alternative to the inadequate current treatment regimens. ILs are essential components of the human im-

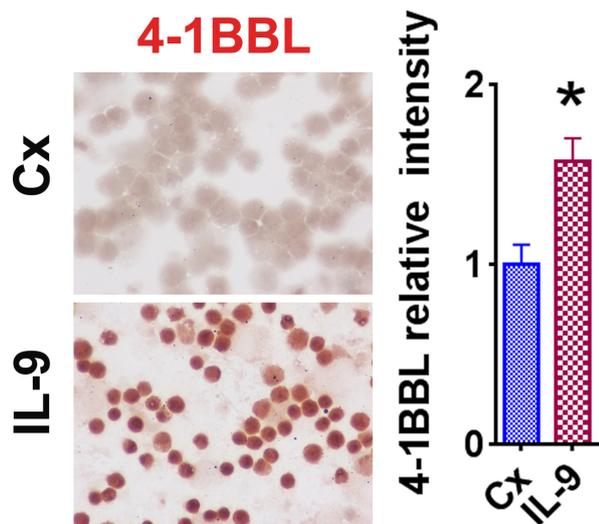


Fig. 4. IL9 upregulates expression of 4-1BBL on ovarian cancer cells, evaluated by IHC. Shown are representative pictures of IHC. Results are expressed as the average staining intensity relative to that in control group. A significant difference in staining intensity between both groups is indicated by the asterisk ($p < 0.05$). Original magnification: $\times 400$.

immune system, and Ox40L and 4-1BBL are important immune molecules involved in recognition and destruction of tumor cells by T cells [26–28]. This research is the first in the immuno-oncology field to examine treatment of ovarian cancer with IL9 and to examine the effects IL9 has on Ox40L and 4-1BBL.

In our study, ovarian cancer cells treated with IL9 had decreased proliferation, and the effects of IL9 on expression of costimulatory molecules were examined. IL9 was found to upregulate expression of Ox40L and 4-1BBL. Tumor cells are killed by cytotoxic T lymphocytes, and agents that increased CD8+ T cell activation are ideal candidates for antitumor immunity [19]. T cells express Ox40 (CD134), and many cells including endothelial cells, natural killer cells, B cells, activated T cells, and regulatory T cells express Ox40L (CD252) [29,30]. Ox40/Ox40L regulates cytokine production and signaling. Ox40/Ox40L binding enhances the survival and function of CD4+ and CD8+ T cells, and it inhibits the immunosuppressive activity of Treg cells [31]. These properties of Ox40/Ox40L give it promise as an antitumor treatment. Ox40 was discovered to be expressed on tumor-infiltrating lymphocytes in ovarian cancer, breast cancer, colorectal cancer, gastric cancer, and head/neck squamous cell carcinoma [30]. In a study examining treatment of metastatic ovarian cancer with agonistic anti-Ox40 antibodies, cytotoxic T cell infiltration was increased and tumor promoting cells were blocked [32]. An article describing treatment of advanced cancer, treatment with agonistic anti-Ox40 increased T cells, B cells, and intratumoral Tregs resulting in an enhanced tumor specific immune response [33]. The current article was the first research con-

ducted to examine how IL9 impacts Ox40L expression in ovarian cancer. Our results revealed that treating ovarian cancer cells with IL9 increased the expression of Ox40L. This finding is vital, as it is imperative to discover ways to stimulate the immune system to implement novel ovarian cancer immunotherapy.

4-1BB is not expressed on resting T cells, however 4-1BB expression on T cells is upregulated after T cells have been activated [19]. 4-1BBL is present on various antigen presenting cells such as dendritic cells, B cells, and macrophages [19]. Stimulation of 4-1BB (CD137) by 4-1BBL generates a robust CD8+ T cell memory response, and because CD8+ T cells are essential to cancer immunotherapy, the 4-1BB/4-1BBL pathway is of great interest to the immune-oncology field [17]. The pioneering study of Melero *et al.* [19] first demonstrated that administration of agonistic anti-4-1BB antibodies has potent antitumor properties against poorly immunogenic sarcoma and highly immunogenic mastocytoma by inhibiting tumor growth by increasing CTL activity [19]. In prostate cancer, combining 4-1BB activation and CTLA-4 blockage have an antitumor effect and is a strategy being examined for prostate cancer immunotherapy [34]. Mice bearing colon adenocarcinoma were injected with anti-4-1BB, and regression of the poorly immunogenic tumors was found [35]. Research is also examining the therapeutic efficacy of 4-1BBL as a cancer vaccination [36]. Our current study expanded on the antitumor properties of 4-1BBL in hopes to expand its use in treatment of ovarian cancer. RT-PCR determined that treatment of ovarian cancer cells with IL9 increased the expression of 4-1BBL. 4-1BBL is a potent stimulator of innate and adaptive antitumor immunity, and because IL9 increased 4-1BBL, IL9 is a paramount cytokine in ovarian cancer immunotherapy.

There are some limiting factors involved in our experiment. First, an animal study was not conducted to examine these effects *in vivo*. Also, only one type of cancer cell line was examined. In future research, it would be important to study other types of female cancer such breast cancer and endometrial cancer. This study only examined IL9, however, a variety of ILs play a role in the pathogenesis and treatment of ovarian cancer. Exploring the impact other ILs have on ovarian cancer cell growth is something to further investigate. Additionally, since our study indicates IL9 upregulates Ox40L and 4-1BBL, it would be of interest to investigate if silencing Ox40L and 4-1BBL by SiRNA technique could reverse the inhibitory effect of IL9 on growth of ovarian cancer.

5. Conclusions

In summary, ovarian cancer cells treated with IL9 were shown to have decreased proliferation. Then by conducting RT-PCR, ovarian cancer cells treated with IL9 were shown to have increased expression of Ox40L and 4-1BBL. Ox40L and 4-1BBL are essential costimulatory molecules

of the immune system that might have potent antitumor effects due to their influence on enhancing T cell activation and survival. Past research demonstrated IL9's antitumor effects on many different types of cancer, such as melanoma, breast, lung, and colon cancer. Proudly, our research is the first to discover the vital antitumor effects of IL9 on ovarian cancer. This study examines the proposed mechanism that decreased proliferation of ovarian cancer cells treated with IL9 may be due to IL9's ability to increase Ox40L and 4-1BBL. Ovarian cancer is a gruesome diagnosis that has an alarming survival rate. Current treatment methods of ovarian cancer with surgery and chemotherapy are unsatisfactory. Interleukin based cancer immunotherapy is a modern approach to treating a variety of cancers, and this article proudly contributes to the specific analysis of ovarian cancer immunotherapy.

Author Contributions

YF and MRW designed the study. YF, ECK, ML, ZEH, AJH, KPD, HX and QB performed the experiment, YF, ZZ, ECK analyzed the data. YF, ZZ, MRW interpreted the data. ECK wrote the draft and YF revised the manuscript.

Ethics Approval and Consent to Participate

The use of human ovarian cancer cells was approved by the Des Moines University Institutional Biosafety Committee (# 4-14-2).

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Conflict of Interest

The authors declare no conflict of interest. YF is serving as one of the Editorial Board members of this journal. We declare that YF had no involvement in the peer review of this article and has no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to Kenny Chitcholtan.

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