Combination immunotherapy with IL-4 *Pseudomonas* exotoxin and IFN- α and IFN- γ mediate antitumor effects *in vitro* and in a mouse model of human ovarian cancer

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Aim: We have shown that IL-4 fused to *Pseudomonas* exotoxin (IL-4-PE) is cytotoxic to ovarian cancer cell lines. The antineoplastic properties of IFN- α , IFN- γ and IL-4-PE have been studied and showed some promise in the clinical trials. Here, we investigated whether the combination of IL-4-PE, IFN- α and IFN- γ will result in increased ovarian cancer cell death *in vitro* and *in vivo*. **Materials & Methods:** Ovarian cancer cells were tested *in vitro* to analyze the cytotoxic effects of IL-4-PE, IFN- α and IFN- γ , and the combination of all three. Tumor-bearing xenograft mice were treated with the combination of IL-4-PE, IFN- α and IFN- γ to monitor their overall survival. The JAK/STAT phosphorylation signaling pathways were studied to delineate the mechanism of synergistic antitumor activity. **Results:** The combination of IL-4-PE with IFN- α and IFN- γ resulted in increased ovarian cancer cell death *in vitro* and *in vivo*. Mechanistically, the synergistic antitumor effect was dependent on interferon signaling, but not IL-4-PE signaling as determined by signaling specific chemical inhibitors. The combination therapy induced the activation of critical mediators of apoptosis. **Conclusion:** The combination of IL-4-PE with interferons increased overall survival of mice with human ovarian cancer xenograft. These data suggest that this novel combination could provide a unique approach to treating ovarian cancer.

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Despite advances in the treatment of many malignant cancers, ovarian cancer remains largely refractory to current treatments [1]. Most patients are diagnosed at Stage 3, indicating an advanced state of the disease with penetration of the primary tumor into the peritoneal cavity and metastatic seeding of the organs in the peritoneum [2]. Although ovarian cancer patients show a positive response to the first-line combination chemotherapy of taxane plus carboplatin, a large number have relapsing disease. There are no US FDA-approved second-line therapies and patients with disease relapse have a high mortality rate, and an overall cure rate of approximately 30% [2]. Therefore, increasing the number of therapeutic options for the treatment of late stage ovarian cancer is urgently needed.

We have reported that the IL-4 receptor α (IL-4R α) also known as CD124 is aberrantly overexpressed in ovarian cancer tissues and cell lines derived from ovarian cancer [3]. Normally, IL-4R expression is limited to B cells, T cells,

monocytes, endothelial cells and fibroblasts [4]. The level of expression of IL-4R is much lower in normal ovary than in tumor tissue [3]. To target IL-4R, we have developed a fusion protein comprised of a circularly permuted version of IL-4 and truncated *Pseudomonas* exotoxin (IL-4-PE). This fusion protein can selectively target cancer cells bearing the IL-4R α [3,5,6]. Upon binding the cell surface IL-4R, IL-4-PE is endocytosed and during the endocytic processing, the PE is activated and translocates to the cytosol, where PE blocks protein synthesis at the translational step. Protein synthesis arrest results in cell death [7]. It has been previously demonstrated that PE induces both the apoptotic and necroptotic cell death; however, the exact mechanism of cell death has not been fully elucidated [8].

Interferons (IFNs) are pleiotropic cytokines that regulate cell cycle, cellular differentiation, cell proliferation and antiviral, bacterial, fungal and parasitic responses [9]. Although IFN- α and IFN- γ production is limited to a small number of cell types primarily of immune origin, the receptors are expressed on almost every cell in the body. IFNs have had limited efficacy in the treatment of malignancies [10–12]. Currently IFN- α 2a and IFN- α 2b are licensed for the treatment of a number of cancer indications, for example, hairy cell leukemia and melanoma. Although IFN- γ is used in the clinic; there are no current indications for its use in the treatment of cancer. The almost ubiquitous expression of IFN- α/γ receptors leads to off target effects and is thought to be responsible for the toxicities associated with therapy [11,12].

Interestingly, the bulk of the metastatic disease in ovarian cancer is confined to the peritoneal cavity, making it a candidate for local-regional therapy through the infusion of drugs into the peritoneal cavity [13]. Indeed, those patients with a high clinical score are candidates for the standard of care, ip. infusion of the chemotherapeutic carboplatin. Studies have shown that ip. infusion of carboplatin increases progression free survival and overall survival [13–15]. However, the use of the therapy is limited by the toxicities associated with carboplatin. There is evidence that loco-regional administration of interferons could limit toxicity and increase therapeutic efficacy [14].

Although single agent therapy can be beneficial, it is widely accepted that combination therapy provides the best treatment options for metastatic ovarian cancer [16]. The ability to simultaneously stimulate distinct cellular pathways may increase the amount of cell death or inhibit tumor cell growth. The use of multiple drugs also decreases the potential for the cancer to mutate and become drug resistant [17]. We and others have shown that the combination of interferons results in the death of ovarian cancer cell lines [18–20]. We have also shown that IL-4-PE is cytotoxic to ovarian cancer cells expressing IL-4R α [3]. Based on these observations, we hypothesized that the combination of all three agents would mediate a better antitumor effect.

Using two human derived ovarian cancer cell lines, we show that IFNs and IL-4-PE mediate a synergistic killing *in vitro*. We utilized a preclinical model of human ovarian cancer by intraperitoneal administration of the A2780 human cancer cell line in immunodeficient mice. We show that the combination of all three agents can result in complete responses (CR), despite a conservative dosing schedule. Western blot analysis of ovarian cancer cells treated with IFNs and IL-4-PE showed robust activation of the IFN and IL-4 signaling pathways, and subsequent activation of molecules critical for inducing apoptotic cell death. Finally, using chemical inhibitors of the IFN and IL-4 signaling pathways, we showed that cell death is dependent on IFN signaling, but not on IL-4 signaling, indicating that these molecules are acting through distinct, nonredundant pathways. Taken together, these data provide evidence for a novel clinical approach to the treatment of ovarian cancer.

Materials & methods

Cell lines, IFNs & chemical inhibitors

OVCAR-5 cells were obtained from Christina Annunziata at the National Cancer Institute, National Institutes of Health (NIH), MD, USA. A2780 cell line (broadly defined as epithelial ovarian cancer to include, serous, clear cell and endometrioid cancer) was obtained from ATCC (VA, USA). All cell lines were verified via short tandem repeat analysis [21]. Cell lines were maintained in RPMI-1640 (Life Technologies Cooperation, NY, USA) supplemented with 10% FBS, 1% L-glutamine. No antibiotics or antifungal agents were added to the cultures.

Human IFN- α 2a, was a gift of Hoffmann LaRoche (NJ, USA), pegylated IFN- α 2b (Sylatron[®]) was purchased from Merck (NJ, USA) and IFN- γ purchased from Intermune Pharmaceutical Inc. (CA, USA). Ruxolitinib (Tocris Bioscience, MN, USA) and Tofacitinib (Tocris Bioscience) were purchased from Selleckchem (TX, USA) suspended in DMSO. Chemicals were stored at -20°C.

Recombinant IL-4 *Pseudomonas* exotoxin production

Recombinant chimeric protein comprised of human IL-4 and *Pseudomonas* exotoxin (IL-4-PE38KDEL) was produced by fusing a circularly permuted IL-4 mutant gene encoding IL-4 amino acids 38–129, the GGNGG

linker and IL-4 amino acids 1–37 and truncated *Pseudomonas* exotoxin gene encoding PE38KDEL. This chimeric gene was expressed in *Escherichia coli* and highly purified protein was isolated on ion exchange and gel filtration columns [22–24]. Recombinant IL-4-PE38KDEL (referred here as IL-4-PE) was reconstituted in phosphate-buffered saline (PBS) and stored at -80°C . IL-4-PE was not used after 1 freeze–thaw cycle.

Cytotoxicity assays

Cell lines were seeded at 10^4 cells/well in a 96-well plate in 100 μl of media and incubated until adherence (4 h, 37°C , 5% CO_2). Serial dilutions IL-4-PE, IFN- $\alpha 2\text{a}$ or IFN- γ were added and incubated for 3 days. IFNs were diluted using serial dilutions in cRPMI to obtain a final concentration of 200 ng/ml of both IFN- $\alpha 2\text{a}$ or IFN- γ . For Ruxolitinib and Tofacitinib studies, 10 μM (final concentration) was added to the plates and allowed to incubate at 37°C for 2 h before addition of IFNs or IL-4-PE. Media was then removed, and cell viability was determined by crystal violet dye. Crystal violet is a tri-arylmethane dye that binds to ribose type molecules such as DNA in nuclei. The dye staining is directly proportional to the cell biomass. Dye absorbance was read at 570 nm using a spectrophotometer.

Western blots

Cell pellets were snap-frozen and stored at -80°C . Pellets were thawed and lysed with MPER buffer supplemented with protease inhibitors. Cell lysates were centrifuged at 13,000 RPM at 4°C for 20 min to clarify the lysate. Clarified lysate was removed and placed in a new vial. Protein concentration was measured using a NanoDrop Spectrophotometer (Thermo Scientific, DE, USA). Lysates were mixed with reducing loading buffer (Life Technologies, MA, USA) and heated at 90°C for 10 min. Samples were allowed to cool and either used immediately or stored at 4°C for future analysis. Samples were run on 10% Bis-Tris gels and transferred to nitrocellulose membranes per manufacturer's directions. Nitrocellulose blots were first blocked with 4% milk in TBST for 1 h at room temperature. Membranes were incubated with primary antibody overnight in 4% milk in TBST. Membranes were developed using ECL reagent (Pierce, MA, USA) as directed. Images were acquired on a Licor Odyssey (LI-COR Biosciences, NE, USA).

Antibodies

Antibodies to Caspase-3 (#9662), Caspase-7 (#12827) and PARP (#9542) were obtained from Cell Signaling Technologies Inc. (MA, USA), and antibodies to Heat Shock Protein 90 (sc-7947) and, p38 (sc-535) were obtained from Santa Cruz Biotechnology (TX, USA).

Human ovarian cancer xenograft model

The peritoneal ovarian tumor model was developed in female nude nu/nu mice of 4–6 weeks of age (about 20 g in body weight). Mice were obtained from the National Cancer Institute-Frederick Cancer Center Animal Facilities, MD, USA, and were maintained in a barrier facility on HEPA-filtered racks in pathogen-free conditions with 12-h light/12-h dark cycles. All animal studies were conducted under an approved protocol (#2000-06) in accordance with the principles and procedures outlined in the program description of Animal Care and Use Program of the Center for Biologics Evaluation and Research, US FDA, MD, USA.

For tumor cell injection, for ovarian cancer model, ovarian cancer cells, A2780-Gluc cells ($2 \times 10^6/200 \mu\text{l}/\text{mouse}$) were injected directly into peritoneum. On day 4 post-tumor cell transplantation, mice were randomly divided into different therapeutic groups and a control group (5–6 mice in first and second experiment and 12 mice per group in the third experiment). These mice had similar weight in various groups. Mice were injected with excipient PBS or IL-4-PE (50 $\mu\text{g}/\text{kg}$ resuspended in 0.2% human serum albumin) or mixture of PEGylated IFN- $\alpha 2\text{b}$ and IFN- γ both in PBS at a conc. of 200 ng/ml each, on alternated days for a total of three injections. One group of mice was injected with the combination of all three agents simultaneously at the same concentrations. All injections were given ip.

In earlier experiments, mice were imaged weekly to monitor the tumor growth in real-time. But unfortunately, the noninvasive quantitative measurement of external visible bioluminescence area (total photon flux) did not correlate well with actual tumor size. We discontinued the *in vivo* imaging and performed later experiments for overall survival of mice. In the third experiment ($n = 12$), two mice were sacrificed 1 week after the last injection for monitoring early growth of tumors. The remainder of the mice were monitored for their survival for up to 175 days. Body weights of mice were measured and mice with extremely distended abdomens were sacrificed and

Table 1. Synergistic antitumor effect of combination of IL-4-PE and interferons in ovarian cancer cell lines.

OVCAR-5		A2780	
IL-4-PE (ng/ml)/IFNs (ng/ml)	CI values	IL-4-PE (ng/ml)/IFNs (ng/ml)	CI values
0.04/0.16	1.25 (A)	0.32/1.6	0.50 (S)
0.2/0.8	0.4 (S)	1.6/8	0.06(S)
1/4	0.11 (S)	4/20	0.02 (S)
5/20	26.2E-06 (S)	40/200	4.5E-04 (S)
EC ₅₀	0.58 (S)	EC ₅₀	0.24 (S)

Synergy analysis is presented as a combinatorial index (CI) number for each concentration group tested where A indicates antagonism, and S is synergistic. Values were generated from data presented in Figure 1 using CompuSyn software.

photographed immediately, tumors and organs were harvested for tumor weight, toxicological and histological studies.

Histology

Organs and tumors were removed and placed in 4% Paraformaldehyde in buffered saline. Tissues were imbedded in paraffin blocks and sectioned. Sections were stained with hematoxylin and eosin and stained with antibodies against Caspsase-3 (Clone #9661, Cell Signaling) and Ki-67 (Clone #MIB-1, Dako, CA, USA).

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean obtained from at least three different experiments. Significance of datasets for drug treatments was analyzed by Two-way ANOVA with Bonferroni post-test analysis with p-values ≤ 0.05 (*p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001). Statistical analysis was performed using GraphPad 7 Software (Prism, CA, USA).

Synergism, additive effects and antagonism for multiple drug treatments were quantified by combination index values (CI) obtained from CompuSyn software [25].

The *in vivo* data were analyzed for statistical significance using Student's t-test and ANOVA. Survival curves were generated by Kaplan–Meier method and compared by using a two-tailed log rank test. The animal experiments were repeated three-times.

Results

IL-4-PE, IFN- α & IFN- γ mediate synergistic cytotoxic effect in ovarian cancer cell lines *in vitro*

We tested two ovarian cancer cell lines to analyze the cytotoxic effects of IL-4-PE, IFN- α and IFN- γ , and the combination of all three. OVCAR-5 cells represent an IL-4-PE sensitive, IFNs sensitive cell line, and A2780 a IL-4-PE low sensitive, IFNs low sensitive cell line. For both cell lines cytokine concentrations above and below the individual EC₅₀s (half maximal effective concentration) for IL-4-PE and IFNs were chosen; concentrations ranging from 0.04 to 40 ng/ml for IL-4-PE, and 0.16 to 200 ng/ml for the IFNs were used.

In the OVCAR-5 cells treated with the lowest cytokines concentration there was a statistically significant difference between IL-4-PE alone, and the combination treatment (Figure 1A). However, this difference was not mathematically synergistic (Table 1). There was a statistically significant difference in the amount of cell death between both IL-4-PE (0.2 ng/ml) alone, IFNs alone (0.8 ng/ml) and the combination. IFNs were added at equal concentrations (i.e., 0.8 ng/ml IFN- α 2a and 0.8 ng/ml IFN- γ). The combination of the three agents resulted in slightly more than 50% cell death. Combination analysis showed that this affect was synergistic (CI: 0.4). Treatment with 1.0 ng/ml IL-4-PE and 4.0 ng/ml IFNs alone resulted in greater than 50% killing of the OVACR-5 cells. Combination treatment showed statistically significant killing compared with the single agents and caused 97% cell death. This effect was highly synergistic (CI: 0.11). At the highest concentrations tested (IL-4-PE, 5.0 ng/ml, 20.0 ng/ml IFNs), there was 100% killing with the combination treatment, which was also highly synergistic (CI: 26.2×10^{-6}).

Compared with the OVCAR-5 cell line, the A2780 cell line is more refractory to both IL-4-PE and IFNs, with EC₅₀s of the individual agents approximately eightfold and tenfold higher (Figure 1B). Similar to the OVCAR-5, there was a statistically significant difference between IL-4-PE and combination treatment that was also synergistic (CI: 0.5) (Table 1). At the three higher concentrations, there was significant and synergistic killing of the A2780 cells, with 100% cell death at the highest concentrations (IL-4-PE 40 ng/ml, IFNs, 200 ng/ml). Despite requiring

Figure 1. Cytotoxic effect of IFN- α 2a, IFN- γ and IL-4-PE on human ovarian cancer cell lines *in vitro*. OVCAR5 (A) and A2780 (B) cells were treated with increasing concentrations of IL-4-PE (black bars), IFN- α and IFN- γ (open bars), or the combination of IL-4-PE, IFN- α and IFN- γ (grey bar). Concentrations are presented on the abscissae with IL-4-PE concentration first, and IFN- α and IFN- γ concentration second. Cell viability is presented as percent of control on the ordinate. Statistics were calculated by two-way ANOVA, with p-values ≤ 0.05 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

high concentrations of both agents, the CI values of the A2780 treatment group were more synergistic in the A2780 cells than the OVCAR-5 cells (Table 1). A2780 cells also had a greater EC_{50} synergistic effect (CI: 0.24) than OVCAR-5 cells (CI: 0.58) (Table 1). The *in vitro* killing assays show that IL-4-PE and IFNs act in a synergistic fashion to kill two ovarian cancer cell lines.

Combination of IL-4-PE, IFN- α & IFN- γ increased overall survival of tumor-bearing mice

We choose A2780 cells for *in vivo* studies as this cell line produced consistent intraperitoneal metastasis. An ip. injection of 2×10^6 A2780 cells into nude mice results in tumor formation throughout the peritoneal cavity that models ovarian cancer in patients. To test the effect of IL-4-PE and IFNs *in vivo*, we created a model that would follow the dosing schedule of IL-4-PE in clinical trials (Figure 2A). Mice received ip. injections of saline, IL-4-PE, IFNs or the combination of both on days 5, 7 and 9. This model was repeated three-times (Figure 2B–D).

In the first experiment, five mice were randomized per group. We injected animals with 2×10^6 A2780 cells ip. (Figure 2B). On days 5, 7 and 9, the animals received ip. injections of saline, IL-4-PE, IFNs or the combination of both. By day 120, the entire IFN treatment group had died. At the termination of the experiment on day 174, one of five animals in both the saline and IL-4-PE group were alive. Three of the five animals in the combination treatment group survived until the termination of the experiment and were scored as having a CR on necropsy. Statistical analysis showed a significant difference between the saline treatment and combination treatment groups ($p = 0.0471$). No statistically significant difference was observed between saline control, IL-4-PE or IFNs alone groups.

Figure 2. *In vivo* therapy model of interferons and IL-4-PE. **(A)** Schematic of dosing: 2×10^6 A2780 cells were injected ip. on Day 0. Animals were treated with either IFN- α and IFN- γ (20 ng/mouse), IL-4-PE (1 μ g/mouse), both IFNs and IL-4-PE or saline ip., total volume of 100 μ l on days 5, 7 and 9. **(B–D)** Kaplan–Meier survival curves for three separate experiments. Graphs show survival of groups treated with saline (black line), IL-4-PE (dotted line), IFNs (dashed line), or IFNs and IL-4-PE (grey line).

In the second experiment (Figure 2C), all the animals ($n = 6$) died in both the IL-4-PE treated group (by day 35) and the saline group by day 42 in the saline group. Five of the six mice died in the IFNs group by day 42, with one animal surviving to day 142. In the dual treatment group, three of the six animals died by day 42, with one dying on day 50. Two remaining mice survived until the termination of the experiment on day 155. There was a highly statistically significant difference between IL-4-PE alone, IFNs alone or saline treatment and combination treatment groups ($p = 0.008$).

In the third experiment (Figure 2D), 10 out of 10 animals had died by day 49 in the IL-4-PE treatment group. Nine out of ten animals had died by day 41 in saline and IFN groups also. In the combination treatment group, three of the ten animals were alive at the termination of the experiment with all three scored as a CR. Statistical analysis showed a significant difference between IL-4-PE alone, IFNs alone or saline treatment and the combination treatment groups ($p = 0.0171$). Saline control, IL-4-PE or IFNs alone treatment groups showed no statistically difference.

Because of fewer number of the animals in the experiment 1, the statistical survival probability analysis showed a little difference among the four groups (global $p = 0.42$). The combination group appeared to be better but not statistical significant. The individual groups in the experiment 2 also appeared to be similar, but because of the rapid events noted compared with the experiment 1, the individual groups exhibited a trend of not being identical (global $p = 0.06$). However, the percent survival in combination group was statistically significant ($p = 0.008$) compared with the other groups. With only six mice per group, the advantage of survival probability in treated groups was inconclusive.

In the experiment 3, we used larger number ($n = 10$) of animals per group. The similar treatment as other two experiments, all individual groups differed from each other and overall survival probability values was highly significant (global $p = 0.0086$). The combination group (IFNs + IL-4-PE) exhibited significantly better survival

Figure 3. Quantification of disease from the *in vivo* therapy model in Figure 2. Total mouse weight (A), tumor weight (B), the ratio of tumor weight to mouse weight (C), and the ratio of tumor weight to days alive (D) from experiments 1 and 3.

than the other three groups (saline, IFNs or IL-4-PE). The three p-values for the combination group compared to either control, IL-4-PE or IFNs group is in between 0.0011 and 0.015. Thus, we conclude that there is clear effect of the combination treatment on overall survival.

Both mouse weight and total tumor weights were measured (Figure 3A & B). There was no difference in mouse weight across groups. However, there was a decrease in total tumor weight in the combination group, but this decrease did not meet statistical significance. As a measurement of cachexia, tumor weight was divided by mouse weight (Figure 3C). Although there was a difference between saline and combination group, the change did not meet statistical significance. We realize that mice tumor weights were measured at different time points, but the data are shown for the last of the experiment. Our results show that despite large tumor burden there was no absolute body mass lost indicating significant cachexia in animals. The inability to directly measure cachexia is important to highlight the limitations of the mouse model when compared with human disease. To measure total tumor burden as a function of time to death, tumor weight was divided by the number of days the animal survived (Figure 3D). Although not statistically significant there was a trend toward a difference between the controls and combination treatment groups. It is to be noted that mice were sacrificed at different days in each group when they reached study end point. Though mice survived longer in combination therapy treated groups, their tumor burden (measured as tumor weight) may not be much different than control mice tumor burden on a day of the sacrifice.

Combination of IL-4-PE, IFN- α & IFN- γ decreased proliferation of tumor cells *in vivo* and did not cause histological damage of vital organs

We performed Ki-67 and Caspase-3 staining on fixed tumor tissue sections. There was a marked decrease in Ki-67 staining in the combination group compared with controls, indicating a decrease in proliferation (Figure 4). Several random areas were picked for neoplastic cells numeration. The degree of proliferative activity was estimated by determining the percentage of Ki-67-positive cells in the total cell population. The marked decrease in Ki-67 in the combination group correlated well with the survival of these mice. However, there was no change in the amount of

Figure 4. Ki-67 staining of tumors. Peritoneal tumors were harvested on day 17 post-tumor implant, fixed and stained for Ki-67. Representative images from one mouse, Control (A), IFNs (B), IL-4PE (C), and Combination (D). A marked decrease in Ki-67 staining (brown cells) was observed in the combination group compared with controls, indicating a decrease in cell proliferation. The figures shown for each group are representatives of all collected tumors stained with Ki-67. The degree of proliferative activity was estimated by determining the percentage of Ki-67-positive cells (brown) in the total cell population. Routinely, several viable areas of each specimen were randomly selected for neoplastic cells examination.

Figure 5. Western blot analysis of interferon and IL-4-PE STAT activation. (A) Western blots for phosphorylated STAT1 in OVCAR-5 and A2780 cells stimulated with IFN-α, IFN-γ, IL-4-PE or all three agents for 20 min at the highest concentrations from Figure 1 (representative of three separate experiments). **(B)** Western blots for phosphorylated STAT6 in OVCAR-5 and A2780 cells stimulated with IFN-α, IFN-γ, IL-4-PE or all three agents for 20 min (representative of three separate experiments). p38 protein kinases were used as a loading control.

Caspase-3 activity between treatment groups (data not shown). To address potential toxicity, the major organs of the peritoneum were fixed and analyzed. Analysis of paraffin-embedded tissue sections from the experiments did not show any gross abnormalities, indicating that the treatment was not toxic to normal tissue (Supplementary Figure 1).

Mechanism of synergistic effect of IL-4-PE & IFNs

IFN-α and IFN-γ signal through the IFN-α and IFN-γ receptors. Both receptors induce STAT1 activation through phosphorylation [10]. Similarly, IL-4 signals through the IL-4Rα and either the common IL-2 receptor γ chain (IL-2Rγc) in immune cells or IL-13Rα1 chain in tumor cells to induce STAT6 activation [26]. We measured both STAT1 and STAT6 phosphorylation in response to IFNs, IL-4-PE or the combination of all three. IFN-α and IFN-γ induced STAT1 phosphorylation in both OVCAR-5 and A2780 cell lines (Figure 5A). IL-4-PE did not induce STAT1 phosphorylation or inhibit IFN induced STAT1 phosphorylation. IL-4-PE induced STAT6 phosphorylation alone and in combination with the IFNs (Figure 5B).

Figure 6. Chemical inhibition of JAK signaling. OVCAR-5 cells (A, B) or A2780 cells (C, D) were treated with either ruxolitinib or tofacitinib for 2 h at a final concentration of 10 μ m before the addition of different concentrations of IFNs, IL-4-PE or the combination of each. Percent viability was measured after 3 days in culture.

Although PE-mediated cell death is independent of IL-4R α signal transduction, it is possible that IL-4R α signaling through STAT6 could influence the observed synergistic killing. Conversely, it is possible that IFNs signaling through STAT1 could influence STAT6 mediated signaling. To test these hypotheses, we used two chemical inhibitors of JAK/STAT signaling. We have previously shown that endocytosis of IL-4-PE is necessary for PE-mediated death. However, the presence of a functional IL-4 receptor could indicate that IL-4 mediated signaling is involved in tumor cell death. Tofacitinib is an FDA approved drug that blocks Jak1 and Jak3 which are protein critical for IL-4 signaling. IFNs do not require receptor internalization to mediate signaling events. As a negative control, we also treated cells with the FDA approved drug Ruxolitinib which blocks Jak1- and Jak2-dependent IFN signaling.

Ruxolitinib blocked all of the IFNs and IL-4-PE-mediated cell death at both 20/4 ng/ml and 200/40 ng/ml IFNs/IL-4-PE (Figure 6A). However, Ruxolitinib did not block any of the IL-4-PE-mediated cell death. Although similar Ruxolitinib-mediated blocking of IFN signaling was observed in A2780 cells (Figure 6C), the addition of

Figure 7. Western blot analysis of interferon and IL-4-PE induction of apoptosis-related proteins. Indicated cell lines were cultured with IFN- α 2a, IFN- γ , IL-4-PE alone or all three drugs for 33 h. Cells were lysed and probed for indicated protein products (A) PARP and cleaved PARP, (B) caspase-3 and cleaved caspase-3, (C) caspase-7 and cleaved caspase-7.

Ruxolitinib slightly increased the amount of IL-4-PE mediated cell death. Tofacitinib experiments showed similar results as the Ruxolitinib experiments in the OVCAR-5 cells (Figure 6B), including a dose-dependent blocking of IFN signaling. Similarly, in the A2780 cells Tofacitinib blocked IFNs-mediated killing at both doses, but not the IL-4-PE-mediated killing in the combination treatment group (Figure 6D).

We and others have shown that IFNs- and IL-4-PE-induce apoptosis in ovarian cancer cell lines. To assess apoptotic cell death, we measured PARP cleavage. PARP is critical for regulating the cellular response to DNA damage. PARP cleavage is both an indication of caspase activation and inhibition of the DNA damage response. Using western analysis, we showed that the IFNs alone, IL-4-PE or the combination of all three induced PARP cleavage in the OVCAR5 cell (Figure 7A). In the A2780 cell line, we only observed PARP cleavage in the IL-4-PE alone or IL-4-PE in combination with the IFNs (Figure 7A). Caspase-3 is an executioner (effector) caspase that mediates protein degradation and subsequent apoptosis. In the OVCAR-5 cells, we found a small amount of Caspase-3 activation with the IL-4-PE groups [8], but not the IFNs (Figure 7B). In the A2780 cells, there was a small cleavage product at 17 kDa (Figure 7B). Of note, there was almost no presence of the whole caspase-3 at 35 kDa. Caspase-7 is also an important protease in apoptotic cell death. Western blot analysis showed Caspase-7 cleavage in all treatment groups in OVCAR-5 cells. Caspase-7 cleavage was also evident in the A2780 cells (Figure 7C). Overall, our apoptosis studies showed that qualitative changes occur in apoptosis-related proteins when cells are treated with IFNs and IL-4-PE.

Discussion

The ability to target multiple pathways at the same time is critical in the treatment of human cancer. Here, we report that the triple combination of IFN- α , IFN- γ and IL-4-PE that targets multiple receptors and pathways is a potential therapeutic modality for ovarian cancer. We show that ovarian cancer epithelial cells are more sensitive to IFNs and IL-4-PE than the individual agents alone. When treated with IFNs and IL-4-PE either alone or in combination, the combination approach mediated synergistic antitumor effects *in vitro* as measured by cytotoxicity assays. To understand the mechanism of synergistic cytotoxicity, we studied intracellular signaling and the activation of cell death (apoptosis) by IFNs and IL-4-PE in ovarian cancer cell lines. Western blot analysis showed that IFNs- and IL-4PE-induced STAT1 and STAT6 phosphorylation, respectively. The JAK inhibitor studies using Tofacitinib and Ruxolitinib showed that the cytotoxicity was dependent on IFN and independent of IL-4 signaling indicating that the two signaling pathways are not interacting with each other to cause cell death. These results confirmed a previous report that IL-4-PE-mediated cytotoxicity is independent of STAT6 signaling [27]. It is known that IL-4 binding to IL-4R induces several signaling pathways in addition to pStat6 such as Akt and Erk, which may also play a role in the cell death [28]. A similar induction is presumably expected of IL-4-PE.

Apoptosis was assessed by western blot analysis for PARP, Caspase-3 and Caspase-7, which showed that both IFNs- and IL-4-PE-activated critical proteins in the apoptosis pathway. Our results are consistent with a previous study

that showed that IL-4-PE induces apoptosis [29]. However, it is unlikely that IL-4-PE would mediate cytotoxicity through IL-4R signaling in animal models. Additional studies are needed to understand the relationship between signaling events and cell death, and the relative contribution of apoptosis to cell death.

To study the antitumor effects of combination therapy *in vivo*, we developed an intraperitoneal metastatic ovarian tumor model in immune deficient animals. One of the more aggressive human ovarian cancer cell line A2780 when injected ip. generated a rapid and dispersed disease within the peritoneal cavity. These animals succumb to disease with median survival time of 35–55 days. Consistent with the *in vitro* results, a combination of IFNs and IL-4-PE when administered *in vivo* mediated synergistic antitumor effects. This antitumor effect was assessed by overall survival of ovarian cancer bearing animals and autopsy findings. Overall, a combination of IFNs and IL-4-PE resulted in statistically significant higher survival of animals compared with individual treatment groups. Despite the aggressive nature of this ovarian cancer model, approximately 33% of animals survived in the combination group with 24% scored as having a CR.

The rationale of using IFN- α and IFN- γ in our combination therapy is based on previous observations that both IFNs themselves are potent antineoplastic proteins [10]. We also demonstrated that IFN- α 2a plus IFN- γ activated monocytes strongly and increased human monocyte cytotoxic activity and eradicated high concentrations of human tumor cells *in vitro* [18]. Based on these studies, we confirmed that monocytes in combination of both IFN- α and IFN- γ resulted in mediating antitumor effect against human ovarian tumors in mouse models [30]. Both IFNs have been given to patients with ovarian cancer. In one study, 39 patients with ovarian cancer were given intraperitoneal IFN- α 2b in combination with chemotherapy, 14/35 patients achieved a pathological CR [31]. In another study, subjects were administered IFN- γ through ip. route which showed CR (21%) and partial response (7%) [32]. In addition, one of the most important observations was that the both IFNs had a tolerable side effect profile. No published studies report the co-administration of both IFN- α 2b and IFN- γ ip. Based on these preclinical and clinical studies, we decided to use both IFNs along with IL-4-PE for an optimal antitumor effect.

To study the timeframe of tumor response and to support the animal survival results, we performed a longitudinal imaging study using gaussia luciferase imaging technology. Ovarian tumor cells were stably transduced with a vector containing luciferase and then chemiluminescence was measured by IVIS imaging system after iv. injection of luciferin. Despite different routes of administration of luciferin (iv. and ip.) and different imaging protocols, we were unable to consistently get strong, reproducible quantification of tumor growth (data not shown). This was due to deep seated tumor nodules. In some animals, the gaussia luciferase levels did not correspond to deep tumor burdens when an autopsy was performed. These inconclusive imaging results led us to explore additional imaging approaches to further study ovarian tumor growth *in vivo*.

To further understand the mechanism of synergistic action *in vivo*, histology of the tumors was performed that showed a decrease in the amount of Ki-67 staining in combination treatment group. This decrease in the proliferation marker suggests that the combination treatment decreases the proliferative capacity of the cells. However, we did not see any difference in Caspase-3 staining between groups. This is most likely due to the advance stage of the tumors. Future studies analyzing the rate of tumor growth and the amount of tumor cell death will provide insight into the antiproliferative effects of the treatments.

The combination therapy did not mediate any visible signs of toxicity as animals being treated did not show changes in body weight or other clinical measurements of animal health. In addition, there was no gross toxicity seen in peritoneal organs as assessed by histology. This is consistent with limited toxicity of IFNs to normal cells *in vitro* despite having antiproliferative effects in cancer cells. Similarly, due to low level expression of IL-4R in normal cells including resting immune cells, IL-4-PE has limited toxicity to normal cells [33]. IL-4-PE has been administered iv. in Phase I clinical trials for the treatment of advanced solid tumors and showed dose limiting reversible liver enzymes elevation [34]. It will be of interest to study biochemical changes in liver function with the combination therapy. In addition, the novel route of administration directly into the peritoneal cavity could change the toxicity profile in the clinical trials by increasing the concentration at the site of disease and decreasing systemic concentrations.

One of the limitations of xenograft models used in the current study is that there is little to no cross-species reactivity with human IFNs and IL-4 and murine IFNs and IL-4 receptors. The human IL-4-PE used in this study is a chimeric protein consisting of human IL-4 and a truncated form of *Pseudomonas* exotoxin which, due to species specificity, is not expected to bind to murine IL-4R [33,34]. Because of this limitation, the immunomodulatory properties of IL-4-PE or IFNs were not assessed on murine immune cells. However, human IL-4 can bind to monkey cells, which by systemic administration in monkeys did not deplete T cells as determined by complete

blood and lymphocyte counts. Additionally, our previous studies have shown that IL-4-PE has no cytotoxic effects on resting T cells [23]. However, when activated, the cells become susceptible to the cytotoxic effect of IL-4-PE, but show no preference between Th1 or Th2 cells. As in an intact host most T cells are in the resting stage, selective killing of Th1 or Th2 cells is not expected. Moreover, in a Phase I clinical study, no changes in leukocytes were reported when IL-4-PE was administered to patients with advanced solid tumors [34]. Based on these observations, we expect that IL-4-PE will not cause any shift in Th1/Th2 response in an intact host.

We have previously assessed safety of IL-4-PE in various preclinical studies [35]. Mice with sc. xenografted human pancreatic carcinoma were injected ip. with IL-4-PE doses up to 200 µg/kg twice a day for 5 days. An additional group of mice was given IL-4-PE up to 500 µg/kg intratumorally (i.t.) every other day for three injections. IL-4-PE was well tolerated in all mice and did not show organ toxicity or mortality [6]. However, three daily iv. injections of IL-4-PE determined the LD₅₀ as 475 µg/kg [36]. The dose-limiting hepatic toxicity seen in our studies was reversible. Monkeys given iv. administration of IL-4-PE at a dose of 50 or 200 µg/kg also tolerated the drug well with reversible hepatic toxicity [37]. Monkeys showed no other systemic toxicities.

In a Phase I clinical study, patients with solid tumors [34] tolerated systemic administration of IL-4-PE at doses up to 16 µg/m² daily for 5 days every 28 days. Similar to preclinical studies, IL-4-PE induced dose limiting elevation of hepatic enzymes in patients. In contrast, localized intracranial administration of IL-4-PE in patients with recurrent brain tumors exhibited no systemic toxicities [37]. Because of no detection of IL-4-PE in serum, we hypothesized that intracranial IL-4-PE did not reach to blood circulation.

The purpose of this study was to demonstrate synergism of two IFNs and IL-4-PE on ovarian tumor cells *in vitro* and animal model of ovarian cancer *in vivo*. We believe that the patient derived xenograft models could provide greater insight. Therefore, future studies are being designed to study the synergism in various patient derived xenograft models and analyze both the efficacy and potential systemic toxicity of combination therapy. In addition, we will examine the effect of combination therapy on the immunomodulatory effects of the host.

Conclusion

The novel combination therapy with IFNs and IL-4 fused *Pseudomonas* exotoxin (IL-4-PE) is a potential new immunotherapy modality for the treatment of ovarian cancer. This combination therapy increased overall survival of mice with human ovarian cancer xenograft. Since the bulk of the metastatic disease in human ovarian cancer is confined to the peritoneal cavity, the intraperitoneal administration of IFNs and IL-4-PE agents could be a suitable approach in managing this disease. The loco-regional administration of IFNs could limit toxicity and increase therapeutic efficacy. Additional studies are planned to support a Phase I clinical trial with IFNs and IL-4-PE.

Future perspective

This combination use, along with the administration of the agents ip., could provide a new, cytokine and fusion toxin protein therapy for the treatment of relapsed metastatic ovarian cancer.

Summary points

- IL-4-PE with IFN-α and IFN-γ resulted in increased ovarian cancer cell death *in vitro*.
- Animals treated with IL-4-PE with IFN-α and IFN-γ showed significantly higher overall survival compared with controls in three independent experiments.
- The combination therapy did not mediate any visible signs of toxicity as animals being treated did not show changes in body weight or other clinical measurements of animal health.
- The synergistic antitumor effect was dependent on interferon signaling, but not IL-4-PE signaling as determined by chemical inhibitors.

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Ethical conduct

All animal studies were conducted under an approved protocol (#2000-06) in accordance with the principles and procedures outlined in the program description of Animal Care and Use Program of the Center for Biologics Evaluation and Research, Food and Drug Administration, Silver Spring, Maryland.

Financial & competing interests disclosure

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Author contributions

Conception, design and writing of the manuscript performed by DS Green, SR Husain, RK Puri, KC Zoon. Data acquisition performed by DS Green, SR Husain, CL Johnson, Y Sato, B Joshi. Data analysis performed by DS Green, SR Husain, CL Johnson, Y Sato, B Joshi, RK Puri, KC Zoon. All authors read and approved the final manuscript.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/imt-2018-0158

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