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ORIGINAL ARTICLE

Phase-I clinical trial of IL-12 plasmid/lipopolymer complexes for the treatment of recurrent ovarian cancer

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A phase-I trial to assess the safety and tolerability of human interleukin-12 (IL-12) plasmid (phIL-12) formulated with a synthetic lipopolymer, polyethyleneglycol–polyethyleneimine–cholesterol (PPC), was conducted on women with chemotherapy-resistant recurrent ovarian cancer. A total of 13 patients were enrolled in four dose-escalating cohorts and treated with 0.6, 3, 12 or 24 mg m^{-2} of the formulated plasmid once every week for 4 weeks. Administration of phIL-12/PPC was generally safe and well-tolerated. Common side effects included low-grade fever and abdominal pain. Stable disease and reduction in serum CA-125 levels were clinically observed in some patients. Measurable levels of IL-12 plasmid were detectable in PF samples collected throughout the course of phIL-12/PPC treatment. In comparison, serum samples either did not contain detectable amounts of plasmid DNA or contained < 1% of the amount found in the corresponding PF samples. Treatment-related increases in IFN-γ levels were observed in PF but not in serum. These data demonstrate that IL-12 gene delivery with a synthetic delivery system is feasible for ovarian cancer patients.
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Introduction

Ovarian cancer is the fifth leading cause of cancer death among women, with more cancer deaths than any other malignancy of the female genital system.[1,2] The 5-year survival of patients with advanced disease is currently about 50%, with 80% of patients eventually relapsing primarily due to drug resistance. Recognition of the limitations of conventional therapy has warranted the search for novel therapies for ovarian cancer.

Most epithelial ovarian cancers express high levels of antigens, which makes them a suitable target for immunotherapy.[3] Treatment strategies involving activation of the immune system offer potential advantages over conventional cytotoxic therapy.[3–6] Interleukin-2 (IL-2), IL-12, interferon (IFN)-α and IFN-γ are the predominant cytokines with immunomodulatory activity in ovarian cancer.[3,5] IL-12 is one of the most active immunocytokines known for its potent ability to activate both innate and acquired immunity against cancer.[7–10] IL-12 has been shown to prime developing T-cells to stably produce IFN-γ,[11] a potent mediator of the antitumor effects of IL-12. Endogenous production of IFN-γ is required for most, if not all, of the IL-12 effects on the immune system.[12–14] In addition to stimulating the innate and acquired immune systems against cancer, IL-12 also exerts antiangiogenic effects through IFN-γ-inducible protein-10 (IP-10) and monokines.[15,16] Administration of recombinant IL-12 (rIL-12) or IL-12-transduced fibroblasts or dendritic cells in ovarian cancer mouse models has been shown to increase IFN-γ concentrations, induce differentiation and proliferation of cytolytic immune cells, inhibit tumor growth and improve animal survival.[17–22] In ovarian cancer patients, exposure of blood or peritoneal lymphocytes to autologous tumors resulted in increased cytolytic activity of the lymphocytes.[23] Intraperitoneal (IP) treatment of patients with peritoneal carcinomatosis originating from gastrointestinal malignancies, mesothelioma, Mullerian carcinoma and ovarian carcinoma resulted in favorable immunological responses, including peritoneal tumor cell apoptosis, decreased tumor cell expression of basic fibroblast and vascular endothelial growth factor, and high levels of immunocytokines in the blood and peritoneal fluid (PF).[24] The initial- and terminal-phase half-lives of rIL-12 in PF were shown to be 1.5 min and 18.7 h, respectively, and resulted in IL-12 treatment-related grade-3 and higher hematological and liver toxicities. Clinical, biological and immunological responses to rIL-12 have also been observed in other types of cancers, along with grade-3 or higher toxicities and downregulation of biological response to repeated delivery.[25–29] Despite aggressive clinical testing, rIL-12 therapy has not advanced to an approved therapy, due to high blood concentrations of IL-12 and IFN-γ resulting in systemic toxicity and downregulation of immunological response. Alternative means of IL-12 delivery are being explored to decrease IL-12 toxicity and improve clinical efficacy.[30] Some of these approaches involve administering

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IL-12 gene or gene-modified cells to achieve therapeutic levels of IL-12 locally to avoid systemic toxicity and downregulation of immunological response. Although effective, these approaches also have limitations, including generation of neutralizing antibodies to viral vector, in addition to lacking the simplicity and versatility required for universal clinical application. Thus, there is significant interest in developing alternative approaches to IL-12 delivery that are safe. We have recently described a gene-based IL-12 therapeutic that produces local concentrations of IL-12 and IFN-γ without causing systemic toxicity in a mouse model of ovarian cancer.[31,32] This IL-12 therapeutic comprises a human IL-12 plasmid (phIL-12) that encodes for functional IL-12 protein and a synthetic DNA-delivery system, polyethyleneglycol–polyethyleneimine–cholesterol (PPC). Comprehensive safety/toxicity and biodistribution studies using normal mice show that phIL-12/PPC delivery is safe and localized at the injection site.[33,34] The spectrum of toxicities was limited to local inflammation.[34] This paper describes the first in human clinical trial of IP phIL-12/PPC in patients with chemotherapy-resistant recurrent ovarian cancer. The study endpoints included safety, preliminary efficacy and biological activity of phIL-12/PPC treatment.

Results

Demographic and other baseline characteristics

Thirteen patients with recurrent ovarian cancer were enrolled in four dose-escalating cohorts. The histological subtype of the ovarian cancer was papillary serous (five patients), mixed papillary serous/endometroid (two patients), endometroid (two patients) and unknown (four patients). Cohorts 1, 2 and 4 (0.6, 3.0 and 24 mg m^{-2} plasmid DNA, respectively) had three patients each while cohort-3 (12 mg m^{-2}) had four patients. One of the patients (412) discontinued the study after the second dose due to diffuse infectious peritonitis. Out of 12 patients who received all four doses and completed the treatment period, one patient in cohort-3 (309) and one patient in cohort-4 (411) was not included for plasmid DNA and cytokine analysis due to sample quality. Demographic data collected at screening were similar across dose cohorts (Table 1). All but one patient was Caucasian. The age range for patients was 41 to 73 years, with a mean age of 60.5 years. Mean age at diagnosis was 57 ± 9 years, and mean years since initial diagnosis was 3.6 ± 1.3 years. Mean years since last recurrence was 0.5 ± 0.6 years. These numbers were similar across dose cohorts. Disease stage at initial diagnosis ranged from III to IV (FIGO system). All patients had been surgically treated and had received chemotherapy prior to screening. The number of prior chemotherapy regimens received by the study patients ranged from 2 to 6, with a median of three regimens. Two patients had received radiation and one patient had received hormonal therapy prior to screening.

Safety assessment

All 13 patients enrolled in the study received at least one dose of the study drug and therefore included in the safety analysis. All patients received four weekly doses of phIL-12/PPC, with the exception of one patient in the 24-mg m^{-2} dose cohort who prematurely discontinued study drug after the second dose due to removal of catheter following an serious adverse event (SAE) of infectious peritonitis. Multiple IP doses of phIL-12/PPC were generally well-tolerated by women with advanced, recurrent epithelial ovarian cancer. There were no apparent dose-related trends in clinical laboratory, ECG or vital sign parameters. The maximum tolerated dose was not achieved.

Patient weight was measured prior to administration of each dose and at the follow-up visits at 1 week and 5 ± 1 weeks after the last dose of study drug (or early termination). No patient's weight changed by $\geqslant 10\%$ from the baseline during the dosing period. The average weight loss for patients in the study was 1.5 kg at the 5 ± 1 week follow-up visit.

Eastern Cooperative Oncology Group (ECOG) performance was assessed at 5 ± 1 week after the last dose of study drug (or early termination) compared with the screening value. The majority (91%) of patients were assessed as fully active at screening. At the follow-up visit, 58% were assessed as fully active and 33% were assessed as able to perform light work. All seven patients (100%) in the 12- and 24-mg m^{-2} dose cohorts were assessed as fully active at follow-up compared with one of six patients (17%) in the two lower dose cohorts.

All 13 patients reported at least one treatment-emergent AE. The most frequent reported AEs were fever (69.2%) and abdominal pain not otherwise specified (53.8%). Most AEs were mild to moderate in severity. Five SAEs were reported in five (38.5%) patients during the study (Table 2). These included peritonitis (3), urinary tract infection (1) and abdominal inflammation (1). The peritonitis events were reported for one patient

Table 1 Demography of patient enrollment

Variable	*Dose cohort*				*All patients* (n = 13)
	0.6 mg m^{-2} (n = 3)	*3.0 mg m^{-2}* (n = 3)	*12 mg m^{-2}* (n = 4)	*24 mg m^{-2}* (n = 3)	
Race *n* (%)					
Asian/Pacific Islander	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	1 (7.7)
Caucasian	3 (100.0)	3 (100.0)	3 (75.0)	3 (100.0)	12 (92.3)
Age					
Mean (s.d.)	63.3 (7.6)	60.3 (3.2)	57.5 (13.2)	61.7 (7.4)	60.5 (8.3)
Range	58–72	58–64	41–73	56–70	41–73

Table 2 Treatment—emergent SAEs

System organ class preferred term	*Dose cohort*				*All patients* (n = 13)
	0.6 mg m^{-2} (n = 3)	*3.0 mg m^{-2}* (n = 3)	*12 mg m^{-2}* (n = 4)	*24 mg m^{-2}* (n = 3)	
Patients with treatment emergent serious adverse events	1 (33.3)	0 (0.0)	1(25.0)	3 (100.0)	5 (38.5)
Gastrointestinal disorders	1 (33.3)	0 (0.0)	0 (0.0)	3 (100.0)	4 (30.8)
Gastritis	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	1 (7.7)
Peritonitis	1 (33.3)	0 (0.0)	0 (0.0)	2 (66.7)	3 (23.1)
Infections and infestations	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	1 (7.7)
Urinary tract infections	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	1 (7.7)

Abbreviation: SAEs, serious adverse events.

in the 0.6-mg m^{-2} dose cohort and two patients in the 24-mg m^{-2} dose cohort. Urinary tract infection was reported for one patient in the 12-mg m^{-2} dose cohort and abdominal inflammation was reported for one patient in the 24-mg m^{-2} dose cohort. Three SAEs were assessed as not related to the study drug (peritonitis (2), urinary tract infection (1)), one SAE was assessed as unlikely to be related (abdominal inflammation) and one SAE was assessed as possibly related (grade-3 fever associated with peritonitis) and was considered a dose-limiting toxicity. No SAE was considered to be definitely related to the study drug. Ten (76.9%) patients reported AEs of special interest. The most frequently reported adverse events of special interest were abdominal pain and discomfort (10, 76.9%) and administration site reactions (3, 23.1%). Two of 20 special interest adverse events (both abdominal pain in the 24-mg m^{-2} dose cohort) were reported as severe. No special interest adverse events were considered to be definitely related to the study drug. Six patients had at least one scheduled laboratory value with CTCAE grade $\geqslant 3$ after receiving the study drug. These laboratory abnormalities were increased glucose (one patient); uric acid (two patients); bilirubin (two patients); and decreased sodium (one patient), WBC (one patient), potassium (one patient) and platelet count (one patient). None of these values was assessed as clinically significant. The system organ classes most affected with AEs were (in decreasing order) gastrointestinal disorders (12 patients, 92.3%), general disorders and administration site conditions (11 patients, 84.6%); nervous system disorders (six patients, 46.2%); and infections and infestations (five patients, 38.5%). The frequency of AEs within these system organ classes was lower in the 0.6-mg m^{-2} dose cohort as compared with that in the other dose cohorts. No clinically significant abnormal findings were reported for any of the blood pressure or heart rate parameters collected during the course of the study. Three patients (one each in the 0.6-, 3- and 12-mg m^{-2} dose group) who had a normal 12-lead ECG at screening had abnormal ECGs at the 5-week follow-up visit. One patient in the 24-mg m^{-2} dose group had abnormal ECG at screening and at the follow-up visit.

RECIST/overall survival/CA-125

Both measurable and non-measurable diseases (as defined in RECIST) were assessed at the screening and

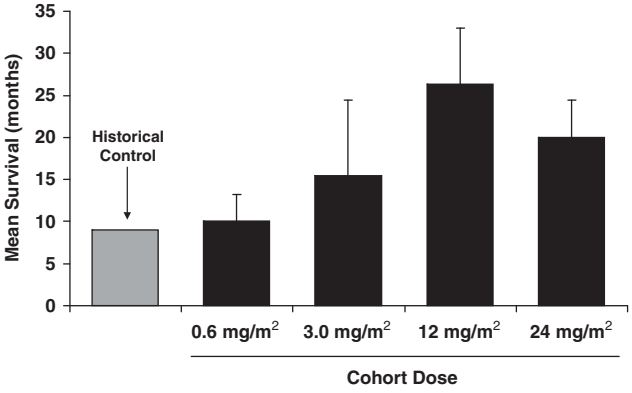

Figure 1 Overall survival of ovarian cancer patients at different phIL-12/PPC doses. Values are means ± s.e.m. phIL-12, human interleukin-12 (IL-12) plasmid; PPC, polyethyleneglycol–polyethyleneimine–cholesterol.

5 ± 1 weeks after the last phIL-12/PPC treatment. All but one patient had a target lesion at baseline. The patient without a target lesion had one non-target lesion (supraclavicular of unknown origin). At the post-treatment follow-up visit, 31% of treated patients had stable disease and 69% had progressive disease. RECIST assessments for target lesions were 46.2% (six patients) stable disease and 46.2% (six patients) progressive disease. Assessments for non-target lesions at the follow-up visit were 7.7% stable/partial response, 46.2% progressive disease and 46.2% had no non-target lesions identified at screening. The combined overall stable disease response for the two low-dose groups (0.6, 3 mg m^{-2}) was 16.7% ($n = 6$) and for the two high-dose groups (12, 24 mg m^{-2}) was 43% ($n = 7$). For target lesions, the combined results were 33.3% ($n = 6$) stable disease in the low-dose group and 57% ($n = 7$) stable disease in the high-dose group.

Patients treated with higher treatment doses also lived longer. The mean survival for patients receiving 0.6, 3.0, 12 and 24 mg m^{-2} of IL-12 plasmid was 10.1, 15.5, 25.5 and 19.7 months, respectively (Figure 1).

The pretreatment CA-125 concentrations were highly variable for individual patients, ranging from 27 to 3739 U ml^{-1}. CA-125 levels decreased or remained stable in six of 13 treated patients at the 5-week follow-up visit (Figure 2). The CA-125 levels decreased by 3% in one of three patients in cohort-1; 36 and 68%

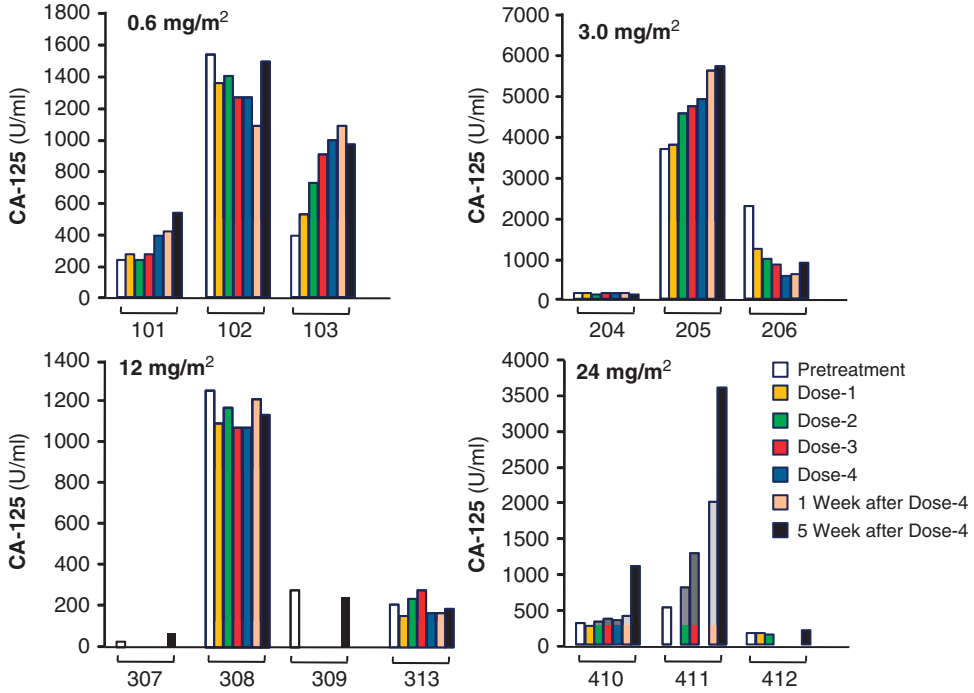

Figure 2 CA-125 levels in the serum of ovarian cancer patients at different phIL-12/PPC doses and study time points. Values are means ± s.e.m. phIL-12, human interleukin-12 (IL-12) plasmid; PPC, polyethyleneglycol–polyethyleneimine–cholesterol.

Table 3 Plasmid distribution in the PF and blood (BL) following phIL-12/PPC treatment. Values that exceed 10^6 copies are above the linear rage of the assay

Patient	*Cohort dose*	*Treatment cycles*							
		1		*2*		*3*		*4*	
		PF	*BL*	*PF*	*BL*	*PF*	*BL*	*PF*	*BL*
101	0.6 mg m^{-2}	712 899	0	$>10^8$	50	$>10^8$	101	$>10^8$	50
102		3 568 850	0	2 196 990	0	$>10^7$	213 351	217 030	0
103		4 011 530	0	18 825 610	0	563 676	0	384 217	0
204	3.0 mg m^{-2}	269 150	0	435 960	50	861 350	0	1 727 000	0
205		458 990	275	921 650	284	865 480	363	74 290	50
206		201 660	0	2 276 450	351765	$>10^6$	0	2 081 400	50
307	12 mg m^{-2}	$>10^6$	12705	$>10^8$	382	291 570	95	$>10^8$	125
308		27 69 860	0	464 030	0	81 102 270	0	$>10^8$	0
313		$>10^6$	50	$>10^6$	0	$>10^6$	50	$>10^6$	50
410	24 mg m^{-2}	$>10^6$	71	$>10^6$	1125	—	705	—	0

Abbreviations: PF, peritoneal fluid; phIL-12, human interleukin-12 (IL-12) plasmid; PPC, polyethyleneglycol–polyethyleneimine–cholesterol; SAEs, serious adverse events.

in two of three patients in cohort-2; and 2, 11 and 16% in three of four patients in cohort-4 at the 5-week follow-up visit. None of the three patients in cohort-4 showed decreased CA-125 concentrations at the 5-week follow-up visit, but two of the three patients had stable concentrations during the treatment period.

Plasmid DNA and cytokine concentrations in blood and PF

The plasmid DNA concentrations in blood and PF samples were measured before treatment and 1 week after each of the four weekly treatments (Table 3). Samples drawn from two of three patients in fourth cohort were not evaluable due to quality concerns. The pretreatment samples did not contain IL-12 plasmid. All post-treatment PF samples contained high concentrations of IL-12 plasmid, roughly 10^4–10^6 times higher than those in the blood. Only 10 of 35 (29%) plasma samples collected after phIL-12/PPC treatment had quantifiable levels of IL-12 plasmid. There was no clear dose effect on plasmid levels in the PF or blood.

The IL-12 and IFN-γ concentrations in all pretreatment blood or PF samples were below the limit of detection of

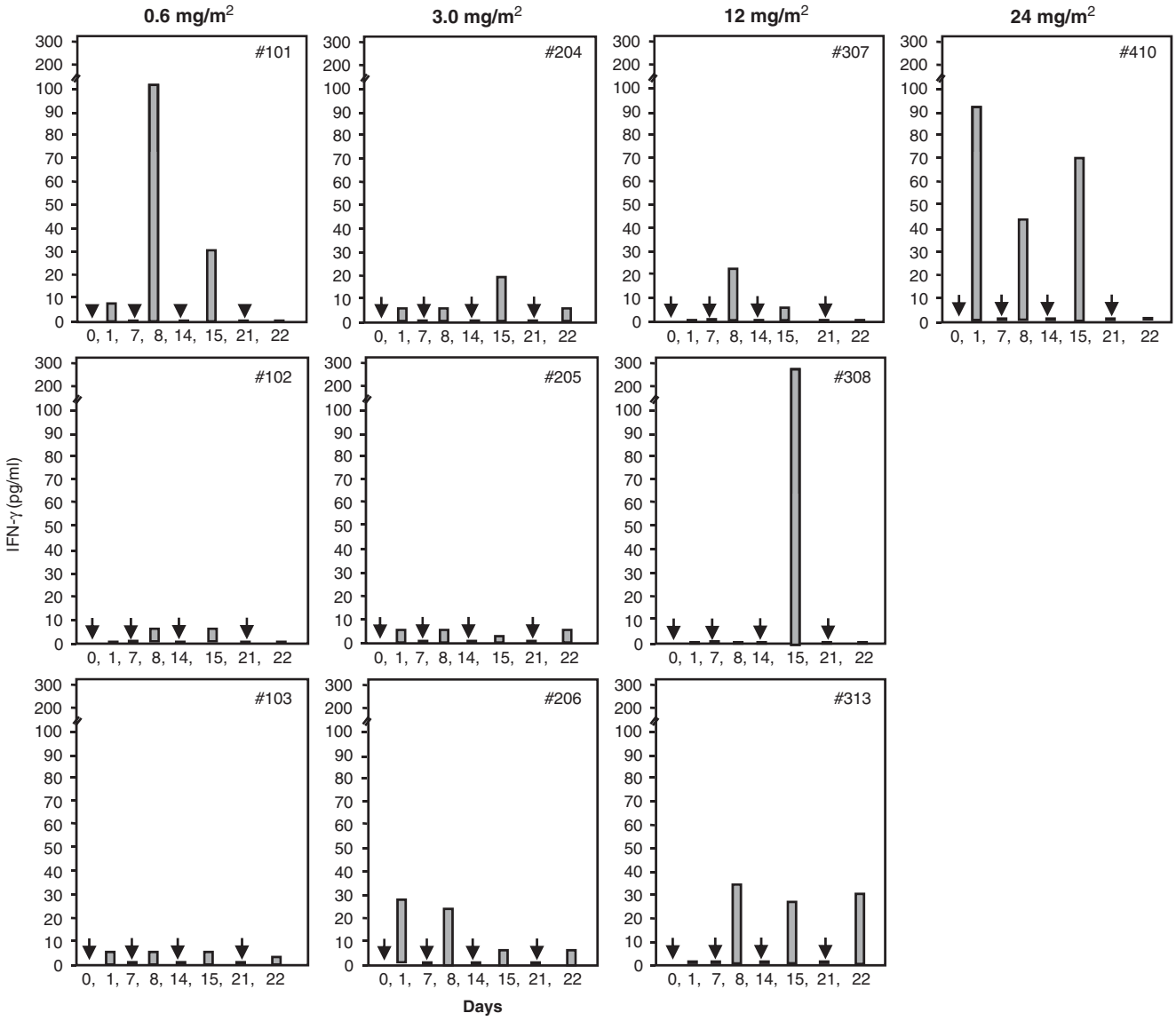

Figure 3 IFN-γ levels in PF samples of ovarian cancer patients before each of the four weekly IP treatments (day 0, 7, 14 and 21) and 1 day thereafter (day 1, 8, 15 and, 22, respectively) with phIL-12/PPC. The data show that IFN-γ levels generally increased 1 day after treatment and returned to baseline in 7 day post-treatment samples. The numbers in the horizontal scale represent study days and arrows indicate treatment days. IFN, interferon; IP, intraperitoneal; PF, peritoneal fluid; phIL-12, human interleukin-12 (IL-12) plasmid; PPC, polyethyleneglycol–polyethyleneimine–cholesterol.

the assay. One patient in the 3-mg m^{-2} dose cohort had measurable IL-12 levels in the PF (11 pg ml^{-1}) 1 day after first of the four treatments. There was no detectable increase in IL-12 levels in any of the blood samples following phIL-12/PPC treatment. The IFN-γ concentrations in the PF of every patient were significantly increased above the baseline 1 day after phIL-12/PPC treatment (Figure 3). IFN-γ levels were not detectable in the blood or PF samples collected 1 week or 5 week after the treatment. In the high-dose-treated groups (12 and 24 mg m^{-2}), 50% of evaluable PF samples contained 20–275 pg ml^{-1} IFN-γ. In comparison, only 16% of PF samples in the low-dose-treated groups (0.6 and 3.0 mg m^{-2}) contained 20–275 pg ml^{-1} IFN-γ. Overall, there were more IFN-γ-positive samples in the high-dose cohorts than in the low-dose cohorts.

Discussion

Intraperitoneal delivery of escalating doses of phIL-12/PPC complexes in women with chemotherapy-resistant advanced recurrent ovarian cancer was safe and well-tolerated. There was no evidence of systemic reagent-specific toxicity or dose-related trends in clinical laboratory, ECG or vital sign parameters following phIL-12/PPC treatment. The most frequent AEs were fever and abdominal pain. Nausea and vomiting were also reported for some patients. Most AEs were mild to moderate, occurred approximately 6 to 12 h after study drug administration and resolved within 24 h. None of the SAEs were definitely related to phIL-12/PPC treatment. One of the three peritonitis cases was not infectious, suggesting that some other factors were

involved. Direct involvement of IL-12/PPC treatment is unlikely due to late onset (12 days after the last plasmid treatment) but cannot be totally ruled out. Peritonitis is a common AE of therapies administered via IP catheters.[17,35,36] Approximately 20–25% of patients who receive treatment via IP catheter experience peritonitis.[35] This underscores the significance of safer catheters and other measures to reduce infectious complications especially for patients who require chronic IP treatments.[35]

The peritoneal localization of IL-12 plasmid and IFN-γ in treated patients is consistent with that in the biodistribution studies conducted using mice where plasmid concentrations in organs directly accessible by peritoneal infusion (mesenteric lymph nodes, spleen and liver) were much higher than those in organs accessible via systemic circulation only,[33,37,38] which demonstrates that there is low systemic contact of IP-administered IL-12 plasmid/PPC and its downstream products. Expressing IL-12 locally in the tumor environment by a biocompatible gene-delivery system appears to minimize systemic toxicity associated with recombinant protein therapy.[17]

The increase in IFN-γ concentrations in ovarian cancer patients shows that IP-administered hIL-12 plasmid is biologically active. The increases in IFN-γ concentrations were quantifiable 1 day after treatment but not 7 days after treatment. In mice, for which detailed pharmacokinetic analysis of IFN-γ levels was feasible, post-treatment IFN-γ levels peaked in the first two days after treatment and returned to near the baseline by 7 days.[37] Therefore, weekly regimen of phIL-12/PPC could provide IL-12 exposure for the entire treatment period. Measurable increase in IL-12 concentrations following phIL-12/PPC treatment was observed only for one patient, suggesting that IL-12 was produced at low levels and/or rapidly cleared by target cells. In treated mice, IP-administered plasmid/PPC complexes expressing mouse IL-12 yielded significantly lower IL-12 levels than the same complexes expressing human IL-12 (biologically inactive in mice), suggesting that the biologically active IL-12 is rapidly associated with its target cells and therefore does not accumulate locally, especially when produced in low amounts. This low pharmacological profile especially of a highly potent cytokine has a safety advantage due to potentially low spillover into systemic circulation. High variability in ascite volume and locoregional differences in cytokine concentrations within the abdominal cavity make it difficult to accurately quantify the biological response. The ascite volume in some instances was too large and required drainage, while in some other instances it was too low and required saline (200 ml) infusion before sample collection.

Encouraging effects on efficacy parameters were observed following phIL-12/PPC treatment. There was an overall clinical response of 31% stable disease and 69% progressive disease at the 5 ± 1 week post-treatment follow-up visit. Analyzing the data by target and non-target lesions show enhanced response in target lesions (46.2% ($n=6$) stable disease and 46.2% ($n=6$) progressive disease) than in non-target lesions (7.7% stable/partial response and 46.2% progressive disease). The CA-125 levels remained stable or decreased from the pretreatment values in six out of 13 patients at the 5 ± 1 week post-treatment follow-up visit. The overall survival of phIL-12/PPC-treated patients was considerably longer than the expected average (8–10 months) for this patient population.

The effects of phIL-12/PPC treatment in ovarian cancer patients appeared to be dose-related. More patients in the higher dose cohorts (12 and 24 mg m^{-2}) had detectable IFN-γ concentrations than in the lower dose cohorts (0.6 and 3 mg m^{-2}). 50% of PF samples in high dose cohorts contained 20–275 pg ml^{-1} IFN-γ as compared to only 16% of PF samples in low dose cohorts in 24 h post-treatment samples. Similarly, the overall response was stable disease in 16.7% of patients in the low-dose cohorts (0.6 and 3.0 mg m^{-2}) and 43% of patients in the high-dose cohorts. Only 17% ($n=6$) of the patients in the low-dose cohorts as compared with 100% ($n=7$) of patients in the high-dose cohorts assessed fully active by ECOG evaluation at final post-treatment analysis. The overall survival was 12.7 months ($n=6$) in the low-dose cohorts and 23 months ($n=7$) in the high-dose cohorts. Although the activity results are encouraging and appeared to follow a dose-dependent trend, the data should be interpreted with caution due to small sample size, possible selection bias and treatment of some of the post-study patients with additional chemotherapies. Nevertheless, the goal of the study was to determine safety and tolerability of the test formulation, and while positive indicators of biological activity are quite promising, no conclusions regarding efficacy should be drawn from this study.

Direct IP administration of anticancer agents as treatment for malignant disease principally confined to the peritoneal cavity has received considerable interest and recognition in recent years.[39,40] IP delivery of rIL-12 protein in patients with peritoneal carcinomatosis[17,41] appeared to be safer than intravenous approach, but some dose-limiting systemic toxicities (for example, neutropenia and elevated transaminases) were also observed. The efficacy results were less than impressive, presumably due to delivery of supraphysiological concentrations in systemic circulation and short half-life of the immunocytokine. IL-12 delivery by IP gene therapy provides for local and durable increase in IL-12 concentration at tumor site, which is not achievable with protein therapy. Easier access to mesenteric lymph nodes is another advantage of peritoneal approach when delivering a DNA-based immunotherapeutic. The paracrine secretion of IL-12 can induce immunity against cancer that can act locally and at distant sites.[9,10] The DNA-delivery system used in this study is designed to facilitate IL-12 gene transfer by protecting plasmid from rapid degradation and promoting plasmid uptake and trafficking across cell membranes.[31] Local delivery of IL-12 by IP administration of IL-12 plasmid/PPC provides IL-12 expression specifically in the tumor environment for achieving increased efficacy while minimizing systemic toxicity. Local gene delivery of IL-12 by intratumoral injection was also found safe and clinically beneficial for some metastatic melanoma patients.[42,43] The choice of gene-delivery system, delivery route and cancer indication is critical to achieving maximal therapeutic benefit from the local approach. The benefit of local delivery of pIL-12/PPC by IP or intratumoral administration has been demonstrated in several mouse models of peritoneal carcinomatosis and

other solid tumors,[31,44,37] demonstrating broad application of IL-12 immunogene therapy for treatment of cancer. The results from the present study warrant further investigation of IL-12/PPC as monotherapy or in combination with other anticancer therapies.

Materials and methods

Patient selection

Patients provided written informed consent prior to participating in the study. The study protocol and amendments to the protocol were approved by the Institutional Review Board and Institutional Biosafety Committees of all participating clinical sites. The major eligibility criteria included females ($\geqslant$18 years age) with chemotherapy-resistant recurrent epithelial ovarian cancer, residual disease detectable by CT scan and performance status score of 0, 1 or 2 per the ECOG. Laboratory requirements included leukocyte count $\geqslant$3000/ml, absolute neutrophil count$\geqslant$1500/ml, platelet count $\geqslant$100 000/ml, total bilirubin within institutional limits, AST (SGOT)/ALT (SGPT) $\leqslant 2.5 \times$ institutional upper limit of normal and creatinine within institutional normal limits or creatinine clearance$\geqslant$60 ml/min/1.73 m^2 for patients with creatinine levels above institutional normal. In addition, eligible patients were required to have an ECG without clinically significant abnormality. Patients also had to be capable of performing IP catheter site care while at home. Major exclusion criteria included serious uncontrolled medical illness or disorder, abdominal surgery or active infection within 4 weeks of study entry, intra-abdominal disease $>$5.0 cm in diameter, prior whole-abdominal irradiation, suspected bowel obstruction or known extensive adhesive disease, recurrence solely outside of the abdominal cavity, known HIV infection, positive HbsAg or HCV serology, prior IP drug administration, life expectancy $<$3 months or prior immunotherapy for ovarian cancer. Screening evaluations were performed within 21 days prior to drug administration.

Manufacturing of phIL-12/PPC

The phIL-12, expressing the p35 and p40 subunits of IL-12 gene under two separate CMV promoters, was constructed as described elsewhere.[31,44] The clinical lot of the plasmid was manufactured under cGMP by Strathmann Biotech (Hannover, Germany). The plasmid was grown in DH10β *Escherichia coli*, isolated by alkaline lysis and purified by ion-exchange chromatography. The delivery polymer PPC was manufactured by Durect Corporation (Birmingham, AL, USA) using the manufacturing scheme described previously.[31] The plasmid was formulated with PPC by mixing the two solutions at a mixing speed of 225 ml min^{-1} using an in-line mixing apparatus. The formulated plasmid/PPC complexes were lyophilized to dry powder and stored at $-80\,^{\circ}$C. The clinical lot was released after QC testing under GLP for visual appearance (before and after reconstitution), sterility, bacterial endotoxin, DNA concentration, pH and moisture content. The physicochemical properties and biological activity of phIL-12/PPC complexes were also determined as described previously.[31]

Study design

This study was conducted as an open-label, non-randomized, phase-I dose-escalation trial at the University of Alabama at Birmingham and Baylor College of Medicine in Houston. The protocol had been approved by the Institutional Review Board and Biosafety Committee of the participating centers. Patients were grouped into four dose-escalating cohorts (0.6, 3.0, 12 and 24 mg m^{-2} DNA), each cohort receiving four weekly doses. The highest dose was restricted to 24 mg m^{-2} since preclinical studies using mice showed transient elevation of liver transaminases at doses above 38 mg m^{-2} (Fewell *et al*., unpublished data). The study endpoints included safety, preliminary efficacy and biological activity. The primary objectives of this study were to determine the maximum tolerated dose and to assess the spectrum of toxicities of phIL-12/PPC when administered by IP infusion to patients with recurrent epithelial ovarian cancer. The secondary objectives were to assess the preliminary efficacy of phIL-12/PPC by monitoring detectable tumor burden, ECOG performance and CA-125 levels. The tertiary objectives were to determine plasmid distribution and IL-12 and IFN-γ concentrations in blood and PF.

Treatment

Eligible patients after enrollment into the study were scheduled for placement of the IP catheter at least 7 days prior to drug treatment to allow adequate time for healing. The procedure and risks associated with placement of the IP catheter was explained to the patient and general surgical/special procedure signed consent was obtained from the patient prior to placement of the catheter. Prior to placement of the IP catheter, patients received a single intravenous dose of the antibiotic Ancef or suitable alternative if allergic to cephalosporins. The peritoneal cavity was punctured with a 16-G cannula under percutaneous ultrasound guidance using a 7.5-Mhz probe. The site of percutaneous approach was selected according to Orsi *et al*.[45] A guide-wire was inserted under fluoroscopic guidance through the cannula and advanced into the cavity. An introducer was advanced over the guide-wire and the percutaneous catheter (Quinton Curl Cath (catalog no. 8811313015; Tyco Healthcare, Mansfield, MA, USA) was inserted into the peritoneal cavity. The final correct positioning and functioning of the IP catheter was confirmed by gamma-camera imaging after catheter infusion of Technetium-99 pertechnetate. Once optimal catheter position was ascertained, ascitic fluid was aspirated by manual suction to obtain PF samples for ancillary biological studies. Patients and caregivers were instructed on proper care of the IP catheter while at home. Prior to study drug administration, each patient was evaluated for presence of ascites that could interfere with adequate absorption of the study drug. If ascites were present, excess PF was removed via the IP catheter and if ascites were absent, 200 ml of 0.9% NaCl for injection, United States Pharmacopeia (USP), was administered via the IP catheter. Over the next 30 min, patients were asked to change position every 5–10 min (upright, Trendelenburg, left and right lateral, dorsal and ventral supine) to assure homogeneous distribution of the saline within the abdominal cavity. The volume of saline left within the

catheter space was removed by aspiration and 10 ml of PF was collected for ancillary biological assessment as described later.

The treatment dose for administration was calculated by surface area.[46] If patient's weight changed by 10%, during the treatment course the body surface area (BSA) (BSA $(m^2) = ([\text{Height (cm)} \times \text{Weight (kg)}]/3600)^{1/2}$ was recalculated and the adjusted dose administered.

All doses of IL-12 plasmid were administered IP on an inpatient basis, with the patient confined for 24 h following study drug administration for safety evaluation and collection of blood and PF samples for plasmid and cytokine analyses. On the day of treatment, the drug vial containing 6 mg of lyophilized DNA complexed with 22.12 mg PPC and 1.2 g lactose was reconstituted with 12 ml sterile water for injection to obtain a final DNA concentration of 0.5 mg ml^{-1}. An appropriate volume of the reconstituted drug corresponding to the intended dose was administered directly through the IP catheter followed by flushing with the remaining volume (up to a total of 200 ml) of 0.9% NaCl for Injection. Patients were to change position (upright, Trendelenburg, left and right lateral, dorsal and ventral supine) every 5–10 min over a period of 30 min (during and following infusion) to facilitate distribution throughout the abdominal cavity. The treatment was scheduled to be repeated on day 7, 14 and 21.

Safety assessment

Patients enrolled in the study who received at least one dose of the study drug and for whom at least one safety evaluation was available were included in the safety analysis. Patients were evaluated for safety 1, 4 and 24 h, and 3 and 7 days following each dose. Patients returned to the clinic for safety evaluations 5 weeks (± 1 week) following the last dose. Changes from baseline were evaluated for AE reporting, including clinical laboratory safety evaluations (chemistry and hematology), 12-lead ECG (standard supine), physical examininations and vital signs (blood pressure, heart rate, respiratory rate, oral body temperature and weight). Twelve-lead ECGs were performed by qualified technicians at screening (within 21 days prior to study drug administration) and at 5 ± 1 week following the last dose of study drug (or early termination). ECGs were assessed for rhythm (normal/abnormal); conduction (normal/abnormal) and PR, QRS, QT and QTc intervals. Clinical laboratory safety evaluations (serum chemistry and hematology) were performed at the site's laboratory from blood draws at screening (within 21 days prior to study drug administration), prior to each dose of study drug, 1 week following the last dose of study drug and 5 ± 1 weeks following the last dose of the study drug (or early termination). Clinically significant laboratory abnormalities or associated diagnoses were reported as AEs. All AEs and serious AEs recorded through the end of the study (that is, 5 ± 1 weeks after the last dose of the study drug, or early termination), regardless of causal relationship to study drug. Patients who prematurely discontinued from the study drug due to an AE (serious and non serious) were followed through the 5 ± 1 week follow-up or until resolution or stabilization. Clinically significant abnormal changes from baseline in physical examination findings, vital signs, ECGs and laboratory evaluations were considered to be AEs. For causal relationship assessment, AEs were considered definitely, probably and possibly related to the use of the study drug. AEs were graded by the investigator based on the National Cancer Institute (NCI) Common Terminology Criteria for Adverse Events v3.0 (CTCAE). Patients were not given any treatment for ovarian cancer (other than phIL-12/PPC) during the entire duration of the study.

Assessment of clinical efficacy

Pelvic and abdominal CT scans were performed at screening (21 days prior to study drug administration) and at 5 ± 1 weeks after the last dose of the study drug (or early termination). The RECIST criteria[47] were used to assess disease and tumor status at these time points. A previous CT scan with contrast was used in place of the screening scan if it was taken $\leqslant 30$ days prior to the day of enrollment. Conventional CT with contrast was performed with cuts of $\leqslant 10$ mm in contiguous slice thickness. All measurable visceral lesions were evaluated. The same method used to document measurable disease and non-measurable disease at screening was used consistently for all evaluations throughout the study. The results of all CT scans were analyzed on site by qualified radiologists.

The ECOG performance status was determined at screening (21 days prior to study drug administration) and at 5 ± 1 weeks following the last dose of the study drug (or early termination). CA-125 levels were measured in serum samples collected at screening (within 21 days prior to study drug administration), 1 week after each of the four treatments and 5 ± 1 weeks after the last treatment (or early termination).

Plasmid distribution

Blood and PF samples were collected for analysis of plasmid IL-12 DNA concentrations just before the first treatment and 1 week after each of the four doses of the study drug (or early termination). Venous blood samples were collected into EDTA Vacutainer tubes, gently inverted to disperse the anticoagulant, then divided into two polypropylene tubes and immediately frozen at $-70\,^{\circ}C$ until analysis. PF was removed from the IP catheter and collected in polypropylene tubes and immediately frozen at $-70\,^{\circ}C$ until analysis. DNA was extracted from the blood and PF samples. A control tissue was included with each batch of specimens to serve as an extraction contamination control (NEC-negative extraction control). Purified DNA from tissue was quantified using a SPECTRAmax 190 spectrophotometer, and then adjusted to 100 ng μl^{-2} with water. Three replicate PCR amplifications were performed on the DNA isolated from each specimen. DNA from the equivalent of 20 µl was analyzed in each reaction. To check for PCR inhibitors, one of the three replicate reactions was spiked with 100 copies of the phIL-12 plasmid DNA. PCR amplification and fluorescence detection were performed using an ABI PRISM 7700 Sequence Detection System. Quantitation of DNA targets was performed using oligonucleotide primers and fluorescence probes designed and qualified to amplify a 95-nucleotide amplicon from the phIL-12 plasmid vector and using DNA standards prepared with plasmid DNA construct. The correlation coefficient of the standard curve (r^2) was $\geqslant 0.980$. The negative extraction control tested below the limit of detection of the assay.

The PCR reagent control (NTC) showed no indication of exponential amplification. A specimen was reported as zero if the duplicate reactions used for quantification showed no indication of exponential amplification (C_t values equal terminal cycle), or if the specimen's mean C_t was greater than the mean C_t of the limit of detection. Results that exceed 10^6 copies are above the upper limit of the assay.

Cytokine assays

A commercially available ELISA kit was qualified for use to detect and quantify the levels of human IL-12 and IFN-γ in human body fluids. The Quantikine Human IL-12 ELISA kit and Quantikine Human IFN-γ ELISA kit was purchased from R&D Systems (Minneapolis, MN, USA). Test samples were run in three sets of duplicate wells and each point of the standard curve was run in triplicate wells on the plate. Standards and test samples were pipetted into the wells of the microplate precoated with recombinant IL-12 or IFN-γ. After room temperature incubation for 2 h, the plate was washed to remove unbound substances and the enzyme-linked polyclonal antibody specific for IL-12 or IFN-γ was added to the wells. The plate was incubated for 2 h at room temperature and again washed to remove any unbound antibody-enzyme reagent. The substrate solution was then added to the wells and color was allowed to develop for 30 min. The stop solution was added and the intensity of the color was measured at 450 nm, with a wavelength correction set at 540 nm correct for imperfections in the plate. The ELISA plate was read with a Molecular Devices SPECTRAmax 190 spectrophotometer using a modified ELISA template from the Softmax PRO 4.0 software. The template automatically interpolated cytokine concentration values of the unknown samples based on analysis of the standards using a non-linear, four-parameter regression analysis to describe the line.

Data analysis

The demographic data and incidence of treatment-related AEs are presented as percent values of the total subjects enrolled in the study. All other data are presented as average value plus standard deviations where indicated.

Conflict of interest

Drs Ronald Alvarez and Mack Barnes received a research grant from EGEN Inc. for this research. Drs Khursheed Anwer, Danny Lewis and Jason Fewell are EGEN employees.

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