Phase II Trial of Interleukin-12 Followed by Interferon Alfa-2b in Patients with Metastatic Malignant Melanoma: Results from CALGB 500001 (Alliance)

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ABSTRACT

The ability of IL12 to stimulate production of IFNγ suggested it might improve the efficacy of low-dose IFNα. In this phase II trial, patients with metastatic malignant melanoma were administered recombinant human (rh) IL12 followed by IFNα2b. Primary endpoints were clinical response and progression-free survival. Secondary objectives were to evaluate the effect of endogenous IFNγ on JAK–STAT signaling and IFN-regulated genes in peripheral blood mononuclear cells (PBMC). Patients with advanced melanoma received rhIL12 on day 1 and IFNα2b on days 2 to 6 of a 14-day cycle. rhIL12 was given intravenously at 300 ng/kg. IFNα2b was dosed at 3×10^6 units subcutaneously. Plasma IFNγ was assayed by ELISA; JAK–STAT signaling was measured in PBMCs by flow cytometry. The proportion of responders was assessed via Simon two-stage design. Thirty-eight patients were enrolled. The regimen was well-tolerated. Two patients achieved a partial response lasting 6 months or longer (5.3%). IL12 administration led to an increase in mean plasma IFNγ from 33.57 pg/mL at baseline to a maximum of 564.86 pg/mL and increased expression of STAT1 and STAT2 in PBMCs. Generation of phosphorylated STAT1 and IFN-simulated gene product 15 in response to IFNα was enhanced following IL12. rhIL12 given prior to IFNα2b stimulated production of IFNγ, which led to increased levels of JAK–STAT signaling intermediates in patient PBMCs. Combination therapy was reasonably well-tolerated but conferred marginal benefit in patients with metastatic melanoma. These results can inform future studies that use recombinant IL12 or novel IL12 constructs.

Introduction

The treatment of patients with malignant melanoma has undergone substantial change within the past decade due to the development of monoclonal antibodies (mAb) that block inhibitory receptors expressed by host T cells (1, 2). However, response rates for treatment with immune checkpoint inhibitors (ICI) such as the anti–PD-1 mAbs nivolumab and pembrolizumab remain in the range of 20% to 40% for patients with melanoma with advanced disease (2–4). Combination therapy with multiple checkpoint inhibitors is one method for improving the effectiveness of this therapy, but an alternative approach to improving patient response would be to combine these drugs with immune stimulatory agents. IL12 is one candidate immune-stimulatory agent.

IL12 is produced primarily by monocytes/macrophages in response to a variety of infectious stimuli such as bacteria, intracellular parasites, and viruses (5). IL12 induces the secretion of IFNγ by both T cells and NK cells and promotes the maturation and activation of type 1 helper (Th1) T cells, thus favoring the development of cellular immunity and immunologic memory over humoral immunity. IL12 also strongly stimulates the proliferation and lytic activity of NK cells and cytotoxic T cells (6). Biomarkers associated with successful response to IL12 therapy include increased levels of IFNγ and TNFα (7). These properties of IL12 suggested that it might be a particularly effective antitumor agent; however, administration of this cytokine as a single agent at high doses led to substantial toxicities and few clinical responses (8). Based upon its ability to stimulate NK cell cytotoxicity and cytokine production, our group has evaluated IL12 in combination with antitumor mAbs such as trastuzumab and cetuximab in patients with metastatic solid tumors. These phase I trials showed increased levels of IFNγ and several chemokines in patients who experienced a clinical response (9–13). The correlation between circulating levels of IFNγ and clinical activity suggested that this action of IL12 was able to prime immune effector cells for increased antitumor activity. Neoadjuvant intratumoral delivery of an IL12 plasmid via electroporation (tavokinogene telseplasmid) in combination with pembrolizumab induced enhanced cytotoxic immune infiltration in patients with locally advanced operable melanoma (14). This agent also demonstrated clinical efficacy in patients with metastatic melanoma (15), although a larger phase II trial (NCT03132675) in patients who had progressed on ICI therapy failed to meet the prespecified overall response rate. An intratumoral lipid nanoparticle-formulated IL12 mRNA is under investigation in patients with advanced solid tumors, including melanoma (NCT03946800; ref. 16), and a phase I study of recombinant IL12 with pembrolizumab in patients with metastatic solid tumors

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(NCT03030378) is ongoing. Patients must have progressed previously on anti–PD-1 or PD-L1 therapy and receive subcutaneous IL12 four times throughout each pembrolizumab cycle up to eight cycles. Additionally, an IL12-Fc fusion protein designed to extend the half-life of IL12 delayed tumor growth and demonstrated sustained IFNγ responses in murine melanoma, colon, and breast cancer models (17). This agent is under investigation in a phase I trial as monotherapy or in combination with nivolumab in patients with locally advanced or metastatic solid tumors (NCT04423029). Thus, IL12-based anticancer agents remain an active area of investigation.

IFNα has documented activity in patients with advanced malignant melanoma. Unfortunately, the toxicities associated with high-dose IFNα therapy have limited its use in the metastatic and adjuvant settings (18). Our group has provided evidence that the antitumor activity of IFNα is dependent on the actions of host immune cells, and that pretreatment of immune effector cells with IFNγ can upregulate levels of JAK–STAT signaling intermediates and sensitize target cells to low doses of IFNα (19–26). Given the importance of IFNα in the generation of host T-cell immune responses via the JAK–STAT pathway (27, 28), it remains important to understand the potential synergy that could be achieved via sequential exposure of immune effector cells to IFNγ and IFNα. The ability of IL12 to modulate the host immune response via the induction of IFNγ (and other cytokines) and promote the activation of T cells and NK cells also has implications in the developing field of ICI therapy, as the IFNγ–JAK–STAT signal transduction axis has been shown to regulate PD-L1 expression in melanoma cells (29, 30). Indeed, a recent study has shown that elevated levels of IFNγ are associated with increased overall survival (OS) and tumor response in cancer patients receiving ICIs as first line therapy (31).

In the present study, it was proposed that pretreatment of patients with melanoma with a low dose of IL12 would induce the endogenous production of IFNγ (11, 12, 30), which in turn would upregulate levels of JAK–STAT signaling intermediates in tumor cells and immune effector cells. It was predicted that this schema of cytokine administration would sensitize patients to low doses of IFNα, leading to increased efficacy of IFN therapy and decreased toxicity. Phase I data from our group had demonstrated that this cytokine combination could be administered safely (30). We therefore conducted a multicenter phase II trial of recombinant (r) human (h) IL12 followed by IFNα2b in patients with advanced melanoma under the auspices of the Cancer and Leukemia Group-B (CALGB) prior to its becoming part of the Alliance for Clinical Trials in Oncology (Alliance). The primary objectives of the study were to estimate the response rate and progression-free survival (PFS) in patients receiving this therapy. In addition, a series of preplanned correlative studies was conducted to elucidate the effectiveness of the IL12 pretreatments in generating the production of IFNγ and modulating IFNα-induced JAK–STAT signal transduction.

Materials and Methods

Objectives and eligibility

This multicenter phase II trial evaluated the combination of rhIL12 followed by IFNα2b in patients with advanced melanoma. Patients were recruited to this study beginning September 2001. The study was terminated in March 2004 because of inadequate response to therapy found at the planned interim analysis. Study submission was delayed because of several factors, including the elimination of the CALGB melanoma working group, the evolution of the CALGB into the Alliance for Clinical Trials in Oncology, the change in location of the statistical headquarters for data analysis, the onset of the COVID-19 pandemic, and the protracted process for obtaining patient-specific study information. This study was registered at ClincalTrials.gov under trial number NCT00026143 and was conducted in accordance with the principles outlined in the Declaration of Helsinki. Institutional Review Board (IRB) approval was obtained from The Ohio State University Biomedical IRB in 1998. The primary objective of this study was to estimate the response rate and PFS in patients with metastatic melanoma receiving rhIL12 and IFNα2b combination therapy. In addition, a series of correlative studies was planned in order to elucidate the effect of this therapy on immune cell activation. All patients provided written informed consent prior to treatment. This trial was open to nonpregnant, nonnursing individuals ages 18 years or older with histologic or cytologic diagnosis of malignant melanoma and clinical evidence of unresectable, distant, or metastatic, regional lymphatic, or extensive in-transit disease. Patients were required to have measurable disease, an Eastern Cooperative Oncology Group performance status of 0 to 1, and adequate hematopoietic, renal, and hepatic function. Subjects with a history of substantial peripheral neuropathy, brain metastases, or other central nervous system disease were excluded, as were subjects with substantial comorbid conditions. Participants were permitted to have received no more than one prior regimen of chemotherapy and were not allowed to have received prior cytokine therapy (e.g., high-dose IL2) for metastatic disease. Prior adjuvant therapy with IFNα was permissible if the patient had remained disease-free for 12 months or longer following the last IFNα treatment.

Treatment protocol

rhIL12 was administered via intravenous push (more than 5–15 seconds) at a dose of 300 ng/kg on day 1 of each cycle. IFNα2b was administered subcutaneously at rotating sites at a dose of 3×10^6 units on days 2, 3, 4, 5, and 6 of each cycle. This cycle of therapy was repeated every 14 days (**Table 1**). Therapy could be administered on an outpatient basis; however, patients were observed for 4 hours following the first injections of both rhIL12 and IFNα2b. Patients were taught to administer the IFNα2b injections at home after the first cycle of therapy. Patients were premedicated with acetaminophen 650 mg orally prior to each injection of rhIL12 and IFNα2b. Ibuprofen or a COX-2 selective inhibitor (e.g., celecoxib) could be used to premedicate patients if rhIL12 symptoms such as fever and chills were not controlled with acetaminophen. All patients received 12 weeks (six cycles) of therapy unless toxicity or disease progression was observed. Disease response evaluation was performed after

Table 1. Treatment schema.

Day	1[a]	2[a]	3[a]	4[a]	5	6	7	8	9	10	11	12	13	14
	IL12	IFN	IFN	IFN	IFN	IFN	–REST–							

14 days constitute a treatment cycle.
IL12[b]: rhIL12 300 ng/kg intravenous injection on day 1 of each cycle.
IFN[b]: IFNα2b 3×10^6 units subcutaneous injection on days 2, 3, 4, 5, and 6 of each cycle.

[a]Peripheral blood for use in correlative studies was obtained immediately prior to injection of rhIL12 and IFNα2b on days 1–4 of cycles 1, 3, 6, and 12.
[b]Patients premedicated with acetaminophen 650 mg prior to each injection of rhIL12 and IFNα2b.

every six cycles, and patients exhibiting a clinical response or stable disease were offered the option of continuing therapy until disease progression.

Management of toxicity

Toxicity was assessed according to the revised National Cancer Institute Common Toxicity Criteria version 2.0 that was in use at the time the study was conducted (32). Patients exhibiting nonhematologic grade 4 toxicity or hematologic/nonhematologic grade 2 or 3 toxicity that did not improve following a 2-week rest period were discontinued from study therapy. Patients were not permitted to receive corticosteroids while on study. Colony-stimulating factors filgrastim and sargramostim could be administered to patients with neutropenia > grade 3.

Procurement of peripheral blood

Peripheral blood for use in correlative studies was obtained immediately prior to the injection of rhIL12 and IFNα2b on days 1, 2, 3, and 4 of cycles 1, 3, 6, and 12 and shipped overnight at room temperature to Dr. Carson's laboratory. Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood by density gradient centrifugation with Ficoll-Paque (Amersham Pharmacia Biotech), and plasma was snap-frozen per laboratory protocol (30).

Quantitation of plasma IFNγ

Patient plasma samples were analyzed in triplicate for IFNγ by ELISA using commercially available mAb pairs as previously described (M700A/M701B, Endogen, Inc.; ref. 30). A separate standard curve was prepared for each plate, and all plasma samples from a single patient were run on the same plate to minimize interassay variability. Samples were read on a BioTek ELx800 microplate reader (BioTek Instruments, Inc.).

Intracellular flow cytometry for STAT1/2 and P-STAT1 levels

PBMCs were cryopreserved and analyzed in batches within a month of sample procurement. PBMCs were assayed for total STAT1/2 and also phosphorylated (P) STAT1 utilizing a flow cytometric assay that was developed by this group (33–35). Briefly, cells were stained with anti-CD3 (#UCHT1) and anti-CD56 (#MY31; BD Transduction Laboratories), fixed and permeabilized (Fix and Perm, Caltag Laboratories), washed in phosphate buffered saline (PBS) containing 5% fetal bovine serum, and then stained for 30 minutes at room temperature with a murine anti-STAT1 (#1/STAT1) or anti-STAT2 (#22/STAT2; BD Transduction Laboratories). Cells were washed, stained with secondary antibody FITC-conjugated goat anti-mouse (BioSource International), and fixed in 1% formalin. Data were acquired on a Coulter Elite II flow cytometer using at least 10,000 cells in the lymphocyte gate based on light scatter properties. Cells stained with an isotype control antibody (#G155-178, #MOPC-21, BD Transduction Laboratories) were used to evaluate background staining. FCS files were analyzed in FlowJo v.3.2. The mean fluorescence intensity for each sample was calculated, and this value was used in the statistical analysis.

To measure phosphorylation of STAT1 in response to IFNα, cells for analysis were treated *ex vivo* with PBS or IFNα2b (10^4 U/mL) for 15 minutes and then permeabilized and stained for 30 minutes with a murine antibody specific for the phosphorylated form of STAT1 (tyrosine 701, Cell Signaling Technology, Inc.; refs. 33–35). Cells were washed once and then stained with secondary antibody, FITC-conjugated goat anti-mouse (BioSource International), and fixed in 1% formalin. Data were acquired on a Coulter Elite II flow cytometer as outlined above. Data were analyzed from total PBMCs, $CD3^+$ T cells, and $CD56^+$ NK cells following IFNα2b treatment with PBS controls subtracted. Representative histograms are included in Supplementary Fig. S2.

IFNα induction of IFN-Stimulated Gene Product 15 transcript

Our group has previously determined that IFN-stimulated gene product 15 (ISG15) is specifically regulated by IFNα in normal PBMCs (21, 22). Moreover, initial experiments performed using patient PBMCs from the phase I study confirmed the specificity and sensitivity of this marker with respect to IFNα treatments (30). To determine the effect of IFNα-induced gene regulation in this patient population, expression of *ISG15* was measured by quantitative real-time polymerase chain reaction (qPCR) using patient PBMCs. Total RNA was extracted by TRIzol phenol–chloroform isolation, and 1 μg RNA was reverse transcribed to cDNA. Samples were run in triplicate in 96-well plates on the ABI PRISM 7700 thermocycler (Applied Biosystems) with predesigned primer/probe sets (Assays On Demand; Applied Biosystems) and 2× Taqman Universal PCR Master Mix (Applied Biosystems). Predesigned primer/probe sets for human *GAPDH* (Applied Biosystems) were used as an internal control. *ISG15* gene expression was normalized to the *GAPDH* mRNA internal control (ΔCt = Ct gene of interest − Ct *GAPDH*). Real-time RT-PCR data were analyzed using Sequence Detector software, version 1.6 (PE Applied Biosystems). Relative mRNA expression for each target was calculated as $2 - (\Delta Ct)$.

Statistical considerations

Data collection and statistical analyses were conducted by the Alliance Statistics and Data Management Center. Data quality was ensured by review of data by the Alliance Statistics and Data Management Center and by the study chairperson following Alliance policies. All analyses were based on the study database frozen on August 12, 2006.

Clinical endpoints

The primary endpoints of this trial were (i) response to treatment with rhIL12 plus IFNα2b [complete response (CR) or partial response (PR)] and (ii) PFS. PR was defined as at least a 30% decrease in the sum of the longest diameter of target lesions by imaging. CR was defined as the disappearance of all target lesions. The null hypothesis, H0, that the response rate, p, to this treatment is ≤0.10 (H0: $P \leq 0.10$), was tested against the alternative, HA, that the response rate is ≥0.20 (HA: $P \geq 0.20$), using a Simon two-stage design. Thirty evaluable patients were to be entered at each stage. An evaluable patient was defined as an eligible patient who had received one complete cycle of therapy. The following decision rule was followed: if among the first 30 evaluable patients entered at stage 1, ≤3 responses were observed, the trial would end concluding that the response rate was ≤0.10. If ≥4 responses were observed, the trial would continue to accrue 30 additional evaluable patients. At stage 2, if ≤8/60 responses were observed, the treatment would be declared inefficacious. If ≥9 responses were observed, the treatment would be deemed efficacious (response rate ≥0.20). The power to test the hypothesis is 0.81 under this design (1-sided $\alpha = 0.12$).

PFS was measured from registration until time of documented progression of disease or death from any cause. If the trial concluded at stage 1 with 30 patients accrued in 6 months and followed for 1.5 years, there would be approximately 83% power to detect an improvement in median PFS of 5.6 months over the historic median

of 3.5 months. A minimum of 28 patients were expected to progress. Power was based on a single sample test of hypothesis for the mean of an exponential distribution (1-sided $\alpha = 0.10$), and 95% confidence interval (CI) estimates were computed using the Greenwood's formula with a log–log transformation. Accrual was expected to be approximately five patients per month over a 12-month accrual period. Patients were to be followed a minimum of 2 years from protocol entry.

Correlative endpoints

Plasma IFNγ levels were measured by ELISA. STAT1, STAT2, and P-STAT1 levels (within total PBMCs, $CD3^+$ T cells, and NK cells) were measured by flow cytometry. ISG15 transcript levels were assessed by qPCR of whole PBMCs. These measurements occurred in a maximum of 38 patients treated on days 1, 2, 3, and 4 of cycles 1, 3, 6, and 12. Only data between cycle 1, day 1 and cycle 12, day 4 were used as only one patient had data beyond cycle 12, day 4. IFNγ was recorded as the average of two independent IFNγ measurements assessed in duplicates taken at each day within each cycle. A linear mixed model using the PROC MIXED procedure in SAS 9.4 was used to model immune correlates by day and cycle and test for potential differences in immune correlate levels over time, cycles, and time by cycle. A compound symmetry covariance structure (constant variance and covariance) was specified for patients within cycle using the repeated statement. Cycles nested within patients were considered as a categorical variable. Time in days was considered as a continuous variable; linear, quadratic, and cubic terms were entered into the models. Models included cycle, day, day^2, day^3 and cycle by day, cycle by day^2, and cycle by day^3 interactions. Mean values were plotted over time using spline interpolation. Patterns are described. A significance level of 0.05 was used for all tests of hypothesis. No adjustments were made for multiple comparisons. Personnel performing correlative studies were blinded to patient clinical data.

Results

Patient demographics

CALGB 500001 was activated on September 15, 2001, and was closed on March 15, 2004, due to inadequate response to therapy at the planned interim analysis. Patient demographics are summarized in **Table 2**. Thirty-eight patients between the ages of 28 and 87 years were enrolled in this study, with a median age of 64 years. The male-to-female ratio was 2.8:1. All patients were White. Thirty-five patients (92.1%) had stage IV melanoma at the time of enrollment, with the remaining three patients being classified as stage III. Patients were classified according to the cutaneous melanoma guidelines in use at the time (American Joint Committee on Cancer Staging Manual, 6th Edition). The most common site of primary tumor was the trunk or pelvis (42.1%), followed by head and neck (29%) and extremities (15.8%). Thirty-one patients were diagnosed with cutaneous primary tumors. One patient had ocular melanoma, and one had mucosal melanoma. The primary site was unknown for five patients. Fourteen patients (36.8%) had undergone surgery prior to enrollment. With respect to systemic therapy, eight patients (21.1%) had received chemotherapy prior to the study, and six patients (15.8%) received prior immune-modulating treatment. Two of these patients received adjuvant IFN therapy. Twenty-four patients (63.1%) received no systemic therapy for their disease prior to study enrollment. All enrolled patients were deemed evaluable. The longest follow-up for any patient in this study was 5 years. Details on the representativeness of the study population in advanced melanoma are included in Supplementary Table S1.

Table 2. Patient characteristics ($N = 38$).

Characteristic		
Age, years		
Median		64
Range		28–87
	N	**Percentage**
Sex		
Male	28	73.7%
Female	10	26.3%
Stage		
III	3	7.9%
IV	35	92.1%
Prior surgery		
No	24	63.1%
Yes	14	36.8%
Prior systemic therapy		
No	24	63.1%
Yes	14	36.8%
Primary tumor location		
Head and neck	11	29.0%
Trunk or pelvis	16	42.1%
Extremity	6	15.8%
Unknown	5	13.1%
Primary tumor type		
Ocular	1	2.6%
Mucosal	1	2.6%
Cutaneous	31	81.6%
Unknown	5	13.2%

Adverse events

The regimen was well tolerated, and severe adverse events (AE) were uncommon. Three patients died of their disease while on treatment. Overall, 16 patients (42.1%) experienced grade 2 AEs, whereas 15 (39.5%) experienced grade 3 AEs. Two patients experienced AEs significant enough to require removal from study. Of these patients, one had grade 4 arachnoiditis/meningitis resulting in confusion and another had grade 4 dizziness/lightheadedness. There were no grade 5 AEs. The less severe AEs that were commonly encountered included grade 2 fatigue (42%), fever in the absence of neutropenia (32%), chills (16%), dehydration (16%), nausea (16%), vomiting (16%), anorexia (13%), and dizziness (11%). Grade 3 fatigue (16%), nausea (11%), dehydration (8%), fever (8%), and myalgia (8%) were also observed. Pretreatment with acetaminophen was routinely used in the trial to control frequency and severity of reactions. Only two of the observed AEs were hematologic: grade 2 hemoglobin (1) and grade 2 hemolysis (1). A complete list of AEs reported during the study is provided in **Table 3**. The listed AEs were felt to be treatment related.

Treatment duration

The median number of cycles received per patient was 5.5 (Q1, Q3: 3, 6; range, 1–70). Seven patients had discontinued therapy by the completion of the second cycle of treatment: three of these patients experienced progressive disease, one patient was removed because of severe AEs, two patients withdrew from treatment, and

one patient died because of their disease during this early time frame before completion of the second treatment cycle. Fifteen patients (39.5%) received six cycles of therapy, and four patients (10.5%) completed at least 12 cycles of therapy (i.e., 6 months). Twenty-seven (71.1%) patients discontinued treatment because of progression of disease.

Efficacy

Two patients achieved a PR for a response rate of 5.3% (95% exact binomial CI, 1%–15%). Both patients had stage IV melanoma. Neither had received previous systemic therapy. One of these patients had metastases to the lungs and distant lymph nodes (stage IVb) from a head and neck primary and developed progressive disease 6 months after experiencing a PR. This patient was classified as TX N2 M1b under the cutaneous melanoma classification system in use at the time. The other patient who achieved a PR had metastases to distant lymph nodes (stage IVa) from a nodular cutaneous primary lesion of the trunk and experienced a PR for 9 months, after which tumor growth led to reclassification as stable disease (SD). This patient was classified as T3b N3 M1a and received 70 cycles before discontinuing treatment due to termination of the rhIL12 supply. Both patients eventually died because of melanoma progression. Clinical features of these responders were otherwise unremarkable. Two patients were classified as having SD and received 12 and 18 cycles of therapy before progressing. The median follow-up was 41.8 months; PFS and OS curves for all patients enrolled in this study are shown in **Fig. 1**. The median PFS was 2.5 months (95% CI, 2–2.7); the median OS was 10.3 months (95% CI, 6.4–14.3). The observed median PFS was less than the hypothesized historic median of 3.5 months. OS (length of time from start of treatment to death from any cause) was not a prespecified endpoint.

Systemic levels of IFNγ

Plasma IFNγ levels were evaluated in all 38 patients prior to each injection of rhIL12 and the first three injections of IFNα2b (**Fig. 2A**). The maximum mean IFNγ within a cycle, 751.80 ± 826.1 pg/mL, was observed on day 2 of cycle 1. Lower average peaks were reached on day 2 of cycles 3, 6, and 12, for an overall maximum mean IFNγ of 564.86 pg/mL. Changes in rates of change were significantly different over cycles (interaction $P = 0.008$), with greater rates of increase and decrease in cycle 1 and lesser rates in cycles 3, 6, and 12. A significant change in the direction of curvature was also observed with average IFNγ levels increasing between days 1 and 2 and decreasing between days 2 and 3 ($P < 0.0001$). This pattern did not differ by cycle. Thus, rhIL12 administration led to significantly increased IFNγ levels at day 2 in every cycle, with the highest effect being seen in cycle 1.

Intracellular levels of STAT1 and STAT2

A flow cytometric assay was used to monitor the levels of STAT1 and STAT2 in patient PBMCs obtained over the course of the study. We tested whether endogenous production of IFNγ following rhIL12 administration correlated with increased expression of JAK–STAT signaling intermediates, as had been observed in a murine model of melanoma and in the previous phase I study (35). As seen in **Fig. 2B**, there was an increase in the mean expression of STAT1 over the course of the first week (days 1–4) of cycles 1, 3, 6, and 12. Average STAT1 levels returned to baseline by the time of next measurement (day 1 of the next cycle). Peak mean STAT1 levels in cycle 1 averaged 170.3 ± 107.1 and occurred on day 4 (vs. baseline at 51.9 ± 28.9). There was a significant increase in mean STAT1 levels over days 1 to 4 ($P < 0.0001$) with significantly decreasing rates of change over cycles ($P = 0.0007$). No significant differences in STAT1 levels over days by cycle were detected. On average, STAT1 levels increased between days 1 and 4; peak mean STAT1 was observed on day 3 or 4. Similarly, there was a significant increase in mean STAT2 levels over days ($P < 0.0001$). On average, peak STAT2 levels were observed on days 3 or 4 of each cycle, as shown in **Fig. 2C**.

Generation of phosphorylated STAT1 in patient PBMCs following *ex vivo* stimulation with IFNα

To determine whether IL12 pretreatment would enhance STAT1 signal transduction in response to IFNα, patient PBMCs obtained before and after the administration of rhIL12 (days 1–4 of cycles 1, 3, 6, and 12) were tested for their responsiveness to exogenous IFNα. **Figure 3** shows P-STAT1 levels for each IFNα-treated sample with the expression in control-treated (PBS) samples subtracted. P-STAT1 levels for total PBMCs, $CD3^+$ T cells, and NK cells were measured. There was a significant difference in mean P-STAT1 levels in total PBMCs across days ($P < 0.0001$). A significant change in the direction of curvature was also observed ($P = 0.0005$). *Ex vivo* IFNα treatments induced a transient increase in the expression of P-STAT1 over baseline in total PBMCs during cycles 3, 6, and 12 [mean (SD) peak P-STAT1 = 49.2 ± 30.0 on day 2, cycle 6]. Similar effects were observed for the $CD3^+$ T-cell and NK-cell subsets (see Supplementary Table S2 for the gating strategy). Mean P-STAT1 levels in $CD3^+$ T cells and NK cells differed significantly by day ($P = 0.0007$ and $P = 0.004$, respectively), and a significant change in the direction of curvature was observed ($P = 0.02$). Average P-STAT1 levels in $CD3^+$ T cells increased between days 1 and 2 of each cycle, with the exception of cycle 1. Similar to $CD3^+$ T cells, mean P-STAT1 levels in NK cells were highest on day 2 of each cycle apart from cycle 1. The enhanced phosphorylation of STAT1 was most prominent at day 2 of the cycle, which is when levels of total STAT1 began to rise (**Fig. 2B**). It should also be noted that within all three cell populations tested there was a less significant change in mean P-STAT1 levels across days for those samples collected in cycle 12, when four patients remained on the study. These findings demonstrate that IL12 administration can modify signal transduction within the PBMC compartment and that this effect can be monitored by flow cytometry.

Activation of IFNα-induced gene expression

To determine the effect of IFNα-induced gene regulation in this patient population, the expression of *ISG15* mRNA was measured by qPCR in patient PBMCs. As seen in **Fig. 4**, there was a marginal trend toward differences in mean change of curvature over days by cycle ($P = 0.1$). Average peak *ISG15* occurred later at day 3 in cycles 6 and 12 versus day 2 in cycles 1 and 3. Peak means for cycles 1, 3, 6, and 12 were 162.0, 90.8, 188.4, and 194.3, respectively. An analysis of variance comparing the means across cycles was significant at $P < 0.0001$. Measurements in these four cycles were made in 38, 23, 14, and 4 patients, respectively.

Cytokine and JAK–STAT signaling response in the partial responders

The IFNγ, STAT1, and P-STAT1 levels of the two partial responders were compared with those of the other study participants. These values did not trend higher than those of the nonresponding patients (Supplementary Fig. S1). However,

Table 3. Patient AEs ($N = 38$).

	Grade of AE						
	2–Moderate		3–Severe		4–Life threat		Total
Type of AE	***N***	**(%)**	***N***	**(%)**	***N***	**(%)**	***N* (%)**
Hematologic AEs							
Blood/bone marrow							
Hemoglobin	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Hemolysis	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Nonhematologic AEs							
Cardiac arrhythmia							
Sinus tachycardia	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Supraventricular arrhythmias	0	(0%)	2	(5%)	0	(0%)	2 (5%)
Cardiovascular							
Hypotension	2	(5%)	1	(3%)	0	(0%)	3 (8%)
Constitutional symptoms							
Constitutional symptoms–other	2	(5%)	0	(0%)	0	(0%)	2 (5%)
Fatigue (lethargy, malaise, and asthenia)	16	(42%)	6	(16%)	0	(0%)	22 (58%)
Fever (in the absence of neutropenia)	12	(32%)	3	(8%)	0	(0%)	15 (39%)
Rigors and chills	6	(16%)	0	(0%)	0	(0%)	6 (16%)
Sweating (diaphoresis)	2	(5%)	0	(0%)	0	(0%)	2 (5%)
Weight loss	3	(8%)	0	(0%)	0	(0%)	3 (8%)
Dermatology/skin							
Bruising	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Endocrine							
Hot flashes/flushes	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Hypothyroidism	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Gastrointestinal							
Anorexia	5	(13%)	1	(3%)	0	(0%)	6 (16%)
Dehydration	6	(16%)	3	(8%)	0	(0%)	9 (24%)
Diarrhea patients without colostomy	1	(3%)	1	(3%)	0	(0%)	2 (5%)
Dysphagia, esophagitis, and odynophagia	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Mouth dryness	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Mucositis/stomatitis	2	(5%)	0	(0%)	0	(0%)	2 (5%)
Nausea	6	(16%)	4	(11%)	0	(0%)	10 (26%)
Vomiting	6	(16%)	2	(5%)	0	(0%)	8 (21%)
Hepatobiliary/pancreatic							
Alkaline phosphatase	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Hepatic–other	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Infection							
Infection without neutropenia	1	(3%)	1	(3%)	0	(0%)	2 (5%)
Metabolic/laboratory							
Aspartate aminotransferase and aspartate aminotransferase	2	(5%)	0	(0%)	0	(0%)	2 (5%)
Creatine phosphokinase	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Glucose serum–high (hyperglycemia)	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Hyponatremia	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Musculoskeletal							
Muscle weakness	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Neurology							
Arachnoiditis/meningismus/radiculitis	0	(0%)	0	(0%)	1	(3%)	1 (3%)
Confusion	0	(0%)	2	(5%)	1	(3%)	3 (8%)
Depressed level of consciousness	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Dizziness	4	(11%)	0	(0%)	1	(3%)	5 (13%)
Hallucinations	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Memory impairment	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Mood alteration	0	(0%)	2	(5%)	0	(0%)	2 (5%)
Neuropathy: sensory	1	(3%)	0	(0%)	0	(0%)	1 (3%)
Syncope (fainting)	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Ocular/visual							
Vision–blurred vision	1	(3%)	0	(0%)	0	(0%)	1 (3%)

(Continued on the following page)

Table 3. Patient AEs ($N = 38$). (Cont'd)

	Grade of AE						
	2–Moderate		3–Severe		4–Life threat		Total
Type of AE	***N***	**(%)**	***N***	**(%)**	***N***	**(%)**	***N* (%)**
Pain							
Arthralgia (joint pain)	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Headache	3	(8%)	0	(0%)	0	(0%)	3 (8%)
Myalgia (muscle pain)	2	(5%)	3	(8%)	0	(0%)	5 (13%)
Pulmonary/upper respiratory							
Dyspnea (shortness of breath)	3	(8%)	1	(3%)	0	(0%)	4 (11%)
Hypoxia	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Pulmonary–other	0	(0%)	1	(3%)	0	(0%)	1 (3%)
Renal/genitourinary							
Incontinence	2	(5%)	0	(0%)	0	(0%)	2 (5%)

levels of IFNγ, STAT1, and P-STAT1 still increased following administration of rhIL12 and IFNα2b at cycle 60 in the patient with the longest PR.

Discussion

A phase II trial was conducted to determine the effectiveness of IL12 when administered in combination with IFNα in patients with advanced melanoma. Patients enrolled in the trial received a median of five cycles of therapy. The regimen did not meet its efficacy goals, as only two patients experienced a PR. The median OS was 10.3 months, whereas the median PFS was 2.5 months. This study provides information on the immune effects of low-dose intravenous rhIL12 when administered sequentially with an activating cytokine. Acquisition of peripheral blood 24 hours following the administration of IL12 permitted the generation of detailed information on the immune response to this cytokine. Correlative studies show that intermittent administration of IL12 led to increased systemic levels of IFNγ over prolonged periods of time. In addition, this regimen led to increased levels of JAK–STAT signaling intermediates and modulated IFNα-induced JAK–STAT signal transduction in patient PBMCs. These results indicate that IL12 can be administered for prolonged periods with measurable proimmune effects while minimizing toxicity if given intermittently at a low dose. Systemic IL12 therapy promoted the production of IFNγ that enhanced JAK–STAT signal transduction in peripheral blood immune effector cells. These findings can be used to predict the immune effects of future studies that use recombinant IL12 or novel IL12 constructs.

This regimen of IL12 and IFNα was reasonably well-tolerated, and preadministration of acetaminophen seemed to improve symptom management. This finding is consistent with the phase I study that preceded this trial (30). Grade 2 fever, chills, nausea, vomiting, anorexia, dizziness, and transient fatigue were common, and grade 3 fatigue, nausea, and myalgias were also observed. These symptoms occurred within hours of IL12 administration and were most severe following the first dose of IL12. Although grade 4 neurologic events have not been commonly observed with IL12 administration, two patients were removed from the study treatment because of arachnoiditis with associated confusion and dizziness, respectively. The coadministration of IFNα may have contributed to this symptom complex. In comparison, patients receiving anti–PD-1 checkpoint blockade have lower rates of grade 3 or above immune-related AEs, at 10% to 15%, although the addition of anti–CTLA-4 or anti–LAG-3 agents increases the AE rate to approximately 55% and 19% to 44%, respectively (3, 4, 36). In contrast to constitutional symptoms observed with IL12 and IFNα therapy, the most common categories of immune-related AEs due to checkpoint blockade are hypothyroidism or thyroiditis, rash and pruritis, fatigue, and colitis. AEs such as pneumonitis, myocarditis, and adrenal and pituitary insufficiency are rarer.

Other phase I/II trials from our group that utilized intravenous or subcutaneous IL12 in combination with FDA-approved therapies (IFNα or trastuzumab) were also well tolerated (11–13, 30). Whereas a study of IL12 in combination with trastuzumab and paclitaxel was associated with a 52% rate of clinical benefit (CR/PR, or stabilization of disease; ref. 11), a more recent study of IL12 in combination with cetuximab in patients with head and neck squamous cell carcinoma showed prolongation of PFS without any clinical responses (13). Although two patients in the present study experienced a PR, it is possible that this result could have been due solely to the actions of IFNα, which has a low but measurable response rate. The antitumor effects of IL12 in multiple studies coupled with the ability to affect cytotoxic effector cells suggests that it might best be used as an adjuvant to approved ICI agents (37–40). An NCI-sponsored phase II study of intratumoral IL12 in combination with PD-1 blockade (NCT02493361) demonstrated a 41% objective clinical response rate in patients with advanced melanoma (41). Studies in murine models of CT26 colon cancer utilized intratumoral injections of an oncolytic virus armed with an anti–PD-1 or murine IL12. Combined injection of these vectors exhibited synergistic antitumor effects (42). Multiple IL12 constructs with novel attributes (such as prolonged plasma half-life and cell-surface tethers) are now in development and may provide a suitable vehicle for exploring the ability of this cytokine to enhance checkpoint blockade (15, 41, 43–46). Although the report of this study was delayed by changes in the structure of the CALGB, the detailed nature of the correlative studies provides important information on the effects of IL12 in a representative population of patients with melanoma.

As in other studies, administration of IL12 had a distinct effect on immune effector cells as measured by the production of high levels of IFNγ by day 2 of all treatment cycles. IFNγ can upregulate levels of STAT1 and STAT2 within patient immune cells (10). Our group has demonstrated that this effect is reproducible and

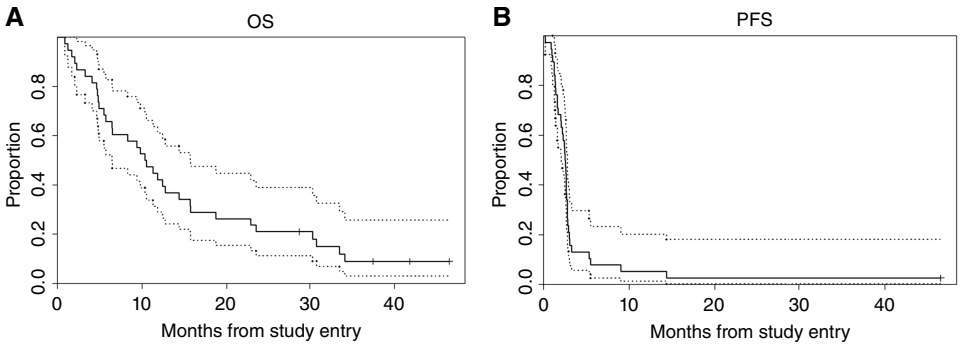


Figure 1.
Patient survival curves. **A,** OS for 38 trial participants (34 events), median 10.3 months. **B,** PFS for 38 trial participants (37 events), median 2.5 months. Error bands represent 95% CI estimates.

associated with enhanced sensitivity to agents such as IFNα that use JAK–STAT signaling intermediates to mediate gene expression (30). As predicted, the administration of IL12 led to a significant increase in levels of STAT1 and STAT2 in patient PBMCs, which indicates that systemic delivery of IL12 can exert a global effect on immune function. STAT1/2 levels returned to baseline by the time of the next cycle. Thus, the immune modulatory effects of rhIL12 seem to be transient but capable of reinduction at these doses. The responsiveness of the $CD3^+$ T-cell and NK cell compartments to subsequent stimulation with IFNα at the level of JAK–STAT signal transduction was enhanced after IL12 treatment, as evidenced by increased levels of P-STAT1. These results suggest that IL12 can augment the immune response to IFNs, yet the role of IL12-induced IFNγ in this process can only be inferred. Whereas the effects of IL12 on IFN-induced JAK–STAT signal transduction were distinct and reproducible, the low antitumor activity of IFNα in melanoma could not be enhanced by this pretreatment. The future role of IL12 in cancer therapy thus remains to be defined.

Although levels of cytokines and JAK–STAT signaling components in the patients who experienced a PR did not trend higher than those for nonresponders during the earlier cycles, administration of rhIL12 and IFNα2b elicited effects even after 32 months of continuous therapy. Therefore, a clinical response to therapy was

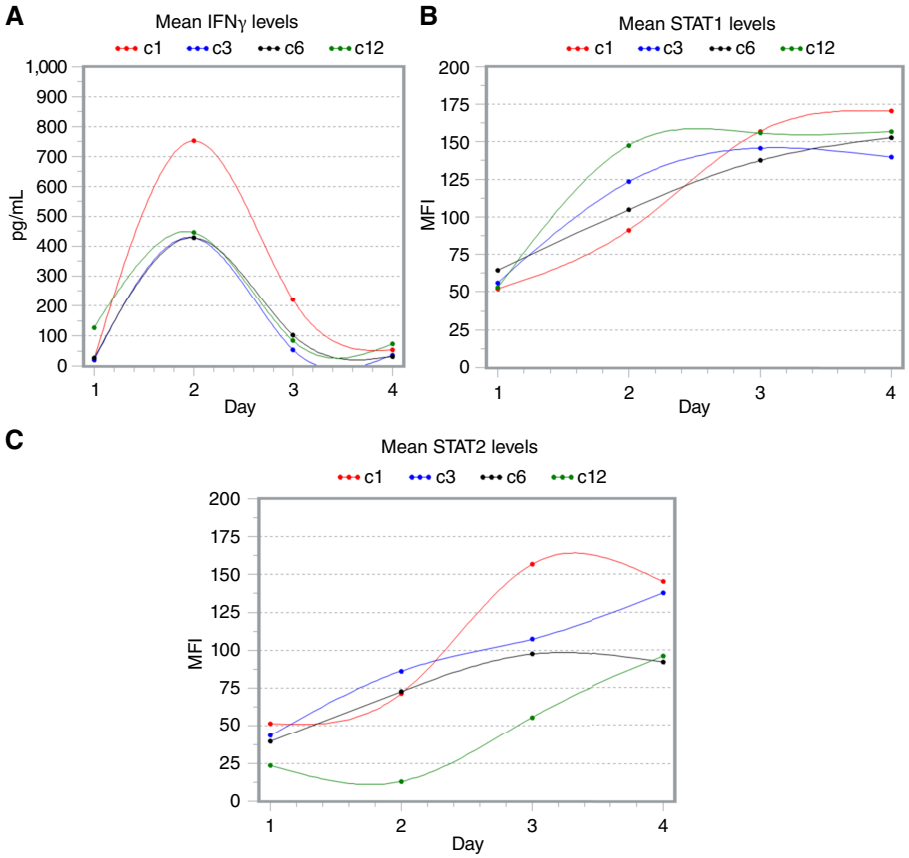


Figure 2.
Mean cytokine expression in 38 patients. Peripheral blood was obtained immediately prior to injection of rhIL12 and IFNα2b on days 1–4 of cycles 1, 3, 6, and 12. (**A**) IFNγ, (**B**) STAT1, and (**C**) STAT2 levels were measured in patient samples. IFNγ levels were measured via ELISA. STAT1 and STAT2 were measured using intracellular flow cytometry. Each data point represents mean expression across all 38 patients sampled. Data from each patient were derived from triplicate measurements. The x-axis represents day in each cycle. The y-axis represents the pg/mL (**A**) and mean fluorescence intensity (MFI; **B**, **C**). Linear mixed model using PROC MIXED in SAS, significance threshold of $P = 0.05$. c, cycle.

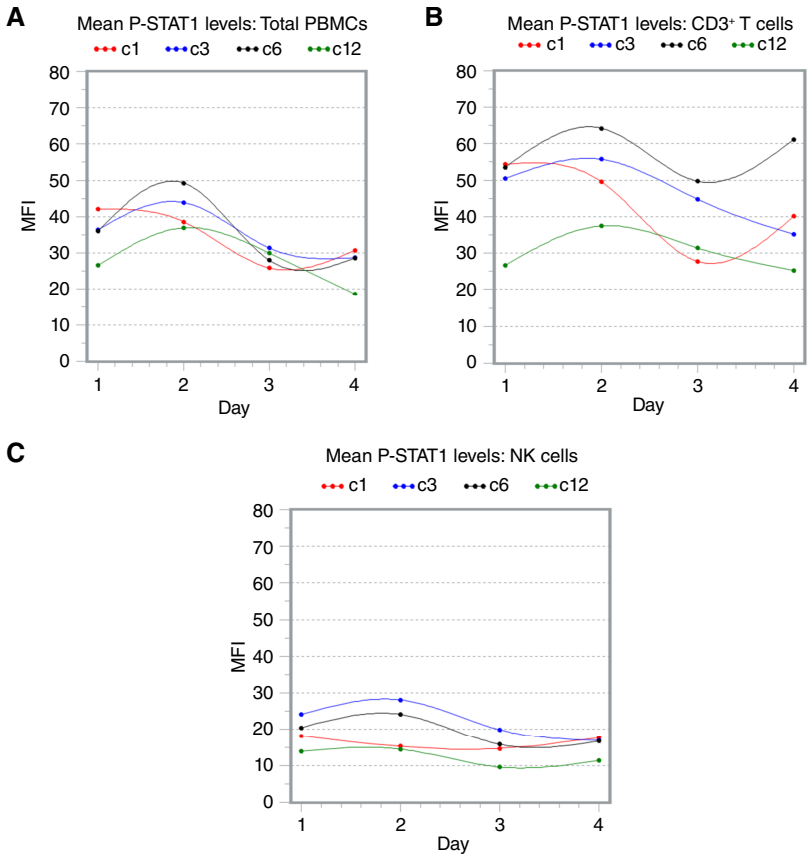


Figure 3.

Effects of IL12 pretreatments on STAT1 phosphorylation. Patient PBMCs obtained prior to the administration of cytokine treatment on days 1–4 of cycles 1, 3, 6, and 12 were treated *ex vivo* with PBS (negative control) or 10^4 U/mL IFNα2b for 15 minutes. Total PBMCs (**A**), $CD3^+$ T cells (**B**), and NK cells (**C**) were then analyzed for intracellular levels of phosphorylated (P) STAT1. Each data point represents the mean expression of P-STAT1 across all patients sampled with PBS control values subtracted. Data from each patient were derived from triplicate measurements. The x-axis represents day in each cycle. The y-axis represents the mean fluorescence intensity (MFI). Linear mixed model using PROC MIXED in SAS, significance threshold of $P = 0.05$. c, cycle.

not associated with a unique pattern of IFNγ induction or JAK–STAT signal transduction. The pattern of cytokine induction over time does suggest that combined administration of IL12 and IFNα2b can modulate the immune response for prolonged periods of time.

A preplanned goal of this study was the use of a relatively low dose of 3×10^6 units of IFNα2b delivered subcutaneously on days 2 to 6 of each cycle. The effects of this low dose of IFNα were expected to be enhanced by IL12; however, despite the observed rise in JAK–STAT signaling components and subsequent increase in activated transcription factors, the clinical activity of combination therapy was not significantly better than that of the individual cytokines. The historic response rate to single-agent IFNα in metastatic melanoma is approximately 10% to 16% (47). The observed increase in STAT1 phosphorylation suggests that the immune compartment was favorably affected by the combination of IL12 and IFNα. In early cycles, there was a rise in expression of the IFN response gene *ISG15* independent of IFNα stimulation on day 2 following IL12 administration. In later cycles, levels of *ISG15* rose later in the week, presumably in response to IFNα dosing in patients with a favorable clinical course. ISG15 is an ubiquitin-like protein that exists as a free intracellular and extracellular molecule. It also acts as a posttranslational modifier in the process of ISG15 conjugation (ISGylation). ISG15 and ISGylation seem to have a strong impact on multiple cellular processes, including immune modulation, cytokine secretion, protein translation, autophagy, exosome output, cytoskeleton organization, DNA damage response, and telomere maintenance. ISG15 favorably affects IFNγ expression and secretion (48, 49), which may underlie the efficacy of IL12 and IFNα in murine and human models.

Although there was a robust induction of IFNγ secretion and JAK–STAT signaling in peripheral immune cells, this study did not characterize potential changes in the intratumoral immune environment elicited by IL12 and IFNα2b therapy. These considerations are relevant given the advent of novel tumor-directed therapies such as personalized neoantigen vaccines and tumor-infiltrating lymphocyte therapy. Genomic sequencing was not performed in this trial due to the embryonic nature of the relevant technology at the time. However, a later report by a separate group performed copy number variation analysis on archived tumors from patients with melanoma metastases treated with IFNα (50). This report found that copy number alterations in IFN-regulated genes were increased in nonresponding patients, suggesting that the unique tumor mutational profile may influence the response to IFNα therapy.

Overall, this study demonstrates that rhIL12 can be combined with IFNα2b in patients with metastatic melanoma. This treatment regimen was reasonably tolerated, but it was not associated with antitumor activity in the majority of patients. The increase in circulating levels of IFNγ and modulation of JAK–STAT signaling pathways confirmed the immunomodulatory activity of IL12. Future studies with IL12 constructs that have been modified to augment half-life are planned or in progress and will determine whether this

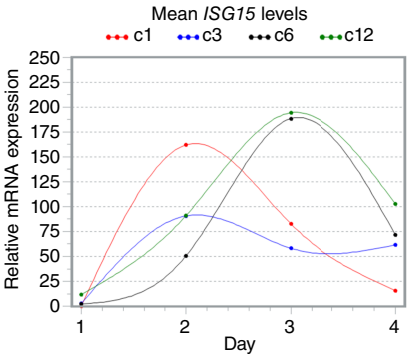


Figure 4.

Mean *ISG15* mRNA expression in patients. Peripheral blood was obtained immediately prior to injection of rhIL12 and IFNα2b on days 1–4 of cycles 1, 3, 6, and 12. *ISG15* expression was measured in patient PBMCs by real-time PCR. Each data point represents the mean expression across all patients sampled. Data from each patient were derived from triplicate measurements. The x-axis represents the day in each cycle. The y-axis represents relative mRNA expression. Linear mixed model using PROC MIXED in SAS, significance threshold of $P = 0.05$. c, cycle.

cytokine can be used in combination with other immune-modulating agents.

Data Availability

To protect patient confidentiality, identifiable data will not be made available. Deidentified data may be requested for reasonable academic use from the Alliance for Clinical Trials in Oncology by submitting a data request form to DataSharing@AllianceNCTN.org. A formal review process will confirm availability of the data, and the investigator must complete a data release form.

Authors' Disclosures

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Authors' Contributions

A. Liu: Formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. **C. Johnson:** Formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. **D. Niedzwiecki:** Conceptualization, data curation, software, formal analysis, validation, investigation, visualization, methodology, writing–original draft, writing–review and editing. **T. Relation:** Formal analysis, investigation, visualization, methodology, writing–original draft, writing–review and editing. **S.V. Yarlagadda:** Investigation, visualization, methodology. **T.F. Gajewski:** Conceptualization, resources, supervision, investigation, methodology, writing–original draft, project administration, writing–review and editing. **W.E. Carson III:** Conceptualization, resources, supervision, funding acquisition, investigation, visualization, methodology, writing–original draft, project administration, writing–review and editing.

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Note

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