

## Spotlight on Clinical Response

# A phase I trial of paclitaxel and trastuzumab in combination with interleukin-12 in patients with HER2/neu-expressing malignancies

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### Abstract

Our preclinical work showed a dramatic synergy between interleukin-12 (IL-12) and trastuzumab for stimulation of natural killer cell cytokine secretion. We aimed to determine the safety profile of IL-12 when given in combination with trastuzumab and paclitaxel to patients with metastatic HER2-overexpressing cancers. Paclitaxel was given i.v. at 175 mg/m<sup>2</sup> every 3 weeks. Trastuzumab was given on day 1 each week (4 mg/kg initially and 2 mg/kg thereafter) in combination with injections of IL-12 on days 2 and 5 starting in cycle 2. This trial accrued 21 patients with metastatic HER2-positive tumors (breast, 7; colon, 6; esophagus, 4; stomach, 2; pancreas, 1; thyroid, 1). The IL-12 component was dose-escalated in cohorts of three patients. The dose-limiting toxicity was grade 3 fatigue at the 300 ng/kg dose level in two patients. The

recommended phase II dose was 200 ng/kg administered s.c. There was one complete response in a patient with breast cancer, partial responses in 4 patients (breast, 2; esophageal, 2), and stabilization of disease lasting 3 months or greater (SD) in 6 other patients. All but one response occurred in patients with HER2 3+ disease. Two SD patients completed 1 year of therapy. Ten patients had progressive disease. There was increased activation of extracellular signal-regulated kinase in peripheral blood mononuclear cells and increased levels of IFN- $\gamma$  and several chemokines in patients with clinical benefit (complete response, partial response, or SD), but not in patients with progressive disease. IL-12 in combination with trastuzumab and paclitaxel therefore exhibits an acceptable toxicity profile and has activity in patients with HER2-overexpressing cancers. [Mol Cancer Ther 2009;8(11):2983–91]

### Introduction

The *HER2/neu* oncogene is overexpressed in approximately 20% of human breast cancers and portends a worse prognosis (1). Trastuzumab is a humanized monoclonal antibody (mAb) that binds to the HER2 protein and mediates growth inhibitory properties on tumors that express HER2 (2). Administration of trastuzumab in combination with cytotoxic chemotherapy leads to improved response rates, longer time to progression, and increased survival in breast cancer patients with HER2-overexpressing metastatic disease (3, 4). The combination of trastuzumab and paclitaxel is a standard chemotherapy regimen for patients with metastatic HER2-positive breast cancer. Prospective randomized clinical trials have shown that the addition of trastuzumab to adjuvant chemotherapy regimens reduces recurrences by approximately one half in patients with early-stage breast cancer (5).

The binding of trastuzumab to HER2-expressing breast cancer cells clearly exerts direct antitumor effects, but it seems that immune effector cells, which bear receptors for the Fc (or “constant”) region of immunoglobulin, may also be involved in the elimination of tumor cells (6). Clynes et al. (7) reported that the antitumor effects of trastuzumab in a murine model of breast cancer required the expression of functional Fc $\gamma$  receptor (Fc $\gamma$ R) by host immune effectors. Although granulocytes and monocytes coexpress both activating and inhibitory Fc $\gamma$ R, natural killer (NK) cells are unique in that they express only the activating, low-affinity Fc $\gamma$ RIIIa (8).

Received 9/4/09; accepted 9/18/09; published OnlineFirst 11/3/09.

**Grant support:** NIH grants K24 CA93670, P01 CA95426, U01 CA76576, T32 CA009338, and P30 CA16058.

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**Note:** T.S. Bekaii-Saab and J.M. Roda contributed equally to this work. Presented in part at the American Society of Clinical Oncology Breast Cancer Symposium, San Francisco, California, September 2007 (abstract no. 174).

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doi:10.1158/1535-7163.MCT-09-0820

NK cells are large granular lymphocytes that contain abundant cytolytic granules, express multiple adhesion molecules, and constitutively display receptors for several cytokines (9). Activated NK cells produce cytokines with antitumor actions [e.g., IFN- $\gamma$  and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )] and chemokines that recruit macrophages and T cells to sites of inflammation (10–12). Of note, expression of Fc $\gamma$ RIIIa enables NK cells to interact with antibody-coated tumor cells and mediate antibody-dependent cellular cytotoxicity and the secretion of IFN- $\gamma$  (13–15).

Our group has shown *in vitro* and in murine tumor models that costimulation of NK cells via the interleukin-12 (IL-12) receptor and Fc $\gamma$ RIIIa activates the extracellular signal-regulated kinase (ERK), which in turn promotes the secretion of IFN- $\gamma$  (16). Based on these preclinical data, we previously conducted a National Cancer Institute (NCI)-sponsored phase I trial of IL-12 and trastuzumab for patients with HER2-positive cancers (17). Elevated levels of IFN- $\gamma$ , TNF- $\alpha$ , macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ; a chemokine), and IP-10 and MIG (antiangiogenic factors induced by IFN- $\gamma$ ) were observed in the patients that exhibited clinical benefit. These results suggested that immunologically active compounds might enhance the patient immune response to therapeutic mAbs.

The aim of the present study was to determine the tolerability of IL-12 when administered in combination with trastuzumab and paclitaxel to patients with metastatic HER2-overexpressing cancers. A secondary goal was to evaluate the immunologic effects of IL-12 administration in this setting and assess, in a preliminary fashion, its correlation with clinical benefit.

## Patients and Methods

### Eligibility

Patients with nonhematologic malignancies that overexpressed HER2 were eligible for enrollment in this NCI-sponsored phase I trial. DAKO HercepTest was used to evaluate HER2 overexpression because it was the standard test in use at the time the trial began accrual. HER2 overexpression was later confirmed by fluorescence *in situ* hybridization, where samples were available. Patients were required to be >18 y of age; have a life expectancy of >6 mo, a Karnofsky performance status index of >70%, a left ventricular ejection fraction of >50%, normal organ function, and measurable disease; and be capable of giving informed consent. Patients were excluded from participation if they had received prior therapy with trastuzumab.

### Treatment Schema and Response Assessment

Treatment cycles were 3 wk long (Table 1). On day 1 of cycle 1, patients received a loading dose of trastuzumab (4 mg/kg i.v.) followed by paclitaxel 175 mg/m<sup>2</sup> i.v. over 3 h. Paclitaxel was administered once every 21 d. Patients were premedicated with dexamethasone (10 mg p.o.) the night before and the morning of paclitaxel administration. A maintenance dose of trastuzumab (2 mg/kg i.v.) was administered on day 1 of weeks 2 and 3 of the first cycle and weekly thereafter. Beginning in cycle 2, IL-12 was given on

**Table 1. Trial schema for a phase I trial of interleukin-12 in combination with paclitaxel plus trastuzumab in patients with HER2-positive malignancies, NCI protocol no. 84**

Cycle I:								
Week 1:	Day	1	2	3	4	5	6	7
Treatment		T/P						
Week 2:	Day	8	9	10	11	12	13	14
Treatment		t						
Week 3:	Day	15	16	17	18	19	20	21
Treatment		t						
Cycles II and up:								
Week 1:	Day	1	2	3	4	5	6	7
Treatment		t/P	C			C		
Week 2:	Day	8	9	10	11	12	13	14
Treatment		t	C			C		
Week 3:	Day	15	16	17	18	19	20	21
Treatment		t	C			C		

NOTE: T, trastuzumab (4 mg/kg i.v.); t, trastuzumab (2 mg/kg i.v.); P, paclitaxel (175 mg/m<sup>2</sup>  $\times$  3 h i.v.); C, IL-12 (100 or 300 ng/kg i.v. or 200 ng/kg s.c.).

days 2 and 5 following the weekly dose of trastuzumab (total of six doses per cycle). IL-12 dose escalation occurred as follows: cohort 1, 100 ng/kg i.v.; cohort 2, 300 ng/kg i.v.; and cohort 3, 200 ng/kg s.c. Patients underwent radiologic evaluation of their disease after cycles 1 and 3 and every 3 mo thereafter. Patients exhibiting a partial response (PR) or stable disease (SD; by Response Evaluation Criteria in Solid Tumors) after three cycles of therapy had the option of continuing therapy for up to 1 y.

### Dose-Limiting Toxicity

Patients who experienced any clearly drug-related grade 3 or greater toxicity (nonhematologic) that did not resolve after a 2-week rest period or any clearly drug-related grade 4 toxicity (nonhematologic) were considered to have experienced a dose-limiting toxicity (revised NCI Common Toxicity Criteria version 3.0) and were removed from the study. Patients resuming therapy after the resolution of grade 3 toxicity that was due to IL-12 or trastuzumab could continue therapy following a 50% dose reduction.

### Procurement of Patient Plasma and Peripheral Blood Mononuclear Cells

Blood for use in correlative studies was drawn just before each injection of trastuzumab or IL-12 (20 mL). Plasma and peripheral blood mononuclear cells (PBMC) were procured from the blood sample and cryopreserved.

### Intracellular Flow Cytometry and Immunoblot Analysis

The production of IFN- $\gamma$  by patient immune cell subsets was analyzed using a FITC-conjugated mAb to human IFN- $\gamma$  (BD Pharmingen) and phycoerythrin-conjugated mAbs to surface markers specific for NK cells (CD56) and T cells (CD3), as previously described (17). The percentage of positively staining cells and mean fluorescence intensity were calculated for IFN- $\gamma$  within the specified cell population. Immunoblot analysis for phosphorylated ERK (p-ERK) and total ERK was conducted as previously described (16). Quantitation of immunoblot bands was accomplished using

NIH ImageJ software. Signal intensity was compared by normalization to total ERK protein levels.

### Cytokine ELISAs

Patient serum samples were thawed on ice and analyzed in triplicate for levels of cytokines (IFN- $\gamma$  and TNF- $\alpha$ ), chemokines [regulated on activation, normal T-cell expressed and secreted (RANTES), IL-8, and MIP-1 $\alpha$ ], and antiangiogenic factors (IP-10 and MIG) by ELISA using commercially available mAb pairs (Endogen, Inc.; ref. 17).

### Statistics

Statistical analyses of binomial data (response/no response) used Fisher's exact test. The Kruskal-Wallis test was used to evaluate the correlation between clinical response and cytokine production when values were greater than zero. Analysis of continuous data including uncensored progression-free survival was done using the nonparametric Mann-Whitney *U* test. Analyses of fold increases in p-ERK levels were done using Student's *t* test. All analyses considered *P* < 0.05 as significant.

## Results

### Patient Characteristics

Demographics for the 21 patients are presented in Table 2. Sixteen patients had more than one metastatic site. The majority of patients (16 of 21) had received at least one prior regimen of systemic chemotherapy, including four who had previously received paclitaxel. Immunohistochemical analysis revealed that 9 patients (43%) had 3+ expression of HER2/neu, whereas the remaining 12 (57%) had 2+ ex-

pression (Table 3). Seven patients had metastatic breast cancer, but 14 patients with other malignancies were also accrued.

### Toxicities

The only grade 4 toxicities encountered were asymptomatic neutropenia in three patients and leukopenia in one patient (Table 4). These toxicities were reversible and did not limit the dose escalation of IL-12 (18, 19). Grade 3 toxicities included fatigue, anorexia, neuropathy, arthralgia, leukopenia, and neutropenia. Dose-limiting grade 3 fatigue was encountered in two of six patients at the 300 ng/kg i.v. dose level (Table 3). Due to poor tolerance of i.v. IL-12 and suboptimal patient compliance with frequent clinic visits, s.c. dosing of IL-12 at 200 ng/kg was initiated. This IL-12 dose and route had been shown to induce IFN- $\gamma$  production and antitumor activity in other studies (20–22). A total of 12 patients were treated at this dose level with no demonstrable dose-limiting toxicity.

### Clinical Responses

One patient had a complete response (CR), four had a PR, and six experienced SD that lasted for 3 months or longer. Patient G, with HER2 3+ breast cancer metastatic to the ipsilateral supraclavicular lymph nodes, had previously undergone neoadjuvant therapy with doxorubicin/cyclophosphamide before surgery. This patient received paclitaxel postoperatively, followed by breast irradiation. She received three cycles of therapy and achieved a CR but was removed from the study when a computed tomography scan revealed an asymptomatic pulmonary embolism. She is disease-free 4 years after being removed from the trial. Patient B, who had HER2 3+ recurrent breast cancer and had previously received adjuvant doxorubicin/cyclophosphamide followed by paclitaxel, maintained a PR for 47 weeks. Patient C, with recurrent HER2 3+ breast cancer following resection and adjuvant therapy with doxorubicin/cyclophosphamide and paclitaxel, maintained a PR that lasted 18 weeks. Patient I, with metastatic HER2 2+ esophageal cancer, received 12 cycles of treatment and experienced a PR that lasted 43 weeks. Patient T, with metastatic HER2 3+ esophageal cancer, received eight cycles of therapy and achieved a PR that lasted for 25 weeks. Patient A, with HER2 3+ metastatic breast cancer, and patient M, with HER2 2+ metastatic gastric cancer, had SD that was maintained for 68 and 62 weeks, respectively. Notably, clinical responses were only observed following the addition of IL-12 to the treatment regimen in the second cycle. Overall, there was a 52% rate of clinical benefit (CR + PR + SD). Ten patients experienced progressive disease.

### IFN- $\gamma$ Production

The only patients with measurable levels of IFN- $\gamma$  at any time during therapy were those who experienced a CR, PR, or SD (see Table 3). IFN- $\gamma$  was not detected (< 10 pg/mL) in the remaining 10 patients with progressive disease (Fisher's exact test, *P* < 0.001; Fig. 1A). Figure 1B shows the peak IFN- $\gamma$  levels for each treatment cycle for the patients experiencing a PR or CR (*top*) and patients with SD (*bottom*). Within any one cycle, IFN- $\gamma$  levels typically peaked following injections of IL-12 (range, 124–1,612 pg/mL) and then decreased

**Table 2. Patient demographics**

Age (y)	
Median	56
Range	41–81
Gender ( <i>n</i> )	
Male	11
Female	10
Karnofsky performance index ( <i>n</i> )	
90–100%	12
80–90%	6
70–80%	3
Tumor type ( <i>n</i> )	
Breast	7
Colon	6
Esophagus	4
Stomach	2
Pancreas	1
Thyroid	1
No. metastatic sites ( <i>n</i> )	
1	5
2	5
$\geq 3$	11
No. prior chemotherapy regimens ( <i>n</i> )*	
0	5
1	4*
$\geq 2$	12

\*Four patients had received prior paclitaxel.

**Table 3. Patient outcomes**

Tumor type	Patient (HER2 level)	Dose level IL-12, 100 ng/kg i.v., 300 ng/kg i.v., 200 ng/kg s.c.	No. cycles received	Best response	Duration of response or SD (d)	Peak IFN- $\gamma$ level (pg/mL)
Breast	A (3+)	100	18	SD*	475	363
Breast	B (3+)	100	15	PR	329	285
Breast	C (3+)	100	6	PR	128	686
Colorectal	D (3+)	300	3	SD <sup>†</sup>	143	174
Breast	E (3+)	300	2	PD <sup>‡</sup>	—	0
Colorectal	F (2+)	300	3	PD <sup>‡</sup>	—	0
Breast	G (3+)	300	3	CR <sup>§</sup>	1,643+	1,448
Breast	H (2+)	300	6	SD	126	124
Esophagus	I (2+)	300	12	PR	303	1,612
Colorectal	J (3+)	200	3	PD	—	0
Colorectal	K (2+)	200	3	SD	100	183
Colorectal	L (2+)	200	3	PD	—	0
Stomach	M (2+)	200	15	SD	435	331
Thyroid	N (2+)	200	2	PD	—	0
Pancreas	O (2+)	200	3	PD	—	0
Breast	P (3+)	200	2	PD	—	0
Stomach	Q (2+)	200	9	SD	191	128
Esophagus	R (2+)	200	3	PD	—	0
Esophagus	S (2+)	200	3	PD	—	0
Esophagus	T (3+)	200	8	PR	177	262
Colorectal	U (2+)	200	3	PD	—	0

\*Patient completed the study and was continued on single agent trastuzumab until disease progression.

<sup>†</sup>Patient was lost to follow-up.

<sup>‡</sup>Experienced grade 3 fatigue that was dose-limiting.

<sup>§</sup>Patient was removed from study because of an incidental pulmonary embolism found on CT scan. The patient is receiving trastuzumab alone and is NED as of this writing.

to baseline (10–200 pg/mL; data not shown). Analysis of patient time-to-progression data using the nonparametric Mann-Whitney *U* test indicated that induction of IFN- $\gamma$  was associated with a statistically significant increase in progression-free survival ( $P = 0.004$ ). Intracellular flow cytometric analysis of cryopreserved PBMCs from day 5 of the

treatment cycle revealed high levels of IFN- $\gamma$  only in those patients who exhibited a clinical response or SD ( $P < 0.0001$ ; Fig. 1C). Furthermore, IFN- $\gamma$  production was only observed within the CD56<sup>+</sup> NK cell population. IFN- $\gamma$  was not observed in CD3<sup>+</sup> T cells of any patient (Fig. 1D).

#### Antiangiogenic Factors

IP-10 and MIG are potent inhibitors of neovascularization (23) that were identified in the circulation of patients that had a CR or SD in the previous trial of IL-12 and trastuzumab (17). In the current study, peak levels of IP-10 and MIG were significantly higher in clinically benefiting patients as compared with those who had progressive disease (Kruskal-Wallis test,  $P = 0.0057$ ; Fig. 2A). Levels of IP-10 and MIG in patients with progressive disease were very close to baseline levels observed in normal individuals (500 and 300 pg/mL, respectively; ref. 17).

#### Other NK Cell Cytokines

We have determined from the previous trial of IL-12 and trastuzumab that the T-cell-attracting chemokines MIP-1 $\alpha$ , IL-8, and RANTES were present at high levels in the sera of patients who experienced a clinical response or SD ( $P < 0.01$ ), but not in the remaining patients who had progressive disease (17). In the present trial, low levels of these three cytokines were detected at baseline in all 21 patients (not shown). Peak levels of MIP-1 $\alpha$  and IL-8 were elevated in patients with favorable clinical outcomes (CR, PR, or SD) as compared with patients that exhibited progressive disease ( $P = 0.046$  and

**Table 4. Treatment toxicities**

Toxicity	Grade 1 (%)	Grade 2 (%)	Grade 3 (%)	Total*
Nonhematologic				
Fatigue	5	76	14	95
Chills	76	5	0	81
Nausea	38	29	0	67
Alopecia	24	33	0	57
Anorexia	18	29	5	52
Neuropathy	33	10	10	53
Arthralgia	24	20	5	49
Myalgia	24	24	0	48
Hematologic				
Anemia	24	20	0	44
Leukopenia	5	10	24	44
Neutropenia	0	0	15	30
Thrombocytopenia	10	0	0	10

NOTE: All toxicities were reversible. Three patients had reversible grade 4 neutropenia and one had reversible grade 4 leukopenia.

\*Includes grade 4 toxicities.



$P = 0.06$ , respectively; Fig. 2B). RANTES levels could not be measured in every patient; however, elevated levels were seen only in clinically benefiting patients (data not shown). Circulating levels of IFN- $\gamma$ , TNF- $\alpha$ , MIP-1 $\alpha$ , and RANTES over time in patient I (PR) are presented in Fig. 2C (17).

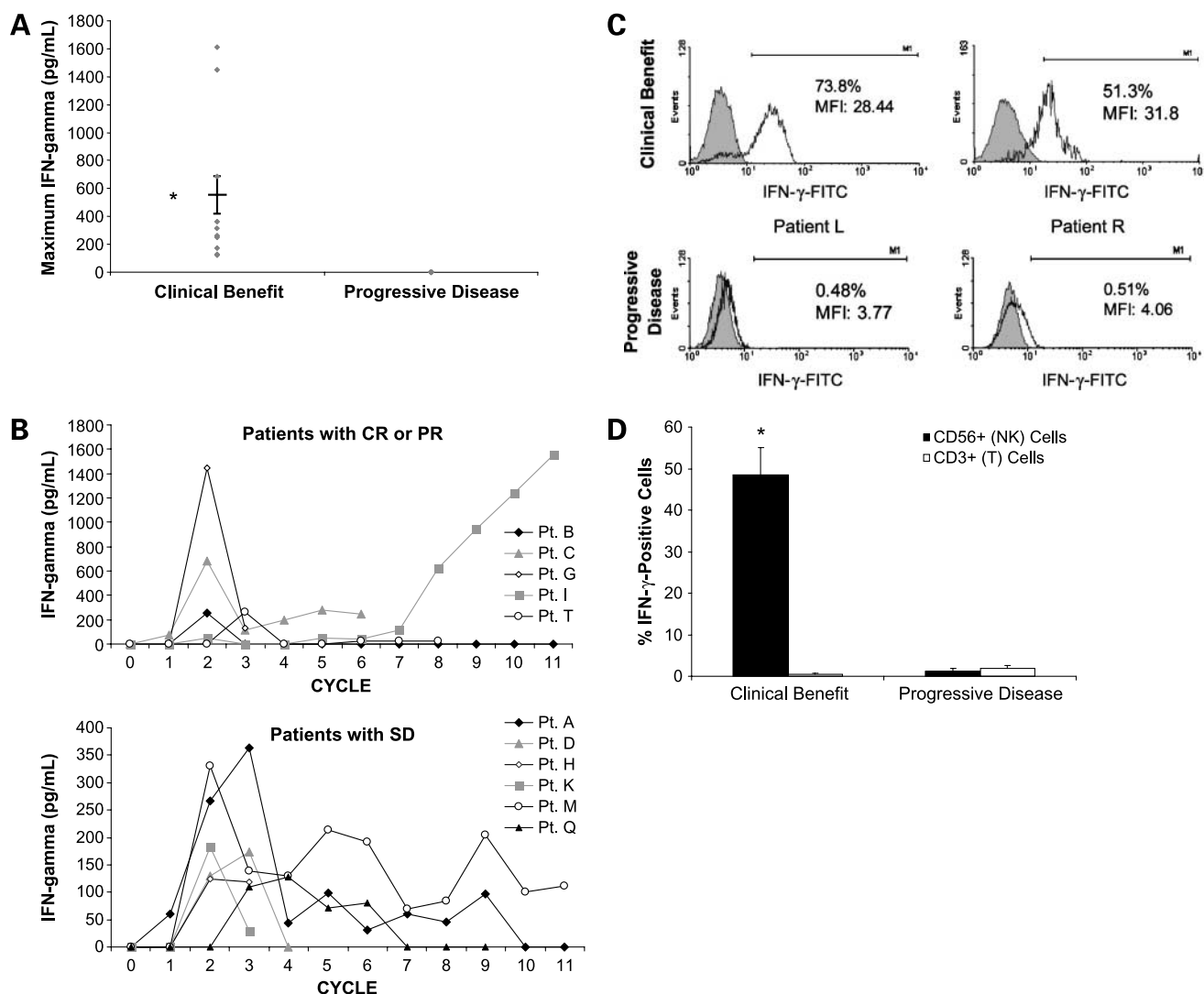
### ERK Phosphorylation in Patient PBMCs

Our group has shown that costimulation of the IL-12 receptor and Fc $\gamma$ RIIIa on human NK cells leads to synergistic activation of ERK, and that this transcription factor is necessary for NK cell production of IFN- $\gamma$  (16). We therefore examined ERK phosphorylation in cryopreserved patient PBMCs obtained before the administration of trastuzumab (day 1) and again after the administration of IL-12 (days 2

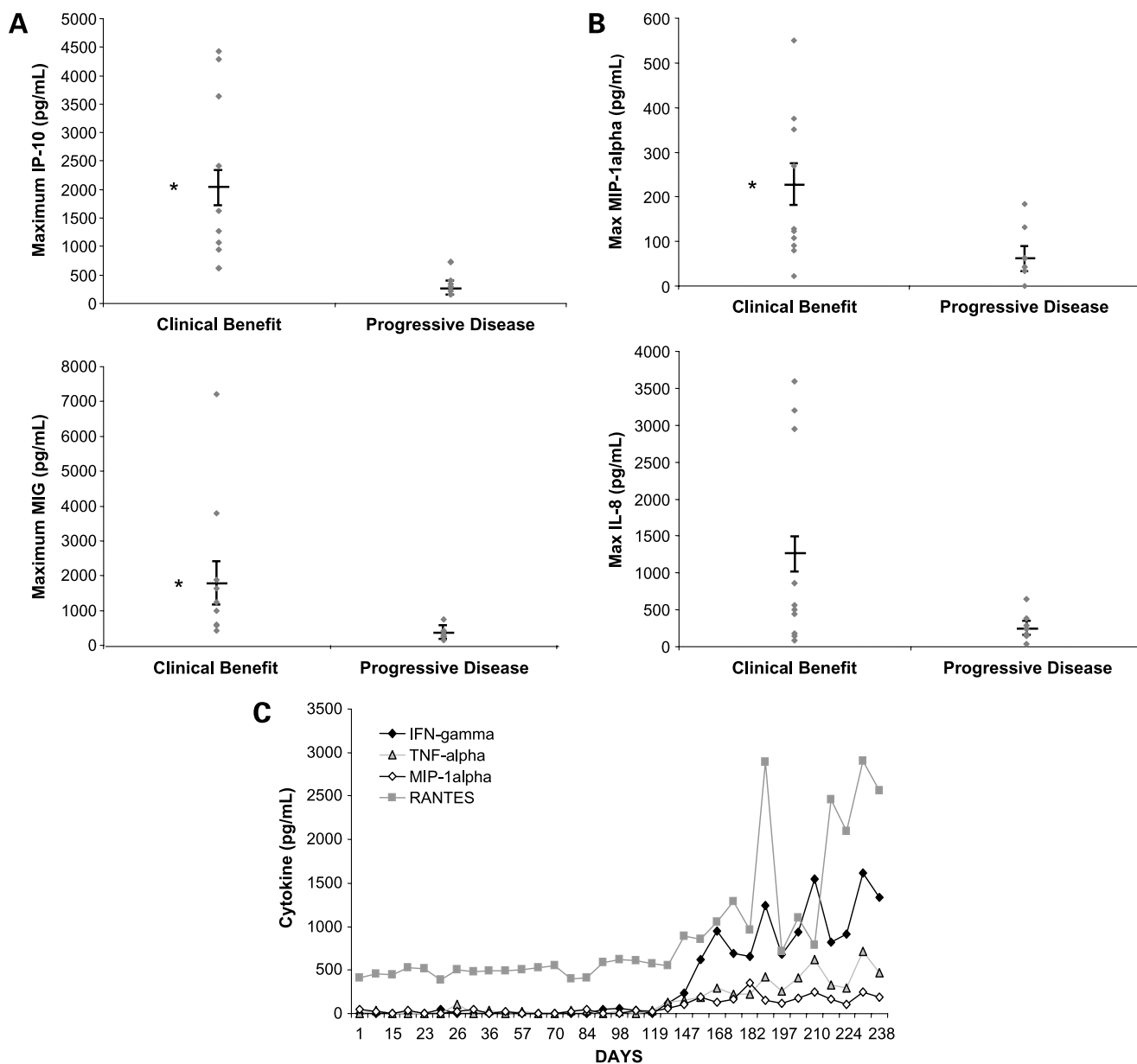
and 5). Enhanced activation of ERK on day 5 was observed only in those patients who exhibited a clinical response to therapy or significant SD (Fig. 3A). An analysis of the densitometric data revealed a statistically significant relationship between clinical response and the induction of p-ERK ( $P = 0.015$ , Fig. 3B).

### Discussion

Herein we describe the results of a phase I trial in which the cytokine IL-12 was administered in combination with trastuzumab and paclitaxel to patients with HER2-overexpressing cancers. This chemo-immunotherapy regimen was



**Figure 1.** **A**, peak circulating levels of IFN- $\gamma$  in patients that experienced clinical benefit from therapy (CR, PR, or SD) as compared with patients that had progressive disease. \*, significant difference between the two groups as determined by Fisher's exact test. **B**, IFN- $\gamma$  levels over time in patients with a CR or PR to therapy (top) or patients with SD (bottom). **C**, dual parametric intracellular flow cytometry for IFN- $\gamma$  and CD56 in representative patients that exhibited clinical benefit from therapy (top) or progressive disease (bottom). The shaded histograms represent the staining of NK cells obtained pre-therapy. The clear histograms represent the staining of NK cells obtained on day 5 of a representative treatment cycle. The percentage of CD56<sup>+</sup> NK cells that stained for IFN- $\gamma$  and their mean fluorescence intensity (MFI) appear next to each histogram. **D**, graphical representation of the flow cytometric data for all patients on trial. \*, significant difference in increase of IFN- $\gamma$  production as determined by Student's  $t$  test ( $P < 0.0001$ ).



**Figure 2.** **A**, peak circulating levels of IP-10 (*top*) and MIG (*bottom*) in patients that experienced clinical benefit from therapy as compared with patients that had progressive disease. **B**, peak circulating levels of MIP-1 $\alpha$  (*top*) and IL-8 (*bottom*) in patients that experienced clinical benefit from therapy as compared with patients that had progressive disease. \*, significant difference between the two groups as determined by the Kruskal-Wallis test. **C**, levels of IFN- $\gamma$ , TNF- $\alpha$ , MIP-1 $\alpha$ , and RANTES in a patient that exhibited a PR to therapy (patient I).

pursued based on our clinical and preclinical data, which showed that IL-12 enhanced the Fc $\gamma$ R-dependent antitumor effects of trastuzumab, and on previous work by other groups, which showed that paclitaxel does not greatly inhibit NK cell function *in vivo* (24). Dose-limiting toxicity was observed when IL-12 was administered *i.v.* at a dose of 300 ng/kg twice weekly, but use of IL-12 at lower doses was well tolerated. Eleven of 21 patients experienced clinical benefit, including five with PR or CR. Patients who experienced clinical benefit in response to therapy exhibited

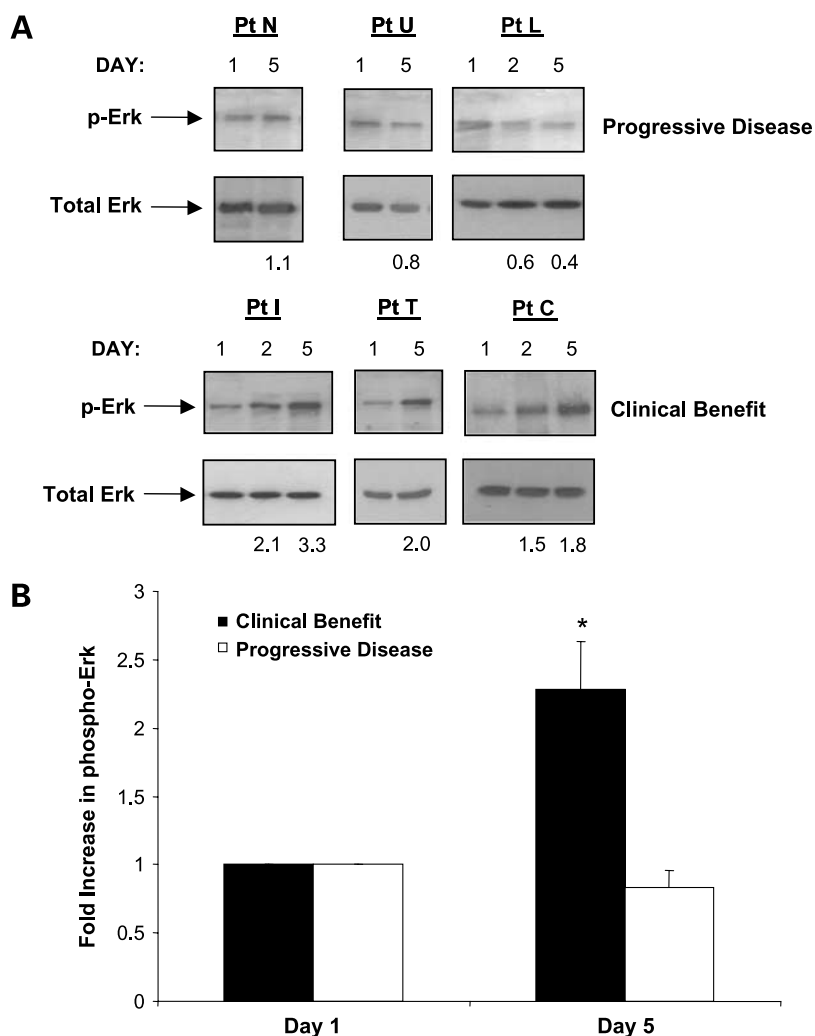
elevated plasma levels of IFN- $\gamma$  and chemokines and showed increased activation of ERK in circulating PBMCs, whereas those with progressive disease had no such immunologic response. These results show that a cytokine can be administered safely in combination with a mAb and cytotoxic chemotherapy and elicit an immunologic response that seems to be associated with clinical outcome.

Overall, this regimen was well tolerated. When IL-12 was given twice weekly *i.v.* at a dose of 300 ng/kg, dose-limiting toxicity in the form of grade 3 fatigue was observed, which

is consistent with previous studies (21, 25). Although the development of severe fatigue was probably due to the concurrent administration of IL-12 with paclitaxel, it seems that trastuzumab may have contributed to the development of this symptom. This conclusion is supported by the observation that fatigue was not observed in a phase I study of twice-weekly i.v. IL-12 in patients with metastatic renal cell cancer and malignant melanoma, but was encountered in 93% of patients who were treated with IL-12 and trastuzumab in our previous study (17, 19). The rationale for proceeding with s.c. dosing of IL-12 lies in the potential for improved tolerance and a reduced requirement for patients to return to the clinic for i.v. infusions. Several groups had previously shown that s.c. administration of IL-12 could induce clinical responses and immune activation in patients with melanoma, renal cell carcinoma, cutaneous T-cell lymphoma, and Kaposi's sarcoma (20, 21, 26, 27). No further instances of dose-limiting fatigue were observed when IL-12 was administered s.c. at a dose of 200 ng/kg. Importantly, significant secretion of IFN- $\gamma$  by NK cells was observed in response to

s.c. administered IL-12 (Fig. 1C and D), and 4 of the 12 patients at this dose level obtained clinical benefit from therapy. Contrary to previous studies of single-agent IL-12 (27, 28), there was no clear evidence that higher doses of IL-12 had greater antitumor activity or immunologic effects because clinical responses and significant IFN- $\gamma$  production were observed at each dose level of IL-12. Subcutaneous dosing of IL-12 has distinct advantages in terms of ease of administration and patient acceptance; however, additional studies will be required to determine the optimal dose for use with trastuzumab-based regimens.

We hypothesized that IL-12, an immune-activating cytokine with actions on NK cells, would be able to enhance the antitumor actions of trastuzumab. Three of six patients with HER2 3+ metastatic breast cancer experienced a clinical response, a result that is consistent with published reports for the combination of trastuzumab and paclitaxel in the first- and second-line settings (3, 29, 30). Responses in two patients with esophageal cancer were also observed. Due to the heterogeneous nature of the patient population



**Figure 3.** **A**, immunoblot analysis of patient PBMCs for levels of p-ERK and total ERK at days 1, 2, and 5 of a representative treatment cycle. Band density as compared with day 1 is provided at the bottom of each lane. **B**, graphical representation of the densitometric data obtained from Fig. 3A. \*, significant difference as determined by Student's *t* test ( $P = 0.015$ ).

under study, the varying levels of tumor HER2 expression, and the multiple dose levels of IL-12 that were used, it cannot be concluded that IL-12 had an appreciable impact on the activity of the trastuzumab/paclitaxel regimen. However, the strong correlation between IFN- $\gamma$  production and clinical benefit suggests that the administration of IL-12 had a distinct immunologic effect in these patients and supports the continued study of immunologic agents that can be given in conjunction with therapeutic antitumor mAbs, such as trastuzumab. Furthermore, our results suggest that the immunostimulatory effects of IL-12 are preserved even when it is administered in combination with potentially immunosuppressive chemotherapeutic agents.

IL-12 was administered with trastuzumab/paclitaxel with the expectation that it would serve as a costimulus to FcR-bearing cells that had bound to antibody-coated cancer cells. Our group has shown that costimulation of NK cells via the IL-12 receptor and FcR leads to colocalization of these two receptors in plasma membrane lipid rafts, which in turn promotes the activation of ERK and secretion of IFN- $\gamma$  (16). In support of this hypothesis, immunoblot analysis of PBMCs from patients that experienced a clinical benefit revealed significantly increased levels of p-ERK on day 5 of the weekly cycle as compared with patients that went on to have progressive disease. Likewise, secretion of IFN- $\gamma$  and chemokines was also limited to patients that had SD or a clinical response. We were able to show via intracellular flow cytometry that NK cells were the source of the IFN- $\gamma$  in these patients. In contrast, the ability of patient NK cells to mediate antibody-dependent cellular cytotoxicity against a trastuzumab-treated HER2/neu-positive breast cancer cell line did not increase following IL-12 administration in either the clinically benefiting patients ( $P = 0.15$ ) or the patients with progressive disease ( $P = 0.192$ ; data not shown). Previous studies of cytokines plus trastuzumab also failed to show a correlation between clinical response and NK cell antibody-dependent cellular cytotoxicity (17, 31). In contrast to a recent report that identified FcR polymorphism as a determinant of responsiveness to trastuzumab-based chemotherapy (32), there was no correlation between the Fc $\gamma$ RIIIa-158 valine/phenylalanine polymorphism and clinical response in the present study (data not shown). Optimal enhancement of cytokine production by FcR-bearing cells might require the administration of multiple cytokines or immune-stimulating agents (33).

In conclusion, we have shown that IL-12 can be given safely in combination with chemotherapy and an anti-HER2/neu mAb. Clinical responses and SD were accompanied by an immune response that was characterized by the secretion of IFN- $\gamma$  and other cytokines. Chemo-immunotherapy represents a novel approach to the treatment of HER2/neu-overexpressing tumors. Further studies of IL-12 in combination with trastuzumab and paclitaxel are indicated.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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*Mol Cancer Ther* 2009;8:2983-2991. Published OnlineFirst November 3, 2009.

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