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Evaluation of natural killer cell expansion and activation in vivo with daily subcutaneous low-dose interleukin-2 plus periodic intermediate-dose pulsing

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Abstract Natural killer (NK) cells may be expanded in vivo with a prolonged course of daily subcutaneous interleukin-2 (IL-2). However, cellular activation requires higher concentrations of IL-2 than are achieved with low-dose therapy. The objective of the current trial was to determine the toxicity and immunological effects of periodic subcutaneous intermediate-dose IL-2 pulses in patients receiving daily low-dose therapy. A group of 19 patients were treated with daily subcutaneous low-dose IL-2 at 1.25×10^6 International Units (1.25 MIU) m^{-2} day^{-1}. After 4–6 weeks, patients received escalating 3-day intermediate-dose IL-2 pulses administered as single daily subcutaneous injections, repeated at 2-week intervals. The maximum tolerated pulse dose was 15 MIU m^{-2} day^{-1}, with transient hypotension, fatigue, and nausea/vomiting dose-limiting. Subcutaneous IL-2 resulted in in vivo expansion of $CD56^+$ NK cells ($796\pm210\%$) and $CD56^{bright}$ natural killer (NK) cells ($3247\pm1382\%$). Expanded NK cells coexpressed CD16, and showed lymphokine-activated killer activity and antibody-dependent cellular cytotoxicity in vitro. Intermediate-dose pulsing resulted in serum IL-2 concentrations above 100 pM. Cellular activation was suggested by rapid margination of NK cells following pulsing, coincident with peak IL-2 levels, with return to baseline by 24 h. In addition, interferon γ production in response to lipopolysaccharide was augmented. Subcutaneous daily low-dose IL-2 with intermediate-dose pulsing is a well-tolerated outpatient regimen that results in in vivo expansion and potential activation of NK cells, with possible application in the treatment of malignancy and immunodeficiency.

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Introduction

Interleukin-2 (IL-2) in high doses has activity against a wide variety of cancers, but is associated with significant clinical toxicity [14]. Recent efforts have focused on the development of less toxic low-dose IL-2 treatment schedules that maintain the antitumor effect [4, 11].

We previously reported on the clinical and immunological effects of low-dose IL-2 administered as a daily subcutaneous injection for 90 days [11]. The schedule in this phase I study was based on several previous observations. First, a protracted venous infusion of low-dose IL-2 selectively expands natural killer (NK) cells in vivo with minimal toxicity, but with the inconvenience, cost and morbidity associated with pump apparatus and central venous catheters [4]. Second, the expanded NK population can be induced in vitro to show antitumor activity [5]. With daily subcutaneous IL-2 delivered at 1.25×10^6 International Units (1.25 MIU) m^{-2} day^{-1}, the NK cell number increased 214% with minimal toxicity [11]. In a variety of advanced malignancies, no antitumor responses were observed. However, our hypothesis was that, while NK expansion resulted from engagement of high-affinity IL-2 receptors during low-dose IL-2 therapy, cytotoxic activity required activation of intermediate-affinity receptors not achieved with the daily subcutaneous low-dose IL-2 therapy [3].

As the next step in the clinical development of an active and well-tolerated outpatient treatment regimen, we conducted a study in which in vivo expansion of NK cells was achieved with daily low-dose IL-2 administered subcutaneously, with interval higher-dose subcutaneous IL-2 pulses administered in an effort to obtain plasma concentrations that would engage intermediate-affinity IL-2 receptors. The clinical and immunological results of this phase I study are reported here.

Materials and methods

Patient eligibility

Patients more than 18 years old, with advanced malignancy for which no further standard therapies were available, were eligible. Requirements also included a performance status of 0–2 (ambulatory for more than 50% of waking hours), a life expectancy of more than 3 months, and more than 3 weeks since previous treatment with chemotherapy, radiation therapy or surgery. Baseline laboratory data included a serum total bilirubin below 3.0 mg/dl (51.3 μmol/l), serum creatinine below 2.0 mg/dl (176.8 μmol/l), an absolute neutrophil count of at least 1000/μl, hemoglobin at least 10 g/dl (6.2 mmol/l), and a platelet count above 100 000/μl. Patients were excluded if they had uncontrolled infection, were pregnant or nursing, had central nervous system metastases, or had an underlying immunological disorder (e.g. autoimmune disease). Concurrent therapy with immunosuppressive agents or immunomodulators was not permitted. Written informed consent was obtained from all patients, and this study was approved by the Institutional Review Board of Roswell Park Cancer Institute.

Treatment schema

IL-2 (aldesleukin, Proleukin, supplied by Chiron Corporation, Emeryville, Calif.; specific activity, 1.8×10^7 IU/mg protein) was administered subcutaneously. Patients received a single dose of IL-2 at 1.25 MIU/m^2 daily (low dose) except on days of intermediate-dose IL-2 pulsing. After 4–6 weeks of daily low-dose therapy (initially 6 weeks was required, but the protocol was amended after the first 7 patients to allow intermediate-dose pulsing after 4 weeks), patients received a daily pulse of intermediate-dose IL-2, administered as a single subcutaneous injection for 3 consecutive days. Intermediate-dose pulses were then repeated at 2-week intervals (14 days between the first day of each pulse sequence).

Dose escalation and determination of the maximum tolerated dose (MTD)

The primary objective of this study was to determine the maximum tolerated 3-day pulse dose of IL-2 that could be safely administered subcutaneously to outpatients who were receiving daily chronic low-dose IL-2 therapy. For each patient, the IL-2 pulse dose was escalated every 2 weeks until dose-limiting toxicity was observed. All patients were treated with 6.0 MIU m^{-2} day^{-1} IL-2 for the first 3-day pulse. Subsequent pulse doses were then increased by 3 MIU m^{-2} day^{-1} (i.e. 9.0, 12.0, 15.0 MIU/m^2, etc.). Dose-limiting toxicity was defined as Common Toxicity Criteria (CTC) grade 3 or four adverse events [6]. If a dose-limiting toxicity was experienced, treatment was discontinued, and a pulse dose halfway between the current dose and the previous dose level was instituted after resolution of toxicity. If a dose-limiting toxicity occurred with the first pulse sequence, the next pulse was given at 3.0 MIU m^{-2} day^{-1}. Dose re-escalation following a dose-limiting toxicity was not permitted, but treatment with the reduced pulse dose was continued at 2-week intervals.

Eleven patients (10 treated) were enrolled in the dose-escalation component of this study. If 2 or more patients developed dose-limiting toxicity at a specific pulse dose level, no future patients were treated at that level. The maximum tolerated 3-day pulse dose of IL-2 was defined as the tested dose level immediately below the level at which 3 consecutive days of IL-2 could not be tolerated (i.e. CTC grade 3–4 toxicity) in at least two-thirds of the patients tested. After the MTD was defined, an additional 6 patients were treated (8 enrolled) at the MTD every 2 weeks, without entering the dose-escalation schema, in an effort to determine the tolerability of repeated pulses at this dose level.

This novel phase I study design was selected to (1) allow rapid determination of the MTD, (2) minimize individual patient treatment at the lowest pulse doses, and (3) provide multiple patients treated at each pulse dose level for accurate toxicity determination.

Tumor measurements were obtained at 10-week intervals in patients with measurable disease. Standard response criteria were used [9]. Treatment was continued until progression of disease was documented, or the patient or treating physician felt that it was no longer in the patient's best interest to continue.

Clinical and laboratory assessments

History, physical examinations, complete blood counts, and serum chemistries were performed at baseline and every 2 weeks during therapy. On the days of intermediate-dose IL-2 pulsing, vital signs were obtained 0, 4, 6, and 8 h following IL-2 injection. Patients were observed for 8 h on day 1 of each pulse sequence, and for at least 4 h on days 2 and 3.

IL-2 pharmacokinetics

Serum samples were obtained for IL-2 pharmacokinetics 0, 1, 4, 6, 8, and 24 h after the first day of each IL-2 pulse sequence. Serum was collected and stored at –70 °C for batch analysis of IL-2 concentrations by enzyme-linked immunosorbent assay (ELISA; Endogen, Woburn, Mass.), according to the manufacturer's instructions.

Lymphocyte phenotyping

Peripheral blood mononuclear cells (PBMC) were collected for phenotypic analysis and cytotoxicity assays at baseline, and every 2 weeks during IL-2 therapy. At the time of intermediate-dose IL-2 pulse sequences, lymphocyte phenotyping was also performed 0, 4, 8, and 24 h after the first of three daily intermediate doses of IL-2. Red blood cells were lysed, and fresh cells were prepared for phenotypic analysis as previously described [22]. Monoclonal antibodies (mAb) were all IgG1 and included fluorescein-isothiocyanat(FITC)-labeled anti-(human CD3) (Becton Dickinson, San Jose, Calif.), peridinin-chlorophyll-protein(PerCP)-labeled anti-(human CD3) (Becton Dickinson), phycoerythrin(PE)-labeled anti-(human CD56) (Coulter, Hialeah, Fla.), and FITC-labeled anti-(human CD16) (Caltag Laboratories, Burlingham, Calif.). Non-reactive isotype control mAb were murine IgG1-FITC, IgG1-PE (Becton Dickinson), and murine IgG1-PerCP (Becton Dickinson). Prior to the addition of specific conjugated mAb, cells were incubated with non-reactive, non-conjugated mouse IgG1 (Sigma, St. Louis, Mo.) to minimize non-specific staining. Forward-scatter, side-scatter, and fluorescence data from each sample were then collected on a FACScan flow cytometer (Becton Dickinson), and 7000 events were analyzed by either Lysis II software program (Becton Dickinson) or the Winlist software program (Verity House, Topsham, Me.). NK cells were identified as CD56$^+$CD3$^-$ lymphocytes, and those showing high-density expression of CD56 (CD5^{bright}) express the high-affinity IL-2 receptor [12]. NK cells were assessed for their expression of CD16 by simultaneously staining with CD56-PE, CD3-PerCP, and CD16-FITC mAb.

Cytotoxicity

The target cell lines utilized were COLO 205 for lymphokine-activated killer (LAK) activity, and P815 (ATCC, Rockville, Md.) for antibody-dependent cellular cytotoxicity (ADCC). Both cell lines were grown in continuous log phase in RPMI-1640 medium supplemented with 10% fetal calf serum (Sigma), and antibiotics (Gibco, Gaithersburg, Md.). Cryopreserved PBMC obtained from patients prior to and during intermediate-dose pulses of IL-2 were thawed, resuspended in RPMI-1640 medium supplemented with 10% human AB serum (C-six diagnostics, Mequon, Wis.) with antibiotics, and cultured for 18 h in a volume of 150 ml plus 1 nM recombinant IL-2 (Hoffman-LaRoche, Nutley, N.J., specific activity 1.5×10^7 U/mg) in 96-well V-bottom plates. Standard 4 h ^{51}Cr-release assays were then performed at an E:T ratio of 25:1, as described [5, 13]. Briefly, after ^{51}Cr-labeled target cells have been incubated with PBMC effectors, supernatant was harvested

Table 1 Patient demographics (n = 19)

Mean age, (years) (range)	57 (38–72)
Male/female	9/10
Malignancy	
Bladder	1
Colorectal	14
Mesothelioma	2
Renal	1
Unknown primary	1
Prior therapy	
Chemotherapy	18
Radiation therapy	10

Table 2 Toxicity of daily low-dose interleukin-2 (1.25×10^6 IU m^{-2} day^{-1}), n = 19

	No. of patients, grade:				
Toxicity	0	1	2	3	4
Anorexia	16	1	2	0	0
Chills	8	10	1	0	0
Diaphoresis	12	6	1	0	0
Diarrhea	14	5	0	0	0
Fatigue	8	6	5	0	0
Fever	1	7	11	0	0
Headache	16	3	0	0	0
Myalgias/arthralgias	13	5	1	0	0
Nausea/vomiting	7	8	4	0	0
Skin local reaction	8	8	3	0	0

from each well and counted on a gamma counter to determine experimental release. Spontaneous release was determined from labeled target cells incubated in medium alone, while maximal release was obtained from labeled targets lysed in 1% NP-40. The percentage specific lysis was calculated as [(experimental release – spontaneous release)/(maximal release – spontaneous release)]×100. ADCC assays were also performed as previously described [5, 13] in a 4-h ^{51}Cr-release assay. P815 target cells were incubated for 1 h, prior to labeling with ^{51}Cr, either in the presence or in the absence of a 1:100 dilution of polyclonal rabbit anti-(mouse lymphocyte) antiserum (Accurate Chemicals, Buffalo, N.Y.). ADCC (%) was determined by subtracting background cytotoxicity against P815 targets which were not incubated with anti-(mouse Ig) antiserum, from that measured against P815 target cells pre-incubated with anti-(mouse Ig) antiserum (both calculated using the equation listed above). In one patient, cytotoxicity was assessed on fresh PBMC obtained immediately before, and 24 h following an intermediate-dose IL-2 pulse, without cyropreservation or additional in vitro incubation with IL-2.

Interferon γ (IFN) production

Cryopreserved PBMC obtained prior to, and during IL-2 therapy, were viably thawed and 2.0×10^5 PBMC were plated in a 200-µl volume of 10% human AB serum in 96-well U-bottom plates. PBMC were then either cultured with 5 µg/ml lipopolysaccharide (LPS) (Sigma) or with phosphate-buffered saline (PBS; control), and incubated at 37 °C in 5% CO_2 for 48 h [8]. Cell-free culture supernatants were then harvested and assayed by ELISA (Endogen) for IFNγ protein production. The sample at each assay time was plated, harvested, and assayed for IFNγ in duplicate.

Table 3 Dose-limiting toxicities with 3-day pulses of subcutaneous interleukin-2 (*IL-2*)

Daily IL-2 dose (MIU m^{-2} day^{-1})	Number treated	Dose-limiting toxicities (number affected
6.0	10	None
9.0	10	Grade 3 hypotension (1)
12.0	9	None
15.0	8	Grade 3 fatigue (1) Grade 3 nausea/vomiting (1)
18.0	2	Grade 3 fatigue Grade 3 nausea/vomiting (1)

Results

A total of 19 patients were enrolled in this trial. Three patients did not receive intermediate-dose IL-2 pulses as they withdrew from study during the initial 4–6 weeks of daily low-dose IL-2 therapy (2 because of progressive disease, and 1 because of patient choice). The last 6 patients were treated with intermediate pulse doses of IL-2 at the maximum tolerated dose every 2 weeks, without dose escalation. Patient demographics are shown in Table 1. All patients had solid tumor malignancies and all except 1 had been previously treated with chemotherapy.

Toxicity

The toxicity of 4–6 weeks of daily subcutaneous low-dose IL-2 before initiation of intermediate-dose pulses is shown in Table 2. All patients experienced grade 1–2 constitutional side-effects, characterized by fever, chills, and fatigue. No severe or life-threatening side-effects occurred during low-dose therapy.

After initial treatment for 4–6 weeks with daily low-dose IL-2, patients received 3-day pulses of intermediate-dose IL-2 at 2-week intervals. Ten patients received escalating pulse doses, to determine the maximum tolerated pulse dose of IL-2 in this schedule. The final 6 patients on study were treated with repetitive 3-day pulses at the MTD to determine the tolerability of prolonged therapy. Table 3 shows the dose-limiting toxicities experienced at each IL-2 pulse dose level for patients in the dose-escalation phase of this trial. Four patients experienced dose-limiting toxicity: 1 patient at 9 MIU/m^2 IL-2, 2 patients at 15 MIU/m^2 IL-2, and 1 patient at 18 MIU/m^2 IL-2. At the time when our definition of the MTD was met, 8 patients had been treated with a 3-day IL-2 pulse dose of 15 MIU/m^2, and 6 of these patients did not have dose-limiting toxicity. Of the 10 patients in the escalation phase, 1 discontinued treatment for progressive disease following the 12-MIU/m^2 pulse sequence, and did not experience dose-limiting toxicity. Therefore, 6 of 9 patients who did not have progressive disease and remained on treatment were able to tolerate a subcutaneous 3-day IL-2 pulse of 15 MIU/m^2. Of 2 patients who received pulse doses of 18 MIU/m^2, one had dose-limiting toxicity.

On the basis of the toxicity experienced by patients treated with 15 MIU/m^2 IL-2, this was chosen as the MTD

Table 4 Toxicity of 3-day pulses of intermediate-dose interleukin-2 (15.0×10^6 IU m^{-2} day^{-1}), $n = 6$

Toxicity	No. of patients, grade:				
	0	1	2	3	4
Anemia	5	0	0	1	0
Anorexia	3	2	0	1	0
Chills	0	3	3	0	0
Diaphoresis	1	3	2	0	0
Diarrhea	4	2	0	0	0
Fatigue	0	0	6	0	0
Fever	0	0	4	2	0
Hypotension	4	0	2	0	0
Nausea/vomiting	0	2	4	0	0
Skin local reaction	3	2	1	0	0

for the IL-2 pulse, and an additional 6 patients were treated with repetitive 3-day pulses at this dose level at 2-week intervals.

In general, toxicities experienced with intermediate-dose IL-2 were similar to those observed with daily low-dose IL-2. Fever and fatigue were more intense, but resolved within 24 h of each subcutaneous pulse dose. All 16 patients had at least grade 2 constitutional side-effects, including fever, chills, and fatigue. In addition, grade 2 hypotension (intravenous fluids required) occurred in 2 patients treated with 15 MIU/m^2 IL-2, and grade 3 hypotension (hospitalization) occurred in 1 patient treated with 9.0 MIU/m^2 IL-2. Hypotension was transient, and responded to intravenous crystalloid, with resolution of baseline blood pressure within 24 h. Vasopressors were not required in any patient. Capillary leak, renal failure, or hepatic failure was not observed. Table 4 lists the toxicities that occurred during intermediate-dose IL-2 pulses in the 6 patients treated with repetitive doses of 15 MIU/m^2 at 2-week intervals. There were no grade 4 toxicities. Two patients had grade 3 fever, and 1 patient had grade 3 anorexia. Each grade 3 toxicity only occurred after several courses of treatment. The mean number of 3-day pulses received by these 6 patients was 3.8 (range 2–5).

Natural killer cell expansion

Figure 1 shows the time course of in vivo NK cell expansion during treatment with IL-2. The number of NK cells had increased with low-dose IL-2 by week 2 of daily therapy, with intermediate pulse dosing resulting in an augmentation of NK expansion that was maintained throughout treatment. The mean absolute number of $CD3^-$ $CD56^+$ NK cells ($\pm$SE) at baseline was 230 ± 60/μl. The mean peak NK cell number was 1410 ± 250/μl, with an average increase in NK cells over baseline of 796 ± 210% ($P<0.0001$, paired t-test). Expansion of $CD56^{bright}$ NK cells was more pronounced, with a 3247 ± 1382% increase over baseline values ($P = 0.0002$). The average baseline $CD56^{bright}$ NK cell number was 35 ± 14/μl, which rose to 498 ± 102/μl. There was a less pronounced expansion of T cells, which rose from a baseline of 1070 ± 200/μl to a mean peak of 1460 ± 270/μl, with a mean increase of 49.2 ± 14.0% ($P = 0.03$). Figure 2 shows the NK and T cell expansion for individual patients.

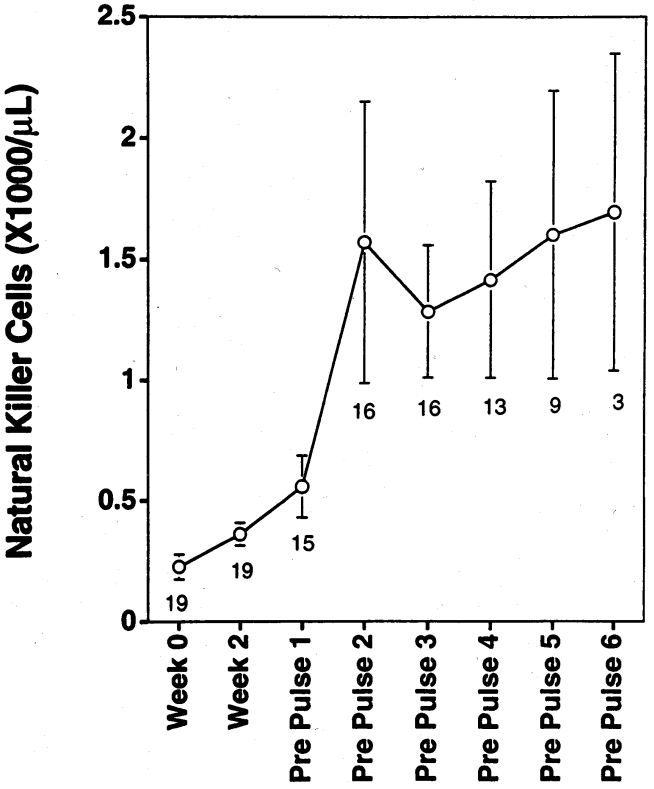

Fig. 1 Time course of natural killer cell expansion with subcutaneous daily low-dose interleukin-2 (IL-2) plus periodic intermediate-dose pulsing. The number of patients at each assay time is noted below standard error bars

In patients treated at the pulse MTD of 15 MIU/m^2, phenotyping was performed at several assay times during the 24 h following the first day of each 3-day IL-2 pulse. The NK cell number decreased 4–8 h following intermediate-dose IL-2 pulsing, but had returned to the pre-pulse baseline by 24 h (Fig. 3). These acute shifts in circulating NK cells coincide with serum IL-2 concentrations. Peak IL-2 concentrations were observed at 4–8 h, and returned to baseline by 24 h (Fig. 4). The peak concentrations ranged from 139 pM to 366 pM in individual patients. There was no apparent alteration in IL-2 pharmacokinetics over time with repeated intermediate-dose pulsing.

$CD56^+CD3^-$ NK cells were assessed over time for coexpression of CD16, the low-affinity immunoglobulin FcγIII receptor. Table 5 correlates CD16 expression with NK expansion, demonstrating that at least 75% of $CD56^+$ NK cells coexpress CD16, in both baseline and expanded populations.

Cytotoxicity

Table 5 lists phenotypic and cytotoxicity data for 5 patients treated at the IL-2 pulse MTD of 15 MIU/m^2. All effector

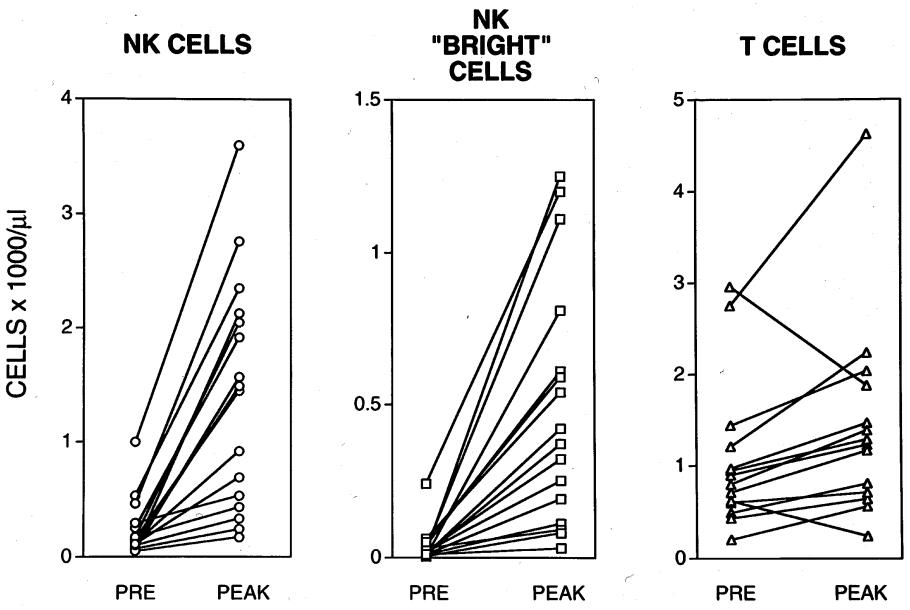

Fig. 2 Relationship between baseline and peak natural killer (*NK*) cell and T cell counts for patients treated with subcutaneous daily low-dose IL-2 plus periodic intermediate-dose pulsing

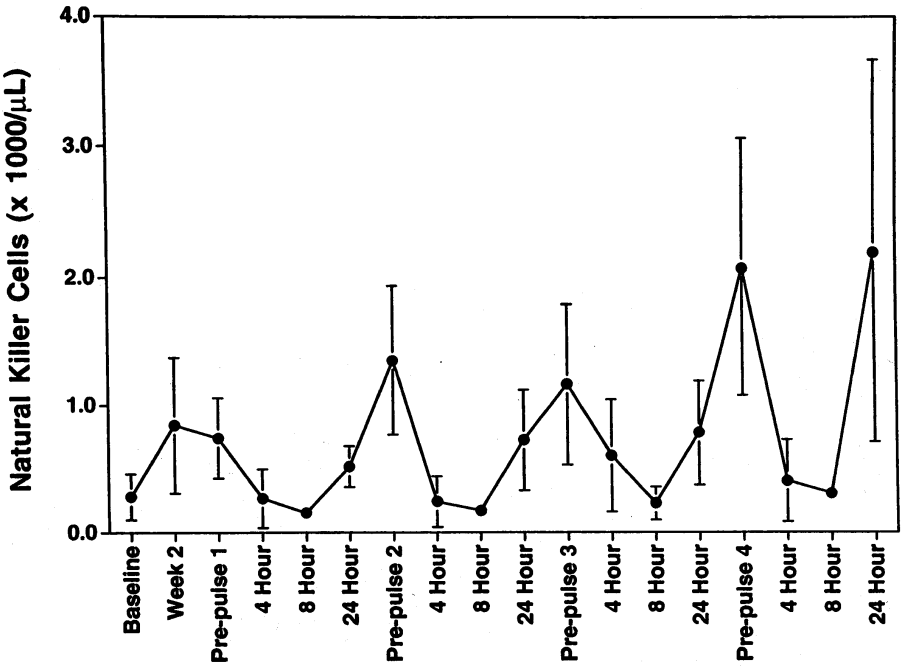

Fig. 3 Acute changes in natural killer cell number in vivo following subcutaneous intermediate-dose IL-2 pulsing with 15.0×10^6 IUm^{-2} day^{-1}. The 3-day pulse sequences were repeated at 14-day intervals. *Bars* standard error

Table 5 Natural killer cell expansion and cytotoxicity at the IL-2 pulse maximum tolerated dose (15.0×10^6 IU m^{-2} day^{-1}). Values represent the average ± SE for 5 patients. Lymphokine-activated killer cells (*LAK*) were assayed by their cytotoxicity against COLO 205. Antigen-dependent cell cytotoxicity (*ADCC*) represents cytotoxicity against P815. *ND* not done

	Level (%) in PBMC			
			After IL-2 pulse:	
Cell type	Baseline	Before IL-2 pulse	8 h	24 h
CD3$^-$CD56$^+$	16.3±3.8	37.7±3.2	10.2±1.9	47.1±4.6
CD3$^-$CD56$^+$CD16$^+$	14.1±3.2	29.5±2.2	*ND*	38.7±4.8
LAK	49.7±8.9	74.4±9.1	9.4±3.2	69.3±11.6
ADCC	49.2±5.7	63.2±2.5	14.8±2.7	57.6±9.9

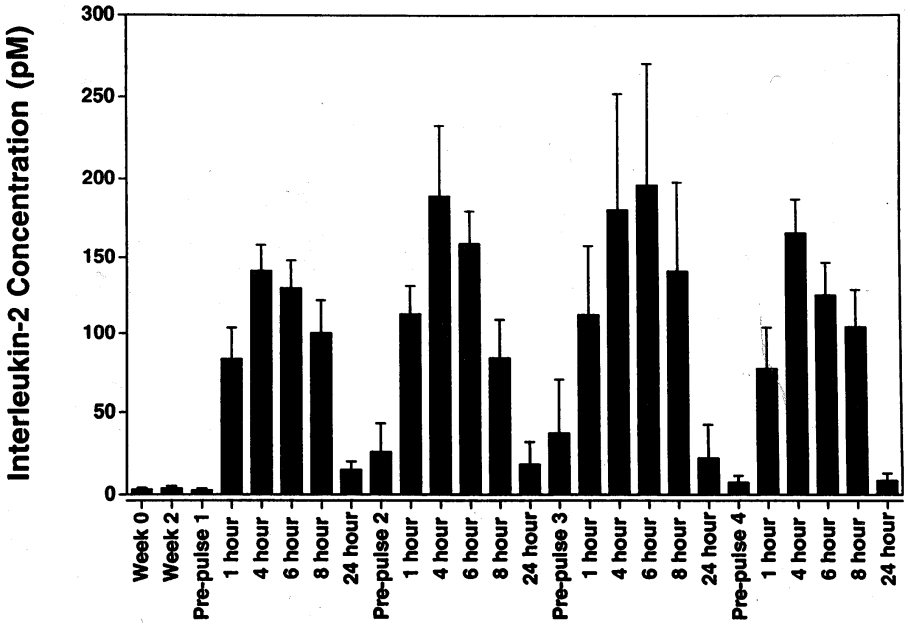

Fig. 4 IL-2 concentrations following subcutaneous pulse doses of 15.0×10^6 IU/m^2. Pharmacokinetics were obtained on the first day of each 3-day pulse sequence, with IL-2 pulsing repeated at 2-week intervals. *Bars* standard error

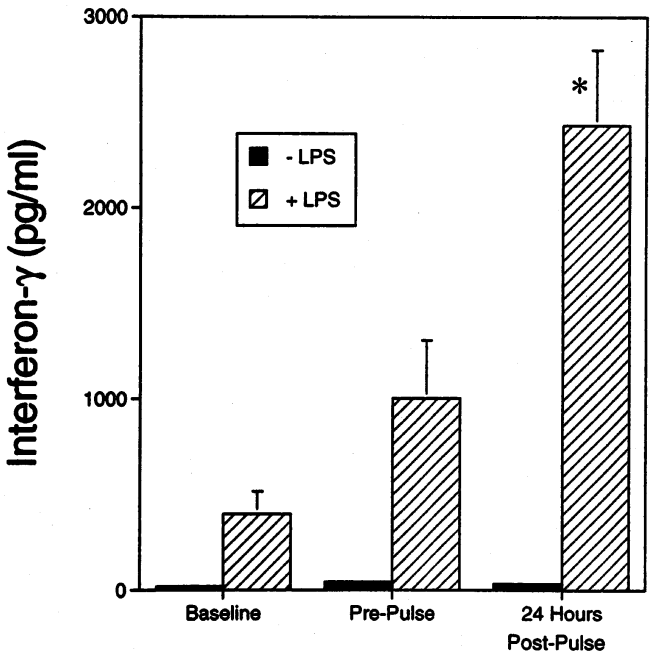

Fig. 5 In vitro assessment of lipopolysaccharide(*LPS*)-stimulated interferon γ (IFNγ) production. Peripheral blood mononuclear cells (PBMC) from various assay times prior to, and during IL-2 therapy were plated and stimulated with LPS or phosphate-buffered saline (control). PBMC were then cultured for 48 h, and cell-free supernatants were assessed for IFNγ protein by enzyme-linked immunosorbent assay. Results represent the mean ± SE of duplicate determinations for 4–5 patients at each assay time. $*P < 0.01$, comparing IFNγ production by PBMC at baseline to those obtained 24 h after an intermediate-dose IL-2 pulse

cells were incubated for 18 h in 1 nM IL-2 prior to NK cytotoxicity and ADCC assays. Each patient was treated for at least 8 weeks prior to this evaluation, accounting for the increased percentage of NK cells immediately prior to the pulse ($P = 0.01$ comparing baseline to pre-pulse values, and baseline to 24-h-post-pulse values for both CD56$^+$ NK cells and CD56$^+$CD16$^+$ NK cells; paired *t*-test). Consistent with this, there was increased NK cytotoxic activity and ADCC at this assay time. In addition, the decrease in NK cells within 8 h of pulse administration coincides with the drop in NK cytotoxic activity and ADCC. By 24 h both NK cell numbers and function had returned to baseline. It is important to note that the NK cytotoxicity and ADCC assays, because they are performed with cryopreserved PBMC which had been incubated in exogenous IL-2, do not measure the in vivo activation of NK cells by bolus IL-2.

In 1 patient treated with IL-2 pulse doses of 15 MIU/m^2, from whom serial samples were available, cytotoxicity assays were performed using fresh PBMC without additional in vitro IL-2 stimulation. Target cells were the NK-resistant cell line, COLO 205. Effectors were obtained immediately before pulse dosing, and 24 h later. The CD56$^+$ NK cell percentage was similar at both assay times (52% before and 55% after the pulse). In this patient, cytotoxicity was increased from a pre-pulse baseline of $17.1 \pm 1.8\%$ to $57.5 \pm 0.37\%$ 24 h after an IL-2 pulse of 15 MIU/m^2, suggesting the in vivo generation of LAK activity with subcutaneous IL-2.

IFNγ

NK cells are responsible for early (i.e. 48 h) production of IFNγ by PBMC stimulated with bacterial extracts such as LPS, a component of the gram-negative bacterial cell wall. This LPS-induced monocyte activation in turn produces monokines required for NK cell IFNγ production [1]. Hence, early IFNγ production by LPS-stimulated PBMC is a measure of NK cell function [8, 23] Here, PBMC obtained from 5 patients treated at the pulse MTD IL-2 dose of 15 MIU/m^2 were assessed in a similar in vitro co-culture

assay, comparing levels of IFNγ protein production before therapy to those during therapy. Equal numbers of cryopreserved PBMC, taken as a baseline sample (week 0), before the intermediate-dose IL-2 pulse (weeks 8–10), and 24 h after the pulse, were cultured with either LPS or PBS (control) for 48 h, and cell-free culture supernatants were then assessed for IFNγ protein production by ELISA. While unstimulated PBMC produced little or no IFNγ, PBMC stimulated with LPS produced measurable amounts of IFNγ protein at all assay times (Fig. 5). A significant increase in IFNγ production was observed 24 h after the intermediate-dose pulse, compared to pre-therapy levels ($P < 0.01$).

Discussion

This study was undertaken as a second step in the development of a well-tolerated outpatient IL-2 regimen for patients with cancer or immunodeficiency. We previously demonstrated that IL-2 could be administered subcutaneously at a daily dose of 1.25 MIU/m^2 for 90 days, and result in a three-fold increase in CD56$^+$CD3$^-$ NK cells in vivo with minimal toxicity [11]. A more dramatic increase in CD56^{bright} cells was observed, consistent with the hypothesis that NK expansion resulted from binding of high-affinity IL-2 receptors (IL-2Rαβγ), which could be achieved with the in vivo IL-2 concentrations (below 100 pM) obtained with our "low-dose" subcutaneous regimen [3]. However, activation of the intermediate-affinity heterodimeric IL-2Rβγ complex (K_d = 1 nM), constitutively expressed by almost all NK cells, appears to be required for significant cytotoxic activity [3, 17]. The current study was performed in order to achieve IL-2 concentrations in vivo that could engage a significant proportion of IL-2Rβγ on an expanded NK cell population.

In patients receiving daily low-dose IL-2 at 1.25 MIU/m^2, the maximum tolerated 3-day subcutaneous "pulse" dose was 15 MIU m^{-2} day^{-1} repeated at 2-week intervals. The dose-limiting toxicities were fatigue, nausea/vomiting, and hypotension. At the MTD, grade 3–4 hypotension did not occur. Transient constitutional symptoms and nausea/vomiting were universal. These symptoms resolved within 24 h and coincided with peak IL-2 levels. Outpatient "intermediate-dose" pulsing resulted in more rapid and more pronounced NK cell expansion than we observed with low-dose therapy alone [11]. The average peak NK levels (1400/μl) obtained with this subcutaneous regimen were comparable to those reported with an intravenous "low-dose plus pulse" schedule [20]. T cells also increased during therapy (approximately 50%), but to a lesser degree than NK cells (approximately 800%). In the current study, peak IL-2 concentrations above 100pM were routinely obtained, which would be expected to saturate high-affinity IL-2 receptors, and engage a significant proportion of intermediate-affinity receptors [17]. Although nanomolar concentrations of IL-2 are required to saturate intermediate-affinity receptors, prolonged exposure to levels that we achieved with 3 days of subcutaneous pulsing may be sufficient for biological activity.

NK cells rapidly disappeared from the circulating compartment following subcutaneous IL-2 pulses, and returned within 24 h. This confirms an earlier report by Soiffer et al. [19] in which an intravenous bolus of IL-2 had a similar effect. It is likely that upregulation of adhesion molecules stimulated by intermediate-dose IL-2 accounts for margination [13]. Whether this manifestation of cellular activation will prove valuable or problematic in the treatment of patients remains uncertain, as it may aid or inhibit delivery of effector cells to tissue sites of disease. NK cell activation was also associated with a transient increase in clinical toxicity. We hypothesize that proinflammatory cytokines produced by NK cells in response to IL-2 might mediate this toxicity.

The cytotoxicity studies we performed during intermediate-dose IL-2 pulsing confirmed that the expanded NK population is functional. This is consistent with our previous reports showing that effectors expanded in vivo with low-dose IL-2 can kill tumor targets in vitro after further in vitro activation with higher concentrations of IL-2 [5, 11]. LAK activity and ADCC were obtained, with more than 75% of expanded NK cells expressing FcγRIII. As an alternative method for measuring NK cell function, we measured the ability of the expanded NK population to produce IFNγ in response to LPS. Intermediate-dose IL-2 pulsing augmented IFNγ production (Fig. 5). This could have resulted from the significant increase in NK cell number and/or a qualitative increase in the amount of IFNγ produced per NK cell. This is consistent with earlier in vitro work demonstrating the ability of IL-2 to augment human NK cell IFNγ production [8]. Although the IFNγ production was not statistically significantly different when PBMC were compared before and 24 h after pulsing, a trend was apparent, and perhaps a larger sample size would have more clearly demonstrated the impact of intermediate-dose IL-2 on NK activation.

In 1 patient in the current trial, we compared the cytotoxicity of fresh PBMC obtained immediately before, to those obtained 24 h following a pulse dose of 15 MIU/m^2 IL-2, without additional in vitro IL-2 exposure. Cytotoxicity increased approximately threefold when an NK-resistant target cell line was used, suggesting that (1) in vivo IL-2 pulses can enhance NK cytotoxic activity, (2) LAK activity is generated, and (3), consistent with the IFNγ assays described above, the effect of subcutaneous IL-2 persists for at least 24 h. While interesting, these data need to be confirmed in a larger population.

Our immunological monitoring took place on the first day of each 3-day intermediate-dose IL-2 pulse sequence. This permitted NK cell sampling before intermediate-dose IL-2 pulsing. It is possible that augmented immunomodulation would have been observed if the assays had been performed following several days of intermediate-dose pulsing. However, as shown in Fig. 3, 24 h after the first day of each pulse sequence, the NK cell number was lower than pre-pulse levels. Therefore, margination of activated NK cells may preclude immunological assessment once pulsing has begun.

This study shows that NK cells may be safely expanded and possibly activated in vivo with a subcutaneous IL-2 outpatient regimen. Recent clinical trials with low-dose IL-2 in renal cell carcinoma [16, 21], melanoma [20], leukemia [15, 18], and acquired immunodeficiency syndrome [2], suggest the potential value of IL-2 immunotherapy in malignant and infectious diseases. Current understanding of the IL-2 receptor complex and the functional consequences of IL-2-receptor/ligand interactions will hopefully lead to effective and well-tolerated treatment approaches. Soiffer et al. recently reported results of a study in which low-dose IL-2 was administered by continuous infusion with weekly higher-dose intravenous bolus pulsing, and NK cell expansion and activation were observed [19]. Weekly bolus doses in the range 0.25–1.0 MIU/m^2 were administered, with constitutional toxicities observed, as in our trial. Hypotension requiring vasopressors was noted at the highest dose level. In addition, 6 of 23 patients had documented bacterial infections. It is unknown whether the level of immunomodulation achieved with our subcutaneous regimen is identical to that of the intravenous program; however, the subcutaneous approach has potential advantages in terms of patient convenience and perhaps decreased risks of infection and hypotension.

It is notable that the expanded $CD56^+$ NK cells in our current study and others [5, 10, 20] coexpress CD16, the FcγIII receptor. Such cells have been shown to participate in ADCC with monoclonal antibodies that recognize tumor-associated cell-surface antigens [7]. Early clinical trials combining IL-2 with murine monoclonal antibodies have been encouraging [20, 24]. As a next step in the development of our program, the Cancer and Leukemia Group B is undertaking a study combining daily subcutaneously administered low-dose IL-2, intermediate-dose pulses, and a humanized anti-HER2 monoclonal antibody in patients with cancers that overexpress HER2.

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