Repeated Administrations of Interleukin (IL)-12 Are Associated with Persistently Elevated Plasma Levels of IL-10 and Declining IFN-γ, Tumor Necrosis Factor-α, IL-6, and IL-8 Responses

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ABSTRACT

Purpose: Repeated administrations of recombinant human interleukin-12 (rHuIL-12) to cancer patients are characterized by a reduction of side effects during treatment. Induction of IFN-γ, considered a key mediator of antitumor effects of IL-12, is known to decline on repeated administrations. We studied whether other immunological effects of rHuIL-12 are tapered in the course of treatment.

Experimental design: In a Phase I study of 26 patients with advanced renal cell cancer, rHuIL-12 was administered s.c. on day 1, followed by 7 days rest and six injections administered over a 2-week time period. Plasma concentrations of various cytokines were monitored, as well as absolute counts of circulating leukocyte and lymphocyte subsets.

Results: The first injection of IL-12 was accompanied by rapid, transient, and dose-dependent increments of plasma levels IFN-γ, tumor necrosis factor-α, IL-10, IL-6, IL-8, but not IL-4, as well as rapid, transient, and dose-dependent reductions of lymphocyte, monocyte, and neutrophil counts. The major lymphocyte subsets, *i.e.,* CD4+ and CD8+ T cells, B cells, and natural killer cells, followed this pattern. On repeated rHuIL-12 injections, IL-10 concentrations increased further, whereas the transient increments of IFN-γ, tumor necrosis factor-α, IL-6, and IL-8 concentrations, as well as the fluctuations of the leukocyte subset counts, were tapered. Dose escalation of IL-12 within clinically tolerable margins did not reduce the decline of these immunological effects.

Conclusions: Induction of pro-inflammatory cytokines and associated fluctuations in leukocyte subset counts decrease on repeated administrations of rHuIL-12. The steady increment of IL-10 plasma levels may mediate the observed down-regulation of clinical and immunological effects.

INTRODUCTION

IL-12[2] is a cytokine with an important function in the regulation of the cell-mediated immune response. In animal and *in vitro* models, IL-12 stimulates CD4+ Th-1 responses, promotes the proliferation and activation of NK cells, and stimulates them to produce IFN-γ (1). IL-12 also stimulates antigen-specific CD8+ T-cell responses (2, 3). Additionally, IL-12 has antiangiogenic properties (4) and a direct growth inhibitory effect on tumor cells (5). The promising antitumor effects that were observed in murine and nonhuman primate models (6) prompted clinical studies in patients with cancer. Efficacy studies of rHuIL-12 have now been performed in patients with ovarian and renal cell cancer, but antitumor responses have been disappointing (7, 8). During Phase I testing of rHuIL-12, we *et al.* (9–12) have observed a decrease of side effects on repeated administrations in conjunction with a reduction of IFN-γ release. Because IFN-γ is considered to be a key mediator of antitumor effects of IL-12 (13), the down-regulation of IFN-γ release observed on repeated IL-12 administrations may be related to the lack of antitumor effects of IL-12.

Here we assessed whether or not the down-regulation of IFN-γ production that occurs on repeated rHuIL-12 administrations: (*a*) comprised other immunological effects; (*b*) was accompanied by up-regulation of an inhibitory immune regulatory mechanism or cytokine; and (*c*) could be prevented by dose escalation of rHuIL-12. To this end, we studied the *in vivo* effects of rHuIL-12 in 26 patients with advanced renal cell cancer, treated in a Phase I study (9). We studied: (*a*) plasma levels of cytokines IFN-γ, TNF-α, IL-10, IL-8, IL-6, and IL-4; (*b*) absolute numbers of circulating neutrophils, lymphocytes, and monocytes; and (*c*) the major lymphocyte subsets CD4+ and CD8+ T, B, and NK cells. We compared the effects of the first rHuIL-12 dose with effects of subsequent repeated administrations at the same or escalated doses.

PATIENTS AND METHODS

Study Design. We studied 26 patients with locally advanced or metastatic renal cell cancer who participated in an open-label, nonrandomized Phase I dose escalation trial. This trial was carried out in the Rotterdam and Mainz cancer centers to evaluate the safety and tolerability of an initial single s.c. injection of rHuIL-12, followed by repeated injections. Patients had a median age of 56 years (range 41–70 years). They had not received more than one previous immunotherapeutic intervention, and all former therapies were terminated ≥6 weeks before the start of treatment with rHuIL-12. Inclusion criteria were a WHO performance score of 0 or 1 and adequate hematological, renal, hepatic, cardiovascular, and pulmonary functions. None of the patients received systemic corticosteroid therapy. All patients had given written informed consent.

rHuIL-12 (Ro 24–7472) was supplied by Hoffmann-La Roche (Nutley, NJ) and administered by s.c. injections. On day 1, a single injection of rHuIL-12 was given, followed by an observation period of 7 days. Subsequently, on day 8, a 2-week cycle was started, with three injections per week. Immunomodulatory effects of the first administration of rHuIL-12 were studied in 26 patients after a dose of 0.1 μg/kg ($n = 3$), 0.5 μg/kg ($n = 19$), or 1 μg/kg ($n = 4$). The immunological effects of repeated injections were studied in 18 patients who received all seven injections each. Twelve of these patients received the same dose of rHuIL-12 for the initial as well as the repeated injections: 0.1 μg/kg ($n = 3$), 0.5 μg/kg ($n = 7$), or 1 μg/kg ($n = 2$). The remaining 6 patients started with an initial dose of 0.5 μg/kg rHuIL-12, and repeated injections were administered at a dose of 1 μg/kg ($n = 4$) or 1.25 μg/kg ($n = 2$).

Cytokines. EDTA-anticoagulated venous blood samples for measurement of cytokines were obtained directly before and 4, 8, 12, 24, 48, and 72 h after the first and seventh administration of rHuIL-12. In 12 patients, blood was also obtained 96 and 168 h after the first injection. Plasma was obtained after centrifugation of blood for 10 min at $1300 \times g$. Plasma samples were stored at −70°C until tested. Serum concentrations of bio-active IL-12 were measured by a method of antibody capture followed by a cell proliferation assay with a lower limit of detection of 50 pg/ml (14). IFN-γ, TNF-α, IL-10, IL-8, and IL-6 concentrations were determined with commercially available enzyme amplified sensitivity immunoassays (Medgenix EASIA; Biosource Europe, Fleurus, Belgium). The lower limits of detection of the assays were IFN-γ, 0.03 IU/ml; TNF-α, 3 pg/ml; IL-10, 1 pg/ml; IL-8, 1 pg/ml; IL-6, 2 pg/ml; and IL-4, 0.2 pg/ml.

Determination of Absolute Numbers of Peripheral Blood Leukocyte Subsets. Blood samples for determination of absolute numbers of peripheral blood leukocyte subsets were obtained from 22 patients after an initial dose of 0.1 μg/kg ($n = 3$), 0.5 μg/kg ($n = 15$), or 1 μg/kg ($n = 4$). Samples were obtained directly before and 1, 2, 3, 4, and 7 days after the first administration of rHuIL-12, before every subsequent administration, and 7 days after the last administration. Leukocyte concentrations and differential counts were determined in EDTA-anticoagulated blood samples using a Technicon H1 automated cell counter (Technicon, Tarrytown, NY).

Lymphocyte Immunophenotyping. Immunophenotyping was performed on blood specimens from 9 patients. All patients studied received an initial injection of 0.5 μg/kg rHuIL-12. The repeated injections were dosed at 0.5 μg/kg ($n = 4$), 1 μg/kg ($n = 3$), or 1.25 μg/kg ($n = 2$). Heparinized venous blood samples for immunophenotyping were obtained directly before (day 0) and 1 day after the first administration (day 1) and directly before (day 19) and 1 week after (day 26) the last administration of rHuIL-12. For immunostaining, the erythrocytes were lysed by ammonium chloride. The remaining leukocytes were washed and stained using the following mixtures of mAb conjugated either with FITC or PE: (*a*) CD45 FITC + CD14 PE; (*b*) CD3 FITC + CD16 PE + CD56 PE; (*c*) CD3 FITC + CD4 PE; (*d*) CD3 FITC + CD8 PE; and (*e*) CD19 PE. Isotype control mAb (mouse IgG2a FITC, mouse IgG1 FITC, and mouse IgG1 PE) were used to visualize nonspecific antibody binding. The CD8 PE mAb was obtained from DAKO (Glostrup, Denmark). All other mAb were purchased from BD Biosciences (San Jose, CA). Sample processing and flow cytometry were performed as described elsewhere (15).

Statistical Analyses. To test differences for any parameter between paired samples, for statistical significance, two-tailed paired Student's *t* tests were performed. To test differences for any parameter between nonpaired samples, two-tailed nonpaired Student's *t* tests were performed. *P*s < 0.05 were considered statistically significant.

RESULTS

Cytokines. The first administration of rHuIL-12 was followed by increments in plasma concentrations of IFN-γ, TNF-α, IL-6, IL-8, and IL-10, whereas virtually no elevation of IL-4 concentrations was observed (Fig. 1). Table 1 shows the baseline and peak levels of cytokines as measured in individual patients during 7 days after the first rHuIL-12 injection, stratified by rHuIL-12 dose level. The pharmacokinetics of rHuIL-12 in this study have been reported previously (9). Samples from 25 patients were available for IL-12 assessments. At the lowest dose, *i.e.,* 0.1 μg/kg rHuIL-12, plasma concentrations of IL-12 remained undetectable in all 3 patients. After 0.5 μg/kg, IL-12 became detectable in 10 of 18 patients, and after 1 μg/kg, IL-12 became detectable in all 4 patients.

The initial induction of IFN-γ, TNF-α, IL-6, IL-8, and IL-10 occurred in a rHuIL-12 dose-dependent way. After administration of the lowest dose, *i.e.,* 0.1 μg/kg, only a significant increment of TNF-α plasma levels was observed. On administration of 0.5 μg/kg, peak levels of IFN-γ, TNF-α, IL-6, IL-8, and IL-10 were significantly higher than the corresponding levels before therapy. At the highest rHuIL-12 dose level, *i.e.,* 1 μg/kg, peak levels of IFN-γ, TNF-α, IL-8, and IL-10 were even higher than those observed after administration of 0.5 μg/kg. Of note, no increments of IL-4 plasma levels were observed at the 0.1 and 0.5 μg/kg dose levels, whereas these became just detectable within 24 h after administration of 1 μg/kg rHuIL-12.

The first rHuIL-12 injection resulted in increments of plasma concentrations of IFN-γ and TNF-α in all patients, whereas increments of IL-10 were demonstrated at the 0.1 μg/kg dose level in 1 of 3 patients (33%), at the 0.5 μg/kg dose level in 11 of 19 patients (58%), and at the 1 μg/kg dose level
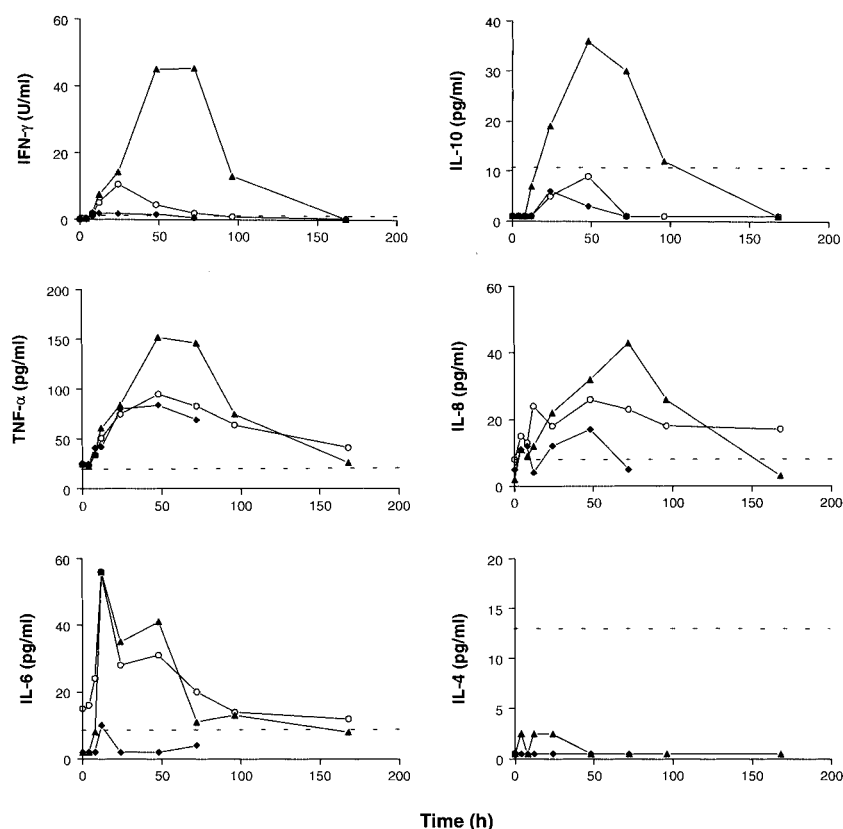
Fig. 1   Plasma concentrations of IFN-γ, IL-10, TNF-α, IL-8, IL-6, IL-4, and IL-12 after the first s.c. injection of IL-12. Median plasma concentrations of IFN-γ, IL-10, TNF-α, IL-8, IL-6, and IL-4 were assessed immediately before and during 7 days after the first s.c. injection of 0.1 μg/kg rHuIL-12 (◆, 3 patients), 0.5 μg/kg (○, 19 patients), or 1 μg/kg (▲, 4 patients). *Dotted lines,* top limit of the normal range.

Table 1   Baseline and peak cytokine concentrations after the first s.c. administration of rHuIL-12

	0.1 μg/kg ($n = 3$)				0.5 μg/kg ($n = 19$)				1 μg/kg ($n = 4$)			
	Baseline		Peak		Baseline		Peak		Baseline		Peak	
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
IL-12 (pg/ml)	<50		<50		<50[a]		298[g]	116–830	<50		548	475–2630
IFN-γ (IU/ml)	<0.03	<0.03–0.4	2.6[h]	1.4–2.9	<0.03	<0.03–1.4	13[g]	1.5–41	0.4	<0.03–0.5	55	12–184
IL-10 (pg/ml)	<1[b]		15		<1[c]	<1–6	22[g]	4–49	<1		37[h]	27–81
TNF-α (pg/ml)	26	21–40	84[h]	73–108	25	8–83	95[g]	60–143	25	7–33	168[h]	149–223
IL-8 (pg/ml)	5	<1–15	24	11–53	11[d]	<1–35	38[h]	14–223	<1	<1–8	53[h]	32–87
IL-6 (pg/ml)	6[e]	<2–10	25	22–27	16[f]	<2–79	82[g]	17–280	<2		83[h]	42–137

As described in "Results," induction of the various cytokines did not occur in all patients. Only data from patients with increments of cytokine concentrations post-IL-12 in comparison with baseline are shown. Symbols denote numbers of patients in which increments of cytokine concentrations have been observed.

[a] $n = 10$.
[b] $n = 1$.
[c] $n = 13$.
[d] $n = 14$.
[e] $n = 2$.
[f] $n = 16$.
[g] $P < 0.001$; paired comparison between peak and baseline levels.
[h] $P < 0.05$.

in all 4 patients (100%). The first rHuIL-12 injection resulted in increments of IL-8 in all but 5 patients, 3 of whom already had elevated baseline levels of IL-8 (between 26 and 58 pg/ml). Increments of IL-6 levels were observed in all but 4 patients, 1

of whom already had a strongly elevated baseline level of 91 pg/ml. IL-6 plasma concentrations were the first to peak at a median of 12 h postinjection (range 8–72 h in individual patients), followed at 24 h by IFN-γ (range 12–72 h) and at 48 h
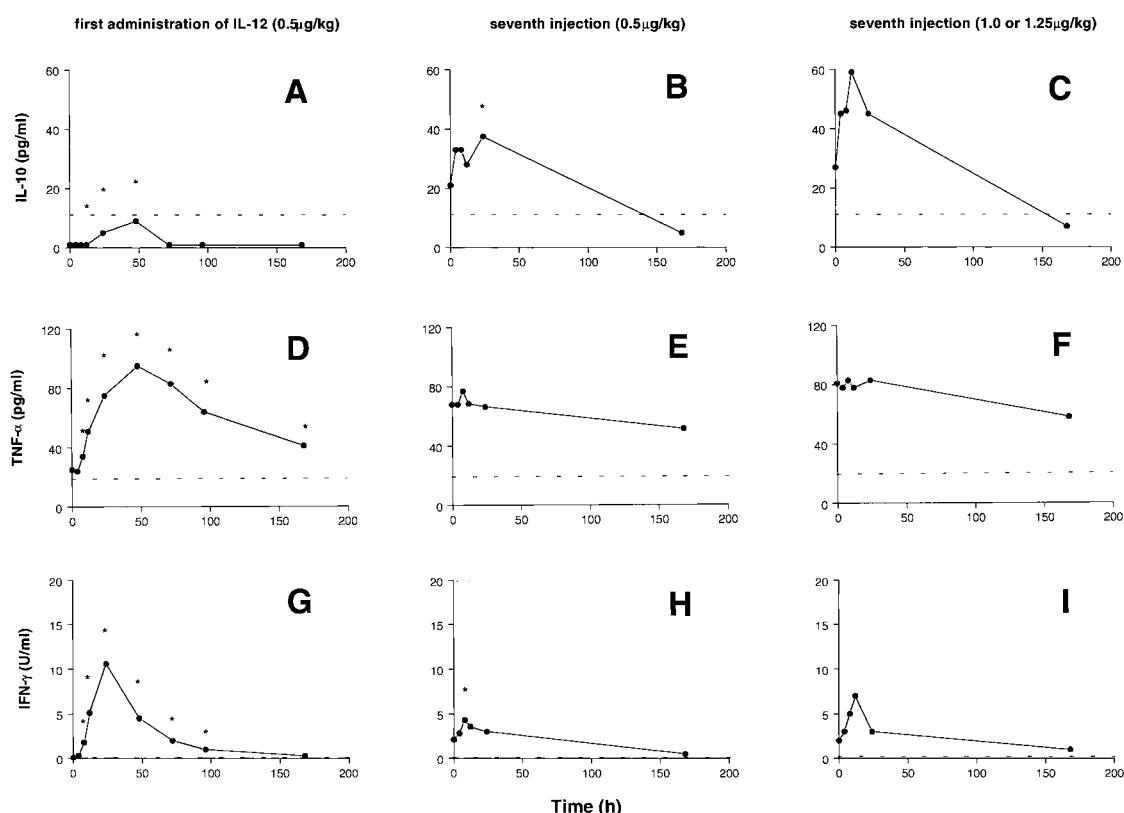
Fig. 2 Cytokine concentrations after the first injection of IL-12 (*A*), compared with concentrations after the seventh injection at the same (*B*) or escalated dose of IL-12 (*C*). Median plasma concentrations of IL-10 (*top panels*), TNF-α (*middle panels*), and IFN-γ (*bottom panels*) were assessed immediately before and during 7 days after the first and seventh injections of rHuIL-12. The first injection of 0.5 μg/kg was given to 13 patients (*A, D,* and *G*). Seven of these patients received subsequent injections at the same dose level (*B, E,* and *H*), and the remaining 6 patients received subsequent injections at the 1 or 1.25 μg/kg dose levels (*C, F,* and *I*). Significant differences (*P* < 0.05) between cytokine concentrations before and after each IL-12 injection are indicated by *asterisks. Dotted lines,* top limit of the normal range.

by TNF-α (range 24–72 h), IL-10 (range 8–72 h), and IL-8 (range 8–168 h; Fig. 1). Thereafter, levels of TNF-α, IFN-γ, IL-10, IL-8, and IL-6 gradually declined. IFN-γ and IL-10 became undetectable at 7 days after rHuIL-12 injection, whereas TNF-α, IL-6, and IL-8 remained detectable around the upper limits of their normal ranges.

Comparison of the effects of the first and seventh injection of rHuIL-12 on cytokine plasma levels revealed that IL-10 still showed a significant increment after seven injections at the same dose level (*i.e.,* 0.5 μg/kg; Fig. 2, *A* and *B*). In contrast, IFN-γ showed only a marginally significant increment after the seventh rHuIL-12 injection (Panels *G* and *H*), whereas TNF-α (Panels *D* and *E*), IL-8, and IL-6 (data not shown) showed hardly any increments as compared with the corresponding increments after the first injection of the same dose of rHuIL-12. The reduced increments of plasma levels of these cytokines were not only observed at the 0.5 μg/kg dose level but also at the 0.1 and 1 μg/kg dose levels (data not shown).

The effect of IL-12 dose escalation was further studied in 6 patients who received, after the initial injection of 0.5 μg/kg rHuIL-12, subsequent injections at doses of 1 or 1.25 μg/kg rHuIL-12. These dose escalations resulted in an even stronger increment of IL-10 plasma levels as compared with the 0.5

μg/kg dose level (Fig. 2, *A–C*). However, increments of IFN-γ (Panels *G–I*), TNF-α (Panels *D–F*), IL-6, and IL-8 plasma levels (data not shown) remained clearly less after the seventh than after the first rHuIL-12 injection.

Leukocyte Subsets. The administration of rHuIL-12 was followed by a rapid and transient decrease of absolute numbers of lymphocytes, monocytes, and neutrophils in the peripheral blood. Table 2 shows the baseline values and nadirs of these cell counts after the first injection of rHuIL-12. The reduction of lymphocytes, monocytes, and neutrophils occurred in a rHuIL-12 dose-dependent way. The lymphocytes reached their nadir at 1–2 days after rHuIL-12 administration, followed by the monocytes at 2–3 days, and the neutrophils at 3–4 days. At the 0.1 μg/kg dose level, all leukocyte subsets had returned to their baseline levels within 7 days. However, at the 0.5 and 1 μg/kg dose levels, neutrophils were still below baseline in 18 of 19 patients at 7 days postinjection, whereas monocytes and lymphocytes were still below baseline levels in 10 of 19 patients at that time (data not shown).

Repeated injections of rHuIL-12 had only minor effects on the numbers of circulating leukocyte subsets as compared with the first injection. The subsequent injections at the 0.1 μg/kg dose level had no significant effects. At the 0.5 and 1 μg/kg dose levels, neutrophil counts were still below baseline levels,

Table 2 Leucocyte subsets after the first s.c. injection of rHuIL-12

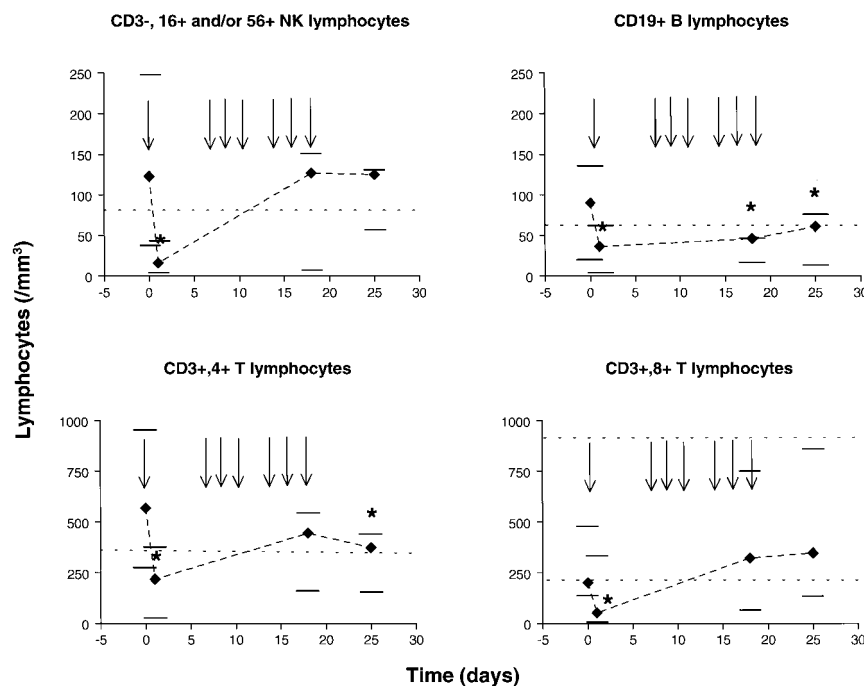| | 0.1 μg/kg ($n = 3$) | | | | 0.5 μg/kg ($n = 15$) | | | | 1 μg/kg ($n = 4$) | | | |
| | Baseline cell count[a] | | Nadir[a] | | Baseline cell count[a] | | Nadir[a] | | Baseline cell count[a] | | Nadir[a] | |
	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range
Lymphocytes	1.8	1.5–1.9	0.8[b]	0.5–0.8	1.3	0.5–2.9	0.5[b]	0.1–1.1	1.4	1.0–2.3	0.3[c]	0.2–0.5
Neutrophils	4.0	3.5–4.1	0.9		4.2	2.7–6.8	1.5[b]	0.7–4.7	3.7	2.9–5.3	1.4[c]	1.1–1.7
Monocytes	0.53	0.46–0.66	0.21	0.17–0.38	0.30	0.17–0.58	0.15[b]	0.03–0.43	0.35	0.29–0.5	0.11[c]	0.02–0.16

[a] Absolute numbers are expressed as (number of cells $\times 10^9$/liter).

[b] $P < 0.001$; paired comparisons between nadirs and baseline counts.

[c] $P < 0.05$.

Fig. 3 Lymphocyte subsets after repeated injections of rHuIL-12. Absolute numbers of NK cells (CD3−, 16+, and/or 56+), B cells (CD19+), T-helper cells (CD3+ and 4+), and cytotoxic T cells (CD3+ and 8+) were assessed immediately before and during 26 days after the first rHuIL-12 injection of 0.5 μg/kg in 9 patients. Subsequent injections were at the 0.5 μg/kg ($n = 4$), 1 μg/kg ($n = 3$), or 1.25 μg/kg ($n = 2$) dose levels. *Arrows,* rHuIL-12 injections. For each time point, the median result of the 9 patients is depicted as a *diamond,* and the interquartile range is depicted with *horizontal dashes. Dotted lines,* bottom and (for CD3+ and 8+ T cells only) top limits of the normal range as assessed in 61 healthy controls. Significant differences ($P < 0.05$) between baseline lymphocyte subset counts and the respective counts during or after rHuIL-12 treatment are indicated by *asterisks.*

yet within normal ranges at 48 h after the sixth rHuIL-12 injection. However, at the highest dose levels, *i.e.,* 1 and 1.25 μg/kg, a marked lymphopenia persisted during the entire period of follow-up (data not shown).

Lymphocyte Subsets. Fig. 3 shows the absolute numbers of NK lymphocytes (CD3−, 16+, and/or 56+), B lymphocytes (CD19+), T-helper cells (CD3+ and 4+), and cytotoxic T cells (CD3+ and 8+) before and after treatment with rHuIL-12. Before treatment, all lymphocyte subsets were in the normal range. The first administration of 0.5 μg/kg rHuIL-12 induced significant reductions of all lymphocyte subset counts to levels below their normal ranges. At 24 h after rHuIL-12 injection, NK cells had decreased from a median of 123 (range 38–314) to 16 (3–42) cells/mm³, B cells from a median of 90 (range 21–240) to 36 (3–178) cells/mm³, T-helper cells from a median of 569 (range 66–1262) to 219 (22–637) cells/mm³, and cytotoxic T cells from a median of 193 (range 240–854) to 59 (8–261) cells/mm³. Immediately before and 7 days after the last injection of rHuIL-12, NK and cytotoxic T cells had returned to

their baseline levels, whereas T-helper cells and, in particular, B-cell counts were still below these levels.

DISCUSSION

Clinical side effects and IFN-γ induction decrease in the course of repeated IL-12 administrations to humans (9, 11, 16–20). Here, we show that other immunological effects of IL-12 are also down-regulated in the course of systemic IL-12 treatment, such as declining TNF-α, IL-8, and IL-6 responses and diminishing effects on leukocyte subsets, and that maintenance of detectable concentrations of IFN-γ, as well as TNF-α, IL-8 and IL-6, cannot be achieved by dose escalation of IL-12. A previous Phase I study has shown an association between antitumor response and the maintenance of IFN-γ concentrations after repeated injections (20). These combined results indicate that a generalized down-regulation of immunological effects, possibly including antitumor effects, occurs on repeated administrations of IL-12.

We showed that the plasma levels of the inhibitory cytokine IL-10 remained elevated or further increased on repeated IL-12 injections. This observation has also been made in other clinical trials (17, 19, 21). An important role has been proposed for IL-10 in the down-regulation of IFN-γ production, as anti-IL-10 antibodies neutralized the down-regulation of side effects on repeated IL-12 administrations to mice, and IL-10 inhibited IL-12-mediated production of IFN-γ by human lymphoid cells (12, 17, 19, 22, 23). On the basis of our results, we suggest that IL-10 down-regulates the IL-12-mediated production of other cytokines as well. Indeed, *in vitro* studies have shown that IL-10 inhibits TNF-α production by lymphocytes (22), IL-6, and IL-8 production by monocytes and macrophages (24, 25) and IL-8 production by neutrophils (26). In various pathological states, endogenously produced IL-10 has an important function in the abrogation of ongoing inflammatory responses by inhibiting the effects mediated by endogenously produced IL-12 (27–30). Hence, IL-12-induced IL-10 production appears to be a protective feedback mechanism. The induction of IL-10 seems to be independent of IFN-γ, because neutralizing anti-IFN-γ antibodies had no effect on IL-12-induced IL-10 synthesis *in vitro* (31). On the other hand, TNF-α possibly plays a role in the increased IL-10 production after IL-12 administration, because it induced high levels of IL-10 mRNA expression and release of IL-10 by human peripheral blood monocytes (32). Moreover, TNF-α was shown to inhibit IFN-γ-mediated effects on human macrophages, and the inhibition of these effects by anti-IL-10 antibodies confirmed the intermediate role of IL-10 (25).

IL-18 is a pleiotropic cytokine that initially was discovered as an IFN-γ-inducing factor derived from liver cells (33). IL-18 synergizes with IL-12 to stimulate IFN-γ production by T cells (34). In a Phase I study of IL-12 to cancer patients, induction of IL-18 and IFN-γ were not correlated; on repeated IL-12 administrations, IL-18 induction was sustained, whereas IFN-γ induction was down-regulated (20). In addition, IL-18 plasma levels peaked later than those of IFN-γ (20). Extrapolation of these observations to our results would therefore suggest that the kinetics of IFN-γ plasma levels in our study are independent of IL-18.

Finally, the down-regulation of IL-12-mediated effects on repeated IL-12 administration may be attributable to the specific down-regulation of its own signaling (35). *In vitro,* prolonged stimulation of T cells by IL-12 results in depletion of the signal transducer and activator of transcription 4 protein. Down-regulation of signal transducer and activator of transcription 4, a critical IL-12 signaling component, resulted in decreased IFN-γ production (35). In line with these findings, we observed a generalized reduction of biological effects *in vivo* on repeated administration of IL-12 at doses approximating the maximum tolerated dose (9).

We have shown that the administration of IL-12 to humans resulted in the release of TNF-α, IL-8, and IL-6, in addition to that of IFN-γ and IL-10. The dose-dependent induction of multiple cytokines after the first injection of IL-12 is probably a combination of direct and indirect IL-12-mediated effects. Activated T and NK cells and, as shown recently, neutrophils, eosinophils, and dendritic cells (36–39) express IL-12 receptors, and IL-12 potentially mediates the production of secondary cytokines by binding to these cell populations. With respect to TNF-α, our results are in accordance with previous reports of elevated plasma TNF-α concentrations and increased TNF-α mRNA expression in peripheral blood mononuclear cells of patients with advanced cancer treated with IL-12 (19, 40). *In vitro,* IL-12 stimulates the production of TNF-α by activated T and NK cells (41, 42). Because mAb to TNF-α abrogate IL-12-mediated IFN-γ production in response to various stimuli *in vitro* (22, 43), TNF-α may be an essential costimulator of IFN-γ production and therefore an important intermediate in the antitumor effects of IL-12.

One study has addressed the issue of IL-8 concentrations after IL-12 in humans and described inconsistent patterns of stimulation (17). In contrast, we observed induction of IL-8 in the vast majority of patients. TNF-α is a physiological stimulant of IL-8 production in humans (44), and therefore, the induction of IL-8 may be secondary to TNF-α induction by rHuIL-12 in our study. Cells from the monocyte and macrophage compartment, endothelial cells, and neutrophils are among the cells that can be stimulated by TNF-α to produce IL-8 (45, 46). Alternatively, IL-8 induction may be a direct effect of rHuIL-12, because this cytokine was shown to induce the production of IL-8 from purified NK cells (47).

We also demonstrated that IL-6 peaks within 12 h after s.c. IL-12 administration. Previous studies that addressed IL-6 reported a large variation of plasma concentrations among patients and lack of IL-12 dose dependency (17, 40). IL-6 peak levels may have been missed in these studies, because the first blood samples were not taken until 24 h after IL-12 administration. Although TNF-α can stimulate IL-6 induction in humans (48), it probably had no major impact on IL-6 production in our patients, because IL-6 already reached peak levels 36 h before TNF-α. Rather, stimulation of IL-6 production may be directly mediated by IL-12, because binding of IL-12 to its receptors on dendritic cells has been shown to stimulate the production of IFN-γ, TNF-α, and IL-6 at the transcription level (39). IL-6 may also be considered as a natural feedback inhibitor of IL-12 production, because IL-6 inhibits both T cell-dependent and -independent induction of IL-12 production in humans (49).

The transient reduction of lymphocyte, monocyte, and neutrophil counts after the first IL-12 injection confirms previous observations (16, 17, 50). We consider this pattern to reflect the transient redistribution of leukocytes, which adhere to endothelium and migrate into the tissues. Indeed, postmortem examination of animals treated with IL-12 showed massive infiltrates of leukocytes in lymph nodes, lungs, liver, and spleen (51). The accumulation of NK and T cells in tumor nodules after IL-12 administration seems relevant to explain the putative antitumor effects of IL-12 (2, 52, 53). Although *in vitro* studies have demonstrated that IL-12 directly promotes interactions between endothelial and T cells, NK cells, and neutrophils (52, 54), secondary cytokines such as IFN-γ, TNF-α, and IL-8 may also contribute to the redistribution of leukocytes, because these cytokines enhance the expression of a wide range of molecules that regulate leukocyte adhesion and migration (55). In addition, chemokines induced by IFN-γ, such as IFN-γ-inducible protein 10 and monokine induced by IFN-γ, have potent chemotactic effects on T cells and may have contributed to their distribution (19). As with IFN-γ, induction of these chemokines declines on repeated IL-12 administrations (19).

In patients with advanced melanoma treated with s.c. IL-12, serum levels of the soluble endothelial adhesion molecules E-selectin, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 were transiently increased after IL-12 administration (2). In parallel, the expression of their respective ligands, *i.e.,* cutaneous lymphocyte antigen, very late antigen-4, and lymphocyte function associated antigen-1, was increased on circulating T cells. Thus, the enhanced expression of both receptors and ligands on endothelial cells and T lymphocytes may have promoted the marked infiltration of the melanoma lesions by tumor-specific CD8+ T cells observed in this study (2).

The development of IL-12 as a cancer therapeutic has followed the classical approach, starting with Phase I studies, followed by efficacy studies with the maximum tolerated dose that was defined previously. Our results indicate that the disappointing antitumor effects observed in Phase II studies are possibly attributable to a generalized reduction of biological effects that occurs when IL-12 is repeatedly administered at doses and in schedules that approximate the maximum tolerated dose.

We conclude that the systemic administration of IL-12 results in direct and indirect induction of multiple cytokines. On repeated IL-12 administration, levels of pro-inflammatory cytokines diminish as well as effects on peripheral blood leukocyte subsets, whereas IL-10 production increases and likely contributes to the down-regulation. Dose escalation of IL-12, within tolerable margins, does not prevent the down-regulation of immunological effects. At present, IL-12 is being studied as an adjuvant for cancer vaccination. The present study indicates that the effects of IL-12 are down-regulated when it is administered at dose levels near the maximum tolerated dose. Therefore, additional investigations are required to define the dose and schedule of IL-12 with optimal immunological effects in the vaccination setting.

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