Clinical Cancer Research

Tumor-Infiltrating Lymphocytes Genetically Engineered with an Inducible Gene Encoding Interleukin-12 for the Immunotherapy of Metastatic Melanoma

Ling Zhang, Richard A. Morgan, Joal D. Beane, Zhili Zheng, Mark E. Dudley, Sadik H. Kassim, Azam V. Nahvi, Lien T. Ngo, Richard M. Sherry, Giao Q. Phan, Marybeth S. Hughes, Udai S. Kammula, Steven A. Feldman, Mary Ann Toomey, Sid P. Kerkar, Nicholas P. Restifo, James C. Yang, and Steven A. Rosenberg

Abstract

Purpose: Infusion of interleukin-12 (IL12) can mediate antitumor immunity in animal models, yet its systemic administration to patients with cancer results in minimal efficacy and severe toxicity. Here, we evaluated the antitumor activity of adoptively transferred human tumor-infiltrating lymphocytes (TILs) genetically engineered to secrete single-chain IL12 selectively at the tumor site.

Experimental Design: Thirty-three patients with metastatic melanoma were treated in a cell dose–escalation trial of autologous TILs transduced with a gene encoding a single-chain IL12 driven by a nuclear factor of the activated T cells promoter (NFAT.IL12). No IL2 was administered.

Results: The administration of 0.001 to 0.1 $\times$ 10^9 NFAT.IL12–transduced TILs to 17 patients resulted in a single, objective response (5.9%). However, at doses between 0.3 and 3 $\times$ 10^9 cells, 10 of 16 patients (63%) exhibited objective clinical responses. The responses tended to be short, and the administered IL12-producing cells rarely persisted at 1 month. Increasing cell doses were associated with high serum levels of IL12 and IFNγ as well as clinical toxicities, including liver dysfunction, high fevers, and sporadic life-threatening hemodynamic instability.

Conclusions: In this first-in-man trial, administration of TILs transduced with an inducible IL12 gene mediated tumor responses in the absence of IL2 administration using cell doses 10- to 100-fold lower than conventional TILs. However, due to toxicities, likely attributable to the secreted IL12, further refinement will be necessary before this approach can be safely used in the treatment of cancer patients. *Clin Cancer Res; 21(10); 2278–88. ©2015 AACR.*

Introduction

Adoptive cell therapy (ACT) using autologous TILs and high-dose IL2 preceded by the administration of a nonmyeloablative lymphodepleting regimen mediates objective tumor regression in 50% to 70% of melanoma patients based on RECIST (1). In addition, ACT using genetic modification of peripheral blood lymphocytes with antitumor receptors can mediate regression in multiple cancer histologies (2–5). In an effort to improve the effectiveness of ACT, we used a strategy to genetically modify TILs to deliver, to the tumor site, molecules that can enhance the antitumor function of the transferred cells. Because of its powerful proinflammatory activities and its multiple roles in bridging innate and adaptive immunity (6), interleukin-12 (IL12) was chosen for study, first in preclinical models and, as reported here, in a phase I clinical trial in patients with metastatic melanoma.

IL12 was the first recognized member of a family of heterodimeric cytokines that includes IL12, IL23, IL27, and IL35. IL12 and IL23 are proinflammatory cytokines that are important to the development of T helper 1 (Th-1) and T helper 17 (Th-17) T-cell subsets, whereas IL27 and IL35 are potent inhibitory cytokines (6). IL12 can directly enhance the activity of effector CD4 and CD8 T cells as well as natural killer (NK) and NKT cells. Preclinical studies in murine tumor treatment models demonstrated powerful antitumor effects following the systemic administration of IL12 (7). In humans, however, attempts to systemically administer IL12 resulted in significant toxicities, including patient deaths and limited efficacy (8).

To improve ACT using TILs and to take advantage of the antitumor properties of IL12, we and others used genetic engineering of antitumor T cells with a gene encoding IL12 to deliver the potent cytokine selectively to the tumor site in murine tumor models (9–12). These animal studies revealed that IL12 had a profound and beneficial effect on the tumor microenvironment. Tumors can have an immunosuppressive environment composed of multiple cell types, including those of myeloid origin. In mice, these myeloid-derived cells can be reprogrammed by IL12 from immunosuppressors to immunostimulatory cells (9). In preclinical studies, the transduction of murine antitumor T cells with a

Surgery Branch, Center for Cancer Research, National Cancer Institute, Bethesda, Maryland.

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L. Zhang and R.A. Morgan share first authorship.

Corresponding Author: Steven A. Rosenberg, Center for Cancer Research, National Cancer Institute, Building 10-CRC, Room 3W-3940, 10 Center Drive, Bethesda, MD 20892. Phone: 301-496-4164; Fax: 301-402-1738. E-mail: sar@mail.nih.gov

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Translational Relevance

The adoptive cell therapy (ACT) of autologous tumor-infiltrating lymphocytes (TILs) plus IL2 can mediate objective responses in up to 70% of patients with metastatic melanoma. On the basis of the improved effectiveness of ACT using IL12 gene–modified cells in preclinical murine tumor models, we performed a first-in-man clinical trial evaluating therapy with TILs genetically engineered with an inducible gene encoding the secretion of IL12. The administration of these TILs mediated cancer regression using 50- to 100-fold fewer cells compared with standard TIL protocols and in the absence of IL2 coadministration. However, the transferred cells did not persist long term and were associated with severe dose-limiting toxicity. Although IL12 gene–modified TILs exhibit clinical promise, further modification of this approach is required before it can be safely applied.

gamma-retrovirus encoding IL12 substantially increased the ability of low numbers of cells to mediate the destruction of invasive cancers in the absence of IL2 administration. The impact of IL12 was dependent on the expression of the IL12 receptor on host cells and not on its expression on the transferred cells, further emphasizing the role of IL12 in altering the tumor microenvironment.

The toxicity resulting from the constitutive production of IL12 by antitumor reactive cells, however, led us to design an expression cassette using a single-chain IL12 driven by a nuclear factor of activated T cells (NFAT) inducible promoter (13). When inserted into antitumor T cells, this construct was designed to lead to IL12 secretion when the T-cell receptor was engaged by antigen at the tumor site. Antigen-reactive murine T cells engineered with this NFAT-regulated IL12 vector (NFAT. IL12) effectively treated large established murine tumors at cell doses that had minimal adverse effects and in the absence of any other cytokine administration (14). On the basis of data from murine models, we conducted a first-in-man clinical trial to evaluate the use of autologous human TILs transduced with an NFAT.IL12 (14) vector to treat patients with metastatic melanoma.

Materials and Methods

Patients

Patients eligible for this study were 18 years or older with evaluable metastatic melanoma and a melanoma lesion suitable for resection to generate TIL cultures. Patients had good clinical performance and normal liver and kidney function, were free from infections and had more than 3-month life expectancy. All patients signed an informed consent approved by the Institutional Review Board of the National Cancer Institute.

Clinical trial design

This clinical trial (National Cancer Institute, NCI, 11-C-011) was conducted in the Surgery Branch of the NCI and was reviewed and approved by the NIH Institutional Biosafety Committee, the NCI Institutional Review Board, the National Institutes of Health Office of Biotechnology Activities, and the FDA (all Bethesda, MD). All patients received a bolus i.v. infusion of TILs genetically modified by a retroviral vector encoding NFAT.IL12 following a lymphodepleting chemotherapy regimen, consisting of 60 mg/kg/d cyclophosphamide for 2 days followed by 5 days of 25 mg/m^2/d fludarabine as previously described (1, 15). This protocol was designed as a cell dose–escalation starting with an initial cohort at 1×10^6 cells and increasing numbers of cells by half-log increments. Patients received baseline CT and/or MRI and PET before treatment. Tumor size was evaluated monthly for 3 months and at regular intervals thereafter by CT, MRI, or documented with photography for cutaneous/subcutaneous lesion. Tumor measurements and patient responses were determined according to RECIST. Duration of response in responding patients were calculated from the time of cell infusion.

Gene transfer

Patients with metastatic melanoma underwent resection of tumor and fragments were individually cultured to generate TILs as previously described (1). The gamma-retroviral vector–expressing IL12 under an NFAT-responsive promoter has been described before (14). In brief, MSGV-1 is derived from the MSGV vector that uses the murine stem cell virus long-terminal repeat and contains an extended gag region and Kozak sequence. The gene encoding human single-chain IL12 was synthesized with the order IL12 p40, linker G6S, and IL12 p35 driven by an NFAT-responsive promoter and inserted into the MSGV-1 vector reverse to the 5′ LTR direction. A high-titer PG13 cell–based producer cell line was generated, and retroviral supernatant was produced by the NCI Surgery Branch Vector Production Facility (Bethesda, MD) under good manufacturing practice conditions. The vector supernatant was tested and passed all currently required FDA guidelines for the production of recombinant gamma-retroviral vectors for clinical application.

The transduction procedure was initiated by stimulating TILs with 30 ng/mL anti-CD3 mAb Orthoclone OKT3 (Centocor Ortho Biotech), 3,000 IU/mL recombinant human IL12 and 4 Gy irradiated allogeneic PBMC feeder cells at a ratio of 200 feeder cells for every TIL. Cells were harvested for transduction on days 4 and/or 5 using RetroNectin (CH-296; Takara Bio Inc.) coated non-tissue culture 6-well plates. Vector supernatant was "spin loaded" onto coated plates by centrifugation at $2{,}000 \times g$ for 2 hours at 32°C. Retroviral vector supernatant was aspirated from the wells and 2×10^6 stimulated TIL cells were added each well followed by centrifugation at $1{,}000 \times g$ for 10 minutes. Plates were incubated at 37°C overnight and cells were harvested for the second transduction the following day. Cells for the first 21 patients underwent two transductions. Cells for patients 22 to 33 underwent only one transduction.

Growth of cells

After transduction, cells were transferred from the 6-well plates into 175 cm^2 Nunc Cell Culture Treated Flasks with Filter Caps (Thermo Fisher Scientific) at a concentration of 0.5×10^6 cells/mL. Cells were cultured in AIMV media (Life technologies) supplemented with 3,000 IU/mL Aldesleukin (IL2; Novartis), 2 mmol/L Glutamax (Life technologies), 5% human serum (Gemini BioProducts) 100 U/mL penicillin, and 100 μg/mL streptomycin (Lonza). Penicillin and streptomycin were excluded from the cell culture of patients with allergies to these reagents. Because IL12 can inhibit lymphocyte proliferation, our usual procedure for lymphocyte expansion was modified. Every day, for the first 8 days of culture, cells were harvested, pooled, and centrifuged in a table top centrifuge (Sorval Legend; 590G for 10 minutes). The

old media were aspirated and the resultant cell pellets were resuspended in fresh media at a concentration of 0.5×10^6 cells/mL. Cells were maintained in culture and expanded in this manner for a total of 10 days after the initial OKT3 stimulation.

Before treatment, the function of transduced cells (1×10^5) was evaluated by overnight activation with phorbol myristate acetate (PMA; 10 ng/mL; Sigma) and ionomycin (2.2 μmol/l; Sigma) and an ELISA measurement (Thermo Fisher Scientific) of IL12 produced in the culture supernatant over the next 18 hours. Cells were tested for sterility by bacterial culture, fungal culture, and *Mycoplasma* PCR testing. Endotoxin was performed by limulus amoebocyte lysis assay. The IL12-transduced TILs were washed in saline before infusion and resuspended in 125-mL saline containing 5% human serum albumin (Grifols), then administered to the patient i.v. over 30 minutes.

Flow-cytometry analysis

For intracellular IL12 detection by flow cytometry, cells were stimulated for 6 hours with PMA (10 ng/mL; Sigma) and ionomycin (2.2 μmol/L; Sigma) in the presence of GolgiStop and GolgiPlug (BD Biosciences). After 6 hours, cells were surface stained with FITC anti-CD3 and APC-Cy7 anti-CD8 antibodies (BioLegend). Cells were subsequently stained intracellularly with PE-anti–IL12 antibody (BD Biosciences) using the Cytofix/Cytoperm Kit (BD Biosciences).

For phenotype characterization of infusion bag cells, cells were stained with the following commercial reagents: anti-CD45RA APC (BioLegend), anti–CD8-APC-Cy7 (BD Biosciences), anti-CD62L FITC (BioLegend). Cell events were acquired on a BD FACS Canto II. For analysis, gates were set using fluorescent minus one controls; a combination of hierarchal and Boolean gating strategies was used. The final data were processed using FlowJo (Treestar) and graphs were generated using Pestle and SPICE software programs (NIAID).

IL12 enzyme-linked immunosorbent spot assay

A 96-well filtration plate (Millipore) was coated with 10 μg/mL anti-hIL12 mAb in PBS (MabTech) overnight at 4°C. The plate was then loaded with NFAT–IL12-transduced cells and stimulated with PMA (10 ng/mL) and ionomycin (2.2 μmol/L) overnight. The plate was washed with PBS five times and 1 μg/mL anti–hIL12mAb-Biotin (MabTech) was added. Two hours later, the plate was washed with PBS and diluted streptavidin (1:3,000; MabTech) was added and incubated for 1 hour. The distinct spots were developed after adding substrate BCIP/NBT for up to 10 minutes. The plate was read and counted using immunoSpot Micro Analyzer (Cellular Technology Ltd.).

Real-time polymerase chain reaction. A specific real-time PCR probe targeting the NFAT.IL12 vector was designed to determine the NFAT.IL12–modified cells in patients after adoptive therapy. For each patient, DNA was extracted from PBMC collected before treatment and at different time points after treatment using the DNeasy Blood and Tissue Kit (Qiagen). The DNA extract from infused T cells of each patient was serially diluted 1:2 as the standard. The percentage of PBMC that contained the IL12 gene at each time point was determined by comparing the qPCR results obtained with DNA of PBMC from each time point to the qPCR results from the standard DNA. All samples were normalized to β-actin (TaqMan Gene Expression Assays; Applied Biosystem). Real-time PCR was carried out in duplicate using the TaqMan 7900 real-time PCR machine (Applied Biosystem).

Multiplex cytokine analysis. The serum samples were collected for each patient pretreatment and every day after treatment. Serum levels of IL2, IL4, TNFα, IL12, IFNγ, GM-CSF, GCSF, IL8, IL6, IL10, IL15, and IL7 in treated patients at multiple time points were analyzed by Aushon Biosystems.

Results

Clinical trial and patient characteristics

This clinical trial was designed to evaluate the safety of the administration of increasing numbers of autologous NFAT.IL12–engineered TILs to patients with metastatic melanoma following a nonmyelobative lymphoid–depleting preparative regimen. ACT in humans was performed in the absence of exogenous IL2 administration. Toxicity concerns associated with previous studies using the systemic administration of the cytokine IL12 (8) necessitated a trial design that involved cell administration starting at low cell doses (10^6/patient) and a slow dose-escalation preceding in half-log increments in consecutive cohorts as described in the Materials and Methods of this article. Patients 1 to 9 were treated with CD8-selected TILs and patients 10 to 23 received unseparated bulk TILs. The switch from CD8-selected TILs to bulk TILs was made following our observations in a randomized clinical trial of the administration of TIL in patients with metastatic melanoma that enriching for CD8 T cells did not increase response rates or safety of TIL therapy (16). In this trial, however, we have not ruled out the possible impact of enriched $CD8^+$ cells.

Table 1 summarizes the patient characteristics, cell doses, and clinical findings of the 33 patients with metastatic melanoma sequentially treated in this phase I trial. Their ages ranged 23 to 67. There were 9 females and 24 males and all had been extensively pretreated, including 4 patients previously treated with conventional TILs and IL2. Twenty-nine of the 33 patients had visceral metastases, and 4 patients had metastatic disease limited to lymph nodes and subcutaneous tissue.

Properties of the infused cells

The *in vitro* biologic activity of each cell culture was determined to meet protocol release criteria. The gene transfer efficiency was measured by IL12 intracellular cytokine staining following OKT3 stimulation and ranged from 2% to 52% (Table 1; mean 14.1% ± 2.2% SEM) with representative staining shown in Supplementary Fig. S1. Although the same viral supernatant lot was used for all transductions, the variations in transduction efficiency were likely due to the varied growth rates and cellular composition of the TIL culture at the time of transduction. The characteristics of the cells administered to patients are shown in Table 2. The 9 patients that received CD8-enriched cultures received an average of 3.6% ± 1.3% (mean ± SEM) CD4 cells compared with the remaining 24 patients whose TIL contained 29.0% ± 5.9% CD4 cells. The fold expansion of the CD8-enriched and bulk TIL cultures following transduction was similar (27.3-fold vs. 31.3-fold) as was the baseline IL12 production by cells at the time of cell infusion (421 and 389 pg/mL/10^5 cells; Table 2). When induced with PMA and ionomycin, IL12 production by 10^5 cells *in vitro* increased about 100-fold to 33,160 and 55,626 pg/mL/10^5

Table 1. Patient demographics treatment and response

Patient	Age/ gender	Sites of disease	Prior treatment	Infusion cell number $\times 10^9$	Transduction efficiency (%)	Infusion IL12 Td cell number $\times 10^9$	Response (mo)
1	56 F	lu, sc, ln	IL2, surgery	0.001	3	0.00003	NR
2	47 M	lu, li, ln	IL2, IMTOX	0.003	21	0.00063	NR
3	30 M	lu, sc, ln	IL2, YT	0.01	35	0.0035	NR
4	50 M	lu, ln, bo	IL2	0.01	2	0.0002	NR
5	20 F	sp, ad, ki, lu, li, sc, ln	YT, ipi	0.01	3	0.0003	NR
6	42 M	lu, sc	IL2	0.01	7	0.0007	NR
7	40 M	ln, sc	IL2	0.01	5	0.0005	NR
8	48 M	lu, ln	IL2	0.01	52	0.0052	NR;
9	47 M	lu, ln	YT	0.03	6	0.0018	CR (38+)
10	60 M	lu, ad	IL2, ipi	0.01	29	0.0029	NR
11	61 M	ln, lu, sc	IL2	0.03	31	0.0093	NR
12	63 M	liver, sc	IL2	0.03	24	0.0072	NR
13	64 M	neck, lu	IL2	0.03	38	0.0114	NR
14	41 F	R toe, lu, sc, ln	IL2	0.03	2	0.0006	NR
15	34 F	li, panc, ln	IL2	0.1	17	0.017	NR
16	63 F	sc, ln, li	Surgery, xrt, IL2	0.1	2	0.002	NR
17	50 M	sp, panc, lu, li, brain, sc	IL2	0.1	32	0.0032	NR
18	63 M	ln, lu	IL2	0.3	16	0.048	PR (4)
19	45 M	sc, ln, brain	IFN, surgery, IL2, TIL	0.3	14	0.042	PR (6)
20	60 F	lu, ln	IFN, xrt, IL2	0.3	12	0.036	PR (21)
21	58 M	sc, ln	YT	1	17	0.17	PR (27+)
22	30 F	ln, lu, brain	Surgery, IFN, xrt, IL2	1	16	0.16	NR
23	59 M	sc, ln	Surgery, MART-F5	1	4	0.04	NR
24	62 F	lu, thigh	Surgery, IL2	3	4	0.12	PR (11)
25	65 M	sc, ln, adrenal	IL2, ipi	3	15	0.45	PR (7)
26	67 M	sc, ln. lu, li, adrenal	Surgery, IL2, ipi, Bor/Sor	1.1	8	0.088	NR
27	38 M	ln, sc	xrt	3	10	0.3	CR (21)
28	58 F	sc, kidney, lu.ln	Surgery	3	14	0.42	PR (4)
29	51 M	sc, bo, spine, lu, ln	IFN, IL2	1	10	0.1	NR
30	61 M	ln, li	Surgery, MART F-5 adj trial +IL2	1	6	0.06	PR (5)
31	65 M	lu, brain, sc	Surgery	1	3	0.03	NR
32	23 M	lu, brain	Surgery	1	4	0.04	PR (12+)
33	36 M	li, ln	Surgery, xrt, IFN, IL2, VFN, anti-PD-1	1	4	0.04	NR

Abbreviations: abd, abdominal; bilat, bilateral; Bor, Bortezomib; F, female; IMTOX, anti-CD25 immunotoxin; ipi; ipilimumab; L, left; li, liver; ln, lymph node; lu, lung; M, male; MART-F5; NR, no response; PR, partial response; R, right; sc, subcutaneous; Sor, sorafenib; T-cell receptor–engineered cells; VFB, vemurafenib; xrt, radiation; YT, short-term cultured TIL.

cells, respectively. When sufficient tumor cells were available, cell infusion cultures were tested for recognition of autologous fresh tumor digests using a coculture assay with and without the addition of an MHC class 1 blocking antibody. Fifteen of the 23 cultures tested showed reactivity against the fresh autologous tumor that could be blocked by an anti–class 1 antibody (Table 2).

In each cell culture, the phenotype of NFAT.IL12–engineered TILs was compared with TILs treated identically, but without the transduction. The differentiation status of the infused cells determined by the expression of CD62L and CD45RA revealed an increase in cells with a central memory–like phenotype and a decrease in effector memory–like cells in cultures transduced with the NFAT–IL12 gene compared with the nontransduced cells (Supplementary Fig. S2). Central memory and effector memory cells comprised 18.8% ± 3.1% and 72.5% ± 2.8%, respectively, in the NFAT.IL12–transduced cultures compared with 5.7% ± 1.6% and 86.9% ± 2.2% in the nontransduced cultures ($P = 0.0001$ and 0.0004, respectively). There were no differences in the percentage of cells with the phenotypes associated with naïve or terminally differentiated effector memory cells (effector memory $CD45RA^+$) in the transduced compared with nontransduced cultures ($P > 0.3$). Neither the expression of these differentiation markers nor the *in vitro* measurement of antitumor activity was correlated with clinical responses in patients receiving these cell infusions.

Clinical responses following infusion of NFAT.IL12–engineered TILs

Eleven of the 33 patients treated in this dose-escalation trial achieved an objective cancer response by RECIST (Table 1). There appeared to be a threshold number of cells needed to mediate reproducible clinical responses. In the 17 patients treated at 0.1×10^9 or fewer cells, only a single objective response was seen (5.9%). Of note, this patient (#9) had previously been treated with 3×10^{10} conventional nontransduced TILs, plus 7 doses of IL2 (720,000 IU/kg) and tumors progressed. Using TILs expanded from the same original culture, the patient was retreated with a culture of 3×10^7 NFAT.IL12 gene–modified TILs (1,000-fold fewer cells containing approximately 1.8×10^6 NFAT.IL12 gene–modified TILs) and has an ongoing complete regression at 38 months of disease metastatic to lung and lymph nodes (Fig. 1).

In contrast with the results at lower cell doses, 10 of the 16 patients treated with 0.3 to 3×10^9 NFAT.IL12 cell cultures exhibited objective responses (62.5% vs. 5.9% at lower cell doses; $P = 0.0008$). Nine patients exhibited a partial regression though only 2 of these patients have ongoing responses (12 and 27 months). One patient exhibited a complete regression but relapsed at 21 months. Regressions were seen at multiple sites, including the peritoneum, brain, lung, lymph nodes, and subcutaneous tissue (Table 1 and Fig. 1). Of the 16 patients in this group, 13 had visceral disease and one (#32) has an ongoing response. Three of the 16 patients had disease localized to lymph

Table 2. Characteristics of the infused NFAT.IL12 cells

Patient	CD8 (%)	CD4 (%)	Fold expansion	Base line IL12 (pg/mL)	Induced IL12 (pg/mL)	Fresh tumor recognition	Class I block	Response
1	97	3	11	27	7,263	N/A	N/A	NR
2	93	7	10	503	37,427	N/A	N/A	NR
3	98	2	14	205	19,717	No	N/A	NR
4	99	1	63	670	13,637	N/A	N/A	NR
5	99	1	12	394	24,956	Yes	N/A	NR
6	98	2	10	400	28,636	N/A	N/A	NR
7	99	1	49	352	32,400	N/A	N/A	NR
8	98	2	50	930	47,800	Yes	No	NR
9	86	13	27	310	86,600	N/A	N/A	CR (38+)
Mean	96.33	3.556	27.33	421.2	33,160			
SEM	1.43	1.334	7.008	87.24	7,826			
10	2	98	15	440	86,600	N/A	N/A	NR
11	15	85	23	210	140,300	Yes	No	NR
12	12	66	3	670	100,100	Yes	No	NR
13	35	64	28	930	81,400	Yes	No	NR
14	83	8	12	510	37,800	Yes	Yes	NR
15	95	4	9	450	57,000	Yes	Yes	NR
16	90	10	8	280	6,000	Yes	Yes	NR
17	87	11	71	270	22,300	Yes	Yes	NR
18	72	28	7	680	52,900	Yes	Yes	PR (4)
19	70	28	4	320	33,100	No	No	PR (6)
20	94	5	22	500	47,300	Yes	Yes	PR (21)
21	85	16	31	10	34,100	Yes	No	PR (27+)
22	87	9	32	490	70,600	Yes	Yes	NR
23	46	65	16	370	32,400	No	No	NR
24	95	4	64	130	46,600	Yes	Yes	PR (11)
25	97	2	118	580	63,400	Yes	Yes	PR(7)
26	50	59	7	80	5,330	Yes	No	NR
27	95	4	64	130	46,600	Yes	Yes	CR (21)
28	93	1	58	900	124,600	Yes	Yes	PR (4)
29	65	33	34	0	48,800	Yes	Yes	NR
30	84	16	14	650	147,800	Yes	Yes	PR (5)
31	70	29	14	35	4,600	N/A	N/A	NR
32	90	10	92	614	28,100	Yes	Yes	PR (12+)
33	60	40	5	85	17,300	Yes	Yes	NR
Mean	66.67	28.96	31.29	388.9	55,626			
SEM	5.87	5.869	6.275	55.85	8,227			

nodes, and subcutaneous tissue and 1 of these 3 (#21) has an ongoing response.

Thus, objective cancer responses could be seen when TILs transduced with the NFAT.IL12 gene were administered at cell doses 10- to 100-fold lower than in our conventional TIL treatments (1) and in the absence of IL2 administration. Response durations tended to be relatively short compared with published results using larger numbers of nontransduced TILs administered in conjunction with IL2 (1). In a recent clinical study, in patients with metastatic melanoma who received larger numbers of nontransduced TILs in conjunction with IL2 administration, objective responses were seen in 23 of 51 patients with 18 of the 23 responders ongoing when followed for a comparable period of time (unpublished data).

Post-infusion studies of cells and serum

Serum levels of IL12 and IFN gamma (IFNγ) were determined daily throughout the first 2 weeks after infusion. Peak levels are shown for each patient in Table 3, and a characteristic example of the pattern of these serum levels are shown in Supplementary Fig. S3. Peak serum IL12 and IFNγ levels occurred between 6 and 15 days after cell administration and tended to occur earlier at higher cell doses. A general cell dose–dependent increase in peak serum IL12 and IFNγ levels was observed, although serum cytokine levels within an individual cohort were variable and sporadic and very high levels were sometimes seen (Table 3). For example, in the cohort receiving 1×10^9 cells, peak serum IL12 levels varied from 46 to 18,052 pg/mL and peak IFNγ levels varied from 299 to18,810 pg/mL. Similarly, at the 3×10^9 cell dose, peak IL12 levels varied from 429 to 30,500 pg/mL, and peak IFNγ levels varied from 1,915 to 72,050 pg/mL. Because of the very high and potentially lethal levels of serum IL12 that were sporadically seen at the higher cell doses required to achieve cancer regression, patient accrual was closed after the patient 33. With the exception of patient 33 (see below), serum IL12 levels returned to undetectable levels within 2 weeks. Serum samples were also subjected to multiplex cytokine analyses for IL2, IL4, IL6, IL7, IL8, IL10, IL15, TNFα, GMCSF, and GCSF. In patients with elevated IL12 and IFNγ, only IL6 and IL10 were consistently elevated after treatment (Supplementary Fig. S4).

At 1 month after cell administration, the persistence of IL12-modified cells in the peripheral circulation was measured by ELISpot assay following stimulation of CD3-enriched cells with PMA/Ionomycin as well as by real-time PCR using vector-specific probes. As shown in Table 4, only 8 of the 33 patients tested showed any detectable persisting IL12 ELISpot activity in blood at 1 month, including 5 of the 16 patients treated at cell doses of 0.3×10^9 or greater. There was no correlation with cell persistence and

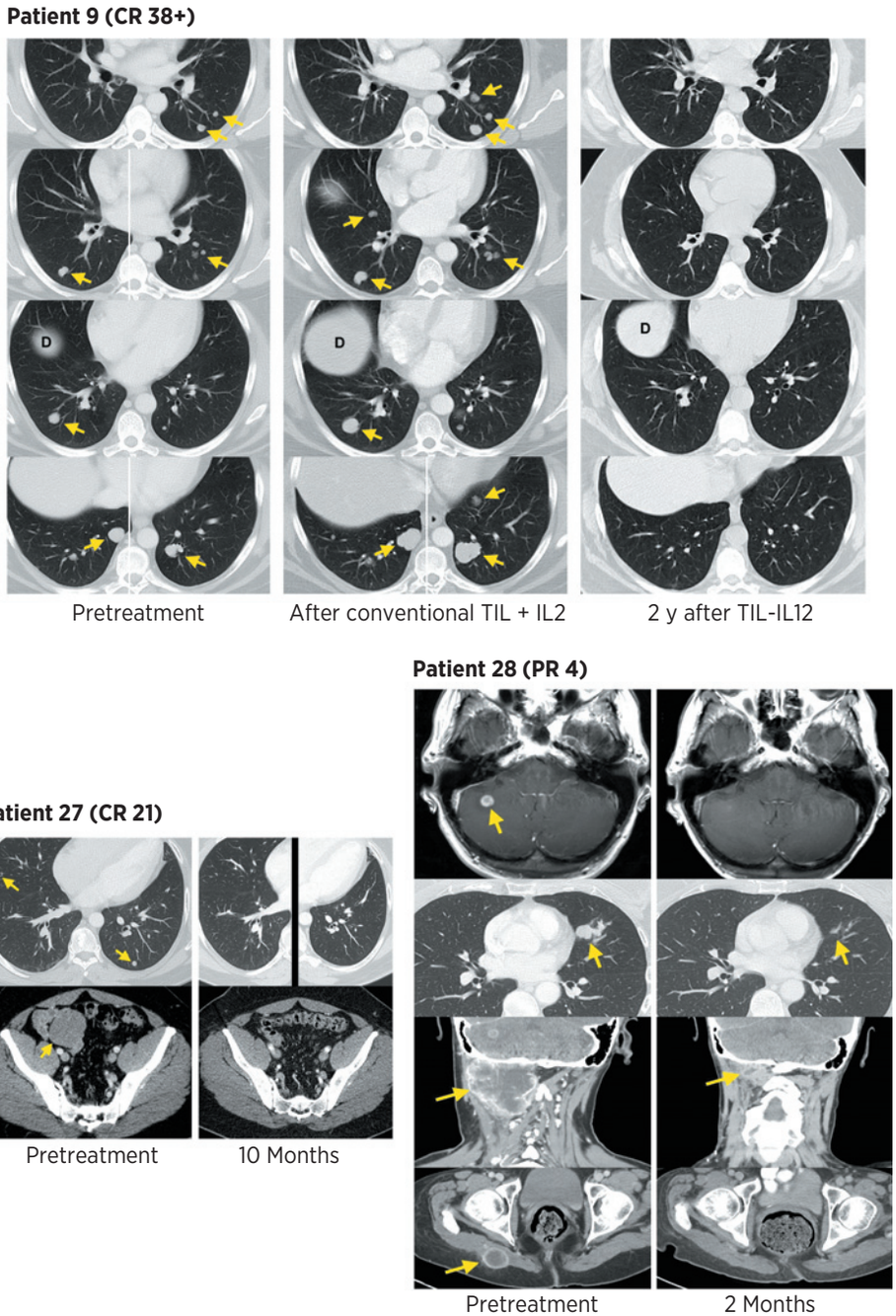

Figure 1.
Antitumor affect in patients with metastatic melanoma of ACT using TILs genetically engineered to secrete single-chain IL12. (Top), Regression of lung metastases in a patient that progressed after receiving 3×10^{10} conventional TIL plus IL2, but achieved a complete regression after receiving 3×10^{7} TILs genetically engineered with an inducible IL12 gene (NFAT.IL12). D, diaphragm. (Bottom) Examples of tumor regression at multiple sites in patients that received NFAT.IL12 gene–engineered cells.

the likelihood of clinical response. Physiologic reconstitution of lymphocyte counts was in accordance with prior experience with ACT of nontransuduced TILs. The two highest values were from patient 9 (67 spots/10^5 $CD3^+$ cells) who had a complete response (CR) and from the nonresponding patient 23 (250 spots/10^5 $CD3^+$ cells). Similarly, determination of vector presence by qPCR demonstrated poor persistence of vector transduced TILs in blood (Table 4), again, with no correlation between response and vector presence. These levels of persistence were lower than those seen in our published conventional TIL trials where larger numbers of cells (usually $5–10 \times 10^{10}$) were given in association with IL2 administration (1, 17). The low persistence of IL12-transduced cells may be due in part to the absence of IL2 administration or to the antiproliferative impact of IL12.

We previously reported that rapid reconstitution of circulating regulatory T cell (Treg) was negatively correlated with clinical response in standard TIL protocols (18). We therefore determined the percentage of $CD4^+/CD25^+/FoxP3^+$ cells in the blood at 1 and 4 weeks in patients who received NFAT.IL12–transduced TILs. At 1 week, those levels were comparable with the levels of reconstitution seen in prior trials of patients receiving large numbers of nontransduced TILs without IL2 administration (Supplementary

Table 3. Peak values of serum IL12, IFNγ, serum ALT, and fevers in each cohort

Patient	Infusion cell number $\times 10^9$	Peak IL12 (pg/mL)	Peak IFNγ (pg/mL)	ALT (U/L)	Temperature (°C)	Days Tm > 39
1	0.001	nd	nd	28 (d5)	37.4 (d2)	0
						—
2	0.003	0	nd	50 (d10)	39.5 (d6)	5
3	0.01	2,773 (d15)	2,864 (d15)	37 (d3)	39.9 (d6)	9
4	0.01	nd	nd	50 (d5)	37.9 (d7)	0
5	0.01	0	220 (d8)	33 (d3)	38 (d4)	1
6	0.01	53 (d10)	96 (d8)	157 (d11)	37.6 (d6)	0
7	0.01	nd	0	208 (d6)	39.6 (d7)	1
8	0.01	0	0	36 (d7)	39.8 (d5)	2
10	0.01	0	303 (d9)	79 (d9)	39 (d4)	1
	Mean	565.2 (d8.3)	580.5 (d10)	85.7 (d6.2)	38.8 (d5.5)	2.0
	SEM	552.0	459.3	26.3	0.4	1.2
9	0.03	786 (d11)	2,220 (d11)	228 (d14)	40.6 (d11)	11
11	0.03	302 (d11)	3,415 (d12)	160 (d18)	39.3 (d5)	3
12	0.03	0	347 (d7)	82 (d8)	39.6 (d9)	5
13	0.03	649 (d10)	384 (d8)	668 (d14)	38.9 (12)	1
14	0.03	347 (d13)	1,494 (d12)	68 (d13)	39.6 (d5)	3
	Mean	416.8 (d11.2)	1,572 (d10)	241.2 (d13.4)	39.6 (d8.4)	4.6
	SEM	138.2	580.3	110.5	0.3	1.7
15	0.1	nd	0	558 (d12)	37.2 (d8)	0
16	0.1	97 (d10)	585 (d6)	42 (d6)	39.1 (d8)	1
17	0.1	nd	Nd	40 (d2)	39.1 (d10)	2
	Mean	97 (d10)	292.5 (d6)	213.3 (d6.6)	38.5 (d8.6)	1.0
	SEM	0	292.5	172.3	0.6	0.6
18	0.3	0	208 (d9)	55 (d11)	39 (d7)	1
19	0.3	0	312 (d9)	317 (d8)	40.2 (d8)	8
20	0.3	255 (d9)	396 (d6)	23 (d14)	38.8 (d6)	0
	Mean	85 (d9)	305.3 (d8)	131.7(d11.0)	39.3 (d7.0)	3.0
	SEM	85	54.37 (d1)	93.1 (d1.7)	0.4	2.5
21	1	73 (d8)	1,340 (d6)	194 (d4)	39.7 (d8)	5
22	1	185 (d7)	1,521 (d9)	58 (d12)	40.2 (d7)	4
23	1	254 (d10)	749 (d7)	281 (d8)	39.2 (d7)	7
29	1	44 (d11)	299 (d7)	130 (d9)	38.2 (d7)	0
30	1	171 (d8)	726 (d4)	539 (d7)	39.6 (d7)	3
31	1	46 (d10)	714 (d8)	68 (d5)	38.8 (d5)	1
32	1	618 (d8)	614(d4)	507 (d4)	38.4 (d6)	0
33	1	18,052 (d11)	6,698 (d15)	4,764 (d11)	40.2 (d9)	6
26	1.1	2,930 (d13)	18,810 (d13)	168 (d18)	40.8 (d10)	16
	Mean	2,486 (d9.5)	3,497 (d8.1)	745 (d8.6)	39 (d7.3)	4.7
	SEM	1,970	202	505	0.3	1.7
24	3	1,852 (d8)	1,915 (d6)	63 (d9)	40.1 (d7)	4
25	3	30,500 (d6)	72,050 (d6)	152 (d14)	40 (d2)	3
27	3	429 (d6)	3,595 (d5)	100 (d10)	40.1 (d3)	6
28	3	556 (d7)	8,485 (d5)	166 (d3)	40.3 (d3)	2
	Mean	8,334 (d6.7)	21,511 (d5.5)	120 (d9.0)	40.1 (d3.7)	3.8
	SEM	7,396	16,904	23	0.1	0.9

Fig. S5; ref. 18). However, data obtained at 4 weeks after infusion in the current trial were consistent with an increase in cells with a phenotype associated with Tregs (Supplementary Fig. S5).

Toxicity following infusion of NFAT.IL12–engineered TILs

All patients received the initial cell infusion with no immediate complications. Hematopoietic reconstitution occurred in a pattern similar to prior patients who received nontransduced TILs with this lympho-depleting regimen (16).

The adverse events seen in these patients are summarized by cohort in Tables 3 and 5. Liver function abnormalities as evidenced by increases in serum ALT (normal 6 to 41 U/L) and AST (normal 9 to 34 U/L) were seen in virtually all cohorts with the incidence tending to be higher at the higher-dose levels. Five of the 16 patients receiving 3×10^8 cells or greater (doses at which tumor regressions were seen) exhibited grade 3 liver function toxicity and 3 patients exhibited grade 4 liver toxicity.

Fevers were common throughout the treatment course in patients in all cohorts, although fevers tended to be higher at cell doses of 3×10^8 or greater (Table 3). In this latter group of 16 patients (10 of whom had objective responses), 14 patients had fevers exceeding 38.5°C and 8 patients had fevers over 40°C lasting 8, 4, 4, 3, 16, 6, 2, and 6 days, respectively. Peak fevers tended to occur between 5 and 8 days following the cell infusion and were present during the periods of neutropenia as well as after neutrophil recovery. Use of antipyretics such as acetaminophen exacerbated the hepatic dysfunction.

The highest levels of serum IL12 and IFNγ were potentially lethal and required intensive care unit management in some patients. The treatment course of patient 33 who received $1 \times$

Table 4. *In vivo* persistence

Patient	Elispot (spot/10e5 $CD3^+$)	Vector PCR (% of infusion)
1	0	0
2	0	0.007
3	ne	0.376
4	ne	0.007
5	5	ne
6	ne	ne
7	0	ne
8	0	ne
9	67	6.93
10	0	0.002
11	0	0.02
12	0	0.1
13	28	0.47
14	0	0.003
15	0	ne
16	0	ne
17	6	0.04
18	0	ne
19	0	0.02
20	0	0.3
21	0	0.01
22	0	0.01
23	250	6.0
24	21	0.3
25	65	0.8
26	0	ne
27	27	ne
28	15	0.2
29	0	ne
30	0	ne
31	0	ne
32	0	ne
33	0	Ne

NOTE: Samples from patients obtained 1 month after infusion were evaluated biologic activity and gene-marked cell persistence. Elispot was performed on CD3-enriched T cells, which do not normally express IL12 (normal $CD3^+$ were always negative). Cells were activated by PMA/ionomycin to stimulate IL12 production. In parallel, DNA was extracted and subject to Q-PCR using vector-specific primers and probes, in which the infusion bag (set as 100%) was used as a standard.
Abbreviation: ne, not evaluated.

10^9 NFAT.IL12 cells was particularly complicated and illustrated the danger of high levels of circulating IL12. On day 2 after cell infusion, this patient developed febrile neutropenia and was started on antibiotics. His fevers progressed and as his serum IL12 and IFNγ levels increased, he required aggressive volume support and multiple transfusions of platelets, packed red blood cells, and cryoprecipitate. He developed acute renal insufficiency and grade 4 liver toxicity. Transaminase levels rose by day 11 with peak asparate aminotransferase of 5,162 U/L (normal 9–34 U/L) and an alanine aminotransferase of 4,764 U/L (normal 6–41 U/L) on day 15. At that time, a liver biopsy revealed acute hepatitis with mild cholestasis and an infiltrate rich in macrophages. On hematoxylin and eosin staining, multiple small foci of hepatic necrosis were seen with marked increases in activated $CD68^+$ macrophages diffusely infiltrating the hepatic parenchyma, portal areas, and hepatic sinusoids. There were very few scattered lymphocytes. Most of the inflammatory infiltrate was composed of macrophages. Serum IL12 levels peaked at 18,052 pg/mL on day 11 and IFNγ levels at 6,698 pg/mL on day 15. Life-threatening hemodynamic changes required intensive intervention and he was treated with the anti-IL12 antibody, ustekinumab, followed by high-dose steroids and administration of alemtuzumab to eliminate lymphocytes. Although production of new IL12 and IFNγ appeared to cease with these interventions, the long serum half-life of the single-chain IL12 and the continued toxicities in this patient required multiple plasmaphereses to reduce serum IL12 levels. Hemodynamic instabilities slowly resolved. The patient recovered and was discharged 20 days after cell administration with normal laboratory values and no further sequelae. These sporadic and life-threatening levels of serum IL12 and IFNγ combined with the relatively short durations of response seen in most patients led us to halt further accrual to this trial.

Discussion

In the present study, we report the first-in-man evaluation of the insertion of a gene encoding–secreted IL12 into T cells used for the ACT of patients with metastatic cancer. IL12 was selected for these studies because of its central role in bridging innate and adaptive immunity as well as the impressive antitumor effects seen in murine models of ACT using antitumor T cells transduced with the gene encoding IL12 (6, 9–12).

For these studies, we used a single-chain IL12, first described by Anderson and colleagues (19) consisting of the p35 and p40 subunits of IL12 connected by a flexible linker. This construct enabled both subunits to assume their natural structure, thus maintaining the normal biologic activity of human IL12. *In vitro* studies by Wagner and colleagues (20) demonstrated that the retroviral transduction of this single-chain IL12 into human lymphocytes resulted in the secretion of the single-chain IL12 molecule with maintenance of a high degree of biologic activity. To attempt to minimize the systemic levels of IL12, an NFAT-responsive promoter was used in our trial to enable the selective secretion of IL12 when the antitumor TCR was activated at the cancer site (14). As shown in Table 2, the baseline secretion of IL12 in these transduced cells was approximately 400 pg/mL/10^5 cells in an overnight coculture assay, and increased to approximately 50,000 pg/mL/10^5 cells following activation with PMA/ionomycin.

Because of the potential toxicity resulting from IL12, a slow cell dose–escalation protocol was used. As shown in Tables 1 and 2, only a single antitumor response was seen in 17 patients treated at 0.1×10^9 or fewer cells. This ongoing CR in patient 9 was highly suggestive of a direct role for an enhanced immune effect of NFAT.IL12–engineered TILs because this patient had rapid progression of disease after treatment using our standard TIL treatment regimen of 3×10^{10} cells with systemic IL2, but had an excellent clinical response after receiving 3×10^7 NFAT.IL12–modified TILs (1,000-fold fewer cells than in the first treatment) and no IL2 (Fig. 1).

In contrast with this single objective response in 17 patients treated at low cell doses, 10 of 16 (63%) patients treated with 0.3 to 3×10^9 NFAT.IL12 cells achieved an objective response. Tumor regression was seen at multiple anatomic sites (Fig. 1). Thus, our data indicate that NFAT.IL12 gene–modified TILs can be associated with cancer regression at cell doses 10- to 100-fold less than standard TIL protocols, and do so in the absence of high-dose IL2 administration.

Several problems emerged, however, in the course of this study. IL12 can inhibit the proliferation of lymphocytes in culture and the low levels of constitutive secretion of the IL12 into the growth medium led to considerable difficulty in growing sufficient cells for administration. It was, thus, necessary to harvest and wash the cells every day to remove any IL12 that was present in the growth

Table 5. Adverse events

Cell dose ($\times 10^9$)	Number of patients treated	Adverse event	Number of grade 3 events	Number of grade 4 events
$CD8^+$ 0.001	1	None		
$CD8^+$ 0.003	1	None		
$CD8^+$ 0.01	6	Fever	1	0
		Elevated LFT[a]	1	0
		Idiopathic thrombocytopenia Purpura	1	0
$CD8^+$ 0.03	1	Elevated LFT	1	0
		Fever	1	0
Bulk 0.001	1	None		
Bulk 0.03	4	Elevated LFT	1	0
Bulk 0.1	3	Prolonged myelosuppression 1[b]	0	1
Bulk 0.3	3	Elevated LFT	1	0
		Fever	1	0
Bulk 1.0	9	Elevated LFT	2	2
		Creatinine[c]	0	1
		Fever	1	0
		Hypoxia	1	0
Bulk 3.0	4	Fever	1	0
		Thrombotic microangiopathy	1	0
		Interstitial pneumonitis	1	0
		Elevated LFT	1	0

[a]Includes ALT, AST, and/or bilirubin.
[b]Prolonged/transfusion dependent (effected had patient prior radiation to left groin, no prior chemotherapy).
[c]Requiring dialysis. $CD8^+$, patients received CD8-enriched TILs.

medium. This soluble IL12 in the medium may also have been responsible for the increased number of central memory cells compared with concomitantly grown nontranduced cells (Supplementary Fig. S2). The antiproliferative property of IL12 may also have accounted for the poor persistence of these cells *in vivo* as evidenced by both ELISpot as well as RT-PCR measurements of the transferred cells in blood (Table 4).

The duration of antitumor clinical responses appeared to be relatively short with only 3 of the 11 total responders still sustaining their response. Although this observation could simply be due to the small numbers of patients treated, data in this report and from others suggest that the antiproliferative effect of IL12 and the complexity of immunoregulatory mechanisms attributed to different doses of IL12 could contribute to the lack of short response durations seen in some patients. For example, low-dose treatment with IL12 enhanced cellular immune responses to lymphocytic choreomeningitis virus infection in mice whereas high-dose IL12 treatment resulted in enhanced virus burdens (21). An IL12-negative feedback mechanism involves the production of IL10 (22), which can inhibit T-cell proliferation, and our data demonstrate (Supplementary Fig. S4) that the NFAT.IL12 T-cell transfer induced a 10- to 100-fold increase in serum IL10 levels in 4 of 5 patients studied. Whether this accounts for the generally poor persistence of our NFAT.IL12–engineered TILs is unknown.

In a clinical trial treating patients with follicular non-Hodgkin's lymphoma, rituximab plus IL12 had a lower response rate than rituximab alone, suggesting that IL12 had a detrimental effect on clinical outcome (23). Studies showed that prolonged *in vitro* exposure of human $CD4^+$ T cells to IL12 caused these cells to lose their ability to produce IFNγ, and induced expression of T-cell immunoglobulin and mucin domain protein (TIM-3; ref. 24). TIM-3 has been shown to impair T-cell function and leads to T-cell exhaustion in models of viral infection and tumors (25–27). TIM-3 as well as other markers of exhaustion have been associated with T-cell dysfunction in melanoma patients (26). Thus, a more balanced or regulated expression of IL12 by gene-engineered TILs may be needed to improve antitumor efficacy.

Significant toxicities were seen in patients receiving cell doses (0.3×10^9 or greater) capable of causing tumor regression. Severe liver function abnormalities and prolonged fevers often exceeding 40°C were seen (Tables 3 and 5). Most disturbing, however, was the unpredictability of peak serum levels of IL12 and IFNγ. Peak IL12 levels in the serum varied from 44 to 18,052 pg/mL and IFNγ levels varied from 299 to 18,810 pg/mL. These values led to potentially lethal hemodynamic instability and were not related to the level of transduction of the cells. High levels of IL12 secretion *in vivo* may have been due to the random introduction of the IL12 gene into T cells with other reactivities such as those against viral antigens capable of stimulating the cells *in vivo* or the possible transduction of cells in the TIL that had a high proliferative capacity in the lymphodepleted homeostatic environment.

The liver toxicities, the high fevers and especially the sporadic and possibly lethal toxicities due to the unpredictable high levels of secreted IL12 have led us to cease accrual to this protocol in its present form. The cancer regressions seen using low NFAT.IL12 cell doses in the absence of IL2 administration, however, provide a stimulus to try to overcome some of the problems in the application of this approach. If the observed toxicities are caused by circulating levels of IL12 produced by engineered TILs, then it may be possible to modify the vector to reduce this circulating IL12. The low but significant (100s of pg/mL) amounts of IL12 produced by unstimulated NFAT.IL12 gene–modified TILs indicate that the promoter has a basal level of activity. Further vector modification to remove adjacent enhancer sequences (such as in a self-inactivating, SIN, vector design) or the use of a small-molecule regulatable promoter would also be possible. An alternate approach might include manipulation of the single-chain IL12 gene itself. Protein engineering to reduce the serum half-life of the protein could potentially preserve the benefits of localized paracine expression, but decrease activity in the circulation. More directly applicable to clinical application would be the use of

IL12 constructs similar in concept to those reported by Pan and colleagues (28), in which a fusion protein was made using the murine single-chain IL12 linked to the transmembrane and cytoplasmic domains of protein B7-1. In that report, the membrane-bound IL12 had minimal shedding and negligible amounts of IL12 were detected in the serum of mice successfully treated by intratumoral injection of an adenovirus vector expressing the membrane-bound IL12.

The 63% response rate observed in patients treated with 0.3×10^9 or greater NFAT.IL12–engineered T cells compares favorably with previous TIL response rates using 10 to 100 higher numbers of cells along with high-dose IL2 and demonstrates, for the first time in humans, that genetic modification of lymphocytes with genes designed to influence the tumor microenvironment can affect the antitumor efficacy of ACT. Current efforts are being devoted to the use of transduced T cells selected for antitumor activity as well as to evaluate additional inducible promoters to control IL12 expression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L. Zhang, R.A. Morgan, M.E. Dudley, R.M. Sherry, M.S. Hughes, S.A. Feldman, S.P. Kerkar, N.P. Restifo, J.C. Yang, S.A. Rosenberg
Development of methodology: L. Zhang, Z. Zheng, M.E. Dudley, S.H. Kassim, A.V. Nahvi, S.A. Feldman, N.P. Restifo, J.C. Yang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.D. Beane, M.E. Dudley, S.H. Kassim, L.T. Ngo, R.M. Sherry, G.Q. Phan, M.S. Hughes, U.S. Kammula, S.P. Kerkar, J.C. Yang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Zhang, R.A. Morgan, J.D. Beane, Z. Zheng, M.E. Dudley, S.H. Kassim, A.V. Nahvi, M.S. Hughes, S.A. Feldman, N.P. Restifo, J.C. Yang, S.A. Rosenberg
Writing, review, and/or revision of the manuscript: L. Zhang, R.A. Morgan, J.D. Beane, M.E. Dudley, S.H. Kassim, R.M. Sherry, G.Q. Phan, M.S. Hughes, U.S. Kammula, S.A. Feldman, S.P. Kerkar, N.P. Restifo, J.C. Yang, S.A. Rosenberg
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.D. Beane, M.E. Dudley, A.V. Nahvi, L.T. Ngo, M.A. Toomey, S.A. Rosenberg
Study supervision: R.A. Morgan, M.A. Toomey, N.P. Restifo, S.A. Rosenberg
Other (manufactured IL12 gammaretroviral vector): S.A. Feldman

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