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IL-7 Administration to Humans Leads to Expansion of CD8⁺ and CD4⁺ Cells but a Relative Decrease of CD4⁺ T-Regulatory Cells

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Summary

Lymphopenia is a serious consequence of HIV infection and the administration of cancer chemotherapeutic agents. Although growth factors can be administered to patients to increase circulating neutrophils, there is no effective method to stimulate CD8⁺ lymphocyte production in humans, *in vivo*. This report is the first to describe the administration of recombinant interleukin-7 to humans and demonstrates the ability of this cytokine to mediate selective increases in CD4⁺ and CD8⁺ lymphocytes along with a decrease in the percentage of CD4⁺ T-regulatory cells. These studies suggest an important role for interleukin-7 in the treatment of patients with lymphopenia.

Keywords

interleukin-7; regulatory T cells; human lymphocytes; lymphopoiesis

Interleukin (IL) 7 is a 17.5 kd cytokine produced by a variety of stromal cells, as well as by keratinocytes, dendritic cells, neurons, and endothelial cells but is not produced by lymphocytes.^{1,2} The IL-7 receptor contains a unique 75 kd α chain and a γ chain shared with IL-2, 4, 7, 9, 15, and 21. Homeostatic control of lymphocyte levels in mice seems to be dependent on the cytokine, IL-7. IL-7 receptor knockout mice exhibit thymic atrophy, arrest of T-cell development at the double positive stage, and severe lymphopenia.³ Administration of IL-7 to mice results in an increase in thymic emigrants, increases in B and T cells, and increased recovery of T cells after cyclophosphamide administration or after bone marrow transplantation.^{4–7} Transgenic mice overexpressing IL-7 exhibit expansion of immature B cells and can develop lymphoproliferative disorders.^{8,9}

In vitro studies have suggested a role for IL-7 in human immune function. The addition of IL-7 to neonatal human thymic cultures increases the number of immature and mature T cells.¹⁰ A mutation in the IL-7 receptor α chain in humans leads to an unusual form of combined immunodeficiency disease characterized by severe T-cell defects but normal B cells and natural

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killer cells.¹¹ An inverse relationship exists between circulating numbers of CD4⁺ lymphocytes and IL-7 serum levels in humans.^{1,2} Thus, on the basis of these and other preclinical studies, IL-7 seems to be involved in the homeostasis of both B and T cells in the mouse but only T cells in the human.

Nonhuman primates administered IL-7 showed histologic evidence of hyperplasia in Peyer patches and lymph nodes with increased circulating levels of lymphocytes.¹² We have now assessed the immunologic impact of IL-7 administration in humans and have found that IL-7 results in a rapid and selective increase in circulating CD4⁺ and CD8⁺ lymphocytes.

MATERIALS AND METHODS

Patients

All patients were treated in the Surgery Branch, National Cancer Institute in a protocol approved by the Institutional Review Board, NCI. Eleven patients had metastatic melanoma and one (patient 7, Table 1) had metastatic sarcoma. All patients were HLA-A*0201⁺ and had measurable disease. Eligibility criteria included creatinine <1.4 mg/dL, liver function enzymes <3 times the normal limit, absolute neutrophil count >1000/mm³, absolute lymphocyte count >200/mm³, platelet counts >100,000/mm³, and coagulation parameters <1.5 times the upper limit of normal. Patients were not eligible if they had any form of immunosuppressive disease or had resting blood pressure >140/90.

IL-7

IL-7 (CYT 99-007) was produced according to good manufacturing practice and supplied to the National Cancer Institute under a Cooperative Research and Development Agreement with Cytheris Corp (Issy les Moulineaux, France). IL-7 was purified from inclusion bodies of recombinant *Escherichia coli* containing DNA encoding the human protein. Purity levels were 100% by SE-HPLC and >98.5% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The IL-7 is a non-glycosylated 153 amino acid protein expressing a methionine at the N-terminal position of the natural 152 amino acid human sequence. It was supplied as a lyophilized powder in vials and reconstituted with water for subcutaneous injection.

Treatment

Patients were treated in 4 sequential cohorts of 3 patients each at 3, 10, 30, or 60 µg/kg of IL-7 administered subcutaneously every 3 days for 8 doses. Patients thus received subcutaneous injections on days 0, 3, 6, 9, 12, 15, 18, and 21. Starting on day 0 and repeated on day 7, 14, and 21, patients received the gp100:209–217(210 M) and MART-1:26–35(27 L) peptides emulsified separately in incomplete Freund adjuvant and injected subcutaneously in different extremities. Patients underwent lymphopheresis before the beginning of the protocol and on day 28. Before and on day 28 all patients underwent complete physical examination and radiologic studies to determine the extent of metastatic cancer.

Immunologic Studies

In vitro sensitization boost assays, Elispot assays, and tetramer assays were performed to detect reactivity to the administered peptides as previously described. Serum was obtained at varying intervals for measurements of IL-7 and anti-IL-7 antibody measured by enzyme-linked immunosorbent assay.

Assessment of the impact of IL-7 administration on hematopoietic and lymphoid cells was performed using fluorescence-activated cell analysis (FACS) using commercial antibodies reactive with CD3, CD4, CD8, CD19, CD56, CD127, and other hematopoietic markers as previously described.

Bone Marrow Analysis

Bone marrow aspirates were collected into sterile sodium heparin, prelysed with ammonium chloride, and stained for 30 minutes at room temperature with antibodies directed against CD13, CD33, and CD36 (Beckman-Coulter, Miami, FL); CD3, CD10, CD14, CD16, CD19, CD22, CD34, CD56, CD45, and CD71 (BD Biosciences, San Jose, CA); CD64 (Caltag Laboratories, Burlingame, CA); and CD20, Kappa, Lambda (Dako-Cytomation, Carpinteria, CA). All cells were fixed in 1.0% paraformaldehyde and stored at 4°C for up to 12 hours before acquisition. Four color cytometry was performed with a BD (Becton-Dickinson, San Jose, CA) FACSCalibur flow cytometer. The sensitivity of fluorescent detectors was set and monitored using Calibrite beads (Becton-Dickinson, San Jose, CA) according to the manufacturer's recommendations. Data were analyzed with CellQuest software (BD). Granulocytes, monocytes, mature lymphocytes, nucleated red blood cells, and immature hematopoietic precursors were determined based upon levels of CD45 expression, side scatter, and pattern of antigen expression.

Reverse Transcriptase-polymerase Chain Reaction Measurement of Foxp3 Expression in CD4⁺ T Cells

Peripheral blood mononuclear cells obtained before and on day 28 after treatment were simultaneously thawed and CD4⁺ cells were isolated using a CD4 Negative Isolation kit as per the manufacturer's instructions (DynaL Biotech, product no. 113.17). Total RNA was extracted using an RNeasy mini kit (Qiagen) and RNA was reverse transcribed using the ThermoScript reverse transcriptase-polymerase chain reaction (RT-PCR) system (In Vitrogen Life Technologies). cDNA was amplified using specific primers and probes for Foxp3 and β -actin. Foxp3 was performed using the predeveloped TaqMan Gene Expression Assays from Applied Biosystems. Primers and probes for β -actin were the following: β -actin TaqMan probe 5'FAM-CCAGCCATGTACGTTGCTATCCAGGC-TAMRA-3', forward primer 5'-GCGAGAAGATGATGACCCAGATC-3', and reverse primer 5'-CCAGTGGTACGGCCAGAGG-3'. Real-time RT-PCR was performed using the ABI PRISM 5700 Sequence Detection System (Applied Biosystems). The mRNA for Foxp3 and β -actin were determined by comparing the unknown samples to the standard curves that were generated by using a serial dilution of plasmids that carried Foxp3 and β -actin genes.

Intracellular Detection of Foxp3 Protein by FACS Analysis

Intracellular staining for Foxp3 protein was carried out by using fixation and permeabilization buffers provided by the Foxp3 kit (clone PCH101, eBioscience) according to the manufacturer's instructions, followed by visualization with PE-conjugated or PerCP-conjugated streptavidin antibody.

RESULTS AND DISCUSSION

Twelve patients with metastatic cancer (11 with melanoma and 1 with sarcoma) were treated with 4 different doses of IL-7 in cohorts of 3 patients each along with immunization using 2 melanoma antigen peptides (Table 1). Patients ranged in age from 20 to 59 years. All patients received all 8 planned doses of IL-7 given every 3 days for 21 days.

Serum levels of IL-7 at 24 hours after the first subcutaneous injection showed a direct relationship to the dose of IL-7 injected (Table 1). The calculated half-life of disappearance of IL-7 from the serum in the 3 patients treated at 60 μ g/kg were 12.7, 12.5, and 12.3 hours, respectively. IL-7 levels were undetectable in serum by 72 hours after injection.

The impact of IL-7 administration on changes in the absolute neutrophil and lymphocyte counts is shown in Figure 1A and on CD4⁺ and CD8⁺ lymphocyte subsets in Figure 1B. The absolute

levels of CD3, CD4, CD8, CD19, and CD56 lymphocytes throughout treatment are shown in Table 2. Absolute neutrophil, eosinophil, and basophil counts were unaffected by IL-7 administration although absolute lymphocyte counts as well as the CD4 and CD8 lymphocyte subsets increased in a dose-dependent manner over the 21-day period. Both CD4⁺ and CD8⁺ lymphocytes increased at about the same rate and the CD4/CD8 ratio remained constant (Fig. 1C). No changes were seen at the 3 µg/kg dose, moderate increases in CD4 and CD8 lymphocytes were seen at the 10 µg/kg dose that increased at 30 and 60 µg/kg. At the 60 µg/kg dose, both CD4 and CD8 counts increased 3 to 7-fold by day 21. By day 28, 7 days after the last IL-7 injection at 60 µg/kg, lymphocyte counts were decreasing but still had not reached baseline levels. There was no consistent impact on B cells (CD19⁺) or NK cells (CD56⁺) (Table 2).

Phenotypic analysis of CD4⁺ and CD8⁺ lymphocytes at day 28 from the 3 patients receiving 60 µg/kg IL-7 revealed a trend toward increase of CD45RA⁺ and decrease in CD45RO⁺ cells in both CD4⁺ and CD8⁺ lymphocytes thus suggesting an increase in naive relative to memory cells. These differences were not significant but may become more impressive as additional patients are evaluated. No consistent changes were seen in the percentage of cells expressing HLA-DR and CD25. There was a trend toward decreased expression of CD127 (the IL-7 receptor α chain) at the 60 µg/kg dose.

A subset of CD4⁺ lymphocytes, commonly expressing cell surface CD25, and expressing high levels of Foxp3, has been shown in murine models and in humans to be a potent suppressor of immune function and to play a role in the control of autoimmunity.¹³ These T-regulatory cells can suppress the ability of antitumor T cells to effectively treat established murine cancers.¹⁴ Compelling evidence for a role for Foxp3-expressing T-regulatory cells in humans comes from patients with a mutation in the Foxp3 gene that leads to an X-linked disease characterized by immunodysregulation, polyendocrinopathy, and enteropathy (IPEX syndrome).¹⁵ These patients suffer from autoimmune manifestations in early infancy including the development of auto-antibodies, severe enteropathy, type 1 diabetes mellitus, and hypothyroidism.

Because of the potentially negative impact of the generation of these immunosuppressive Foxp3 expressing T-regulatory cells in humans, we analyzed the expression of Foxp3 on the increased levels of CD4⁺ cells in patients after IL-7 administration (Table 3) (Fig. 2). A decrease in Foxp3 expression in CD4⁺ cells was seen using semi-quantitative RT-PCR measurements in 7 of 8 patients tested. At the 60 µg/kg dose, Foxp3/ β -actin message in CD4⁺ cells was reduced by 29%, 73%, and 47% in the 3 patients, respectively (Table 3). Measurement of intracellular Foxp3 expression by FACS analysis also revealed a decrease in the percentage of CD4⁺ cells expressing Foxp3 that was more marked in patients with the highest levels of increase of CD4⁺ cells (patients treated at 60 µg/kg) (Fig. 2A). After IL-7 administration, these 3 patients showed an increase in CD4⁺ cells but a decrease in Foxp3-expressing cells of 32%, 20%, and 70%, respectively (Fig. 2A and Table 3). Interestingly, Foxp3 expressing cells before IL-7 administration (about 3% to 10% of CD4⁺ cells) did not express CD127, the IL-7 receptor α chain, which helps explain the reduced ability of IL-7 to increase the level of these regulatory cells (Fig. 2B).

These results are in marked contrast to results seen in patients receiving IL-2. IL-2 is a T cell growth factor that is predominantly involved in the generation and survival of T-regulatory cells.¹⁶ Administration of IL-2 leads to significant increases in T-regulatory cells in patients with cancer.^{17,18} Intermittent administration of IL-2 to HIV-positive patients has little, if any, impact on CD8⁺ lymphocytes and seems to increase CD4⁺ cells expressing Foxp3 suggestive of T-regulatory cells.^{19,20} In contrast, IL-7 in our study selectively increased CD8⁺ T cells and CD4⁺ T cells that lacked expression of Foxp3.

The administration of IL-7 was well tolerated in all patients. Except for mild and transient fevers and mild erythema at the injection site few toxicities were seen. One patient treated at 10 µg/kg had a transient grade 3 hypocalcemia probably related to the apheresis procedure and 2 patients (1 at 30 µg/kg and 1 at 60 µg/kg) had a transient grade 3 rise in liver function enzymes that rapidly returned to baseline levels. No grade 4 toxicities were seen. All side effects promptly returned to normal after the IL-7 administration ceased. No patient experienced an objective clinical cancer response.

In this trial, patients also received immunization with 2 melanoma peptides to determine whether immunization might be enhanced by the concomitant administration of IL-7. We have treated over 300 patients with these melanoma peptides alone and have not seen changes in overall levels of CD8⁺ or CD4⁺ cells.^{21,22} The 4 peptide injections administered over 21 days did not result in the generation of immune precursors against the peptides as measured using Elispot, tetramer, or in vitro sensitization assays, which is consistent with our prior experience with this limited immunization in the absence of IL-7 administration. The immunocompetence of these patients, however, was demonstrated by their ability to react against influenza peptide, a memory response that was not affected by IL-7 administration at any dose (data not shown). Thus, in this limited study, we could derive no evidence that the simultaneous administration of peptides plus IL-7 increased immune reactivity against class 1 restricted peptides.

In contrast to results in murine models, we saw little change in circulating B-cell levels in patients although there was a suggestion of a decrease at the 60 µg/kg IL-7 dose. However, because of the proliferative impact of IL-7 on B cells in mice and the lymphoproliferative disorders seen in IL-7 transgenic mice,^{8,9} we performed bone marrow biopsies before and after treatment in the 6 patients receiving 30 or 60 µg/kg of IL-7. In 2 of the 3 patients treated at 30 µg/kg, CD19⁺CD10⁺ progenitor B cells increased from 3.8% and 3.4% of marrow mononuclear cells at baseline to 30% and 15%, respectively. In 2 of the 3 patients receiving a dose of 60 µg/kg, the progenitor B cells increased from 8% and 3% to 44% and 8%, respectively. These B cells were normal maturing polyclonal B cells. In the other 3 patients, B-cell progenitor levels did not change. There was no evidence of hematopoietic neoplasms. Thus, in humans, IL-7 administration led to expansion of B-cell progenitors in the bone marrow of some patients that was not reflected in the circulation. Mature T cells were increased in the bone marrow of all 6 patients evaluated.

The IL-7 administered to these patients was produced in *Escherichia coli* and thus did not contain the normal glycosylation seen in eukaryotic IL-7. Because of the possibility that nonglycosylated IL-7 might be immunogenic in humans, we measured anti-IL-7 antibody titers before and after IL-7 administration (Table 1). All patients receiving 10 to 60 µg/kg of IL-7 developed low titer binding antibodies detected by enzyme-linked immunosorbent assay by day 28, seven days after the last injection of IL-7. However, when tested in a specific bioassay to measure neutralization potential, sera from several patients were positive but none of these sera reached the positive threshold defined within the protocol (>1/400 by day 28 or at the last measure at day 56). Furthermore, these antibodies did not seem to affect circulating lymphocyte levels because these levels remained normal with prolonged follow-up of patients as they proceed to other treatments for their cancer (data not shown). Nevertheless, a new formulation of fully glycosylated IL-7 is currently being produced in eukaryotic cells for future studies, that will enable us to explore the effect of higher doses and/or more prolonged treatments by repeated cycles of IL-7 injections.

The studies reported here demonstrate that IL-7 is a potent lymphopoietic factor in humans and has substantial potential for use in the treatment of patients developing lymphopenia from HIV infection or from chemotherapy used in cancer treatment. The selective increase in non-T-regulatory CD4⁺ T cells represents a significant advantage of the use of this cytokine.

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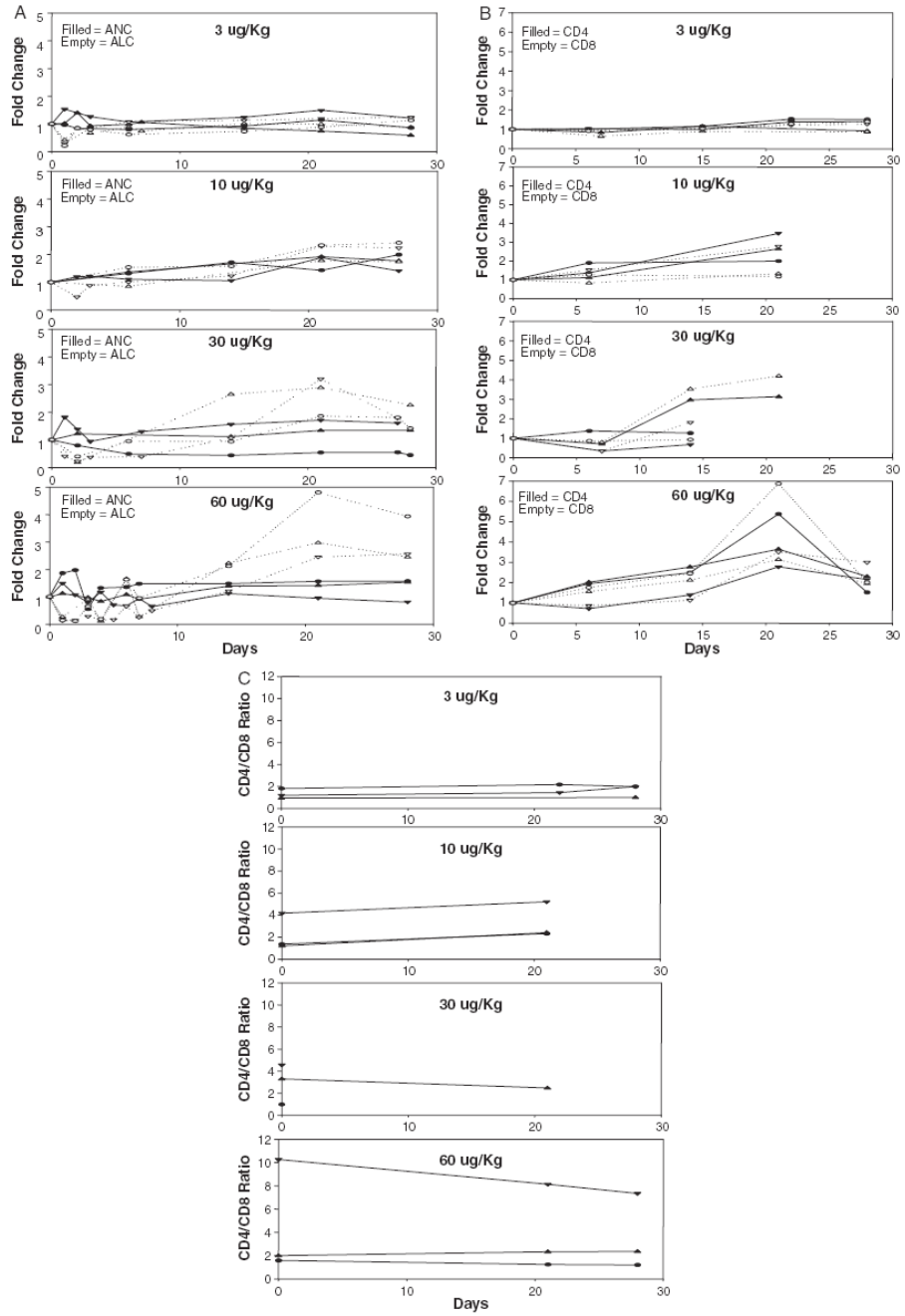


FIGURE 1. Changes in circulating levels of hematopoietic cells in patients receiving IL-7. Each line represents an individual patient. A, Absolute neutrophil (filled symbols) and absolute lymphocyte (empty symbols) counts. B, CD4⁺ (filled symbols) and CD8⁺ (empty symbols) cells. C, CD4/CD8 ratio for each patient.

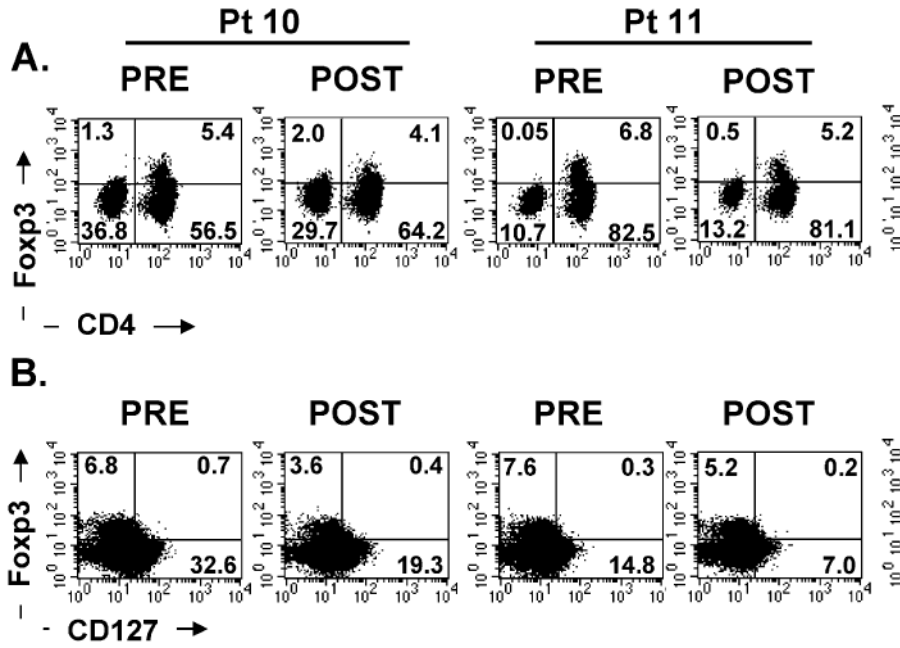


FIGURE 2. Peripheral blood mononuclear cells from 3 patients (60 $\mu\text{g}/\text{kg}$ dose) (A) were stained with fluorescein isothiocyanate-conjugated CD4, PerCP-conjugated CD3, and APC-conjugated CD25 antibodies followed by intracellular staining with biotinylated Foxp3 antibody. The dot plots were gated on CD3⁺ T lymphocytes. B, Peripheral blood mononuclear cells from these patients were also stained with fluorescein isothiocyanate-conjugated CD4, PE-conjugated CD25, and antigen presenting cells-conjugated CD127 (IL-7R α chain) followed by intracellular staining for Foxp3 protein. The dot plots were gated on CD3⁺CD4⁺ T cells. The quadrants were set based on isotype control antibodies as well as negative control, and the numbers represent the percentage of cells in each quadrant.

TABLE 1

Patient Characteristics

Patient [†]	IL-7 Dose (µg/kg)	Age/Sex	Prior Treatment [‡]	Serum IL-7 Levels* (pg/mL)			Serum Neutralizing Antibody	
				Pre	24 h	48 h	Pre	Day 28
1	3	42/F	S,I	—	—	—	0	0
2	3	43/F	S,C,I	—	—	—	0	0
3	3	20/F	S,I	—	—	—	0	0
4	10	66/F	S,C,I	0	64.5	0	0	0
5	10	67/M	S,I	0	51.3	0	0	0
6	10	45/M	S,C,I	0	53.9	0	0	0
7	30	58/M	S	0	194	0	0	1:100
8	30	59/M	S	0	809	275	0	1:100
9	30	49/F	S,I	0	102	0	0	0
10	60	51/M	S	0	622	14.3	0	1:200
11	60	57/M	S	0	497	183	0	1:100
12	60	33/M	S	0	946	52.6	0	1:50

* The limit of detection in this assay was 12.5 pg/mL.

[†] All patients had metastatic melanoma except for patient 7 with metastatic sarcoma.

[‡] S indicates surgery; C, chemotherapy; I, immunotherapy.

TABLE 2

Hematologic Effects of IL-7 Administration

Patient	IL-7 Dose (µg/ kg)	Day	Neutrophils	Total	Lymphocytes					
					CD3	CD4 (cells/µL)	CD8	CD19	CD56	
1	3	0	7763	2473	1029	1080	50	198		
		21	5715	2448	ND	ND	ND	ND		
		28	4598	2166	2003	944	41	121		
2	3	0	2247	2279	1876	800	245	193		
		21	3351	2718	2384	969	141	200		
		28	2843	2610	2425	1016	217	222		
3	3	0	4103	2180	1549	537	344	345		
		22	6365	2798	2193	1494	325	317		
		28	4822	3569	2247	745	487	367		
4	10	-1	2261	1931	1479	815	302	104		
		27	4332	3435	3055	890	213	154		
		27	4000	3368	ND	ND	ND	ND		
		2826	1814	1065	199	265	410	616		
5	10	0	5354	4178	3458	554	143			
		21	3989	4038	ND	ND	ND	ND		
		27	2531	1080	511	374	27	173		
6	10	-1	3614	2513	1499	44	111	183		
		21	5040	2609	1024	ND	ND	ND		
		27	3764	1525	891	271	145	167		
7	30	0	5058	4404	3981	1138	172	280		
		21	5090	3450	ND	ND	ND	ND		
		28	3342	1258	802	176	101	186		
8	30	0	5544	3949	963	ND	ND	ND		
		21	5221	2203	ND	ND	ND	ND		
		27	2696	2661	1027	1051	48	99		
9	30	0	1450	4949	2155	ND	ND	ND		
		21	1215	3791	ND	ND	ND	ND		
		28	3461	1179	587	293	164	126		
10	60	-1	4923	3519	3182	921	82	237		
		21	5346	2905	1979	572	105	124		
		28	4200	1757	1346	118	305	78		
11	60	-1	4004	4301	3861	414	127	246		
		21	3387	4503	2956	354	109	159		
		28	3769	2053	903	568	65	176		
12	60	-1	5881	9853	1778	3910	44	240		
		21	5915	8076	2822	1139	22	47		

ND indicates not done.

TABLE 3
T-Regulatory Cell Markers in Patients Receiving IL-7

Patient	Dose of IL-7 ($\mu\text{g}/\text{kg}$)	Semiquantative RT-PCR		FACS Analysis	
		Pre	Day 28 (Foxp3/106 β actin)	Pre (% of CD4 ⁺ cells Expressing Foxp3)	Day 28
1	3	2217	1332 (-40%)	11.05	9.96 (-10%)
3	3	2289	1391 (-39%)	8.64	9.25 (+7%)
5	10	2323	1418 (-39%)	5.94	4.16 (-30%)
6	10	1340	1527 (+14%)	6.38	5.20 (-18%)
7	30	582	253 (-57%)	3.15	1.91 (-39%)
10	60	1219	985 (-29%)	8.68	6.05 (-32%)
11	60	1337	365 (-73%)	7.58	6.06 (-20%)
12	60	1501	801 (-47%)	5.31	1.57 (-70%)