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Regulatory effects of interleukin-7 on renal tumor infiltrating lymphocytes

P. Ditonno*, C. L. Tso, T. Sakata, J. B. de Kernion, and A. Belldegrun

Immunotherapy Laboratory, Division of Urology, UCLA School of Medicine, and the Jonsson Comprehensive Cancer Center, Los Angeles, California, USA

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Summary. Biological therapy using a combination of lymphokine and tumor infiltrating lymphocytes (TILs) is a new approach to the treatment of patients with advanced cancer. To improve the potency of TILs, new cytokines with T-cell stimulatory effects used alone or in combination with interleukin-2 (IL-2) are currently being investigated. We have studied the effect of interleukin-7 (IL-7) on TILs derived from renal cell carcinoma. Our data demonstrated that five of ten TILs proliferated in response to IL-7 alone. This proliferative response was 73–90% less than that obtained with IL-2 alone. The use of IL-7 plus IL-2 resulted in a 1.2- to 4.7-fold increase in proliferation of six of ten TILs compared with IL-2 alone. IL-7-driven TIL growth was consistently blocked by anti-IL-2, anti-IL-2R and anti-IL-7 antibodies (37.2%, 41.6% and 82.2% suppression, respectively). The expression of IL-2 receptors was also significantly increased in the presence of IL-7 or IL-7 phytohemagglutinin (40.6 + 3.8 and 72.5 + 1.5). In comparison with IL-2, IL-7 treatment was associated with a decrease in CD56 (46.3% ± 19 vs 10% ± 4.9) and increase in CD3 (29.3% ± 12 vs 73% ± 6.4) and CD4 (19.3% ± 15 vs 58% ± 10). These studies suggest that in some renal TILs, IL-7 and IL-2 can have a synergistic proliferative effect. The IL-7 stimulatory effect appears to be mediated via both an IL-2 pathway and an IL-7-independent pathway.

Key words: Tumor infiltrating lymphocyte – Interleukin-7 – Renal cell carcinoma

Exogenous recombinant interleukin-2 (rIL-2) supplementation has been used in vitro for tumor infiltrating lymphocyte (TIL) expansion and in vivo as a systemic adjuvant to enhance TIL activity. The high toxicity of interleukin-2 (IL-2) and the low antitumor specificity of renal TILs are the main drawbacks of TIL immunotherapy [3, 4, 12]. New cytokines, alone or in combination with IL-2, have been tested as a means of improving the therapeutic efficacy and reducing the toxicity of IL-2 [16].

IL-7 is a cytokine involved in the regulation of lymphoid cell maturation and proliferation [10]. Initially, IL-7 was characterized as a pre-B-cell growth factor [15]. More recently it has been demonstrated that IL-7 can influence the proliferation and differentiation of T cells. Adult resting thymocytes, including double-negative and single-positive subsets, were stimulated to grow in response to IL-7 [7]. Fetal thymocytes also proliferated in response to IL-7 [20]. Concavalin A stimulated mature T lymphocytes to proliferate in the presence of IL-7 with increased expression of IL-2R and production of IL-2 [14]. In another assay, IL-7 in association with phorbol myristate acetate (PMA) stimulated mature T cells to proliferate in a partial IL-2-dependent pathway [6]. IL-7 was able to generate cytolytic T lymphocytes and activated killer cells from peripheral blood mononuclear cells [1] and from thymocytes [5]. Owing to these multiple activities of IL-7 on T lymphocytes, we have investigated the effects of IL-7 on in vitro proliferation and the phenotype of TILs originated from renal cell carcinoma (RCC) as a first step in the evaluation of the role of IL-7 in TIL immunotherapy.

Materials and methods

Human TIL preparation

Methods for growing TILs have been published in detail elsewhere [18]. Briefly, ten consecutive renal tumors obtained at surgery were minced into small pieces (0.5 cm^3) and digested overnight in RPMI 1640 culture medium (Biofluids, Rockville, Md.) containing 0.01% hyaluronidase type V, 0.002% DNase type I, 0.1% collagenase type IV (Sigma, St. Louis, Mo.) and 50 mg/ml gentamicin (Biofluids, Rockville, Md.). The cell suspension obtained was washed twice in HBSS (Biofluids) and the tumor and mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation (LSM, Litten Bionetics, Charleston, S. C.). After Ficoll separation, two washings with HBSS and a final washing with AIM-V (Gibco, Chagrin Falls, Ohio) or 10% AB serum supplemented RPMI were performed.

Single tumor/TIL cell suspensions were diluted in culture medium at 2.5–5.0 × 10^5 viable cells/ml. (The number of viable cells

* *Present address:* Division of Surgical Nephrology, University of Bari, Bari, Italy

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Table 1. Interleukin-7 (IL-7) induces proliferation of tumor infiltrating lymphocytes (TILs) alone or in combination with phytohemagglutinin (PHA) or anti-CD3 mAb

TIL no.	IL-2	IL-7	IL-7 + PHA	IL-7 + anti-CD3 Mab	Medium
1	5175 (468)	NG	3076 (1185)	NG	107 (28)
2	9843 (1593)	NG	NG	NG	103 (26)
3	10863 (1037)	3150 (1110)	1854 (665)	1105 (82)	60 (10)
4	9568 (1037)	NG	NG	NG	82 (9)
5	4091 (892)	NG	2452 (327)	1725 (278)	55 (5)
6	10770 (4523)	1025 (254)	N/A	N/A	65 (28)
7	3702 (1105)	NG	2443 (23)	NG	184 (32)
8	6929 (2660)	1379 (302)	N/A	NG	106 (43)
9	11792 (528)	1307 (198)	4558 (454)	2147 (137)	105 (43)
10	16807 (1209)	1615 (324)	N/A	N/A	98 (29)

Human rIL-2 and rIL-7 were used at 10 ng/ml, PHA at 0.1 μg/ml and anti-CD3 mAb at a dose of 5 U/ml. N/A, Not available; NG, no growth. Experiments were performed at 3–4 weeks after tumor extraction. All experiments were done in triplicate. Datas are expressed as cpm (SD)

was determined using trypan blue dye exclusion.) Bulk cultures were maintained in 175 cm^2 flasks, lying flat at 37°C, in 5% CO_2. Cultures were supplemented with fresh medium and fresh cytokines on a weekly basis. TIL cultures were distributed in 5-ml aliquots in 6-well culture plates (Costar, Cambridge, Mass.) for experimental purposes.

Separation of CD4+ and CD8+ cell subpopulations

Bulk cultures, following cell expansion, were incubated in 275 cm^2 CELLctor flasks coated with anti-CD8 IgG (AIS, Menlo Park, Calif.). Following 1 h incubation at room temperature, nonadherent cells were separated by several washings with 10 ml HBSS. Adherent cells were expanded in rIL-2 supplemented AIM-V or 10% AB serum supplemented RPMI 1640. The cells were recovered from the capture flask after 3 days.

Cytokines

Human rIL-2 was kindly provided by Hoffman-La Roche (Nutley, N.J.). rIL-2 specific activity was 1.2×10^7 NU/mg with a protein content (IL-2 + human serum albumin) of 25.6 mg/vial. rIL-2 was used at a final concentration of 10 ng/ml. Human rIL-4 was kindly provided by Immunex (Seattle, Wash.). Purity was above 98%, specific activity $1.8 \pm 0.4 \times 10^7$ U/mg and protein concentration 97.4 μg/vial. rIL-4 was used at a final concentration of 400 U/ml. Human rIL-6 was kindly provided by Interpharm Laboratories, Inc. (Ness Ziona, Israel). The specific activity of IL-6 was 10×10^6 U/mg and the protein concentration 296 μg/ml. rIL-6 was used at a final concentration of 5 U/ml. Human rIL-7 was kindly provided by Sterling (Malvern, Pa.). The specific activity of IL-7 was 4.0×10^7 U/mg protein and the protein concentration 0.76 mg/ml. rIL-7 was used at a final concentration of 10 ng/ml. Tumor necrosis factor alpha (α-TNF) and γ-interferon (γ-IFN) were kindly provided by Genentech (San Francisco, Calif.). The specific activity of α-TNF was 27×10^6 U/mg and the protein concentration 1.25/ml. α-TNF was used at a final concentration of 200 U/ml. The specific activity of γ-IFN was 4×10^7 U/ml and the protein concentration 0.99 mg/ml; it was used at a final concentration of 500 U/ml.

Neutralizing antibodies

The anti-IL-2 polyclonal and anti-IL-2R monoclonal antibodies were purchased from Genzyme (Boston, Mass.). Anti-IL-7 polyclonal antibody, a heat-hyperimmune rabbit serum, was kindly provided by Sterling.

Proliferation assays

The medium used for the cultures was AIM-V or 10% AB serum supplemented RPMI. TILs were cultured at 2×10^4 cells/well in 200 μl culture medium. Cultures were pulsed with 1 μ Ci/well of thymidine (75 Ci/mmol) during the last 12 h of a 72 h culture period and then harvested onto glass fiber filters. Radioactivity incorporation was measured by liquid scintillation spectometry.

Flow cytometry

TIL were washed with cold staining medium (HBSS without phenol red, with 5% heat-inactivated fetal calf serum and 0.02% sodium azide) and resuspended at a concentration of 5×10^5 to 1×10^5 cells/ml. Undiluted fluorescein isothiocyanate (FITC) – conjugated monoconal antibodies to TILs were added to 100 μl volumes of cell suspension at a concentration of 5%. The antibodies used included CD3 (pan T cells), CD4 (T helper cells), CD8 (T cytotoxic/suppressor cells), CD56 (NK marker) and CD25 (IL-2 receptor). After staining for 30–60 min at 4°C, cells were washed with staining medium and fixed with 1% paraformaldehyde, washed again and resuspended in 0.5 ml of staining medium. Cell surface antigens were detected using flow cytometry on a FACS 440 (Becton Dickinson, Mountain View, Calif.).

Statistical analysis

All experimental samples were plated in triplicate. Datas are expressed as the mean ± 1 SD and Student's *t*-test was used to determine whether two experimental values were significantly different.

Results

IL-7 induced proliferation of renal TILs

Five out of ten TIL sets proliferated in response to IL-7. This proliferative response was between 90.4% and 71.1% less than that obtained with IL-2. When a suboptimal dose of phytohemagglutinin (PHA; 0.1 μg/ml) was added to IL-7-supplemented cultures, three TILs proliferated that had not show any growth in the presence of IL-7 alone. Considering the proliferative response of all TIL popula-

Table 2. TIL proliferation in the presence of cytokine combinations

TIL no.	IL-2	IL-7	IL-2 + IL-7	IL-4 + IL-7	Medium
1	5175 (468)	NG	7757 (1239)	1115 (125)	107 (28)
2	9843 (1593)	NG	16940 (4968)	1156 (425)	103 (26)
3	10863 (1037)	3150 (1110)	5295 (1806)	1492 (484)	60 (10)
4	9568 (1037)	NG	11153 (3842)	NG	82 (9)
5	4091 (892)	NG	19268 (1176)	2123 (383)	55 (5)
6	10770 (4523)	1025 (254)	27648 (455)	1322 (88)	65 (28)
7	3702 (1105)	NG	2943 (406)	NG	184 (32)
8	6929 (2660)	1379 (302)	19545 (6415)	NG	106 (43)
9	11792 (528)	1307 (198)	7682 (2192)	1422 (244)	105 (43)
10	16807 (1209)	1615 (324)	16052 (2653)	N/A	98 (29)

TILs were cultured with IL-7 (10 ng/ml), IL-2 (10 ng/ml) and IL-4 (400 U/ml). Experiments were performed at 3–4 weeks after tumor extraction. All experiments were done in triplicate. N/A, Not available; NG, no growth. Datas are expressed as cpm (SD)

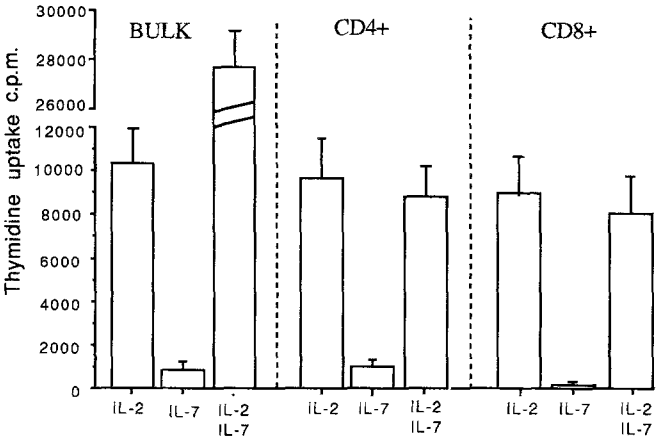

Fig. 1. The effect of interleukin-7 (IL-7) on proliferation of CD4+ and CD8+ tumor infiltrating lymphocyte (TIL) subsets. Bulk cultures, following cell expansion, were separated with an anti-CD8 IgG-coated flask. After a 5-day period of resting in AIM-V medium, TIL subsets were cultured in the presence of either rIL-7, rIL-2 or rIL-2 + rIL-7. After 1 week of growth, TIL samples were transferred to 96-well flat-bottomed microtiter plates at 2×10^4 cells/well in 200 µl culture medium. Cultures were pulsed with 1 µ Ci/well of thymidine (75 Ci/mmol) during the last 12 h of a 72 h culture period and then harvested

tions, IL-7 + PHA stimulated a higher level of thymidine incorporation than did IL-7 alone. This proliferative difference was close to significance (P:0.08). TILs were also primed with anti-CD3/TCR mAb and evaluated for responsiveness to IL-7. Only three of eight TILs proliferated, with no statistically significant change in thymidine incorporation with respect to those grown in IL-7 alone (Table 1).

To verify whether IL-7 was active on a particular subset, we separated TILs in CD4+ and CD8+ subpopulations with an anti-CD8 IgG-coated flask. Cell purity was 99%. At 1 week after plating, very low thymidine incorporation could be recorded in CD8+ TILs grown in IL-7 alone, and no statistical difference from TILs grown in medium alone could be observed. IL-7 proliferation of CD4+ TILs appeared to promote a better growth than bulk cultures grown in IL-7 (not significant). In bulk cultures the combination of IL-2 + IL-7 appeared to have an additional proliferative effect with respect to cultures supplemented with IL-2 alone. This difference in proliferation could not be observed in CD4+ and CD8+ TIL subsets (Fig. 1).

The combination of IL-2 and IL-7 had a synergistic proliferative effect

All the TILs tested with IL-7 in combination with IL-2 proliferated. Of these, six out of ten TILs showed a thymidine incorporation higher than the respective combined values of thymidine incorporation in the presence of IL-2 and IL-7 separately. In these TILs, a 1.2- to 4.7-fold increase in thymidine incorporation was observed ($P<0.05$). However, when we took all the TIL populations into account, this statistical significance was not reached. No correlation was found between TILs which responded to IL-7 and those which responded synergistically to IL-2 + IL-7.

IL-7 in combination with IL-4 supported proliferation in five of nine TIL bulk cultures, but the thymidine incorporation was comparable to that obtained with IL-7 alone (Table 2).

IL-7 increased IL-2R expression

The level of IL-2R expression was examined by flow cytometry with FITC-conjugated monoclonal antibodies (CD25). IL-7 alone, but especially in combination with PHA or an anti-CD3, induced the expression of IL-2R in 40.6% ± 3.8, 72.5% ± 1.5 and 88.5% ± 9.5, respectively. The level of expression of IL-2R in cultures supplemented with exogenous IL-2 or IL-4 was significantly lower than that of IL-7 cultures (Fig. 2).

IL-7 proliferation is partially influenced by blocking the IL-2 proliferative pathway

In TIL cultures that responded to IL-7, an anti-IL-2 polyclonal and anti-*Tac* monoclonal antibodies were used

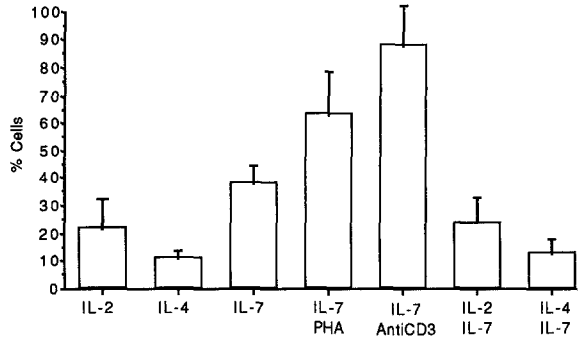

Fig. 2. IL-2R expression in TILs grown in different cytokine combinations. Phenotype analysis was done 3–4 weeks after the extraction of TILs from the tumor

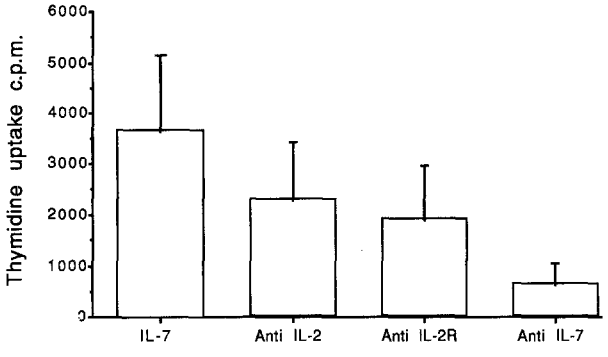

Fig. 3. Anti-IL-2 polyclonal and anti-monoclonal IL-2R antibodies partly inhibited the IL-7-driven TIL proliferation. This represents three TILs. Only anti-IL-2R inhibition was statistically significant ($P<0.05$). Anti-IL-7 polyclonal antibody caused 82.2% suppression in blocking experiments. Anti-IL-2 and anti-IL-2R (*Tac*) antibodies caused 37.2% and 41.6% suppression, respectively. Anti-IL-7 polyclonal antibodies caused 82.2% suppression (Fig. 3). Only anti-IL-2R and anti-IL-7 polyclonal antibody inhibition was statistically significant ($P<0.05$).

IL-7 induced phenotypical changes in TILs

At 3 weeks after TIL extraction and plating, IL-7 caused significant changes in the phenotype of TILs compared to IL-2, decreasing the number of CD56-positive cells (10% ± 4.9 vs 46.3% ± 19) and increasing the number of CD3-positive cells (73% ± 6.4 vs 29.3% ± 12) with CD4 dominance (59.5% ± 10 vs 14.5% ± 4.5). This IL-7 activity was similar but greater than that obtained with the administration of IL-4. However, in association with IL-2, the phenotypical changes obtained with IL-7 were lost (Fig. 4).

Discussion

Our results demonstrate that IL-7 is a growth factor for some TILs derived from RCC. Five out of ten TILs responded to IL-7 alone. In those TILs that responded, IL-7 stimulated a lower level of thymidine incorporation than did IL-2 [19]. The heterogeneous response of TIL to IL-7 can be attributed to several causes. Firstly, the different cell composition of the TIL population with differentiated sensibility to the action of IL-7 can be

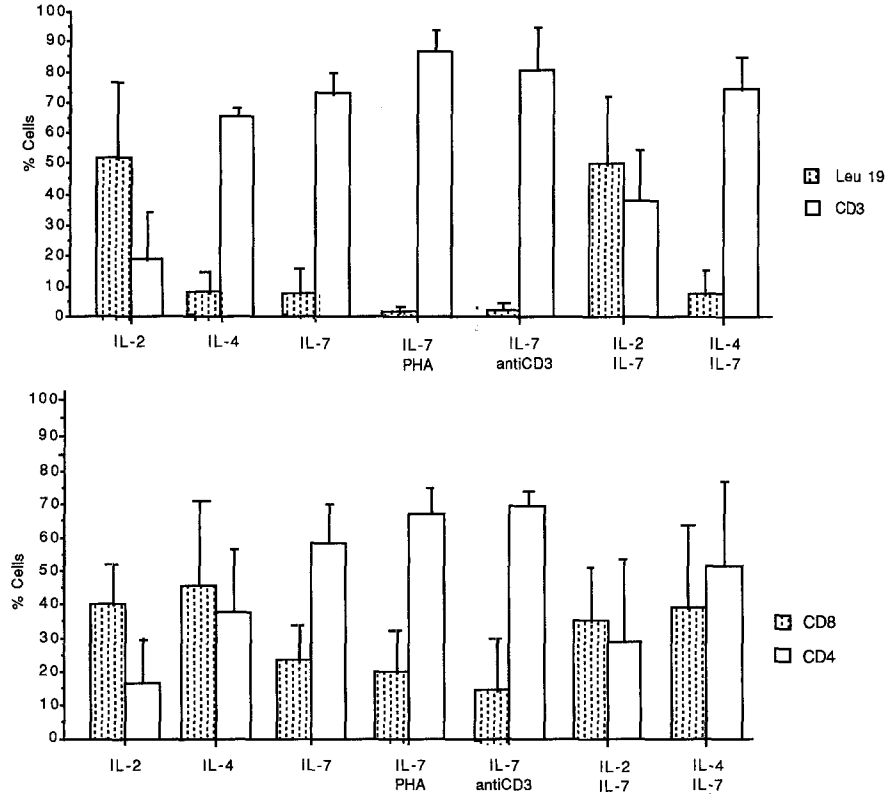

Fig. 4. CD56/CD3 *(above)* and CD4/CD8 *(below)* phenotype expression in different culture conditions. This represents four TILs. Experiments were done 3–4 weeks after tumor extraction. TIL samples were analyzed as described in Materials and methods

accounted for. In fact, in the presence of 10 ng/ml IL-7, a better proliferative response of CD4+ and bulk cultures than CD8+ cultures was observed. Secondly, inhibitory factors secreted by the renal tumor might be involved. For example, transforming growth factor (TFG)-beta, which has a wide inhibitory activity on cell proliferation and cytokine functions [17], is highly expressed in renal tumors [8]. Lee et al. [11] reported that TGF-beta can inhibit the IL-7 proliferative activity of pre-B cells.

The addition of a suboptimal dose of PHA induced the proliferation of TILs which were unresponsive to IL-7 stimulus alone. This costimulatory activity can be attributed to the fact that PHA binds to several receptor molecules on the T-cell membrane, including the T cell receptor (TCR) [2], and thus, activating an unknown signal pathway, which enhances cell proliferation. However, when we stimulated the TCR with anti-CD3 mAb, only three TILs proliferated in response to IL-7, and the thymidine incorporation was substantially lower than that obtained with the costimulatory activity of PHA. It is possible that, besides the activation of the CD3/TCR complex, additional signals or additional cytokines are required to render TIL responsive to the IL-7 stimulus. These signals appear to be provided by the costimulatory activity of PHA or other mitogens.

In view of previous experiments showing that IL-7 induces the expression of IL-2R on the T-cell membrane [2, 9], phenotypical analysis and blocking studies to determine the involvement of the IL-2 pathway on IL-7-driven TIL proliferation were performed. TIL proliferation in IL-7-supplemented cultures was partly blocked by anti-IL-2 polyclonal and anti-IL-2R monoclonal antibodies. These blocking studies and the higher frequency of IL-2R+ cells in cultures stimulated with IL-7, alone or in combination with PHA, substantiated a partial dependence of IL-7 TIL proliferation on the activation of the IL-2 pathway. Our data are consistent with the report of Morrisey et al. [14] that IL-7 stimulation of concanavalin A activated T cells is associated with the production of IL-2 and IL-2R expression. This partial dependence on IL-2 in our assay can be explained by the fact that endogenous IL-2 is produced by T cells when they are activated by an antigenic stimulus, as occurs in TIL cultures. Alderson et al. [1] also noted that the IL-7 generation of cytotoxic T lymphocytes in mixed lymphocyte culture (and therefore in the presence of antigenic stimulus) is partly dependent on the IL-2 pathway. The combination of IL-2 with IL-7 cultures increased the thymidine incorporation in all TILs tested and was 10-fold greater than that with IL-7 alone. Moreover, six out of ten TILs showed an additional proliferative response in the presence of IL-2 + IL-7 as compared with IL-2 alone. These data were confirmed in bulk cultures only, while no proliferative differences in CD4+ and CD8+ TILs could be found.

Other cytokine combinations (IL-7 + IL-4, IL-7 + IL-6, IL-7 + TNF, IL-7 + γ-IFN) did not yield any proliferative enhancement with respect to IL-7 alone (data not shown).

Interesting phenotypical modifications were observed in TIL bulk cultures. At 3 weeks of plating, all the TILs that responded to IL-7 showed a depletion of CD56+ cells with a predominant expression of CD3+ cells. This pattern was similar to, but more evident in magnitude than, that obtained with exogenous IL-4. Moreover, in the CD3+ cell population the CD4+ cells outnumbered the CD8+ cells. These data contrast with those of Welch et al. [21], who reported no change in the ratio between CD4+ and CD8+ cells when cultured for 3 days with 1000 U/ml of IL-7 alone or in combination with PHA. Our assay, using only 10 ng/ml IL-7 (90 U/ml), could have been responsible for a more selective proliferation of some T-cell subsets, while at higher doses IL-7 probably supports a more widespread cell growth.

A recent report has pointed out the absence of side effects when IL-7 was injected in high doses in a mouse model for up to a month [13]. If this is confirmed in humans, IL-7 in combination with IL-2 may have a role in supporting TIL survival and proliferation in vivo. Smaller doses of IL-2 in combination with IL-7 should be sufficient to obtain a cell expansion comparable to that obtained with high doses of IL-2 alone, and thus decreasing the IL-2 toxic side effects.

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Arie Belldegrun, MD
Division of Urology
UCLA School of Medicine
10883 Le Conte Avenue
Los Angeles, CA 90024-1738
USA