Differential Quantitative Effects of Interleukin (IL)-2 and IL-15 on Cytotoxic Activity and Proliferation by Lymphocytes from Patients Receiving in Vivo IL-2 Therapy


ABSTRACT

Lymphocytes from patients receiving in vivo interleukin (IL)-2 therapy possess enhanced in vitro proliferative and cytotoxic responses to IL-2. The cells from these patients that respond to exogenous IL-2 are CD56+ natural killer cells expressing intermediate-affinity IL-2 receptor βγc complexes. Because IL-15 activates cells via these same βγc receptors, we hypothesized that IL-15 would also activate lymphocytes from patients treated with in vivo IL-2 therapy and therefore that IL-15 might potentially be useful as an immunotherapeutic agent alone or in combination with IL-2. We report here that peripheral blood mononuclear cells (PBMCs) from patients receiving in vivo IL-2 therapy do proliferate in response to IL-15. However, a greater dose of IL-15 is needed to reach the same level of proliferation stimulated by IL-2. The EC50 for IL-2 is 0.04 ± 0.16 nM (mean ± SE; n = 18), whereas the EC50 for IL-15-stimulated proliferation is 1.16 ± 0.16 nM (n = 18). In contrast to the proliferative response, equivalent doses of IL-2 and IL-15 stimulate patient PBMCs to mediate similar levels of cytotoxicity against Daudi, K562, and LA-N-5 tumor targets. Notably, low concentrations of IL-15 that do not stimulate a substantial proliferative response (e.g., 1.0 ng/ml) do boost PBMCs to mediate cytotoxicity against these tumor targets. These distinct dose-response curves for proliferation compared to cytotoxicity suggest that IL-15 should be evaluated for its potential as an immunotherapeutic agent to treat cancer, particularly in regimens providing doses that might minimize the proliferative response (associated with cytokine release and toxic side effects) while maintaining the cytolytic antitumor response.

INTRODUCTION

Although a consistent but small fraction (10–20%) of renal cell carcinoma and melanoma patients treated with IL-2 experience antitumor responses (1, 2), all patients have dose-limiting side effects that appear to be mediated against normal tissues (such as endothelial cells) by IL-2-activated lymphocytes and by cytokines released by these lymphocytes (3–6). The major toxicity of IL-2 therapy is a vascular leak syndrome that results in widespread tissue edema and can lead to organ failure (5–7). Functional and molecular studies of PBMCs from these IL-2-treated patients have been undertaken in an attempt to elucidate the mechanisms of in vivo activation, toxicity, and antitumor effects induced by this treatment.

Following IL-2 treatment, the absolute number of all lymphocytes increases in the peripheral blood, and CD56+ NK cells are particularly expanded (1). Expression of the IL-2Rα chain on lymphocytes also increases, although this reflects increased IL-2Rα expression by CD3+ T cells, as opposed to CD56+ NK cells. In fact, CD56+ NK cells activated by in vivo IL-2 are found to express only the intermediate-affinity IL-2R complex (βγc) and not IL-2Rα chains (8). PBMCs from IL-2-treated patients mediate enhanced cytotoxic and proliferative responses measured in vitro when compared to pretreatment lymphocytes or PBMCs from healthy donors (1, 9, 10). These cytotoxic and proliferative responses by PBMCs from IL-2-treated patients are boosted further in vitro in the presence of exogenous IL-2. Paradoxically, circulating CD3+ T cells become less responsive to mitogenic stimuli after treatment with in vivo IL-2 (11). These CD3+ lymphocytes have up-regulated expression of IL-2Rα chains and secrete IL-2Rα, although they have low levels of the signaling IL-2Rβ subunit on the cell surface as measured by flow cytometry, which may partially explain their relative inability to respond to IL-2 (12).

Because of the limited number of patients experiencing antitumor responses following IL-2 therapy and the potentially severe dose-limiting side effects, clinical investigators continue to search for new immunological approaches to treat cancer, including the search for other cytokines that may be more efficacious than IL-2. IL-15 has several functional similarities with IL-2, as well as substantial differences, to merit examination of its potential as an immunotherapeutic agent. Both IL-2 and IL-15 are known to stimulate proliferation of T cells, B cells, and NK cells, as well as cytotoxicity by NK cells (13–16).
Unlike IL-2, IL-15 is produced primarily by activated monocytes rather than by T cells and appears to have a greater influence on some NK cells than IL-2 does (17). These facts, along with the suggestion that IL-15 may potentially have certain advantages over IL-2 in the immunotherapy of cancer. Because IL-2 and IL-15 share the use of the IL-2Rβ and γc subunits for binding and signaling, IL-15 might be able to activate a patient's NK cells, which bear intermediate-affinity IL-2R (βγc), without substantial activation of T cells bearing high-affinity IL-2R (αβγc). IFN-γ secreted by T cells and TNF-α secreted by monocytes are two of the cytokines primarily implicated in promoting the capillary leak syndrome associated with IL-2 therapy (19–22). Because some of the dose-related side effects are mediated via cytokines secreted by activated T cells, IL-15 might be expected to induce fewer side effects than IL-2, while maintaining the ability to activate a cytotoxic NK cell response against tumor cells.

The antitumor efficacy of IL-15 in conjunction with cytokine secretion has been addressed previously in the context of TILs. Lewko et al. (23) reported that TILs could be expanded in vitro by IL-15, although not as well as when stimulated by IL-2. However, addition of either IL-2 or IL-15 resulted in elimination of tumor cells in these in vitro TIL cultures (23). Interestingly, IL-15 did not activate TNF-α secretion in these TIL cultures, whereas high-dose IL-2 boosted TNF-α secretion 10-fold over background (23). Low-dose IL-2 and IL-15 in combination had an inhibitory effect on IFN-γ and GM-CSF secretion, whereas high-dose IL-2 and IL-15 had a synergistic effect on TNF-α secretion (23).

Although these effects of IL-15 on cytokine secretion suggest that certain concentrations of IL-15 might promote less vascular leak in vivo compared to IL-2, further studies examined this question more directly. Additional evidence in vivo indicates that IL-15 activates less pulmonary vascular leak than a comparable dose of IL-2 does when tested in a metastatic mouse tumor model (24). When the antitumor effect was accounted for in this in vivo model, IL-15 caused 3-fold less pulmonary edema than IL-2 at doses with similar antitumor effects.

To further investigate the potential of IL-15 as an anticancer treatment, lymphocytes from melanoma patients treated with IL-2 in vivo were further activated in vitro with IL-2 or IL-15. Cell functions including proliferation, induction of the activation marker CD69, and cytotoxic responses were analyzed. Magnetic cell sorting was used to isolate CD56+ and CD3+ enriched subpopulations from these lymphocytes, which were also examined for cytotoxic and proliferative responses induced by IL-15 stimulation.

**MATERIALS AND METHODS**

**Cytokines.** Recombinant human IL-2 with a specific activity of 1.5 × 10^7 IU/mg was provided by Hoffman-La Roche (Nutley, NJ) via the Biological Response Modifiers Program of the National Cancer Institute (Frederick, MD). Each vial contained one million IU of lyophilized IL-2, 5 mg of mannitol, and 25 mg of human albumin. The conversion equation for IL-2 is as follows: 225 units/ml of IL-2 = 15 ng/ml, or 1.0 nm. Recombinant human IL-15 was provided by Immunex Research and Development Corp. (Seattle, WA). All IL-15 stock concentrations were determined at Immunex by amino acid analysis using a Beckman 6300 amino acid analyzer. Several different lots of IL-15 were used for these studies, and each lot showed comparable stimulation of proliferation and cytotoxicity. F42K is an IL-2 variant that does not bind the IL-2Ra chain due to a point mutation substituting lysine for phenylalanine at the 42nd amino acid residue (25–27) and was generously provided by Dr. G. Ju of Hoffman-La Roche. The concentration of the stock F42K was determined by dividing the A_{280 nm} by the extinction coefficient (0.665; determined experimentally for IL-2). As IL-2 and IL-15 are of the same molecular weight (M, 15,000), the following equation was used to convert IL-2 and IL-15 concentrations: 15 ng/ml IL-15, IL-2, or F42K = 1.0 nm.

**Patient Blood Samples.** All patients from whom samples were obtained were enrolled in a Phase 1 clinical trial at the University of Wisconsin Comprehensive Cancer Center testing the combined treatment of IL-2 with antganglioside GD3 monoclonal antibodies ch14.18 and R24 (CO9391). These patients all had biopsy-proven malignant melanoma with distant metastatic disease or multiple local recurrences, and all patients signed consent forms approved by the University of Wisconsin Committee for the Protection of Human Subjects. IL-2 was administered to patients as a 96-h continuous infusion for 3 consecutive weeks at a dose of 1.5 × 10^7 units/m^2/day. During the second week, patients were given 2 mg/m^2/day of the ch14.18 antibody administered as a 4-h i.v. infusion, and 1 mg/m^2/day of the R24 antibody administered as an 18-h i.v. infusion. 1 h following the ch14.18 infusion. Subsequent groups of patients received escalating doses of both antibodies to determine the maximum tolerated dose. Blood samples for harvest of fresh PBMCs were drawn 24 h after completion of the IL-2 infusion (days 13 and/or 20). PBMCs were isolated from heparinized venous blood by centrifugation over a Ficoll-Hypaque density gradient. Patients with stable or regressing disease were eligible to receive a second course of IL-2 and antibody therapy with the same doses and schedule as in course 1.

The R24 and ch14.18 antibodies administered in vivo are not believed to influence the lymphocyte responses observed in vitro. PBMCs obtained at day 13 or 20 (following 2 or 3 weeks of in vivo IL-2) from patients enrolled in this study of IL-2 combined with monoclonal antibodies ch14.18 and R24 had in vitro responses similar to those of PBMCs from patients receiving IL-2 therapy alone in prior studies (1, 2, 28). The toxicity, clinical effects, and antitumor activity of this clinical trial of IL-2 with R24 and ch14.18 monoclonal antibodies are distinct from the scope of this in vitro analysis with IL-15 and were reported separately (28).

**Target Cell Lines.** Daudi is a Burkitt’s lymphoma cell line obtained from the American Type Culture Collection (Rockville, MD). Daudi cells were maintained in RPMI complete medium consisting of RPMI 1640 (BioWhittaker, Inc., Walkersville, MD) supplemented with 10% heat-inactivated FBS (HyClone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 25 mM HEPES (pH 7.4). Daudi cells were cultured at 1–10 × 10^6 cells/ml and split every 2–3 days when the concentration exceeded 10^6 cells/ml. K562 is a chronic myelogenous leukemia cell line obtained from the
American Type Culture Collection. K562 cells were maintained in RPMI complete medium with 10% FBS by culturing at 1–5 × 10^5 cells/ml and splitting every 3–4 days when the concentration exceeded 6 × 10^5 cells/ml. LA-N-5, a neuroblastoma cell line kindly provided by Dr. R. Seeger (Los Angeles, CA), was maintained as an adherent monolayer in Leibovitz’s medium (L15) supplemented with 10% heat-inactivated FBS. A trypsin-EDTA solution was used to harvest the cells every 3–4 days once the flask had reached confluence.

**In Vitro Proliferative Assay.** Responder cells (1 × 10^5) were added to triplicate wells of 96-well microtiter plates in a final volume of 200 μl of RPMI 1640 complete medium and increasing concentrations of IL-2, IL-15, or F42K. Cells were incubated for 48 h at 37°C with 5% CO₂, followed by 12–16 h of pulse labeling with 1 μCi of [3H]thymidine per well. Cultures were harvested onto glass fiber filters using a 96-well Packard FilterMate cell harvester (Packard Instrument Co., Meriden, CT). Dry filters were then counted for 5 min by gas ionization detection on a Packard Matrix 9600 direct β counter (Packard Instrument Co.). Values are reported as total counts in 5 min. EC₅₀ (effective concentration of cytokine necessary to achieve 50% of maximal stimulation) were calculated using the ALLFIT program provided by J. Rossio at the National Cancer Institute (Frederick, MD). Results were analyzed by blocked one-way ANOVA.

**In Vitro Cytotoxicity Assay.** PBMCs from patients and healthy donors were used as effector cells in 51Cr-release assays as described previously (4, 9). Fresh PBMCs were resuspended in RPMI complete medium with 10% human serum and plated into 96-well microtiter plates in a 100-μl volume at 50:1, 25:1, and 12.5:1 E:T ratios. Fifty μl of IL-2 or IL-15 at various dilutions were added to each well. Daudi, K562, and LA-N-5 tumor targets were labeled with 51Cr for 2 h at 37°C in 5% CO₂, washed, and resuspended at 1 × 10^5 cells/ml in RPMI complete medium with 10% human serum. Labeled target cells were added to triplicate wells of 96-well microtiter plates in a 100-μl volume at 50:1, 25:1, and 12.5:1 E:T ratios. Fifty μl of IL-2 or IL-15 at various concentrations were added to each well. Daudi, K562, and LA-N-5 tumor targets were labeled with 51Cr for 2 h at 37°C in 5% CO₂, washed, and resuspended at 1 × 10^5 cells/ml in RPMI complete medium with 10% human serum. Labeled target cells were added to quadruplicate wells (5000 target cells/well) in a 50-μl volume for a final well volume of 200 μl. Plates were centrifuged for 5 min at 800 rpm to gently sediment targets and effectors. After a 4-h incubation at 37°C in 5% CO₂, the supernatant from each well was harvested using Skatron harvesting filters (Skatron, McLean, VA). Filters were dried overnight and centrifuged for 5 min. Aliquots of supernatants were counted in a Packard four-channel gamma counter (Packard Instrument Co.). Spontaneous release was determined by incubating 50 μl of targets in RPMI complete medium alone. Maximum release is the number of counts released when 50 μl of targets are incubated in cetrimide detergent to achieve 100% lysis (Sigma Chemical Co., St. Louis, MO). One lytic unit is defined as the number of effector cells causing 20% lysis of 5000 target cells; these data are expressed as lytic units/10⁷ effector cells.

**CD69 Induction Assay.** CD69 is an early activation marker induced on the surface of T cells, B cells, NK cells, monocytes, and neutrophils following cellular activation (29–31). CD69 is not detectable on fresh, resting PBMCs, but it can be induced following 60 min of in vitro stimulation. As an early indicator of cellular activation, CD69 expression does not always correlate with late activation events such as proliferation (32). The studies reported here used CD69 induction as a functional response, which could be used to compare the dose-response relationships induced by IL-2 and IL-15.

Fresh or thawed PBMCs were resuspended at a concentration of 10⁶ cells/ml in RPMI complete medium with 10% human serum. Cells were incubated overnight (between 18 and 24 h total incubation time) at 37°C in 5% CO₂, with varying concentrations of IL-2 or IL-15. Approximately 0.5–1 × 10⁶ cells were aliquoted into flow cytometry tubes and washed once with cold FACS buffer (PBS with 1% BSA). Cells were stained on ice in a volume of 100 μl with anti-CD69-FITC and anti-CD56-PE or with isotype control antibodies (Becton Dickinson, Mountain View, CA) for 45–60 min. Cells were washed in cold FACS buffer to remove unbound antibody, and propidium iodide was added at 1 μg/ml to allow exclusion of dead cells during analysis on a FACSscan flow cytometer (Becton Dickinson).

**MACS.** Prior to initiating a negative selection protocol, fresh PBMCs were washed two to three times in cold PBS. To sort for CD56⁺ cells, 200 × 10⁶ PBMCs were incubated with anti-CD5-biotin (8 μl/10⁶ cells), anti-CD3-biotin (8 μl/10⁶ cells), anti-CD14-biotin (2.5 μl/10⁶ cells), and anti-CD19-biotin (2.5 μl/10⁶ cells). To sort for CD3⁺ cells, 200 × 10⁶ PBMCs were incubated with unconjugated anti-CD56 (8 μl/10⁶ cells), anti-CD14-biotin (2.5 μl/10⁶ cells), and anti-CD19-biotin (2.5 μl/10⁶ cells). All of the antibodies were obtained from Becton Dickinson. The final volume of each reaction was brought to 20 ml with PBS, so that the cell concentration was 10⁷/ml. Cells were incubated on ice for 60 min with periodic mixing and then washed twice with cold PBS. Because the anti-CD56 antibody was unconjugated, 0.5 μl of goat antimouse-biotin per 10⁶ cells was added to those washed cells that were to be sorted for CD3⁺ cells. These cells were incubated for an additional 30 min on ice and washed in PBS. Those cells to be sorted for CD56⁺ cells did not require staining with goat antimouse-biotin, because only biotin-conjugated antibodies were used to stain these cells. Meanwhile, MACS streptavidin magnetic microbeads (Miltenyi Biotech) were washed by resuspending 500 μl of bead suspension in 10 ml of PBS and washing the beads through a type C MACS separation column while they were engaged in the magnet. The column was rinsed with 10 ml of PBS, and the beads were eluted by removing the column from the magnet and rinsing them again with 20 ml of PBS. Ten ml of beads were added to each resuspended cell pellet, mixed, and incubated for 15 min on ice. Each cell suspension was loaded onto a type C MACS separation column locked into the magnet apparatus. Cells binding the biotin-conjugated antibody should also bind the streptavidin microbeads and therefore should be retained in the column by the magnet, whereas cells not binding the antibody should not be retained by the magnet. The volume that flowed through was reloaded onto the column a second time. The final volume collected was then washed, centrifuged, and resuspended in RPMI complete medium with 10% human serum, and cells not retained by the magnet were counted on a hemacytometer. A small fraction of each sorted sample was...
stained with anti-CD56-PE or anti-CD3-PE to determine the purity of the sorted populations. Greater than 90% purity could reproducibly be obtained using this method (Table 1).

### RESULTS

**IL-2 and IL-15 Stimulate Proliferation of PBMCs Obtained from Patients Treated with in Vivo IL-2 Therapy.**

Unsorted PBMCs activated *in vivo* by IL-2 were incubated *in vitro* with increasing doses of IL-2 or IL-15 and, after 48 h of stimulation, were pulsed with [3H]thymidine. These PBMCs activated *in vivo* with IL-2 require a greater dose *in vitro* of IL-15 than IL-2 to achieve a comparable level of response (Fig. 1). The results were similar to those observed previously with other cell lines and primary cells (16, 33). This difference in dose-response curves for IL-2 and IL-15 is not entirely a result of the prior *in vivo* treatment with IL-2, because PBMC samples from the same melanoma patients collected prior to IL-2 treatment or from healthy donors also revealed a similar dose-response differential for IL-2 and IL-15 (Table 2).

The effective concentration needed to reach a 50% maximal response (EC50) was calculated for IL-2 and IL-15 stimulation of PBMCs from healthy control and patient donors (Table 2). The unsorted PBMCs activated *in vivo* by treatment with IL-2 displayed significantly lower EC50 values for IL-2 (0.21 ± 0.04 nM) than for IL-15 (1.16 ± 0.16 nM), as determined by one-way ANOVA (P < 0.0001). Likewise, PBMCs obtained from patients prior to treatment with IL-2 therapy had significantly different EC50s for IL-2 and IL-15, 0.57 ± 0.03 and 3.3 ± 1.2 nM, respectively (P = 0.032; Table 2). This difference was not unique to PBMCs from melanoma patients, because fresh PBMCs from healthy donors exhibited EC50s similar to those for PBMCs prior to treatment with IL-2 *in vivo*; for IL-2, the EC50 was 0.54 ± 0.14 nM, and for IL-15 the EC50 was 3.14 ± 0.72 nM (P = 0.046).

**The Subpopulation of Cells Activated in Vivo by IL-2 That Proliferate in Response to IL-15 Are CD56+ NK Cells.**

Magnetic cell sorting was used to isolate both CD3+ and CD56+ enriched subpopulations from PBMCs of patients treated with *in vivo* IL-2 therapy. As measured by flow cytometry in one representative experiment, the sorted populations were 89% CD3+ and 92% CD56+, respectively (Table 1). These sorted populations, as well as the unsorted cells, were compared in a standard proliferation assay, shown in Fig. 2. As has been observed previously, a greater dose of IL-15 than IL-2 was necessary to reach a maximal response for each population of responding cells. However, the CD3+ enriched cells displayed an extremely blunted response, in that they did not proliferate well in response to either IL-2 or IL-15 (Fig. 2). The maximum level of [3H]thymidine incorporation by CD3+ cells was approximately 2000 counts. In contrast, the CD56+ enriched cells exhibited a strong proliferative response to both IL-2 and IL-15, with the level of [3H]thymidine incorporation reaching 50,000 counts. The level of response reached by the unsorted population was intermediate between the CD3+ and CD56+ sorted populations. These data document that the CD56+ lymphocytes, and not the CD3+ lymphocytes, activated *in vivo* by IL-2 were capable of responding to IL-2 or IL-15 by proliferation. In fact, the slight proliferative response noted in the CD3+ sorted population could be attributed to the small percentage (6%) of contaminating CD56+ cells (Table 1); the CD3+ cells themselves may be incapable of a detectable proliferative response. Note that the EC50s for the CD56+ cells are lower for both IL-15 and IL-2 responses when compared to the EC50s for the unsorted cells (P = 0.0097 and 0.0075 for IL-2 and IL-15, respectively; Table 2). This suggests that the presence of the nonresponsive CD56− cells in the unsorted population has an inhibitory effect on the dose-response curve for CD56+ cells comprising approximately 50% of the unsorted population.

The mean EC50s for IL-2 and IL-15 stimulation of the CD3+ enriched population were not significantly different (P = 0.096); however, the responses were so weak, as shown in Fig. 2, that these EC50s may not be meaningful. In contrast, IL-2 and IL-15 did stimulate strong yet significantly different proliferative responses by CD56− enriched lymphocytes (P = 0.0019; Table 2).

**Induction of Cytotoxicity by Patient Lymphocytes Results in Similar Dose-Response Curves for Both IL-2 and IL-15.** The lytic activity of these melanoma patients’ unsorted lymphocytes obtained after *in vivo* IL-2 was tested against a

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**Table 1**

<table>
<thead>
<tr>
<th>Cells</th>
<th>CD56+</th>
<th>CD3+</th>
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</thead>
<tbody>
<tr>
<td>Unsorted</td>
<td>53%</td>
<td>42%</td>
</tr>
<tr>
<td>CD56+ enriched</td>
<td>92%</td>
<td>6%</td>
</tr>
<tr>
<td>CD3+ enriched</td>
<td>6%</td>
<td>89%</td>
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</table>

*In this representative experiment, PBMCs obtained following 3 weeks of *in vivo* IL-2 treatment were tested for CD3 and CD56 expression before MACS separation and after CD3+ or CD56+ enrichment. Percentages of cells positive for each marker were determined using flow cytometry. These are the same cells used for the representative experiments depicted in Figs. 2 and 5.*

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**Fig. 1**

PBMCs from melanoma patients following *in vivo* IL-2 treatment proliferate in response to IL-2 or IL-15. PBMCs were obtained from a patient following 3 weeks of *in vivo* IL-2 therapy (course 1, day 20) 24 h after completion of IL-2 treatment. Cells (1 × 10⁷/well) were incubated with increasing concentrations of IL-2 (○) or IL-15 (□) for 48 h, followed by 16 h of pulse labeling with [3H]thymidine. Each point on the graph depicts the mean of triplicate wells from a single representative experiment. A greater dose of IL-15 than IL-2 was necessary to stimulate the same level of proliferative response. The mean EC50s for all 18 experiments are reported in Table 2.
were determined by comparing IL-2 and IL-15 responses in a one-way ANOVA. The proliferative and cytotoxicity dose-response curves for IL-2 and IL-15, as described in "Materials and Methods." The EC_{50} (effective concentration of cytokine necessary to achieve 50% of maximal stimulation) were calculated using the ALLFIT program. Data are presented as the mean EC_{50} ± SE, with the number of experiments given in parentheses. P values were determined by comparing IL-2 and IL-15 responses in a one-way ANOVA.


determined as an increased mean percentage of CD69 NK cells. The induction of CD69 on CD56 NK cells was measured days after initial cytokine stimulation, whereas cytokine secretion and incorporation of [3H]thymidine to measure proliferation, and expression of CD69, which is induced within a few hours following stimulation with IL-2 (31). The ability of IL-15 and IL-2 to up-regulate CD69 expression was tested following overnight stimulation (18–24 h) with increasing doses of IL-2 or IL-15. The up-regulation of CD69 on CD56^+ NK cells was determined as an increased mean percentage of CD69^+ NK cells. As depicted in the representative experiment in Fig. 6, when patient PBMCs are incubated with IL-2 or IL-15 overnight, the dose-response curves resemble the proliferation dose-response curves. A given dose of IL-15 induces significantly less CD69 expression than the same dose of IL-2 (P < 0.0001; Fig. 6).


toxicity is measured in a matter of hours, we also compared IL-2 and IL-15 induction of an earlier marker of cellular activation, i.e., induction of CD69 expression. NK cell activation by IL-2, can be measured using flow cytometry to detect surface expression of CD69, which is induced within a few hours following stimulation with IL-2 (31). The ability of IL-15 and IL-2 to induce CD69 expression was tested following overnight stimulation (18–24 h) with increasing doses of IL-2 or IL-15. The up-regulation of CD69 on CD56^+ NK cells was determined as an increased mean percentage of CD69^+ NK cells. As depicted in the representative experiment in Fig. 6, when patient PBMCs are incubated with IL-2 or IL-15 overnight, the dose-response curves resemble the proliferation dose-response curves. A given dose of IL-15 induces significantly less CD69 expression than the same dose of IL-2 (P < 0.0001; Fig. 6).


discussion

To further evaluate the potential of IL-15 as an immunotherapeutic agent, these preclinical studies analyzed lymphocytes activated in vitro with IL-2 for their ability to respond to IL-2 or IL-15 treatment in vitro. These in vitro-activated lymphocytes were already known to be responsive to IL-2 and to mediate tumour lysis in vitro, making them a clinically relevant population of cells to study.

Three cellular responses were examined in these studies: induction of cytotoxic activity, induction of CD69 surface expression, and incorporation of [3H]thymidine to measure proliferation. IL-2 and IL-15 induce significantly different responses for proliferation (P < 0.0001) and CD69 expression (P < 0.0001), but cytotoxic responses are not significantly different (P = 0.06). Finally, using magnetic cell sorting, we have demonstrated that CD56^+ NK cells are responsible for these proliferative and cytotoxic responses stimulated by IL-2 and IL-15, whereas CD3^+ T cells are relatively unresponsive.

The fact that CD3^+ T cells activated in vivo with IL-2 were relatively unresponsive has been documented previously (11). Because T cells are known to mediate MHC-restricted cytotoxicity against tumor cells in other model systems, this induction of relative T-cell anergy by systemic IL-2 therapy implies that non-MHC-restricted cytotoxicity would be preferentially used when patients are treated with this regimen. It is unknown at this

<table>
<thead>
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<th>Cells</th>
<th>IL-2</th>
<th>IL-15</th>
<th>P</th>
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<tr>
<td>Unsorted healthy donor PBMCs</td>
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<td>3.14 ± 0.72 nm (4)</td>
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<td>Unsorted patient PBMCs (pre-IL-2)</td>
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<td>Unsorted patient PBMCs (post-IL-2)</td>
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<td>1.16 ± 0.16 nm (18)</td>
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<td>CD3^+ enriched patient PBMCs (post-IL-2)</td>
<td>0.22 ± 0.11 nm (3)</td>
<td>0.96 ± 0.14 nm (3)</td>
<td>0.096</td>
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<tr>
<td>CD56^+ enriched patient PBMCs (post-IL-2)</td>
<td>0.027 ± 0.013 nm (3)</td>
<td>0.34 ± 0.07 nm (3)</td>
<td>0.0019</td>
</tr>
</tbody>
</table>

* The mean EC_{50} for IL-2 with unsorted patient PBMCs and CD56^+ enriched patient PBMCs were significantly different; P = 0.0007.

* The mean EC_{50} for IL-15 with unsorted patient PBMCs and CD56^+ enriched patient PBMCs were also significantly different; P = 0.0075.

A given dose of IL-15 induces significantly less CD69 expression than the same dose of IL-2 (P < 0.0001; Fig. 6).

Low Doses of IL-15 Capable of Boosting a Cytotoxic Response in Vitro Are Not Sufficient to Induce Proliferation of PBMCs Activated in Vivo with IL-2. Interestingly, if the proliferation and cytotoxicity dose-response curves for IL-2 and IL-15 are overlaid, a striking difference is observed. Whereas the two responses to IL-2 (proliferation and cytotoxicity) show similar dose-response curves, responses to IL-15 are quite different. Notably, at low doses of IL-15, such as 1 ng/ml, the cytotoxic response is clearly boosted above baseline levels, although the incorporation of [3H]thymidine is only slightly greater than background at this low dose (Fig. 5). Because proliferation and cytokine secretion can be simultaneously induced when a cell is activated, this observation suggests that IL-15 might not stimulate as much cytokine release or toxicity as IL-2 when used as an immunotherapeutic agent, while still maintaining comparable cytotoxic activity.

Induction of CD69 Expression on CD56^+ Cells in Response to IL-2 or IL-15. Because proliferation can only be measured days after initial cytokine stimulation, whereas cytotoxicity is measured in a matter of hours, we also compared IL-2 and IL-15 induction of an earlier marker of cellular activation, i.e., the induction of CD69 expression. NK cell activation by IL-2 and IL-15 induction of an earlier marker of cellular activation, i.e., the induction of CD69 expression. NK cell activation by IL-2 can be measured using flow cytometry to detect surface expression of CD69, which is induced within a few hours following stimulation with IL-2 (31). The ability of IL-15 and IL-2 to induce CD69 expression was tested following overnight stimulation (18–24 h) with increasing doses of IL-2 or IL-15. The ability of IL-15 and IL-2 to up-regulate CD69 expression was tested following overnight stimulation (18–24 h) with increasing doses of IL-2 or IL-15. The up-regulation of CD69 on CD56^+ NK cells was determined as an increased mean percentage of CD69^+ NK cells. As depicted in the representative experiment in Fig. 6, when patient PBMCs are incubated with IL-2 or IL-15 overnight, the dose-response curves resemble the proliferation dose-response curves. A given dose of IL-15 induces significantly less CD69 expression than the same dose of IL-2 (P < 0.0001; Fig. 6).

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Three cellular responses were examined in these studies: induction of cytotoxic activity, induction of CD69 surface expression, and incorporation of [3H]thymidine to measure proliferation. IL-2 and IL-15 induce significantly different responses for proliferation (P < 0.0001) and CD69 expression (P < 0.0001), but cytotoxic responses are not significantly different (P = 0.06). Finally, using magnetic cell sorting, we have demonstrated that CD56^+ NK cells are responsible for these proliferative and cytotoxic responses stimulated by IL-2 and IL-15, whereas CD3^+ T cells are relatively unresponsive.

The fact that CD3^+ T cells activated in vivo with IL-2 were relatively unresponsive has been documented previously (11). Because T cells are known to mediate MHC-restricted cytotoxicity against tumor cells in other model systems, this induction of relative T-cell anergy by systemic IL-2 therapy implies that non-MHC-restricted cytotoxicity would be preferentially used when patients are treated with this regimen. It is unknown at this


time whether in vivo IL-15 also induces T cells to become relatively unresponsive to ex vivo IL-2 or IL-15.

The observed differences in the IL-2 and IL-15 dose-response curves might be attributable to changes in IL-2 and IL-15 receptor expression over time, secondary to the cytokine stimulation. Previously published data have shown that IL-15 binding is quickly down-regulated on phytohemagglutinin blasts following stimulation by IL-15 (18). In contrast, this IL-15 binding increases after stimulation with IL-2 (18). These data suggest that IL-15 inhibits expression of its own high-affinity receptor on activated T cells (the IL-15Rα chain), whereas IL-2 stimulation may activate increased IL-15Rα expression. If these same modulations of IL-15Rα occur on NK cells and prolonged activation with IL-15 stimulates a corresponding decrease in IL-15Rα expression, then perhaps the differences in the dose-response curves presented above could be explained by the varying lengths of time required for IL-15 stimulation in each of the three assays. Stimulation with IL-15 over a few hours, as in the cytotoxicity assay, might not be sufficient to decrease the IL-15Rα expression substantially on these NK cells, so that the expression of the high-affinity IL-15R on these cells allows them to respond to IL-15 with a boost of cytotoxicity comparable to that achieved with the same dose of IL-2. However, after 20 h or more of IL-15 treatment, as in the CD69 induction or proliferation assays, the expression of IL-15Rα may be down-regulated. The hypothesized decreased IL-15Rα expression could potentially shift the IL-15 dose-response curve to the right, requiring greater concentrations of IL-15 to achieve the same effect as IL-2, because the intermediate-affinity IL-15Rα...
The proliferative response of PBMCs activated in vivo by IL-2 overlaid with the cytotoxic response of these same effector cells against LA-N-5 tumor targets. PBMCs obtained from a patient following 3 weeks of in vivo IL-2 therapy (course 1, day 20) were incubated with increasing concentrations of IL-2 (○) or IL-15 (▲) for 48 h, followed by 16 h of pulse labeling with [3H]thymidine. Each point on the graph depicts the mean of triplicate wells from a single representative experiment. The results of 15 separate experiments testing cytotoxicity against the LA-N-5 tumor target are overlaid on the same graph. The data are expressed as mean lytic units (bars, SE). PBMCs were incubated with 51Cr-labeled LA-N-5 tumor targets as described in “Materials and Methods.” Lysis was enhanced similarly by the same dose of either IL-2 (○) or IL-15 (▲). One-way ANOVA performed using data from all 18 proliferation experiments showed that at a low dose of IL-15, such as 1 ng/ml, the difference between IL-2- and IL-15-stimulated proliferation is significant (P < 0.0001). However, statistical analysis of data from the 15 cytotoxicity experiments indicated that the difference between the cytotoxicity response curves for IL-2 and IL-15 is not significant (P = 0.06).

Corresponds to a greater EC50 value than the high-affinity IL-15R.

The observation that the EC50s for IL-2 and IL-15 are lower for PBMCs obtained following in vivo IL-2 activation, as compared to the EC50s for those PBMC samples obtained prior to IL-2 treatment, suggests that IL-2R and IL-15R are up-regulated on the surface of PBMCs following in vivo IL-2 treatment (Table 2). In addition, we have documented that these responses are mediated by NK cells, suggesting that in vivo IL-2 treatment causes an increase in IL-2 and IL-15 receptor mechanisms on NK cells. Perhaps in vivo IL-2 also up-regulates the IL-15Rα chain on the surface of these activated NK cells, thereby contributing to enhanced IL-15 responsiveness. Currently, there is no method available to directly quantitate the IL-15Rα protein expressed on a cell surface. Once reagents allowing surface IL-15Rα protein detection become available, patient PBMCs, pre- and post-IL-2 treatment, should be examined for IL-15Rα surface expression, as well as after subsequent in vitro stimulation with varying doses of IL-2 or IL-15, to confirm whether IL-15Rα expression correlates with responsiveness to IL-15 and whether this expression changes following IL-15 stimulation.

Another alternative explanation for the different dose-response curves observed is that different subpopulations of patient PBMCs may express distinct IL-15 receptor complexes, thereby allowing each subpopulation to mediate a different response. If the subpopulation of CD56+ cells that mediates cytotoxicity boosted by IL-15 expresses a greater level of IL-15Rα, then perhaps the cytotoxic response stimulated by IL-15 will have a lower EC50 than the proliferative response mediated by a different subpopulation of CD56+ cells, which may express a relatively lower amount of IL-15Rα chains. This difference in response might also result from the selective expression of a particular signaling molecule by one responding subset that dictates the level of that response. Such signaling differences in proliferation versus cytotoxicity might be related to the functions that are mediated by a particular cell, but they might also be coincidental.

Finally, the possibility cannot be ignored that the antiganglioside antibodies R24 and ch14.18 used to treat these patients in vivo may have contributed to the distinct responses observed. The cell surface ganglioside GD3 is known to be expressed on a small percentage of lymphocytes, including T and NK cells, and this GD3+ subset is statistically larger in patients with disseminated melanoma compared to healthy controls (34). Anti-GD3 antibodies, in particular R24, have been shown to stimulate proliferation, cytokine secretion, and lytic activity of lymphocytes. However, proliferation of CD8+ T cells induced by anti-GD3 is inhibited in the presence of CD16+ NK cells (35). Although preliminary analysis of the clinical data suggests no difference between the lymphocyte responses in this trial and previous studies using IL-2 alone (8–12), activation of PBMCs by anti-GD3 antibodies may have affected the subsequent responses to IL-2 and IL-15 ex vivo. Further studies with these antibodies in vitro may be necessary to determine the exact role of R24 and 14.18 in the activation of patient PBMCs.

These data with patient PBMCs obtained following in vivo activation with IL-2 may be relevant to the development of
future cancer treatments. The population of CD56+ NK cells activated in vivo by IL-2 has been shown to possess lytic capabilities that are boosted in the presence of either IL-2 or IL-15, making this a clinically relevant effector population to study. Also, these differences in dose-response curves suggest that IL-15 may potentially serve as a useful immunotherapeutic agent, possibly in combination with IL-2. IL-15 stimulates a weaker proliferative response than IL-2 at concentrations that cause comparable cytotoxic activation; this may correlate with decreased secondary cytokine production in response to IL-15 and possibly fewer toxic side effects associated with IL-15 compared to IL-2, while not compromising the activation of cell-mediated cytotoxicity. If so, then IL-15 may have certain advantages over IL-2 in immunotherapeutic protocols.

Due to limited availability of CD56+ patient PBMCs activated in vivo by IL-2, experiments have not been performed to directly quantitate and compare the secretion of cytokines (such as IFN-γ, GM-CSF, or TNF-α) by these cells following subsequent ex vivo stimulation by IL-2 or IL-15. However, results consistent with this hypothesis have been published previously by Carson et al. (14). These data show that CD56dim NK cells secrete approximately twice as much GM-CSF in response to 10 ng/ml IL-2 compared to 10 ng/ml IL-15. Although these CD56dim cells have not been activated in vivo with IL-2, they express the IL-2Rβγ receptor, as do the CD56+ patient PBMCs studied here.

Patients pretreated with IL-2 prior to IL-15 administration might perhaps up-regulate expression of IL-15Rα molecules on the surface of responding NK cells. In this case, if IL-15 were then to be administered to these IL-2-treated patients, their PBMCs should respond to IL-15 better than PBMCs not pretreated with IL-2 previously, which is analogous to the in vitro data presented here (Table 2). In addition, because IL-15 treatment may cause down-regulation of high-affinity IL-15 receptors, these cells will be less likely to become overly activated by IL-15, so as to potentially inhibit undesired side effects, in contrast to the activation mediated by a similar dose of IL-2. If IL-2 and IL-15 were alternated in an in vivo treatment protocol, then one might potentially take advantage of the “priming” activity of IL-2 to enhance the response to IL-15, while maximizing the cytolytic activation achieved by IL-15 and minimizing secondary responses such as proliferation, which may be correlated with undesired side effects. Additional experiments to document the expression of IL-15Rα on PBMCs from patients treated with in vivo IL-2 therapy will be critical to understanding the mechanism of these dose-response differences between IL-2...
and IL-15 and to rationally designing in vivo regimens that potentially combine these cytokines.

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