

Increased Expression of the Interleukin 2 (IL-2) Receptor β Chain (p70) on CD56⁺ Natural Killer Cells after In Vivo IL-2 Therapy: p70 Expression Does Not Alone Predict the Level of Intermediate Affinity IL-2 Binding

By Stephan D. Voss,* Richard J. Robb,|| Gilda Weil-Hillman,*
Jacquelyn A. Hank,* Kazuo Sugamura,¶ Mitsuru Tsudo,**
and Paul M. Sondel*‡§

From the Departments of *Human Oncology, ‡Pediatrics, and §Genetics, the University of Wisconsin, Madison, Wisconsin 53792; ||Central Research and Development, Glenolden Laboratory, E.I du Pont de Nemours & Co., Glenolden, Pennsylvania 19036; the ¶Department of Microbiology, Tohoku University School of Medicine, Sendai 980, Japan; and the **Department of Immunology, The Tokyo Metropolitan Institute of Medical Science, Bunkyo-ku, Tokyo 113, Japan

Summary

The expression of the 70-kD β subunit of the interleukin 2 receptor (IL-2R) has been examined on peripheral blood lymphocytes (PBL) obtained from patients receiving systemic infusions of IL-2. Using monoclonal antibodies directed against p70, flow cytometric analyses revealed a greater than threefold increase in expression of the IL-2R β chain on CD56⁺ natural killer (NK) cells from post-IL-2 therapy PBL relative to pre-therapy cells. The level of p70 expression on the post-therapy cells was three- to fourfold greater (based on fluorescence intensity) than the level of p70 expression on YT cells, an NK-like cell line that expresses \sim 12,000 intermediate affinity IL-2 binding sites/cell. Despite the high level of p70 expression, in ¹²⁵I-IL-2 binding assays only 790–1,290 intermediate affinity IL-2 binding sites/cell were detected on post-therapy cells from six patients. These data represent the first report of increased p70 expression after in vivo IL-2 administration and suggest a requirement for at least one additional subunit for the formation of functional intermediate affinity IL-2Rs. Furthermore, the presence on the surface of post-therapy NK cells of excess p70 that does not bind IL-2 with intermediate affinity implies that the formation of intermediate affinity IL-2Rs is not solely determined by the level of p70 expression, and that the response of NK cells to IL-2 might be regulated by altering the expression of p70 or some other IL-2R subunit.

IL-2, originally characterized as the active component in T cell growth factor, has been found to have stimulatory effects on cells of multiple hematopoietic lineages. As such, IL-2Rs have been identified on T cells, B cells, NK cells, peripheral blood monocytes, macrophages, and early granulocytes (1). IL-2Rs are composed of at least two chains, a 55-kD α chain (Tac) (2–8) and a 70–75-kD β chain (p70) (9–14). The chains independently bind IL-2 with either low (Tac; K_d = 5–15 nM) or intermediate (p70; K_d = 0.5–2 nM) affinity, while the noncovalent association of the two chains results in the generation of an IL-2R complex with high affinity binding properties (K_d = 5–10 pM) (9–16). Cellular responses to IL-2, including ligand internalization, are mediated by both high and intermediate affinity receptors and appear to require β chain participation (1, 6, 11, 15–23).

Unstimulated T cells express only very low numbers of high affinity IL-2Rs. Activation of these cells with antigenic

or nonspecific mitogenic stimuli, however, results in increased IL-2 and Tac synthesis, followed by a rapid increase in high affinity IL-2R expression (1, 24). This activation also leads to increased release of soluble Tac protein from the cells (25). Similar results were noted when purified T cells were cultured directly in IL-2 (1, 19, 24, 26). The latter observation suggests that IL-2, when present in sufficient concentrations, directly activates resting Tac⁻ cells through the intermediate affinity p70 β chain, leading to the synthesis of IL-2 and Tac and the formation of Tac/p70 IL-2R complexes. Subsequent autocrine responses to IL-2 would then be mediated by the newly formed high affinity receptors. Consistent with these in vitro observations, increased levels of soluble Tac have been observed in the sera of patients receiving systemic infusions of high-dose IL-2 (27, 28), in the sera of patients experiencing graft rejections (29), and in other forms of in vivo immune activation (30).

Resting NK cells also appear phenotypically Tac⁻, suggesting that IL-2 activation of these cells may occur exclusively through the p70 β chain of the IL-2R (22, 31–33). Recent studies have further demonstrated that the majority of the resting NK cells are CD16⁺CD56^{dim} and bind IL-2 through p70. However, a small subset of resting NK cells is distinguishable phenotypically as CD56^{bright}CD16⁻, and these cells bear high affinity IL-2Rs (34, 35). Whether expansion of one or both of these subsets resulted in the increased Tac expression observed after exposure of resting NK cell populations to IL-2 in vitro (11, 20–22) is not known. The functional role of these distinct NK cell subsets is also unclear, although it has been suggested that the CD16⁻CD56^{bright} subset bearing the high affinity IL-2R may represent a precursor population capable of expanding and differentiating in response to low physiologic concentrations of IL-2 that occur naturally in vivo (34, 35). This hypothesis is also attractive with regard to the in vivo immune activation observed in patients who have achieved steady-state serum IL-2 levels of \sim 30 U/ml during continuous infusions of IL-2 for the treatment of cancer (36).

The exposure of heterogeneous populations of lymphocytes to high concentrations of IL-2 activates a non-MHC-restricted cytolytic activity due to the expansion of a population of large granular lymphocytes with properties of activated NK cells (36–38). The cells mediating this lymphokine activated killer activity effectively lyse fresh tumor targets as well as various NK-resistant cultured tumor cell lines (38). This observation, together with the demonstration of in vivo efficacy in murine tumor models, has led to testing of IL-2 as a therapeutic modality (36, 37, 39). In clinical trials at a number of institutions, patients with renal cell carcinoma and melanoma have shown objective regression of tumor in response to a variety of IL-2 treatment regimens (39–43).

In an effort to better characterize and modify further the in vivo effects of systemically administered IL-2, we have examined changes in lymphocyte function and phenotype after IL-2 therapy. Enhanced proliferative and cytotoxic activity in response to IL-2 treatment in vitro was observed with PBL obtained after IL-2 therapy (44–46). These responses were mediated primarily by the CD56⁺ NK cell subpopulation, a subset of the circulating lymphocytes that is significantly expanded in number during the course of in vivo IL-2 therapy (45–48). The increased levels of soluble Tac detected in patients' sera during IL-2 therapy (27), together with increases in the percentage of Tac⁺ PBL after therapy (45–47), suggested that the majority of the CD56⁺ cells might be responding through receptors containing the Tac protein. Exposure to IL-2 in vitro leads to Tac induction on resting peripheral blood NK cells obtained from control donors, or from patients before beginning IL-2 therapy. However, the CD56⁺ NK cell population activated by IL-2 in vivo, when cultured in IL-2 in vitro, proliferated without subsequent cell surface expression of Tac (44). The proliferative response to IL-2 of lymphocytes obtained after IL-2 therapy was inhibited by the anti-p70 mAb TU27 (49), while an anti-Tac mAb had a negligible effect, suggesting a predominant role for the p70

β chain in the responses of post-IL-2 therapy PBL to IL-2 (44). We now report the direct examination of IL-2R β chain expression before and after IL-2 therapy and present the first evidence for increased expression of the IL-2R β chain in response to in vivo IL-2. We further demonstrate that the high level of expression of p70 is not accompanied by a corresponding high level of intermediate affinity IL-2 binding sites, suggesting a requirement for some additional molecule in the formation of functional intermediate affinity IL-2Rs.

Materials and Methods

Patients. All patients enrolled in this phase I clinical trial had locally recurrent renal cell carcinoma or malignant melanoma that was incurable by surgery. Patients received IL-2 at 1–3 \times 10⁶ U/m²/d as a 96-h continuous infusion for four consecutive weeks. Clinical and immune response data from this treatment regimen have been reported elsewhere (50). All patients signed consent forms for in vivo and in vitro studies approved by the University of Wisconsin Committee for the Protection of Human Subjects. Patient PBMC (PBL) were obtained by Ficoll-Hypaque separation of heparinized blood specimens drawn before initiating, or 24 h after each 4-d cycle of IL-2, cryopreserved by controlled rate freezing in 10% DMSO, and stored at -135°C until later use. Unless stated otherwise, pretherapy PBL refers to PBL obtained just before starting any IL-2 treatment, and post-therapy PBL are cells obtained 24 h after finishing 4 wk of IL-2.

IL-2. rIL-2 was generously provided by Hoffmann-La Roche, Inc. (Nutley, NJ). All IL-2 units are based on the National Cancer Institute-Biological Response Modifiers Program standard for unit determination. The specific activity of HLR IL-2 is 1.5 \times 10⁷ U/mg protein.

Antibodies. FITC- and PE-conjugated mAbs (anti-CD25[Tac]-FITC, anti-CD16[Leu-11a]-FITC, anti-CD5-FITC, anti-CD56[Leu-19]-PE, anti-CD3-PE) and FITC-conjugated goat anti-mouse IgG (GAM-FITC)¹ were purchased from Becton Dickinson Monoclonal Center (Mountain View, CA). GL439 is a mouse IgG1 mAb directed against the p55 Tac chain of the human IL-2R (51). TU27 is a mouse IgG1 mAb directed against the p70 β chain of the human IL-2R (49). Mik β 1 (IgG2a) and Mik β 3 (IgG1) are also directed against the p70 IL-2R β chain (52). Ascites fluid containing IgG1 and IgG2a isotype control mAbs (MOPC21 and UPC10, respectively) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Lines. YT is an NK-like cell line established from a patient with acute lymphoblastic lymphoma and thymoma (53), and was generously provided by J. Yodoi, Kyoto University. YT cells are negative for expression of the T cell antigen CD3 and express NK cell markers detected by the anti-CD16 (FcR) and anti-CD56 mAbs. YT expresses a relatively high number (\sim 12,000/cell) of intermediate affinity IL-2R β chains (12, 13).

Flow Cytometric Analysis. For single-color fluorescence analysis with either TU27, Mik β 1, or Mik β 3, 10⁶ cells were incubated in 100 μ l of a 1:4,000 dilution of ascites fluid containing TU27 or a 1:1,000 dilution of ascites fluid containing either Mik β 1 or Mik β 3. The dilution buffer was HBSS, 1% FCS, 0.2% Na₃N. Serial dilutions of the ascites fluids have been made up to 1:4,000 (TU27) and 1:1,000 (Mik β 1 and Mik β 3) with no change in staining pattern. After 30–60 min on ice, antibody was removed, cells were washed, and resuspended in 100 μ l of dilution buffer. GAM-FITC

¹ Abbreviations used in this paper: GAM, goat anti-mouse; MFI, median log fluorescence intensity.

was added at 1 $\mu\text{g}/\text{ml}$ for 30 min, cells were washed and resuspended, and analyzed immediately. For two-color fluorescence, cells were first stained as described above with appropriate dilutions of ascites fluid containing either TU27, Mik β 1, or Mik β 3. After GAM-FITC staining, the cells were incubated for 10 min on ice in HBSS, 1% FCS, and 0.2% NaN_3 containing 10 $\mu\text{g}/\text{ml}$ purified control mouse IgG1 (MOPC 21; Sigma Chemical Co.) to block any free antibody binding sites on the bound GAM-FITC molecules. After blocking, cells were stained with either anti-CD3-PE or anti-CD56-PE for 30 min on ice, washed, and analyzed by flow cytometry. Control staining of TU27/GAM-FITC-stained cells with a PE-conjugated isotype control antibody showed no nonspecific staining due to recognition of the PE-conjugated antibodies by bound GAM-FITC molecules (unpublished observations). All samples were analyzed on a FACScan flow cytometer (Becton Dickinson & Co.) using FACScan Research and Consort 30 Software. All samples were gated on forward and side scatter. Propidium iodide was added at 1 $\mu\text{g}/\text{ml}$ before analysis to allow exclusion of dead cells.

Median log fluorescence intensity (MFI) calculations were performed by subtracting the MFI of appropriate isotype control antibodies from the MFI of the positively stained samples. Thus, all MFI values presented represent relative increases in log fluorescence intensity over a background value assigned 0, allowing comparisons to be made between individual populations.

FACS. Freshly obtained, Ficoll-Hypaque-separated PBL were resuspended in HBSS containing 100 U/ml penicillin/streptomycin, and 0.3% BSA. 1.8×10^8 cells were resuspended at $2.0 \times 10^7/\text{ml}$ and stained with anti-CD56-PE and anti-CD5-FITC. After rotating for 60 min at 4°C , the cells were washed twice and resuspended at a final concentration of $5 \times 10^6/\text{ml}$. Before cell sorting cells were passed through a Nytex membrane to achieve a single cell suspension. Sorting was performed on a FACStar^{Plus} flow cytometer (Becton Dickinson & Co.). Purity of the CD56⁺- and CD5⁺-sorted populations was >99% upon restaining and reanalysis.

For purification of larger numbers of CD56⁺ cells ($>5.0 \times 10^7$), negative depletions were performed using the anti-CD3 mAb OKT3 (Ortho Pharmaceuticals, Raritan, NJ) and immunomagnetic beads coupled to GAM IgG (Dyna Beads; Dynal, Inc., Great Neck, NY). Bead depletions were performed as described previously (44). Reanalysis of CD3-depleted cells with anti-CD5 and anti-CD56 revealed purity of >95%.

Fc Receptor Blocking. Rabbit Ig (Sigma Chemical Co.) was resuspended at 20 mg/ml in HBSS and heat aggregated for 30 min at 65°C as described (54). Patient PBL (5×10^6) were incubated in 200 μl HBSS containing 10 mg/ml aggregated rabbit Ig for 2 h at 4°C . Lower concentrations of rabbit Ig were not effective at blocking staining by anti-FcR mAbs (anti-CD16[Leu-11] directed against Fc γ RIII present on NK cells; S. Voss, unpublished observations). Aliquots of cells were then removed and stained with either TU27, anti-CD16, anti-CD3, anti-CD56, or isotype control mAbs. FACS analyses were performed as described above. Neither monomeric rabbit or human Ig, nor aggregated human Ig, were found to be as effective as aggregated rabbit Ig in interacting with the FcRs expressed on NK cells (S. Voss, unpublished observations). Incubation of the patient's cells with rabbit Ig also had no effect on staining with anti-CD3 or anti-CD56.

FACS Analysis With Biotinylated IL-2-lys. IL-2-lys-biotin was generously provided by Dr. Paul Simon, Glenolden Laboratory, E.I. DuPont de Nemours & Co. (Glenolden, PA) and has been described previously (55). Cells (10^6) were resuspended in 75 μl HBSS, 1% FCS, 0.1% NaN_3 . 1 μg of either IL-2-lys-biotin or unbiotinylated control IL-2 was added and the cells were incubated at 37°C for 10 min, followed by incubation on ice for an additional 60–90 min.

After washing, cells were stained with PE-conjugated Streptavidin (Becton Dickinson Monoclonal Center) and either IgG1-FITC or anti-CD3-FITC to allow gating on the CD3⁻CD56⁺ NK cell subpopulation. FACS analysis was performed as described above.

¹²⁵I IL-2 Binding Assay. IL-2 binding assays were performed as described previously (6). ¹²⁵I-IL-2 was obtained from New England Nuclear (Boston, MA) and had a specific activity of $0.94\text{--}3.6 \times 10^6$ cpm/pmol. 50 μl of cells were combined with 50 μl of respective serial dilutions of ¹²⁵I-IL-2 in binding buffer (RPMI 1640, 10 mM Hepes, pH 7.2, 5 mg/ml BSA). Patients' cells were at concentrations ranging from 2.5 to $8.8 \times 10^7/\text{ml}$, such that final concentrations in the assay ranged from 1.25 to $4.4 \times 10^6/\text{data point}$. YT cells were at $8 \times 10^6/\text{ml}$. For experiments in which mAb GL439 was present, patient cells and YT cells were pretreated for 20 min on ice with GL439 at a final concentration of 50 $\mu\text{g}/\text{ml}$. For experiments in which the mAb TU27 was also present, a 1:100 dilution of ascites fluid containing the antibody was included in the pretreatment of the cells. The cells and ¹²⁵I-IL-2 were combined for 12 min at 37°C and then spun through a 200- μl layer of silicone/paraffin oil (81:19). Cold competition values were determined by including 2 μg of unlabeled IL-2 (1.3 μM final concentration) in one assay tube with each cell sample and ranged from 0.2 to 0.4% of free counts. These binding assay conditions are sufficient to allow $\geq 80\%$ of the maximal binding observed after incubation for 40 min to occur (unpublished observations), and are consistent with published data demonstrating a $t_{1/2}$ of association to intermediate affinity IL-2Rs of ~ 4.5 min (16). Binding data were analyzed according to the method of Scatchard (56).

Cell Surface Radioiodination and Immunoprecipitation. Before iodination cells were passed over Ficoll/Hypaque to insure good cell viability, 10^7 cells were labeled with 1.0 mCi ¹²⁵I (New England Nuclear) using the glucose oxidase/lactoperoxidase method (Enzymobeads^R), essentially according to manufacturer's (Bio-Rad Laboratories, Richmond, CA) instructions. Briefly, cells were resuspended in a final volume of 125 μl of PBS, followed by addition of 50 μl Enzymobeads, 25 μl 1% β -D glucose, and 1.0 mCi ¹²⁵I. After 20–25 min at room temperature, cells were pelleted, washed four times with PBS, and solubilized in lysis buffer (20 mM Tris, pH 7.5, 140 mM NaCl, 0.1% deoxycholate, and 1% NP-40) containing 1 mM PMSF and 2.5 $\mu\text{g}/\text{ml}$ each aprotinin, leupeptin, and antipain. Nuclei and debris were removed by centrifugation for 30 min at 100,000 g . Lysates were precleared twice by incubation with 25 μl (packed) mouse IgG agarose (Sigma Chemical Co.), followed by two additional preclearings with 25 μl (packed) Gammabind-protein G agarose (Genex, Gaithersburg, MD). Immunoprecipitations were performed by incubating aliquots of precleared lysates with 2–5 μl of ascites fluid containing either control IgG2a (UPC10) or anti-p70 (Mik β 1) mAb. After 2–4 h on ice, 50 μl Gammabind protein G agarose (1:1 in PBS, pH 7.4, 0.05% thimerosal) was added. After 1–2 h at 4°C , precipitates were pelleted, washed four times with lysis buffer, two times with 10 mM Tris, pH 7.5, and resuspended in SDS-PAGE sample buffer. After boiling for 10 min, samples were analyzed on 9% SDS polyacrylamide gels under reducing conditions, as described (57).

Results

Flow Cytometric Analysis of IL-2R p55 and p70 Expression. Pre- and post-IL-2 therapy PBL were examined for expression of both the α (Tac) and β (p70) subunits of the IL-2R. Consistent with our previous observations (44), circulating PBL show a slight increase in Tac expression after in vivo

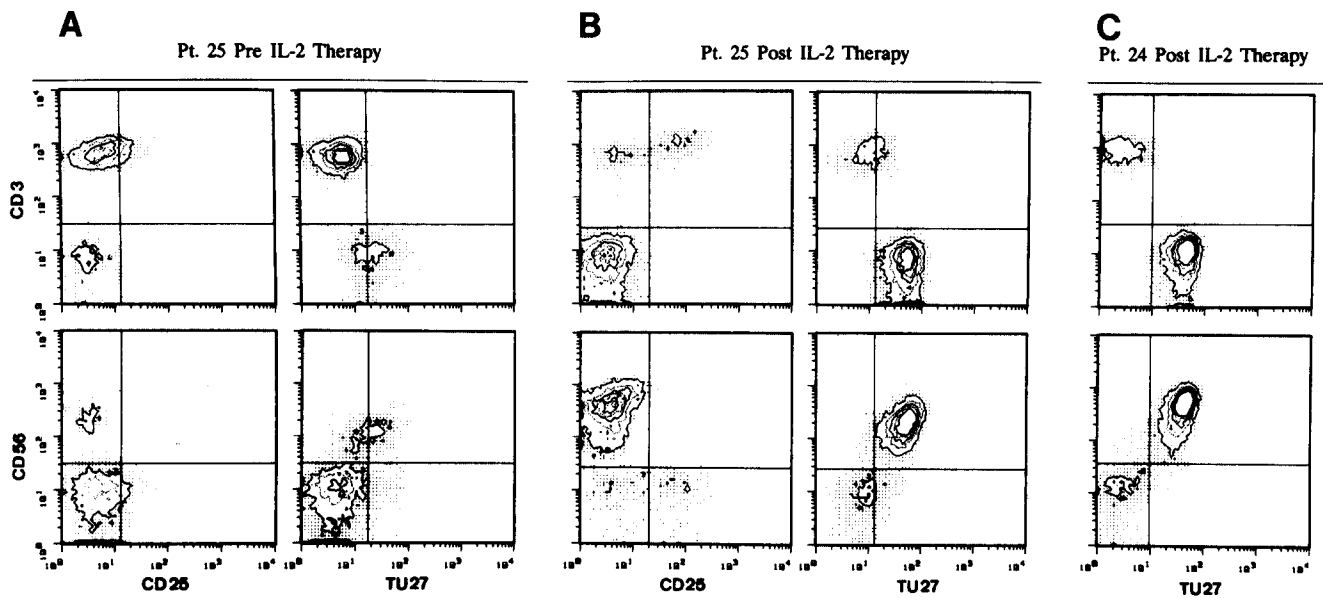


Figure 1. Flow cytometric analysis of IL-2R expression on pre- and post-IL-2 therapy PBL. Cryopreserved PBL from patient 25 obtained before (A) or 24 h after (B) IL-2 therapy and from patient 24 after IL-2 therapy (C) were thawed and stained with the indicated antibodies: anti-CD25-FITC, anti-CD3-PE, anti-CD56-PE, or the anti-p70 mAb TU27. Staining with TU27 was followed by indirect fluorescent staining with GAM-FITC. Two-color fluorescence was performed as described in Materials and Methods. Quadrant settings, distinguishing positive from background fluorescence, were determined for each marker based on staining with isotype control antibodies (not shown). The results shown for patients 24 (C) and 25 (A and B) are representative of comparable data obtained from nine different patients (Table 1).

Table 1. Summary of TU27 Fluorescence and ^{125}I -IL-2 Binding Results from Post-IL-2 Therapy PBL

Patient	Percent CD56 ⁺	TU27 MFI		^{125}I -IL-2 binding	
		CD3 ⁺ subpopulation	CD56 ⁺ subpopulation	K_d	Receptors/cell
				<i>pM</i>	
14	81	0.6	41	510	800
15	42	0.9	39	ND	ND
16	87	1.0	33	1,030	1,780
17	88	2.6	44	730	1,290
19	74	0	45	ND	ND
21	74	0.4	45	ND	ND
22	83	1.5	35	570	690
24	82	0	37	1,820	1,060
25	86	5.5	47	1,330	790
YT	ND		13	1,410	12,800

For post-IL-2-therapy PBL, electronic gates were set on either the CD56⁺ or the CD3⁺ population. For YT cells, all viable cells were analyzed. TU27 MFI was calculated by subtracting MFI of irrelevant control IgG1 from MFI of the TU27-stained cells. An MFI of 0 denotes that TU27 staining was not detectably different from background.

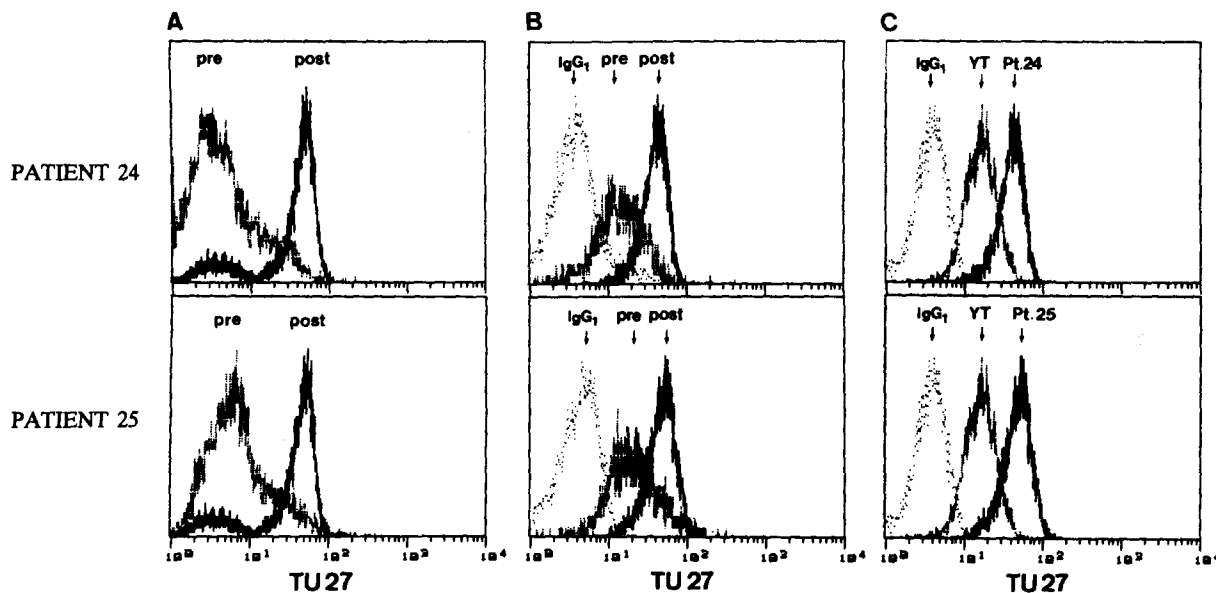


Figure 2. Comparison of TU27 fluorescent staining on pre- and post-therapy PBL and YT cells. The relative levels of TU27 staining of pre- and post-therapy PBL were determined by indirect immunofluorescence. (A) Comparison of pre- and post-therapy PBL stained with TU27 and gated only on forward and side scatter to allow evaluation of the total lymphocyte population. (B) Comparison of the level of TU27 staining of pre- and post-therapy CD56⁺ NK cells, performed by staining cells with anti-CD56-PE and TU27/GAM-FITC. Lymphocyte gates were set based on characteristic forward and side light scatter properties, followed by gating on CD56⁺ cells. TU27 MFI (calculated by subtracting background fluorescence of control IgG1) on the pre-therapy CD56⁺ populations were 10 and 14, as compared with post-therapy TU27 MFIs of 37 and 47 for patients 24 and 25, respectively. (C) Comparison of the level of TU27 staining on post-therapy CD56⁺ NK cells and YT cells. Forward and side scatter gates were set for YT cells; patient cells were gated as described above in B. In B and C, the histogram closest to the ordinate (IgG1) represents negative control staining of post-therapy CD56⁺ PBL (B) and YT cells (C). For clarity, only one control histogram is shown since controls for individual samples were superimposable (not shown). For each sample, the number of events analyzed was similar (7,000–9,000), with the exception of pre-therapy cells gated on the CD56⁺ population (1,500–1,700 events). This difference is reflected in the difference in vertical scale between the pre- and post-therapy samples (B).

IL-2 therapy; all Tac⁺ cells were CD3⁺CD56⁻ T cells (Fig. 1, A and B). No Tac expression was detected on the CD56⁺ CD3⁻ NK cell population.

Using TU27, a mAb directed against the p70 β chain of the IL-2R, we and others have observed low but detectable levels of p70 on fresh NK cells obtained from healthy control donors (33–35, 58, and S. Voss, unpublished observations). Similar results were obtained when PBL from cancer patients were analyzed before *in vivo* IL-2 therapy (Fig. 1 A). These data indicate that the pattern of TU27 staining observed on PBL obtained from patients before initiating IL-2 therapy is similar to that observed with PBL from normal healthy donors. In contrast, when post-therapy PBL were examined, a dramatic increase in p70 expression was observed (Fig. 1 B and C). This increase was limited to the CD56⁺ NK cell subset. Similar results were obtained with PBL from seven other patients receiving continuous infusions of IL-2 (Table 1). In all of the post-therapy PBL samples studied, bright TU27 staining of the CD56⁺ population was observed (Table 1). Identical results were obtained using indirect fluorescent staining with the TU27 mAb (Fig. 1) or with TU27 directly conjugated to FITC (not shown). Although some weak staining of CD3⁺ T cells with TU27 was occasionally observed (Fig. 1 B), this observation has not been seen reproducibly with PBL from other patients (Fig. 1 C, Table 1).

The percentage of CD56⁺ cells in the pre-therapy PBL was usually <20% (not shown), while 42–88% of the post-therapy PBL from these patients were CD56⁺ (Table 1). To compare the level of TU27 staining of the CD56⁺ cells from pre- and post-therapy samples, we double stained cells with anti-CD56 and TU27 and directly compared TU27 fluorescence intensity on the CD56⁺-gated populations. Fig. 2 shows data for TU27 staining of the ungated (A) and CD56⁺-gated (B) PBL from two representative patients. From Fig. 2 B, it is apparent that there is a dramatic increase in p70 expression on the post-therapy CD56⁺ PBL relative to pre-therapy CD56⁺ PBL. Comparison of the TU27 MFI of pre- and post-therapy CD56⁺ cells revealed post-therapy values that were 3.6-fold (Pt.24) and 3.25-fold (Pt.25) greater than the corresponding pre-therapy values. Fig. 2 C shows, as a control population, YT cells: a well-characterized NK-like cell line expressing a relatively high number (\sim 12,000/cell) of intermediate affinity IL-2Rs (12, 13, 16, 17). The MFI for TU27 staining of YT cells (MFI = 13) was less than that for post-therapy cells (average MFI = 41 ± 6 ; Table 1), suggesting that continuous administration of IL-2 *in vivo* might result in a high level of expression on post-therapy PBL of intermediate affinity IL-2Rs (>12,000/cell, based on comparison with YT cells).

Because the level of p70 expression detected by FACS on

the post-therapy PBL samples is considerably higher than the levels reported by others on various cell populations (33–35, 49, 52, 58), it was important to confirm our observations with TU27 using another anti-p70 mAb. We therefore repeated our flow cytometric analyses using Mik β 1, an anti-p70 mAb that was derived independently from TU27 using a different cell line (YT rather than TL-Mor) as an immunogen (52). As presented in Fig. 3, staining with Mik β 1 resulted in the same increase in fluorescence intensity on the post-therapy PBL as that previously observed with TU27. This increase was again restricted to the CD56⁺ NK cell subpopulation. Furthermore, the level of expression of p70 on the patient's cells was again higher than that observed on YT. These experiments have been repeated with PBL from three patients with identical results and confirm our observations with TU27. Further confirmation was also obtained with Mik β 3, another anti-p70 mAb that, in contrast to TU27 and Mik β 1, does not compete with IL-2 for binding to p70 (49, 52). The results of staining with Mik β 3 are presented below as a component of the IL-2 binding analysis with biotinylated IL-2 (shown in Fig. 9).

Effect of Fc Receptor Blocking on TU27 Staining of Post-Therapy PBL. We and others have reported increased numbers of NK cells bearing FcRs (Fc γ RIII, CD16) in post-IL-2 therapy PBL (47, 59). Even though the Fc γ RIII expressed on NK cells binds monomeric IgG poorly and monomeric murine IgG at undetectably low levels (60, 61), it remained possible that the apparent increase in p70 expression observed on the post-therapy CD56⁺ NK cells was due to interaction of the anti-p70 mAbs with the FcRs present on these cells.

To rule out this trivial explanation for the increase in TU27 staining observed, post-therapy PBL were pre-incubated with heat-aggregated rabbit IgG to saturate all FcRs before staining with TU27. This FcR saturation treatment virtually eliminated the ability of these cells to be recognized by the anti-CD16 mAb, but had no effect on TU27 staining (Fig. 4). These results, together with the negligible staining observed with appropriate isotype control mAbs, and the observation that both CD16⁺ and CD16⁻ post-therapy CD56⁺ NK cell subpopulations stain with TU27 with equal intensity (not shown), argue that the increased staining with the anti-p70 mAbs reflects an increase in p70 β chain expression, and not interaction of the anti-p70 mAbs with FcRs.

Proliferative Response of Post-Therapy CD56⁺ PBL to IL-2. To evaluate the functional role of the p70 β chains expressed on the CD56⁺ post-therapy PBL in the response of these cells to IL-2, freshly obtained post-therapy PBL were sorted to 99% purity by FACS using anti-CD56 and anti-CD5 mAbs. The CD56⁺CD5⁻ cells were cultured for 48 h in increasing concentrations of IL-2 in the presence of the anti-p70 mAb TU27, the anti-p55 mAb GL439, or both. Representative results from experiments performed on three separate occasions are presented in Fig. 5. TU27 completely inhibited the proliferative response of the cells to IL-2, while GL439 had no effect. In control experiments, GL439 produced a substantial inhibition of the proliferative response of fresh normal PBL to IL-2 (40–75% inhibition at IL-2 concentrations \leq 0.5 nM [not shown]). CD5⁺CD56⁻ T cells sorted from the

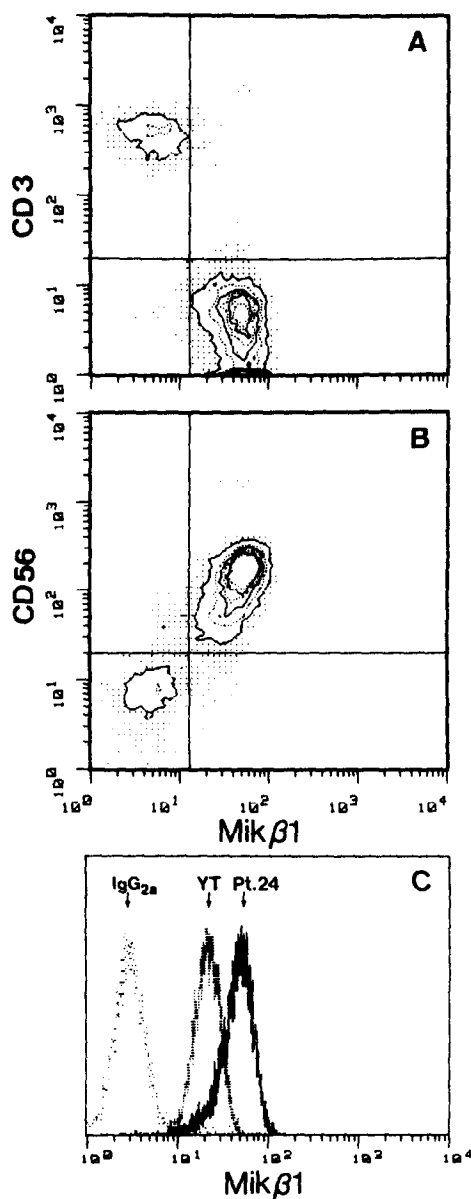


Figure 3. Mik β 1 staining of post-therapy PBL and YT cells. Post-therapy PBL from patient 24 are shown stained with the anti-p70 mAb Mik β 1 followed by GAM-FITC. Two-color fluorescence analysis was performed as described in Materials and Methods with anti-CD3-PE (A) or anti-CD56-PE (B). Quadrant settings were determined based on negative staining of isotype control antibodies (not shown). Comparison of Mik β 1 staining on YT cells and Pt. 24 post-therapy cells gated on the CD56⁺ population is shown in C. The histogram closest to the ordinate (IgG_{2a}) shows isotype control staining on Pt. 24 PBL. Similar isotype control staining for YT cells was observed (not shown).

same post-IL-2-therapy PBL in the same experiment and cultured similarly showed no response to in vitro IL-2 (not shown), consistent with previous results showing decreased T cell responses by post-therapy PBL (44, 48). These results confirm our previous observations, performed with unseparated populations of cryopreserved post-therapy PBL (44, 47), and document that the response of post-IL-2-therapy PBL to subse-

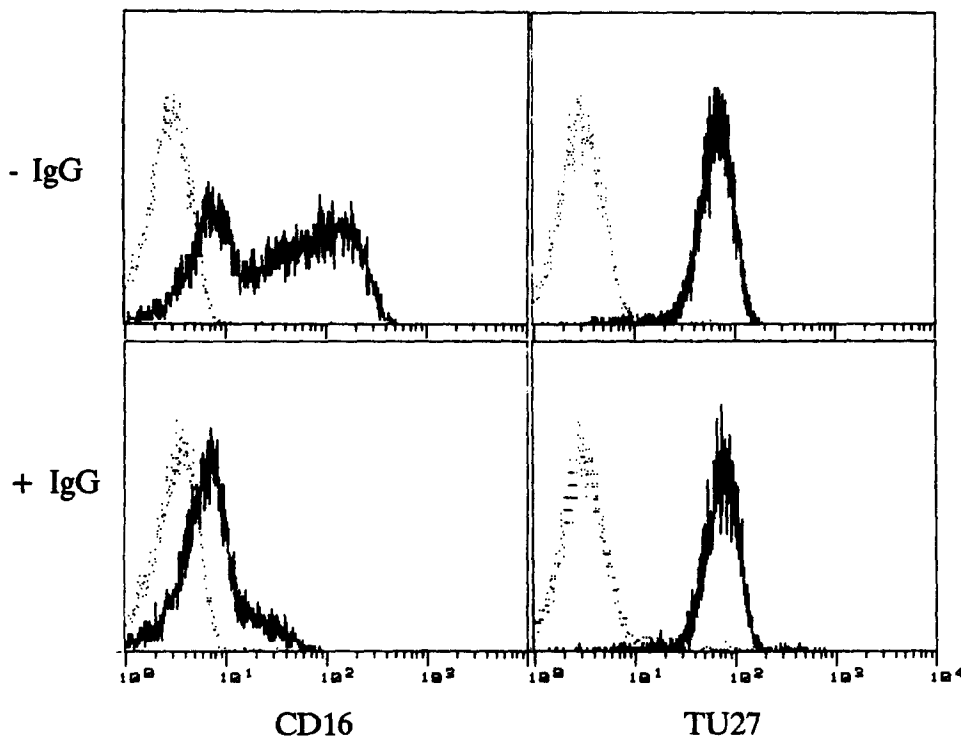


Figure 4. Effect of FcR blocking on TU27 fluorescence intensity. Post-therapy PBL from patient 14 are shown stained with either anti-CD16 (Leu-11a, a mAb that recognizes the Fc γ RIII found on NK cells [53]) or TU27, after preincubation with 10 mg/ml aggregated rabbit Ig (+ IgG) or control FACS buffer (- IgG), as described in Materials and Methods. The histogram outlined by the dotted line represents isotype control staining. The apparent dimly positive staining observed with the anti-CD16 mAb even in the presence of saturating concentrations of rabbit IgG may be due to slight differences in the fluorescein/protein (F/P) ratios of the IgG1-FITC and anti-CD16-FITC antibody/fluorochrome conjugates. For analysis, electronic gates were set on the CD3⁻ NK cell subpopulation, as described in Fig. 2.

quent stimulation by IL-2 *in vitro* is mediated primarily by the CD56⁺ NK cells and occurs predominantly through the p70 IL-2R without involvement of the p55 Tac chain.

¹²⁵I-IL-2 Binding Studies. The observation that GL439 (a mAb that inhibits IL-2 binding to the p55 Tac chain [51]) had no effect on the IL-2-induced proliferative response of the post-therapy CD56⁺ NK cells (Fig. 5) suggested that high affinity IL-2Rs were not contributing functionally in the proliferative response of the cells to IL-2. To determine whether any high affinity binding sites, reflecting Tac involvement, could be detected on post-therapy NK cells, ¹²⁵I-IL-2 binding assays were performed on post-therapy CD56⁺ NK cells (purified by immunomagnetic beads) in the presence and absence of the anti-p55 mAb GL439 (Fig. 6). GL439 inhibited the high affinity component of the ¹²⁵I-IL-2 binding to YT cells, a result consistent with previous studies demonstrating low level expression of Tac on YT cells, predominantly in the form of high affinity IL-2R complexes (12, 13, 17). In contrast, GL439 had no effect on ¹²⁵I-IL-2 binding to the patient's cells. The absence of detectable high affinity binding sites confirms that the post-therapy NK cells do not utilize Tac/p70 high affinity receptor complexes for IL-2 binding. Under these experimental conditions, the ¹²⁵I-IL-2 binding to YT cells demonstrates that even a very low level of high affinity binding on the patient's NK cells could have been detected.

Further ¹²⁵I-IL-2 binding analyses were performed on post-therapy PBL from six additional patients in order to determine whether the increase in TU27 staining on the CD56⁺ cells was accompanied by a corresponding increase in IL-2 binding sites. To reduce the contribution of the small

proportion of T cells (<20%) in these samples, the experiments were performed in the presence of GL439. Since post-therapy CD56⁺ cells express no detectable Tac (Fig. 1), their proliferation in response to IL-2 is negligibly affected by GL439 (Fig. 5), and no effect of GL439 on ¹²⁵I-IL-2 binding could be demonstrated (Fig. 6), we reasoned that the antibody would not alter the number or affinity of IL-2 binding sites on the CD56⁺ subpopulation. In the samples on which subsequent ¹²⁵I-IL-2 binding assays were performed, CD56⁺ cells comprised an average of 85% of the total cell population (Table 1). Representative Scatchard plots of ¹²⁵I-IL-2 binding obtained from two patient samples and from control YT cells are shown in Fig. 7. The patient sample curves were slightly curvilinear, reflecting either minor heterogeneity in receptor site affinities or experimental variability due to the very low level of bound radioisotope. Based on the results shown in Figs. 5 and 6, analysis of these data and that of the four additional patients studied was limited to best fit lines representing a single class of IL-2 binding sites (Table 1).

Comparison of ¹²⁵I-IL-2 binding data for the patient samples and the control YT cells revealed that the patient cells expressed intermediate affinity receptors with roughly the same K_d as that of the receptors on the YT cells (Table 1). Despite the high level of TU27 staining on the post-therapy cells in reference to the control YT cells, suggesting more p70 molecules on post-therapy PBL than on YT, only very low numbers of intermediate affinity IL-2 binding sites were detected on the post-therapy PBL (Table 1). In fact, the number of binding sites was in the range previously observed by others for unstimulated NK cells (22, 58), although lower than that determined by one group of investigators (34). This ¹²⁵I-IL-2

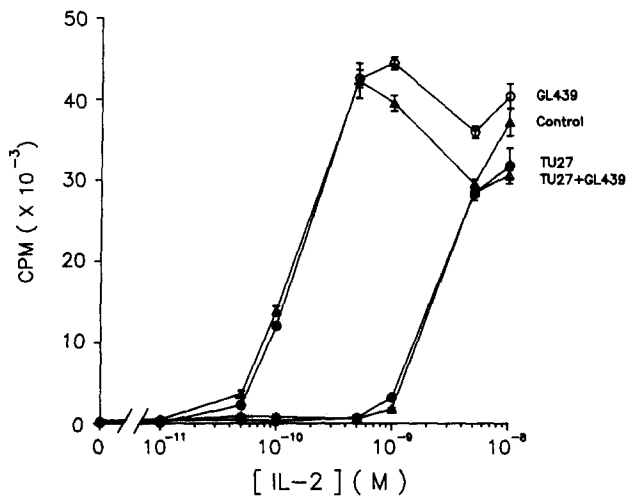


Figure 5. Effect of anti-p55 (GL439) and anti-p70 (TU27) mAbs on proliferation of Pt.23 CD56⁺ post-therapy PBL. CD56⁺ post-therapy cells, obtained after 2 wk of in vivo IL-2 therapy, were sorted by FACS. TU27-FITC staining of the CD56⁺ cells revealed a TU27 MFI of 51 (compare with Table 1). The sorted cells were incubated with increasing concentrations of IL-2 in the presence of control mAb (▲), GL439 (○), TU27 (●), or TU27+GL439 (△). 10⁵ cells/well were incubated with mAbs for 60 min at 4°C before addition of IL-2. After culture for 42 h at 37°C, cells were pulse labeled with 1 μCi of [³H]thymidine for 18 h. Error bars indicate SE of quadruplicate samples. GL439 was added at 50 μg/ml. Ascites fluid containing either TU27 or control IgG1 (MOPC 21; Sigma Chemical Co.) was used at a 1:640 dilution. In control experiments, GL439 inhibited up to 75% of the proliferative response of normal control PBL or anti-CD3-activated T cells to IL-2 (not shown).

binding, like the intermediate affinity IL-2 binding to the control YT cells, was blocked by preincubation of the cells with the TU27 mAb (Fig. 7).

These findings indicate a higher level of p70 expression on the post-therapy NK cells than on YT cells, as determined flow cytometrically using anti-p70 mAbs. These results also demonstrate that only a fraction of the p70 molecules expressed so abundantly on the surface of the post-therapy cells could form functional intermediate affinity IL-2Rs comparable with those on YT cells. One explanation for this result, it could be argued, is that under the experimental conditions used in these studies (i.e., incubation for 12 min at 37°C), equilibrium binding has not occurred, making estimates of receptor number inaccurate. This is not likely to influence our interpretation of the results presented in Table 1, however, which compare the level of IL-2 binding seen on patients' cells with that seen on YT cells handled in parallel under identical conditions. Furthermore, in our experience, >80–85% of the maximal binding (B_{max}) observed after longer incubation periods had occurred by 12 min, consistent with a $t_{1/2}$ of association of ~4.5 min to intermediate affinity IL-2Rs (16). This slight difference in B_{max} observed after longer periods of incubation is unlikely to be the explanation for the 10–15-fold differences in the level of intermediate affinity IL-2 binding seen when comparing the patients' NK cells with YT cells. Furthermore, in one experiment in which

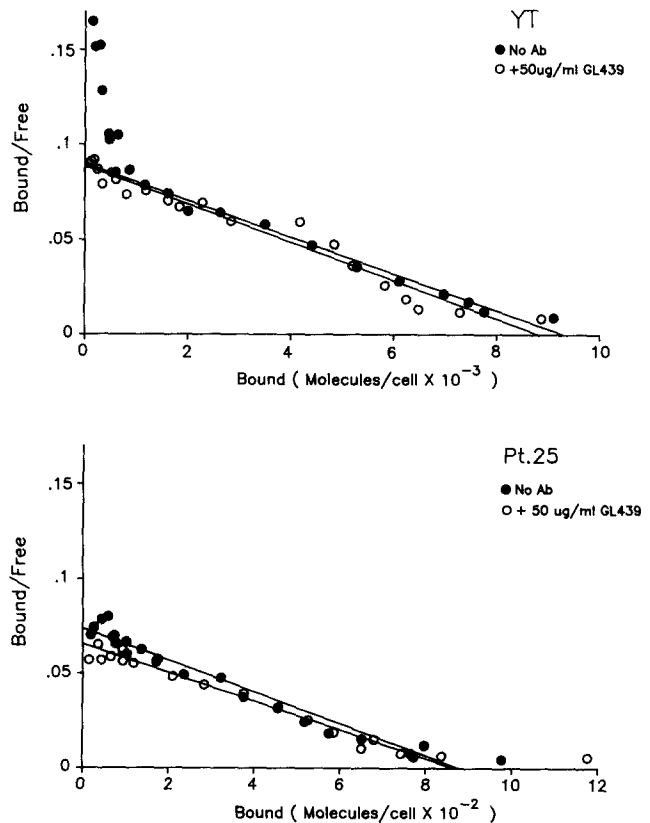


Figure 6. Scatchard analysis of high and intermediate affinity ¹²⁵I-IL-2 binding to YT and Pt. 25 PBL. Patient 25 PBL were depleted of T cells by two rounds of immunomagnetic bead depletion using anti-CD3 (OKT3) and Dynal beads coupled to GAM IgG, as described (44). Upon reanalysis after depletion, the Pt. 25 PBL were >95% pure for CD56⁺ NK cells. Pt. 25 PBL and YT cells were incubated with increasing concentrations of ¹²⁵I-IL-2 for 12 min at 37°C, followed by centrifugation through silicone/paraffin oil. Before addition of ¹²⁵I-IL-2, cells were preincubated for 30 min on ice with 50 μg/ml GL439 (○) or no antibody (●).

incubations were carried out at 37°C for 25 min, a similar low level of IL-2 binding to the patient's NK cells relative to YT was observed. It is thus apparent that the majority of the molecules on the surface of the patients' NK cells recognized by the anti-p70 mAbs were incapable of the level of intermediate affinity IL-2 binding that would have been expected under the experimental conditions used, based on comparison with the level of anti-p70 mAb staining seen on the YT cells.

Competitive Inhibition of TU27 mAb Binding by IL-2. Since the flow cytometric data were collected on individual viable cells and the IL-2 binding assays were performed with large numbers of cells in bulk populations, it is difficult to make direct quantitative comparisons between the apparently discordant results of these two assays. TU27 and Mik β1 binding to post-therapy PBL was greater than that to YT cells by FACS analysis, whereas the ¹²⁵I-IL-2 binding to the patient PBL was considerably less than the binding to YT cells. We therefore felt it was important to evaluate IL-2 binding on the post-therapy PBL relative to YT cells using flow cytometry. Since IL-2 and the TU27 mAb compete with one an-

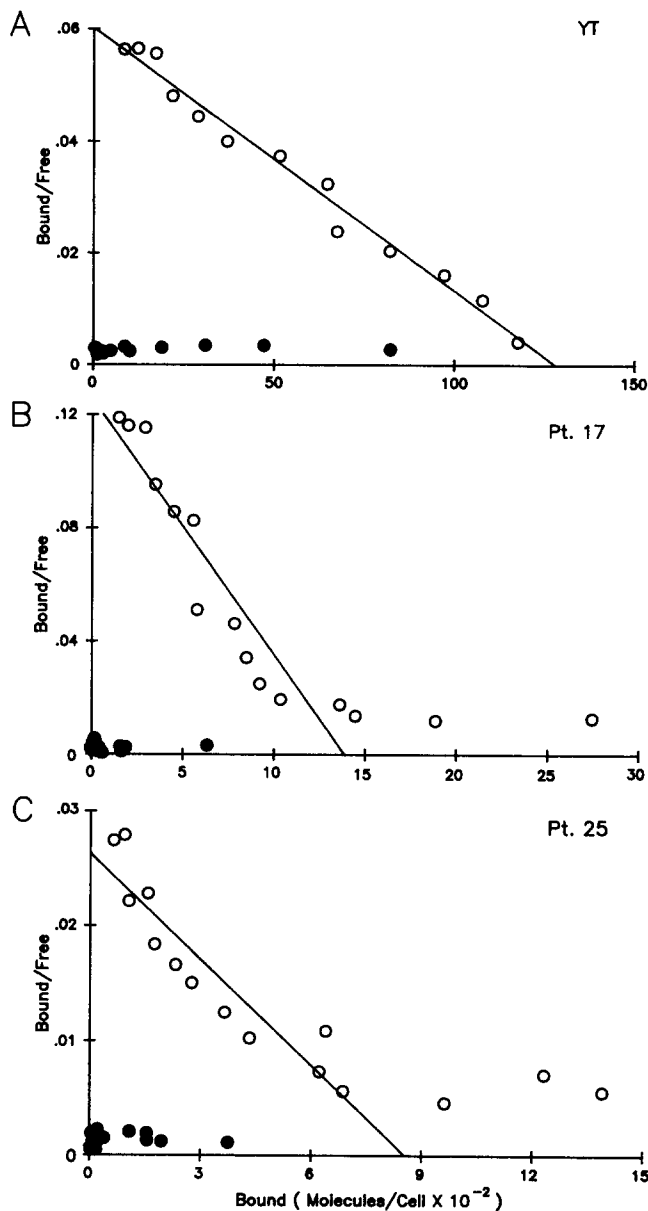


Figure 7. Scatchard analysis of representative ^{125}I -IL-2 binding data. YT cells (A) or freshly thawed cryopreserved patients' cells (B and C) were incubated with increasing concentrations of ^{125}I -IL-2 for 12 min at 37°C , followed by centrifugation through silicone/paraffin oil. Before addition of ^{125}I -IL-2, cells were preincubated for 20 min on ice with $50\ \mu\text{g}/\text{ml}$ GL439 (O) or $50\ \mu\text{g}/\text{ml}$ GL439 + 1:100 dilution of ascites fluid containing TU27 (●). In control experiments, preincubation of MT-1 cells, a Tac $^+$ p70 $^-$ T cell line, with $50\ \mu\text{g}/\text{ml}$ GL439 eliminated all specific ^{125}I -IL-2 binding to the low affinity Tac IL-2R subunit. Preincubation of MT-1 cells with ascites fluid containing TU27 had no effect on the ^{125}I -IL-2 binding (not shown).

other for binding to the p70 β chain (49), preincubation of cells with excess IL-2 should competitively inhibit TU27 binding to functional intermediate affinity IL-2Rs. Indeed, when YT cells were preincubated for 1–2 h at 4°C with 0.4 – $1.6\ \mu\text{M}$ IL-2 and then stained with TU27, the MFI de-

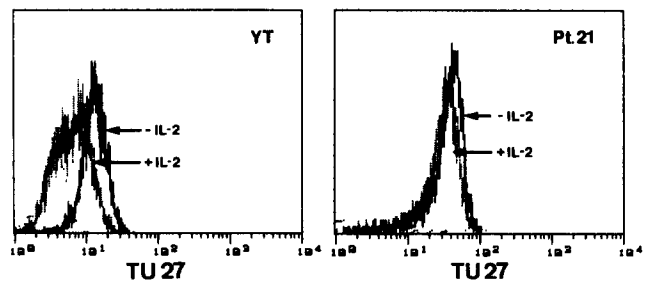


Figure 8. Competition of TU27 staining by IL-2. YT cells and Pt. 21 post-therapy PBL were incubated for 2 h at 4°C in the presence (+) or absence (–) of $1.6\ \mu\text{g}$ IL-2, before staining with TU27 and GAM-FITC. Pt. 21 PBL were also stained with anti-CD56-PE. The effect of IL-2 on TU27 fluorescence was evaluated on YT cells gated only on forward and side scatter. Patient 21 PBL were additionally gated for expression of CD56 after setting lymphocyte gates based on characteristic forward and side scatter patterns. MFI values for the populations shown were calculated as described in Materials and Methods: YT (–IL-2)=10.1; YT (+IL-2)=3.5; Pt21 (–IL-2)=34.9; Pt21 (+IL-2)=29.4.

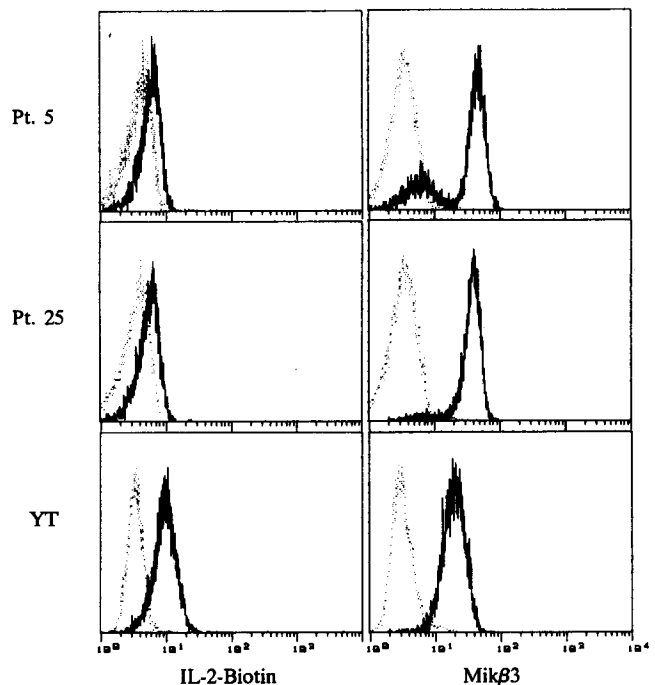


Figure 9. FACS analysis of IL-2 binding using biotinylated IL-2-lys. To examine the distribution of IL-2 binding sites on the post-IL-2 therapy patient PBL, PBL from Pts. 5 and 25, as well as YT cells, were incubated with $1\ \mu\text{g}$ of either biotinylated IL-2-lys or control unbiotinylated IL-2, followed by staining with PE-conjugated Streptavidin. Pt. 5 and Pt. 25 PBL were also stained with anti-CD3-FITC to allow electronic gates to be set on the CD3 $^-$ NK cell subpopulation. All cell populations were gated on forward and side scatter before analysis. Indirect staining of the same cell samples with Mik β 3 and GAM-FITC is shown for comparison. For Mik β 3 staining, the patients' PBL were not gated on the CD56 $^+$ NK cell subpopulation. Control staining with either unbiotinylated IL-2 or IgG1 isotype control mAb is represented in the histograms shown by dotted lines. Staining with either biotinylated IL-2-lys or Mik β 3 is represented by solid lines.

creased by 65% (Fig. 8). Alternatively, if the majority of the p70 β chains recognized by TU27 on post-therapy PBL do not have intermediate affinity IL-2 binding properties, then preincubation with excess IL-2 should minimally inhibit TU27 binding. In fact, when post-therapy cells were stained under identical conditions as the control YT cells, a decrease in MFI of only 16% could be demonstrated (Fig. 8). These experiments were repeated with post-therapy PBL from four different patients on three separate occasions with comparable results. These data confirm the ^{125}I -IL-2 binding data and indicate that the majority of the molecules on the post-therapy CD56⁺ cells recognized by the anti-p70 mAb TU27 do not bind IL-2 with the intermediate affinity binding properties of the majority of the p70 molecules present on control YT cells.

FACS Analysis of Cell Surface IL-2 Binding with Biotinylated IL-2. The ^{125}I -IL-2 binding data and the competitive inhibition data presented thus far, while independently demonstrating a low level of intermediate affinity IL-2 binding on post-therapy NK cells, do not distinguish between uniform distribution of a low number of intermediate affinity binding sites on all CD56⁺ NK cells and a high number of intermediate affinity binding sites expressed on only a few cells in the CD56⁺ population. To address this issue, experiments were performed in which YT cells and post-therapy PBL were incubated with IL-2-lys-biotin (55), followed by fluorescent staining with PE-conjugated streptavidin. Low level, homogeneous IL-2-lys-biotin staining of the patients' post-therapy cells was observed when compared with the positive staining of the control YT cells (Fig. 9), indicating that the low level of IL-2 binding detected in the bulk population in ^{125}I -IL-2 binding assays is distributed uniformly throughout the CD56⁺ NK cell population. As a control, staining with the anti-p70 mAb Mik β 3 was included (Fig. 9), confirming our earlier observations with TU27 and Mik β 1, and demonstrating that despite low levels of IL-2-lys-biotin binding relative to YT cells, high levels of p70 (relative to YT) were expressed on the patients' NK cells.

Immunoprecipitation of p70 From Post-Therapy PBL and YT. The data presented thus far indicate a discrepancy between the number of intermediate affinity IL-2 binding sites present on post-IL-2 therapy NK cells (measured by IL-2 binding) and the level of the p70 IL-2R β expressed on the cell surface, as detected by FACS analysis with anti-p70 mAbs. At least one of the mAbs used in the FACS analyses (Mik β 3) clearly recognizes an epitope on p70 distinct from the epitope(s) recognized by Mik β 1 and TU27, since Mik β 3, unlike Mik β 1 and TU27, does not compete with IL-2 for binding to p70. While it seems, therefore, unlikely that this panel of anti-p70 mAbs is recognizing some molecule other than p70 on the post-therapy PBL, immunoprecipitation experiments were undertaken to prove recognition of p70. As shown in Fig. 10, only the 70–75-kD IL-2R β chain was specifically precipitated by Mik β 1 when tested with either YT cells or post-therapy PBL from Pt.16. Identical results have been obtained with TU27 and Mik β 3 (not shown). The pattern of migration of this 70–75-kD band, specifically precipitated by the anti-p70 mAbs, is distinct from that described for CD16

(62), again making it unlikely that the increased p70 expression observed on the post-therapy patient PBL is due to FcR interaction. Similar p70 immunoprecipitation results have now been obtained with PBL from six different patients (not shown).

Discussion

These studies have evaluated the influence of in vivo IL-2 administration on cell surface IL-2R expression. We and others have previously documented an increase in both the absolute number and the mean percentage of Tac⁺ PBL after IL-2 therapy (28, 36, 45, 46). Nevertheless, subsequent analyses showed that the majority of the post-therapy CD56⁺ NK cells did not express detectable levels of Tac and maintained this minimal Tac expression upon further in vitro culture with IL-2 (44). Furthermore, very low levels of soluble Tac were released during in vitro stimulation, relative to pre-therapy PBL, again suggesting a minimal role for Tac in the response of in vivo activated NK cells to IL-2 (44).

In the studies reported here, we have examined patients' pre- and post-therapy PBL for the possibility of increased expression of the p70 β chain of the IL-2R. When pre- and post-therapy cells from the same patient were stained with either anti-Tac or the anti-p70 mAb TU27, we again observed virtually no Tac⁺ CD56⁺ cells; the majority of the Tac expression was restricted to CD3⁺ T cells. In contrast, nearly all of the CD56⁺ cells stained brightly with TU27, while only dim staining of CD3⁺ cells was seen. When electronic gates were set on the CD56⁺CD3⁻ population, a comparison of the TU27 staining on pre- vs. post-therapy cells revealed an increase in TU27 fluorescence intensity on the post-therapy population, suggesting increased expression of the p70 β chain on CD56⁺ NK cells induced by in vivo IL-2. FcR blocking experiments, as well as immunoprecipitation

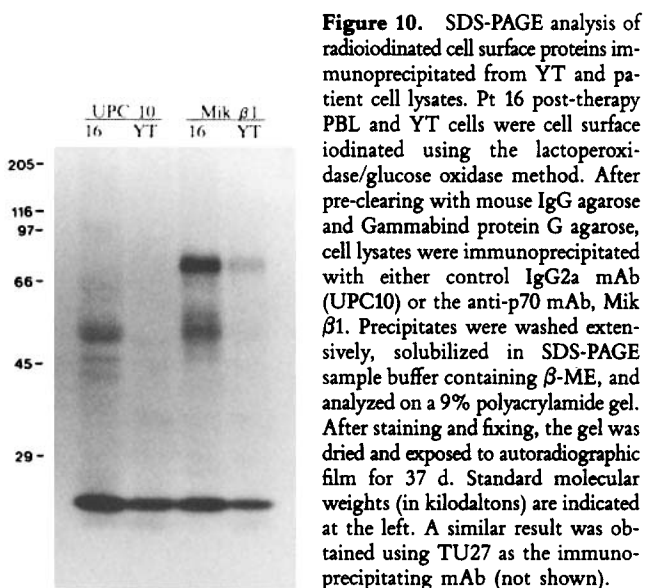


Figure 10. SDS-PAGE analysis of radioiodinated cell surface proteins immunoprecipitated from YT and patient cell lysates. Pt 16 post-therapy PBL and YT cells were cell surface iodinated using the lactoperoxidase/glucose oxidase method. After pre-clearing with mouse IgG agarose and Gammabind protein G agarose, cell lysates were immunoprecipitated with either control IgG2a mAb (UPC10) or the anti-p70 mAb, Mik β 1. Precipitates were washed extensively, solubilized in SDS-PAGE sample buffer containing β -ME, and analyzed on a 9% polyacrylamide gel. After staining and fixing, the gel was dried and exposed to autoradiographic film for 37 d. Standard molecular weights (in kilodaltons) are indicated at the left. A similar result was obtained using TU27 as the immunoprecipitating mAb (not shown).

data, demonstrated that this increased TU27 fluorescence intensity was not due to interaction of the anti-p70 mAb with FcRs.

A comparison of the TU27 staining of post-therapy cells to YT cells revealed, to our surprise, that the post-therapy cells were considerably brighter than YT cells when stained with TU27. When the flow cytometric analyses were repeated with Mik β 1 and Mik β 3, both independently derived anti-p70 mAbs, identical results were obtained. Immunoprecipitation experiments confirmed that the molecule recognized by the anti-p70 mAbs was the 70–75-kD IL-2R β chain. However, only low levels of IL-2 binding to the post-therapy cells could be demonstrated in 125 I-IL-2 binding assays, relative to YT cells. Similar results have been obtained from six additional patients (not shown). Scatchard analyses indicated an average of only 790–1,290 intermediate affinity receptors/cell in the post-therapy population, as compared with an average of 12,000 receptors/YT cell. This unexpected result was verified flow cytometrically when we observed that preincubation of post-therapy cells with excess IL-2 had little effect on TU27 staining, while similar treatment of YT cells resulted in a >60% decrease in TU27 staining. FACS analysis of IL-2 binding to the post-therapy PBL using biotinylated IL-2 further demonstrated uniform distribution of the low number of IL-2 binding sites detected in the 125 I-IL-2 binding assays.

In this report, we have also demonstrated the lack of high affinity IL-2R expression on the post-therapy CD56⁺ NK cells, confirming FACS data from our laboratory (44), and from others (59), showing the lack of cell surface staining by the anti-Tac mAb. In addition, these cells appear to respond to IL-2 exclusively through the p70 β chain and do not appear to synthesize Tac and express high affinity IL-2R after exposure to IL-2 in vitro, since proliferative responses to IL-2 were completely inhibited by the anti-p70 mAb TU27 and were unaffected by the anti-Tac mAb GL439. Nagler et al. (34) and Caligiuri et al. (35) have recently demonstrated the presence of functional high affinity IL-2Rs on a small subset of resting NK cells that bear the CD56^{bright}CD16⁻ phenotype. These authors hypothesized that the high affinity IL-2R-bearing CD56^{bright} population of NK cells may represent a progenitor population capable of responding to low doses of IL-2 by expanding and maturing into FcR-bearing NK cells. In most cases, the majority of the NK cells expanded in vivo are CD56^{bright}, 40–60% of which generally coexpress FcR, as detected by the anti-CD16 mAb. Our in vivo data are thus consistent with this hypothesis and suggest that exposure to continuous infusions of IL-2 (during which steady-state IL-2 levels of \sim 30 U/ml are attained) may lead to the expansion of CD56^{bright} NK cells by virtue of the circulating concentrations of IL-2 being sufficient to saturate high affinity IL-2R. If this is the case, our data would further suggest that chronic exposure to IL-2 in vivo, while possibly leading to the expansion of an NK cell subset originally characterized by the presence of high affinity IL-2Rs, leads to the loss of high affinity IL-2R expression on the NK cells that return to the peripheral circulation from the lymphoid organs after cessation of IL-2 therapy. This result is consistent with

the observation that the CD56^{bright} NK cells purified from fresh peripheral blood that initially express high affinity IL-2Rs rapidly downmodulate cell surface Tac expression upon in vitro culture in IL-2 (J. Phillips and L. Lanier, personal communication). Another possibility, also consistent with these data, would be the in vivo activation by IL-2 of a small NK subpopulation that neither expresses nor utilizes the Tac molecule, with subsequent in vivo expansion enabling this initially small subset of NK cells to be the predominant NK cell phenotype in the post-therapy PBL.

The molecular mechanisms regulating the level of p70 expression on the patients' post-therapy NK cells remain unclear. Our data, demonstrating an elevation in p70 expression without a corresponding elevation in the level of intermediate affinity IL-2 binding, suggest that the presence of the p70 β chain alone may not be the sole determinant of intermediate affinity IL-2 binding. While both previous binding and cross-linking studies indicated that p70 was the IL-2 binding component of the intermediate affinity IL-2R, it appears from our data that some other level of regulatory control may be required to allow the p70 β chain to bind IL-2. One explanation for the apparent discrepancy between the anti-p70 mAb staining and IL-2 binding data might be expression of immature or structurally modified forms of p70 that remain antigenically similar but lack IL-2 binding capabilities. This seems less likely in view of the immunoprecipitation results showing the 70–75-kD β chain as the predominant species specifically precipitated by Mik β 1 (as well as Mik β 3 and TU27) from whole cell lysates prepared from cell surface-iodinated post-therapy patient PBL. Alternatively, it is conceivable that a conformational change in the β chain could be introduced by association with a putative γ subunit or that a putative γ subunit might itself bind IL-2. Crosslinking studies with 125 I-IL-2 have revealed numerous other bands in addition to the 55-kD α and 70-kD β chains (1, 63, 64), any one of which could function to regulate the binding of IL-2 to the p70 β chain. In immunoprecipitation experiments using the anti-p70 mAb TU11, Sugamura and coworkers (65) have demonstrated the association of a 64-kD molecule, p64 (which they have termed IL-2R γ), with the p70 β chain of high affinity IL-2R. Whether p64 is also a component of intermediate affinity IL-2Rs remains to be determined. It is tempting to speculate that p64, which could only be detected when IL-2 was bound to its receptor during the immunoprecipitation, plays a role in regulating the binding of IL-2 to the p70 β chain.

A number of other recent reports have also addressed the possible involvement of additional IL-2R subunits in the formation of functional IL-2Rs (1, 52, 63, 64, 66–69). Transfection experiments in which the cDNA encoding the p70 β chain was introduced into nonlymphoid cells failed to demonstrate intermediate affinity IL-2 binding despite good cell surface expression of p70, while transfection of the p70 cDNA into Jurkat cells, which express neither p55 nor p70, or into MT-1 cells, which express the p55 α chain but little to no p70 β chain, produced functional intermediate and high affinity receptors, respectively (67, 68, and B. Freemark and R. Robb, unpublished observations). Robb and coworkers (69) have also recently characterized the kinetic binding properties of

p55/p70 IL-2R complexes expressed on cDNA-transfected CHO cells. These receptors, while displaying equilibrium binding properties characteristic of true high affinity IL-2Rs, appear to be pseudo high affinity, in that bound IL-2 has a very rapid rate of dissociation from these receptors (at least 10-fold greater than the rate of dissociation seen with HUT 102 cells) (69). Based on these data, one can speculate that at least one function of additional IL-2R subunits might be to stabilize the IL-2/ β chain complex and slow the rate of dissociation of IL-2 from p70. Whether this is the explanation for the discrepant low level of IL-2 binding, despite the high level of p70 expression, observed on the post-IL-2 therapy

patients' cells is unknown. The results presented here, together with the results of others, suggest the existence of at least a γ subunit to facilitate formation of functional β -dependent intermediate and high affinity IL-2 binding sites, and imply that the expression of γ may be restricted to cells of lymphoid origin. Our data further suggest that even in lymphoid cells, increased expression of p70 molecules does not necessarily enable them to function as intermediate affinity IL-2Rs; some other level of control is involved. It will be the goal of future studies to investigate the role of such putative regulatory subunits in the response of lymphocytes to IL-2.

We thank Drs. J. A. Sosman, M. Albertini, C. Gambacorti, as well as K. H. Moore, A. Borchert, K. Schell, and J. Surfus for coordination of clinical care and sample procurement; L. Morrissey, and V. Chittick for their expert assistance with the cell sorting and flow cytometry experiments; T. Talley for assistance with the IL-2 binding assays; D. Peters for helpful discussions; and N. Miller and K. Zaremba for administrative help. Special thanks are due Dr. V. Craig Jordan and the members of his laboratory for allowing use of their facilities for the iodination experiments, and Dr. Steven C. Clark for helpful discussions and advice, and for use of equipment during the course of the iodination and immunoprecipitation experiments.

This work was supported by National Institutes of Health contracts CM-47669 and CM-87290, NIH grants CA-32685, RR-03186, and T35-DK07389, and American Cancer Society grant CH-237. S. Voss is supported by a Lutheran Brotherhood/Life and Health Insurance Medical Research Fund M.D./Ph.D training fellowship.

Address correspondence to Paul M. Sondel, Department of Human Oncology, University of Wisconsin Clinical Cancer Center, 600 Highland Avenue, Madison, WI 53792.

Received for publication 13 December 1989 and in revised form 13 April 1990.

References

1. Waldmann, T.A. 1989. The multi-subunit interleukin-2 receptor. *Annu. Rev. Biochem.* 58:875.
2. Greene, W.C., and W.J. Leonard. 1986. The human interleukin-2 receptor. *Annu. Rev. Immunol.* 4:69.
3. Leonard, W.J., J.M. Depper, T. Uchiyama, K.A. Smith, T.A. Waldmann, and W.C. Greene. 1982. A monoclonal antibody that appears to recognize the receptor for human T-cell growth factor: partial characterization of the receptor. *Nature (Lond.)* 300:267.
4. Leonard, W.J., W.J. Depper, R.J. Robb, T.A. Waldmann, and W.C. Greene. 1983. Characterization of the human receptor for T-cell growth factor. *Proc. Natl. Acad. Sci. USA.* 80:6957.
5. Robb, R.J., and W.C. Greene. 1983. Direct demonstration of the identity of T cell growth factor binding protein and the Tac antigen. *J. Exp. Med.* 158:1332.
6. Robb, R.J., W.C. Greene, and C.M. Rusk. 1984. Low and high affinity cellular receptors for interleukin 2: implications for the level of Tac antigen. *J. Exp. Med.* 160:1126.
7. Leonard, W.J., J.M. Depper, G.R. Crabtree, S. Rudikoff, J. Pumphrey, R.J. Robb, M. Kronke, P.B. Svetlik, N.J. Peffer, T.A. Waldmann, and W.C. Greene. 1984. Molecular cloning and expression of cDNAs for the human interleukin-2 receptor: evidence for alternate mRNA splicing and the use of two polyadenylation sites. *Nature (Lond.)* 311:626.
8. Nikaido, T., A. Shimizu, N. Ishida, H. Sabe, K. Teshigawara, M. Maeda, T. Uchiyama, J. Yodoi, and T. Honjo. 1984. Molecular cloning of cDNA encoding human interleukin-2 receptor. *Nature (Lond.)* 311:631.
9. Sharon, M., R.D. Klausner, B.R. Cullen, R. Chizzonite, and W.J. Leonard. 1986. Novel interleukin-2 receptor subunit detected by cross-linking under high-affinity conditions. *Science (Wash. DC)* 234:859.
10. Tsudo, M., R.W. Kozak, C.K. Goldman, and T.A. Waldmann. 1986. Demonstration of a non-Tac peptide that binds interleukin-2: a potential participant in a multi-chain interleukin-2 receptor complex. *Proc. Natl. Acad. Sci. USA.* 83:9684.
11. Siegel, J.P., M. Sharon, P.L. Smith, and W.J. Leonard. 1987. The IL-2 receptor β chain (p70): Role in mediating signals for LAK, NK, and proliferative activities. *Science (Wash. DC)* 238:75.
12. Teshigawara, K., H.-M. Wang, K. Kato, and K.A. Smith. 1987. Interleukin 2 high-affinity receptor expression requires two distinct binding proteins. *J. Exp. Med.* 165:223.
13. Robb, R.J., C.M. Rusk, J.Y. Yodoi, and W.C. Greene. 1987. Interleukin-2 binding molecule distinct from the Tac protein: analysis of its role in formation of high-affinity receptors. *Proc. Natl. Acad. Sci. USA.* 84:2002.
14. Dukovich, M., Y. Wano, Le Thi Bich-Thuy, P. Katz, B.R.

- Cullen, J.H. Kehrl, and W.C. Greene. 1987. A second human interleukin-2 binding protein that may be a component of high-affinity interleukin-2 receptors. *Nature (Lond.)* 327:518.
15. Wang, H.-M., and K.A. Smith. 1987. The interleukin 2 receptor: functional consequences of its bimolecular structure. *J. Exp. Med.* 166:1055.
 16. Lowenthal, J.W., and W.C. Greene. 1987. Contrasting interleukin-2 binding properties of the α (p55) and β (p70) protein subunits of the human high-affinity interleukin 2 receptor. *J. Exp. Med.* 166:1156.
 17. Robb, R.J., and W.C. Greene. 1987. Internalization of interleukin 2 is mediated by the β chain of the high-affinity interleukin 2 receptor. *J. Exp. Med.* 165:1201.
 18. Fung, M.R., G. Ju, and W.C. Greene. 1988. Co-internalization of the p55 and p70 subunits of the high-affinity human interleukin 2 receptor: evidence for a stable ternary receptor complex. *J. Exp. Med.* 168:1923.
 19. Le Thi Bich-Thuy, M. Dukovich, N.J. Pfeffer, A.S. Fauci, J.H. Kehrl, and W.C. Greene. 1987. Direct activation of human resting T cells by IL-2: The role of an IL-2 receptor distinct from the Tac protein. *J. Immunol.* 139:1550.
 20. Tsudo, M., C.K. Goldman, K.F. Bongiovanni, W.C. Chan, E.F. Winton, M. Yagita, E.A. Grimm, and T.A. Waldmann. 1987. The p75 peptide is the receptor for interleukin-2 expressed on large granular lymphocytes and is responsible for the interleukin-2 activation of these cells. *Proc. Natl. Acad. Sci. USA.* 84:5394.
 21. Kehrl, J.H., M. Dukovich, G. Whalen, P. Katz, A.S. Fauci, and W.C. Greene. 1988. Novel interleukin 2 (IL-2) receptor appears to mediate IL-2-induced activation of natural killer cells. *J. Clin. Invest.* 81:200.
 22. Mohamed-Habib, B.A., N. Moire, D. Metivier, C. Vaquero, O. Lantz, D. Olive, B. Charpentier, and A. Senik. 1989. IL-2 receptors on circulating natural killer cells and T lymphocytes. Similarity in number and affinity but difference in transmission of the proliferation signal. *J. Immunol.* 142:490.
 23. Ishii, T., T. Takeshita, N. Numata, and K. Sugamura. 1988. Protein phosphorylation mediated by IL-2/IL-2 receptor β chain interaction. *J. Immunol.* 141:174.
 24. Smith, K.A. 1988. The interleukin-2 receptor. *Adv. Immunol.* 42:165.
 25. Rubin, L.A., C.C. Kurman, M.E. Fritz, W.E. Biddison, B. Boutin, R. Yarchoan, and D.L. Nelson. 1985. Soluble interleukin-2 receptors are released from activated human lymphoid cells in vitro. *J. Immunol.* 135:3172.
 26. Yagita, H., M. Nakata, A. Azuma, T. Nitta, T. Takeshita, K. Sugamura, and K. Okumura. 1989. Activation of peripheral blood T cells via the p75 interleukin 2 receptor. *J. Exp. Med.* 170:1445.
 27. Voss, S.D., J.A. Hank, C. Nobis, P. Fisch, J.A. Sosman, and P.M. Sondel. 1989. Serum interleukin-2 receptor levels during IL-2 therapy reflect systemic lymphoid mass activation. *Cancer Immunol. Immunother.* 29:261.
 28. Lotze, M.T., M.C. Custer, S.O. Sharrow, L.A. Rubin, D.L. Nelson, and S.A. Rosenberg. 1987. In vivo administration of purified human interleukin-2 to patients with cancer: development of interleukin-2 receptor positive cells and circulating soluble interleukin-2 receptors following interleukin-2 administration. *Cancer Res.* 47:2188.
 29. Colvin, R.B., T.C. Fuller, L. MacKeen, P.C. Kung, S.H. Ip, and A.B. Cosimi. 1987. Plasma interleukin-2 receptor levels in renal allograft recipients. *Clin. Immunol. Immunopathol.* 43:273.
 30. Scollard, D.M., D.K. Wagner, and D.L. Nelson. 1988. Release of soluble interleukin 2 receptors (Tac peptide) in vivo during human immune responses to tuberculin. *Cancer Immunol. Immunopathol.* 46:450.
 31. Ortaldo, J.R., A.T. Mason, J.P. Gerard, L.E. Henderson, W. Farrar, R.F. Hopkins III, R.B. Herbermann, and H. Rabin. 1984. Effects of natural and recombinant IL-2 on regulation of IFN γ production and natural killer activity: lack of involvement of the Tac antigen for these immunoregulatory effects. *J. Immunol.* 133:779.
 32. Trinchieri, G., M.M. Kobayashi, S.C. Clark, J. Seehra, L. London, and B. Perussia. 1984. Response of resting human peripheral blood natural killer cells to interleukin 2. *J. Exp. Med.* 160:1147.
 33. Phillips, J.H., T. Takeshita, K. Sugamura, and L.L. Lanier. 1989. Activation of natural killer cells via the p75 interleukin 2 receptor. *J. Exp. Med.* 170:291.
 34. Nagler, A., L.L. Lanier, and J.H. Phillips. 1990. Constitutive expression of high affinity interleukin 2 receptors on human CD16⁻ natural killer cells in vivo. *J. Exp. Med.* 171:1527.
 35. Caligiuri, M.A., A. Zmuidzinas, T.J. Manley, H. Levine, K.A. Smith, and J. Ritz. 1990. Functional consequences of interleukin 2 receptor expression on resting human lymphocytes. Identification of a novel natural killer cell subset with high affinity receptors. *J. Exp. Med.* 171:1509.
 36. Sondel, P.M. 1989. Cellular immunotherapy of cancer: preclinical and clinical testing utilizing interleukin-2. In *IL-2 Activated Killer Cells in Cancer Treatment*. E. Lotzova and R. Herberman, editors. CRC Press, Inc., Boca Raton. 1-24.
 37. Rosenberg, S.A. 1988. Immunotherapy of cancer using Interleukin-2. Current status and future prospects. *Immunol. Today.* 9:58.
 38. Grimm, E.A., A. Mazumber, H.Z. Zhang, and S.A. Rosenberg. 1982. Lymphokine-activated killer cell phenomenon: lysis of natural killer resistant fresh solid tumor cells by interleukin-2-activated autologous human peripheral blood lymphocytes. *J. Exp. Med.* 155:1823.
 39. Parkinson, D.R. 1988. Interleukin-2 in cancer therapy. *Semin. Oncol.* 15(6 suppl):10.
 40. Rosenberg, S.A., M.T. Lotze, L.M. Muul, A.E. Chang, E.P. Avis, S. Leitman, W.M. Linehan, C.N. Robertson, R.E. Lee, J.T. Rubin, C.A. Seipp, C.G. Simpson, and D.E. White. 1987. A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high dose interleukin-2 alone. *N. Engl. J. Med.* 316:889.
 41. Lotze, M.T., A.E. Chang, C.A. Seipp, C. Simpson, J.T. Vetto, and S.A. Rosenberg. 1986. High dose recombinant interleukin-2 in the treatment of patients with disseminated cancer: responses, treatment-related morbidity, and histologic findings. *JAMA (J. Am. Med. Assoc.)* 256:3117.
 42. West, W.H., K.W. Tauer, J.R. Yannelli, G.D. Marshall, D.W. Orr, G.B. Thurman, and R.L. Oldham. 1987. Constant infusion recombinant interleukin-2 in adoptive immunotherapy of advanced cancer. *N. Engl. J. Med.* 316:898.
 43. Sosman, J.A., P.C. Kohler, J.A. Hank, K.H. Moore, R. Bechhofer, B. Storer and P.M. Sondel. 1988. Repetitive weekly cycles of recombinant human interleukin-2 (IL-2): responses of renal carcinoma with acceptable toxicity. *J. Natl. Cancer Inst.* 80:60.
 44. Weil-Hillman, G., S.D. Voss, P. Fisch, K. Schell, J.A. Hank, J.A. Sosman, K. Sugamura, and P.M. Sondel. 1990. In vivo interleukin-2 induced lymphokine activated killer activity is mediated by Leu19⁺ cells which maintain the Tac negative phenotype. *Cancer Res.* 50:2683.

45. Sondel, P.M., P.C. Kohler, J.A. Hank, K.H. Moore, N. Rosenthal, J. Sosman, R. Bechhofer, and B. Storer. 1988. Recombinant interleukin-2 given by repetitive weekly cycles to patients with cancer: clinical and immunologic effects. *Cancer Res.* 48:2561.
46. Sosman, J.A., P.C. Kohler, J.A. Hank, K.H. Moore, R. Bechhofer, B. Storer, and P.M. Sondel. 1988. Repetitive weekly cycles of recombinant human interleukin-2 (IL-2). II. Clinical and immunologic effects of dose, schedule, and addition of indomethacin. *J. Natl. Cancer Inst.* 80:1451.
47. Weil-Hillman, G., P. Fisch, A.F. Prieve, J.A. Sosman, J.A. Hank, and P.M. Sondel. 1989. Lymphokine activated killer activity induced by in vivo interleukin-2 therapy: predominant role for lymphocytes with increased expression of CD2 and Leu19 antigens but negative expression of CD16 antigens. *Cancer Res.* 49:3680.
48. Hank, J.A., J.A. Sosman, P.C. Kohler, R. Bechhofer, B. Storer, and P.M. Sondel. 1989. Depressed in vitro T cells responses concomitant with augmented interleukin-2 (IL-2) responses by lymphocytes from cancer patients following in vivo treatment with IL-2. *J. Biol. Response Modif.* 9:5.
49. Takeshita, T., Y. Goto, K. Tada, K. Nagata, H. Asao, and K. Sugamura. 1989. Monoclonal antibody defining a molecule possibly identical to the p75 subunit of the interleukin 2 receptor. *J. Exp. Med.* 169:1323.
50. Goldstein, D., J.A. Sosman, J.A. Hank, G. Weil-Hillman, K.H. Moore, A. Borchert, R. Bechhofer, B. Storer, P.C. Kohler, D. Levitt, and P.M. Sondel. 1989. Repetitive weekly cycles of interleukin-2 (IL-2): outpatient treatment with a lower dose of IL-2 maintains heightened non-MHC restricted killer activity. *Cancer Res.* 49:6832.
51. Robb, R.J., C.M. Rusk, and M.P. Neep. 1988. Structure-function relationships for the interleukin-2 receptor: location of ligand and antibody binding sites on the Tac receptor chain by mutational analysis. *Proc. Natl. Acad. Sci. USA.* 85:5654.
52. Tsudo, M., F. Kitamura, and M. Miyasaka. 1989. Characterization of the interleukin 2 receptor β chain using three distinct monoclonal antibodies. *Proc. Natl. Acad. Sci. USA.* 86:1982.
53. Yodoi, J., K. Teshigawara, T. Nikaido, K. Fukui, T. Noma, T. Honjo, M. Takigawa, M. Sasaki, N. Minato, M. Tsudo, T. Uchiyama, and M. Maeda. 1985. TCGF (IL-2)-receptor inducing factor(s). I. Regulation of IL-2 receptor on a natural killer-like cell line (YT cells). *J. Immunol.* 134:1623.
54. Lanier, L.L., A.M. Le, J.H. Phillips, N.L. Warner, and G.F. Babcock. 1983. Subpopulations of human natural killer cells defined by expression of the leu 7 (HNK-1) and leu 11 (NK-15) antigens. *J. Immunol.* 131:1789.
55. Lin, Y., R.J. Robb, J.E. Gray, and P. Simon. 1988. The construction and characterization of a biologically active recombinant IL-2 containing a lysine-rich C-terminal extension. *J. Immunol.* 141:3847.
56. Scatchard, G. 1949. The attraction of proteins for small molecules and ions. *Ann. NY Acad. Sci.* 51:660.
57. Laemli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680.
58. Ohashi, Y., T. Takeshita, K. Nagata, S. Mori, and K. Sugamura. 1989. Differential expression of the interleukin-2 receptor subunits, p55 and p75, on various populations of primary peripheral blood mononuclear cells. *J. Immunol.* 143:3548.
59. Phillips, J.H., B.T. Gemlo, W.W. Myers, A.A. Rayner, and L.L. Lanier. 1987. In vivo and in vitro activation of natural killer cells in advanced cancer patients undergoing combined recombinant interleukin-2 and LAK cell therapy. *J. Clin. Oncol.* 5:1933.
60. Anderson, C.L., and R.J. Looney. 1987. Immunoglobulin G Fc receptors of human leukocytes. *Methods Enzymol.* 150:524.
61. Unkeless, J.C., E. Scigliano, and V. Freedman. 1988. Structure and function of human and murine receptors for IgG. *Annu. Rev. Immunol.* 6:251.
62. Lanier, L.L., J.H. Phillips, and R. Testi. 1989. Membrane anchoring and spontaneous release of CD16 (FcRIII) by natural killer cells and granulocytes. *Eur. J. Immunol.* 19:775.
63. Herrmann, T., and T. Diamanstein. 1987. The mouse high-affinity IL-2 receptor complex. I. Evidence for a third molecule, the putative γ chain, associated with the α and/or β chain of the receptor. *Immunobiology.* 175:145.
64. Saragovi, H., and T.R. Malek. 1988. Direct identification of the murine IL-2 receptor p55-p75 heterodimer in the absence of IL-2. *J. Immunol.* 141:476.
65. Takeshita, T., H. Asao, J. Suzuki, and K. Sugamura. 1990. An associated molecule, p64, with high affinity IL-2 receptor. *Int. Immunol.* In press.
66. Szollosi, J., S. Damjanovich, C.K. Goldman, M.J. Fulwyler, A.A. Aszalos, G. Goldstein, P. Rao, M.A. Talle, and T.A. Waldmann. 1987. Flow cytometric resonance energy transfer measurements support the association of a 95-kDa peptide termed T27 with the 55-kDa Tac peptide. *Proc. Natl. Acad. Sci. USA.* 84:7246.
67. Hatakeyama, M., M. Tsudo, S. Minamoto, T. Kono, T. Doi, T. Miyata, M. Miyasaka, and T. Taniguchi. 1989. Interleukin-2 receptor β chain gene: generation of three receptor forms by cloned human α and β chain cDNA's. *Science (Wash. DC).* 244:551.
68. Tsudo, M., H. Karasuyama, F. Kitamura, Y. Nagasaka, T. Tanaka, and M. Miyasaka. 1989. Reconstruction of a functional IL-2 receptor by the β chain cDNA. A newly formed receptor transduces a negative signal. *J. Immunol.* 143:4039.
69. Ringheim, G.E., B.P. Freemark, and R.J. Robb. 1990. Expression of a pseudo high-affinity IL-2 receptor complex in CHO cells: kinetics of ligand binding and internalization. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4(7):2784. (Abstr.)