Natural Killer Cells Activated by Interleukin 2 Treatment in Vivo Respond to Interleukin 2 Primarily through the p75 Receptor and Maintain the p55 (TAC) Negative Phenotype

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ABSTRACT

Interleukin 2 (IL-2) induced activation of unstimulated resting natural killer (NK) cells or resting T-cells initially occurs following binding of IL-2 through the p75 receptor that is expressed primarily by these cells. However, this IL-2/p75 interaction induces TAC chain synthesis and formation of high affinity IL-2 receptor required for the proliferation of resting peripheral blood lymphocytes. In this study, we present data indicating that NK cells activated by in vivo IL-2 treatment, in contrast to resting NK cells, respond and proliferate to further IL-2 in vitro using primarily the p75 receptor with only a minor component of cells responding through the high affinity receptor. These in vivo activated NK cells minimally expressed the TAC chain and maintained this TAC negative phenotype while proliferating in response to IL-2. The primary involvement of the p75 receptor in the proliferative response of these cells to IL-2 was demonstrated by the need for concentrations of IL-2 higher than 44 pm to obtain a significant response and by the dramatic inhibition of this response by anti-p75 monoclonal antibody. Anti-TAC monoclonal antibody inhibited only the poor proliferation obtained at low doses of IL-2 suggesting a minor role for TAC and high affinity IL-2 receptors. This was in contrast to the partial inhibition of proliferation by anti-p75 or anti-TAC observed in unstimulated pretherapy peripheral blood lymphocytes suggesting that these cells respond to IL-2 through both high affinity receptors and intermediate affinity p75 receptors. The T-cells isolated from in vivo activated peripheral blood lymphocytes, despite expressing TAC, were not responsive to IL-2, suggesting that these cells express predominantly nonfunctional low affinity TAC receptors. NK cells activated by IL-2 in vitro represent a unique model system of IL-2 dependent cells that respond and proliferate to IL-2 essentially through the p75 IL-2 receptor.

INTRODUCTION

Three forms of IL-2R have been described: a high affinity receptor (K_a = 10^{-11} M), a complex of at least two distinct polypeptide chains, the p55 (TAC) and the p75; an intermediate affinity receptor (K_a = 10^{-9} M) consisting of the p75 chain alone; and a low affinity receptor (K_a = 10^{-8} M) consisting of the p55 TAC chain alone (1-6). The p75 subunit alone is able to bind IL-2 (1-5) and mediates its internalization (7, 8) leading to lymphocyte activation (8, 9).

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3 The abbreviations used are: IL-2R, interleukin 2 receptor; IL-2, interleukin 2; NK, natural killer; PBL, peripheral blood lymphocytes; mAb, monoclonal antibody; LAK, lymphokine-activated killer; FITC, fluorescein isothiocyanate; PHA, phytohemagglutinin; PBS, phosphate buffered saline; HS-RPMI, RPMI 1640 supplemented with 25 mm 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer; l-glutamine, penicillin/streptomycin, and 10% heat inactivated pooled nontransfused male serum (Pel-Freez).

However, the TAC chain alone, although capable of binding IL-2 (10), does not internalize it (7) and is not by itself functional unless associated with the p75 chain in the form of the high affinity receptor complex (6, 9). Several chemical cross-linking studies with radiolabeled IL-2 performed on purified resting T-cells (11-13), large granular lymphocytes (11, 14), and purified NK cells (13, 15) separated from normal unstimulated human PBL have shown that these cells express primarily the p75 chain, with only a minimal fraction of cells expressing the TAC p55 chain. Functional studies of these unstimulated lymphocytes following depletion of the small fraction of TAC+ cells have suggested a two step model for cell activation. In the first step, IL-2 (at high concentrations) binds to the p75 chain and initiates a series of events leading to T-cell activation (12) or augmented NK cytolytic activity (15), in addition to subsequent synthesis of TAC mRNA (12-15). Anti-TAC mAb that prevent IL-2 binding to the p55 TAC chain but not to the p75 chain do not inhibit the augmented cytolytic activity (11, 15-17) or the changes in gene induction (12, 13, 15). In the second step, newly synthesized TAC chains are made and secreted by the surface of the cell and associate with p75 resulting in the formation of high affinity IL-2 receptor complexes. The progression of both T and NK cells into a proliferative phase apparently requires IL-2 binding to this high affinity receptor complex, since proliferation can be inhibited by anti-TAC mAb (11-13, 15-17). This two step model has also been demonstrated for TAC+ large granular lymphocyte leukemic cells (14).

LAK cells are generated by the culture of PBL with IL-2 in vivo (18, 19). These LAK cells consist primarily of IL-2 activated NK cells; they express the NK cell markers CD56 (Leu-19) and CD16 (Leu-11) and mediate a wide spectrum of non-major histocompatibility complex restricted cytotoxicity against NK resistant fresh tumor cells and tumor cell lines (18-23). Although IL-2 receptors have been characterized on resting T-cells and T-lymphocytes activated by PHA or CD3 (8, 9) and resting NK cells, little is known about the composition or function of IL-2 receptors on the surface of NK cells activated by IL-2 that mediate LAK activity. Generation of lymphocytes with LAK activity can be induced in vivo following treatment of cancer patients with IL-2 (24-27). We have studied the immune response of cancer patients who received four repetitive weekly cycles of continuous IL-2 infusion in a phase I trial (24, 28). We found that following IL-2 therapy, circulating lymphocytes expressing the NK CD56 antigen mediated significant IL-2 dependent LAK activity (23). In addition, an increase in the number of lymphocytes expressing TAC was noted after 4 weeks of IL-2 therapy (28). Release of high levels of soluble TAC in the serum during the therapy was also observed, suggesting systemic activation of lymphoid cells by IL-2 (29). These observations, coupled with data indicating a two step IL-2 triggering mechanism for T-cells and resting NK cells, sug-
suggested that the LAK cells activated by IL-2 in vivo should express increased levels of TAC to enable high affinity binding of IL-2 and subsequent proliferation of the cells. However, in experiments aimed at studying the role of TAC on LAK cells induced by in vivo IL-2, we found that detectable expression of TAC on the cell surface is not necessary for maintenance of IL-2 induced proliferation or LAK activity of the cells. This study indicates that the signal inducing and maintaining proliferation and cytotoxicity by the majority of PBL activated by IL-2 in vivo is transmitted primarily through the p75 IL-2R.

MATERIALS AND METHODS

Patient PBL. PBL from 8 cancer patients (5 with renal cell carcinoma, 2 with melanoma, and 1 with non-Hodgkin's lymphoma) were used for the in vitro studies reported here. IL-2 at doses of 1.5–3 × 10^6 units/ml/day was administered i.v. to the patients as a continuous 24-h infusion or a combination of bolus and continuous infusion (half the daily dose was given by bolus and half was given by 24-h continuous infusion) for 4 days followed by 3 days of observation (28). Each patient received four repetitive weekly cycles of 4 days of IL-2 treatment. Patients treated with continuous infusion or a combination of bolus and continuous IL-2 infusion showed similar changes in the immunological parameters tested (28). Immunological and antitumor effects of these treatment regimens as well as their toxicity and tolerance have been reported previously (24, 28). All patients treated in this protocol signed consent forms for in vivo and in vitro studies approved by the University of Wisconsin Committee for the Protection of Human Subjects. Patients' PBL were obtained before IL-2 therapy and 24 h after or 3 or 4 cycles of IL-2 therapy (day 20 or 27) and were separated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. The cells were cryopreserved by controlled rate freezing in 10% fetal bovine serum (Gibco, Grand Island, NY). Goat anti-mouse IgG-conjugated magnetic beads (Dynabeads; Dynal, Great Neck, NY) were washed 4 times with PBS containing 0.1% bovine serum albumin at 4°C and resuspended in cold PBS-1% fetal bovine serum. Beads were added to the cells at a bead:cell ratio of 5:1 and incubated for 40 min at 4°C while rotating gently. Cells positive for TAC antigen were depleted from the negative cells using a magnet. The procedure was repeated with the negative cells to increase efficiency of separation. The effectiveness of the separation was assessed by immunofluorescence (see below). Following depletion, cells were resuspended in HS-RPMI. Cells were washed and plated in microtiter plates in medium supplemented with 200 units/ml of IL-2 for at least an overnight incubation prior to functional assessment (23). The rationale for this preincubation in IL-2 is that PBL activated by IL-2 in vivo are IL-2 dependent (23, 25). Patients' PBL behave like activated PBL in their rapid response to IL-2 and are dependent on IL-2 for their activity (23, 25). To determine the role of TAC+ cells in this responsiveness to IL-2, PBL from three patients obtained 24 h after the 3rd or 4th cycle of IL-2 therapy were depleted of TAC+ cells by immunomagnetic bead separation (Table 1). Patient to patient variations in the percentage of TAC+ cells obtained in PBL following IL-2 therapy were noted [Table 1] [the mean percentage of TAC+ cells for 21 patients completing this IL-2 protocol was 16% (28)]. Depletion of TAC+ cells did not affect the LAK activity of these PBL. The lysis of Daudi cells and proliferative response to IL-2 mediated by TAC depleted PBL (TAC-PBL) were comparable to the responses obtained from the unseparated PBL (Table 1).

To assess the function of TAC+ cells, PBL obtained from a patient following 4 weeks of IL-2 therapy were sorted by flow cytometry. The TAC+ cells mediated only a low level of cytotoxicity against the Daudi target and did not proliferate in response to IL-2 (Table 1). This was not due to an inhibitory effect of the anti-TAC mAb present on the surface of the cells following the sorting procedure since unseparated cells labeled with the antibody and tested in the same conditions did mediate Daudi lysis and proliferation to IL-2 (Table 1). The TAC–sorted cells mediated a high level of LAK activity similar to

RESULTS

LAK Activity of Posttherapy Patient PBL Depleted of TAC+ Cells. We have shown previously that post-IL-2 therapy patients' PBL behave like activated PBL in their rapid response to IL-2 and are dependent on IL-2 for their activity (23, 25). To determine the role of TAC+ cells in this responsiveness to IL-2, PBL from three patients obtained 24 h after the 3rd or 4th cycle of IL-2 therapy were depleted of TAC+ cells by immunomagnetic bead separation (Table 1). Patient to patient variations in the percentage of TAC+ cells obtained in PBL following IL-2 therapy were noted [Table 1] [the mean percentage of TAC+ cells for 21 patients completing this IL-2 protocol was 16% (28)]. Depletion of TAC+ cells did not affect the LAK activity of these PBL. The lysis of Daudi cells and proliferative response to IL-2 mediated by TAC depleted PBL (TAC-PBL) were comparable to the responses obtained from the unseparated PBL (Table 1).
The LAK activity of PBL activated by prolonged in vivo treatment with IL-2 does not require TAC expression on the surface of the cells. We have shown previously that in vivo induced LAK activity was mediated primarily by CD56+/CD3− NK cells (23); therefore TAC expression was assessed on the surface of CD56+ NK cells and CD3+ T-cells [only 2–5% cells coexpress CD3 and CD56 antigens (23)]. A two color flow cytometric analysis of PBL from a representative patient (patient 8) isolated before and after IL-2 therapy is presented in Fig. 1. Before therapy, CD3+ T-cells represented the majority of cells and a few of them expressed TAC while no detectable TAC was expressed on CD56+ cells. Following IL-2 therapy, the CD56+ cells were considerably expanded at the expense of the T-cells as observed previously (23). However, these CD56+ cells still exhibited only minimal coexpression of TAC and the majority of the TAC antigen was expressed by a fraction of the CD3+ T-cells. This observation, reproduced with cells from 7 other patients (not shown), indicates that the increase in TAC+ PBL following in vivo IL-2 is due to TAC expression on CD3+ T-cells rather than on CD56+/CD3− NK cells. Therefore, depletion of TAC+ cells from PBL activated by IL-2 in vivo mainly depleted the fraction of T-cells expressing TAC and did not affect the CD56+ cells that are responsible for mediation of LAK activity (Table 1).

**Table 1** Depletion of TAC+ cells from in vivo IL-2 activated PBL does not abrogate their LAK cytotoxic activity or proliferative response to IL-2

PBL obtained from patients on day 20 or 27 of IL-2 therapy were depleted of TAC+ cells by immunomagnetic bead separation. Unseparated and TAC depleted cells were cultured overnight in IL-2 (200 units/ml) and then assayed for cytotoxicity against 14Cr-labeled Daudi targets (data expressed in lytic units (LU/10^6) PBL). Cells were stained with anti-TAC-FITC mAb and analyzed by flow cytometry (mouse IgG1-FITC control antibody was used to monitor nonspecific binding). For proliferation to IL-2, cells were cultured in IL-2 (200 units/ml) for 3 days and then labeled with [3H]thymidine for 6 h (proliferation of these cells in medium was low: 50–400 cpm). Mean ± SD of triplicates are shown. For sorting of LAK activity (Table 1).

<table>
<thead>
<tr>
<th>Donor Separation</th>
<th>Population</th>
<th>% of TAC+ lysis Proliferation to IL-2</th>
<th>TAC+ lysis Proliferation to IL-2</th>
<th>TAC+ lysis Proliferation to IL-2</th>
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<tbody>
<tr>
<td>Patient 1</td>
<td>Beads</td>
<td>Unseparated 7</td>
<td>11,845</td>
<td>29,267 ± 9,906</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAC- 1</td>
<td>10,692</td>
<td>20,376 ± 1,799</td>
</tr>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td>Beads</td>
<td>Unseparated 12</td>
<td>2,805</td>
<td>7,622 ± 714</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAC- 1.5</td>
<td>2,377</td>
<td>16,430 ± 49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td>Beads</td>
<td>Unseparated 31</td>
<td>1,222</td>
<td>7,668 ± 689</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAC- 3</td>
<td>1,945</td>
<td>11,592 ± 1,388</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient 4</td>
<td>FACS</td>
<td>Unseparated 6</td>
<td>2,558</td>
<td>1,703 ± 164</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TAC- 1</td>
<td>2,816</td>
<td>2,335 ± 275</td>
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</tbody>
</table>

Thus, TAC− cells obtained after IL-2 activation in vivo do not express or release TAC following further culture in IL-2 in vitro (from 31% on day 1 to 11% on day 8). On day 8, 5% of the PBL coexpressed CD56 and TAC and could be of T-cell phenotype (CD3+/CD56+) rather than NK (CD3−/CD56+) since all the 11% TAC+ cells coexpressed CD3 (Table 2). The TAC depleted population from in vivo activated PBL maintained the low percentage of TAC+ cells (3–4%) during the 8 days of culture, indicating minimal reexpression of the TAC receptor on activated cells (Table 2).

These data were reproducible with PBL from 3 other patients. Unlike in vivo IL-2 activated PBL, pretherapy resting PBL showed an increase in the percentage of TAC+ cells following 8 days culture in IL-2; much of the TAC expression appeared on T-cells but more than one-third of the CD56+ NK cells were also induced to express TAC, from both unseparated and TAC depleted PBL populations (Table 2). In resting PBL depleted of TAC+ cells, reexpression of TAC was observed as early as 1 day after culture in IL-2 (10% TAC+ cells). TAC expression on T-cells was observed primarily on CD4+ cells and not on CD8+ cells (data not shown).

The pretherapy resting PBL released detectable levels of soluble TAC receptor within 3 days of culture with IL-2 and high levels on day 8 of culture (Table 2). This TAC release occurred to a comparable degree both in unseparated PBL and in TAC depleted PBL and paralleled the increase in the percentage of TAC+ cells in both populations. These results confirm, as previously noted, that exposure of resting PBL to IL-2 will induce expression and secretion of TAC (29). In contrast, this was not observed following culture of PBL activated previously with IL-2 in vivo. In the unseparated population, although the percentage of TAC+ cells was initially 31%, release of TAC was very low. By day 8 of culture the level of TAC release (587 units/ml) was still much lower than that released by parallel cultures of resting PBL activated in vitro (2165 units/ml). Posttherapy PBL from other patients initially expressed fewer TAC+ cells (10–16%) and showed lower levels of TAC release (usually <100 units/ml) following culture in IL-2. In vivo activated PBL depleted of TAC+ cells released substantially lower levels of TAC than the unseparated PBL. Thus, TAC+ cells obtained after IL-2 activation in vivo do not express or release TAC following further culture in vitro with IL-2 as assessed at 3 different time points of the IL-2 culture. However, these findings cannot rule out a transient expression of TAC.

**Phenotypic and Functional Analysis of CD56+ NK Cell and CD3+ T-Cell Subpopulations from Posttherapy Patient PBL.** We have shown in the previous section that the majority of cells expressing the TAC receptor, following in vivo IL-2 treatment, are T-cells. It was of interest, therefore, to study the functional response to IL-2 of the T-cell subset in parallel to the CD56+ NK cell subset. PBL from a patient obtained after 4 weeks of IL-2 therapy were selectively depleted of NK cells to recover a T-cell population or were depleted of T cells to recover a NK cell population. Unseparated populations and the two depleted cell populations were tested for LAK cytotoxic activity, proliferative response to IL-2, TAC receptor release, and phenotype (Table 3). In the unseparated population, 10% of the cells express the TAC marker with 7% coexpressing CD3 and 3% coexpressing CD56. In the separated NK cell population consisting of 87% CD56+ cells, only 7% expressed the TAC antigen. The separated T-cell population consisted of 97% CD3+ cells with 23% coexpressing TAC. Despite this greater expression of TAC by purified T-cells, this population of cells did not seem responsive to IL-2; they were hardly induced to proliferate by IL-2, although they still responded to PHA. Thus...
IL-2 RECEPTORS ON IL-2 ACTIVATED PBL

Fig. 1. Expression of TAC on T-cells and CD56+ NK cells. PBL obtained from a representative patient (patient 8) before IL-2 therapy (A, B) and following IL-2 therapy on day 27 (C, D) were double labeled with anti-TAC-FITC and anti-CD3-phycoerythrin (PE) (A, C) or anti-CD56-phycoerythrin (B, D) and analyzed by flow cytometry. Gates were set for nonspecific binding using cells labeled with mouse IgG1-FITC and mouse IgG1-phycoerythrin.

Table 2. Correlation between release of TAC receptor and TAC expression on the surface of patient PBL before and after IL-2 therapy following culture in IL-2 for 1–8 days

<table>
<thead>
<tr>
<th>Sample Population</th>
<th>TAC IL-2R released (units/ml)</th>
<th>% of cells for markers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TAC</td>
<td>TAC/CD3</td>
</tr>
<tr>
<td>Pre IL-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unseparated</td>
<td>1</td>
<td>13</td>
</tr>
<tr>
<td>3</td>
<td>151</td>
<td>12</td>
</tr>
<tr>
<td>8</td>
<td>2165</td>
<td>34</td>
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<tr>
<td>TAC-</td>
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<td>3</td>
<td>203</td>
<td>9</td>
</tr>
<tr>
<td>8</td>
<td>1610</td>
<td>31</td>
</tr>
<tr>
<td>In vivo IL-2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unseparated</td>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>31</td>
</tr>
<tr>
<td>8</td>
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<tr>
<td>3</td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>151</td>
<td>4</td>
</tr>
</tbody>
</table>

*a Pre IL-2, resting PBL obtained from patient 3 before IL-2 therapy; in vivo IL-2, in vivo activated PBL obtained from patient 3 on day 27 of IL-2 therapy.

expression of TAC on the cells does not necessarily predict that IL-2 will induce these cells to proliferate. In contrast to T-cells, the CD56+ enriched population proliferated strongly in response to IL-2 but not to PHA. LAK cytotoxicity was recovered in the CD56+ NK cell population while virtually no cytolytic activity was detected in the T-cell population, consistent with previous results (23). TAC receptor release by the unseparated PBL was very low after 1 day in culture in IL-2 and increased only slightly after 6 days of culture. Most of this TAC release appeared to be mediated by the T-cell subset while the release of TAC from CD56+ cells was much lower. These CD56+ cells might express a low amount of TAC (undetectable by fluorescence activated cell sorter) and therefore shed in vitro lower amounts of TAC than T-cells. Alternatively, the low level of TAC shed by CD56+ cells might be due to the minor fraction of these cells expressing TAC (7% of 87%).

IL-2 Dose Dependent Proliferative Response of Activated PBL versus Resting PBL. We have shown that the proliferative response to IL-2 by patients' PBL, obtained after IL-2 therapy, was mediated by CD56+ cells even though these cells exhibited minimal expression of TAC and proliferated to the same extent if TAC+ cells were depleted. However, several studies have
suggested that the high affinity IL-2R including the TAC antigen is required for the proliferative response of resting NK or T-lymphocytes to IL-2 (11–13, 15–17). The data presented here suggest that detectable TAC expression is not necessary for proliferation of activated CD56+ NK cells. To clarify this point further, the IL-2 dose dependent proliferative response of *in vivo* activated PBL was compared to the proliferative response of resting PBL obtained from the same patient before therapy. Pre- and posttherapy PBL were incubated for 5 days in increasing concentrations of IL-2 (1–100 units/ml (4.4–440 pM)). Five days of incubation in IL-2 were necessary to obtain significant proliferation of pretherapy resting PBL to IL-2. These pretherapy PBL showed a progressive dose response to IL-2 starting at a concentration as low as 5 units/ml of IL-2 and reaching a plateau at 30 units/ml of IL-2 (Fig. 2). Fourteen % of these cells expressed TAC after 5 days in 100 units/ml IL-2. However, *in vivo* activated PBL from the same patient showed only a very low level of proliferation at low concentrations of IL-2 (1–10 units/ml), with a significant boost in proliferation occurring only at concentrations of IL-2 greater than 10 units/ml (44 pM) (Fig. 2). Sixteen % of these *in vivo* activated PBL expressed TAC after 1 day in 100 units/ml IL-2 but by day 5, at the time proliferation was measured, no TAC antigen was detectable, probably reflecting the considerable expansion of TAC−CD56+ NK cells (94% on day 3) at the expense of T-cells (only 4% CD3+ cells on day 5). A T-cell clone isolated in our laboratory (CD3+, TCR a/ß+) expressing 60% TAC+ cells had an IL-2 dose dependent pattern of proliferation similar to that observed in resting PBL (Fig. 2). At low concentrations of IL-2 (5 and 10 units/ml) the response by pretherapy PBL (*P* = 0.001) or the T-cell clone (*P* = 0.0001) were significantly greater than the response by posttherapy PBL (determined in a two way analysis of variance). This comparison between *in vivo* activated PBL which did not express TAC, resting PBL in the process of *in vitro* activation by IL-2, and a T-cell clone expressing a high percentage of TAC+ cells suggests that *in vivo* activated PBL respond to IL-2 with a lower affinity IL-2 receptor requiring a higher concentration of IL-2 to elicit a response. Resting PBL and the T-cell clone, on the other hand, respond to IL-2 at lower concentrations and appear to bind IL-2 through the TAC/p75 high affinity receptor. To rule out that the low level of proliferation of *in vivo* activated PBL observed at low concentrations of IL-2 was not a result of consumption of IL-2 in the 5-day assay, half of the medium was removed each day from all the wells and replaced by fresh medium containing the same concentration of IL-2 used initially in that culture. Proliferation was tested after 1, 2, 3, 4, and 5 days in IL-2. Data are shown in Fig. 2, inset. Proliferation of the cells was detectable by day 2 at 30 units/ml (132 pM) IL-2 and by days 3, 4, and 5 was more significant, showing a boost in proliferation starting at doses higher than 10 units/ml (44 pM) similar to the response obtained for patient 5 (Fig. 2). Thus the relative inability of these *in vivo* activated PBL to respond to low doses of IL-2 (<10 units/ml) did not result merely from rapid IL-2 utilization by the cells, nor is this pattern simply a reflection of altered response kinetics since

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**Table 3 Phenotypic and functional analysis of CD56+ cells and CD3+ cell subpopulations separated from posttherapy patient PBL.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Daubi lysis (lytic units/10^6)</th>
<th>Proliferation (^[3H]thymidine cpm)</th>
<th>TAC IL-2R released (units/ml)</th>
<th>% of cells positive for markers</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Medium</td>
<td>IL-2</td>
<td>PHA</td>
</tr>
<tr>
<td>Unseparated</td>
<td>1,321</td>
<td>350 ± 24</td>
<td>15,858 ± 622</td>
<td>7,179 ± 802</td>
</tr>
<tr>
<td>CD56+ cells</td>
<td>1,903</td>
<td>214 ± 26</td>
<td>14,011 ± 99</td>
<td>335 ± 88</td>
</tr>
<tr>
<td>CD3+ T-cells</td>
<td>10</td>
<td>169 ± 81</td>
<td>426 ± 185</td>
<td>6,761 ± 665</td>
</tr>
</tbody>
</table>

* NT, not tested.
relatively poor responses at low doses of IL-2 were seen in assays performed every day over a 5-day period.

Effect of Anti-TAC and Anti-p75 mAbs on the Proliferative Response of Pre- and Posttherapy PBL. To distinguish the role of the p75 and the p55 TAC IL-2 receptor subunits in the IL-2 induced proliferative response of posttherapy patient PBL, TU27, a mAb specific for p75 and GL439, a mAb specific for TAC, were included in the proliferative assay. The anti-TAC mAb caused a shift in the IL-2 dose-response curve of unstimulated pretherapy PBL with proliferation starting at concentrations of IL-2 higher than 30 units/ml (132 pM) (Fig. 3A). The response was markedly inhibited (84–99%) by anti-TAC at low concentrations of IL-2 (22–132 pm) and inhibition was still observed at higher concentrations of IL-2 (63 and 44% inhibition at 440 and 1100 pm, respectively). A similar profile of anti-TAC inhibition of proliferation was previously reported for anti-CD3 or PHA stimulated lymphocytes (8, 9) and for human leukemic cell lines (31, 32) which express TAC and respond through high affinity IL-2 receptors. The anti-p75 mAb TU27 inhibited about 50% of the proliferation by pretherapy PBL (Fig. 3A). The combination of anti-TAC and anti-p75 virtually abrogated the response to IL-2 by these cells, as also shown previously for responses by TAC+ leukemic cell lines (31, 32). These data suggest that the proliferative response of unstimulated PBL to IL-2, reflecting the combination of resting T-cells and resting NK cells responding to IL-2, is mediated through both high affinity receptors (TAC associated with p75) and intermediate affinity receptors (p75 alone). Antibody blocking of IL-2 binding to TAC still allows a proliferative signal to be transmitted by p75, especially at higher concentrations of IL-2 necessary to bind to the intermediate affinity p75 receptor (8, 9). These p75-mediated events would be blocked when both anti-TAC and anti-p75 are present.

The proliferative response to IL-2 observed with the posttherapy PBL (Fig. 3B) is primarily mediated by the CD56+/CD3− NK cells (Table 3) since we have shown that CD3+ T-cells isolated after IL-2 therapy did not proliferate after 3 days (Table 3) or 6 days (not shown) in IL-2. Moreover, CD56+/CD3− NK cells sorted from posttherapy PBL showed the same pattern of proliferation to IL-2 than unseparated PBL. Anti-TAC mAb had only minimal inhibitory effect on the proliferative response by posttherapy PBL (Fig. 3B). Especially when compared to the anti-TAC inhibition on pretherapy PBL. Inhibition of the response of posttherapy PBL by anti-TAC occurred primarily at lower concentrations of IL-2 (22–44 pm), at concentrations at which the proliferative response was already low in the absence of anti-TAC. At concentrations of IL-2 greater than 44 pm (10 units/ml), inhibition by anti-TAC decreased and the IL-2 dose-response curves in the presence or absence of anti-TAC appeared quite similar, indicating a minimal role for TAC or the high affinity IL-2 receptors. In contrast, the inhibition by the anti-p75 mAb was dramatic (Fig. 3B). Inhibition was 76–99% over a large range of IL-2 concentrations [22–1100 pm (5–250 units/ml)] in contrast to the lower degree of inhibition (50%) caused by this mAb on the response by pretherapy PBL in the same IL-2 dose range (Fig. 3A). Thus it seems that the majority of the proliferative response by in vivo activated PBL which occurs at IL-2 concentrations greater than 44 pm (10 units/ml) is mediated through the intermediate affinity p75 IL-2R. The response of in vivo activated PBL was completely abrogated by the combination of anti-TAC and anti-p75 mAbs except at 1000 units/ml of IL-2. At this high dose of IL-2, inhibition by anti-p75 was also reduced, probably due to competition between antibody and excess IL-2.

The low level of proliferation observed at low concentrations of IL-2, which was inhibited by anti-TAC, may be mediated by a small cohort of cells remaining after in vivo activation which still function via the TAC molecule. This minor cell population does not express CD56 as suggested by the recent findings on the proliferative response of CD56+ NK cells sorted from posttherapy PBL. The response of CD56+ cells started at 100 pm of IL-2 was not inhibited by anti-TAC but completely abrogated by anti-p75 alone, indicating that CD56+ NK cells respond to IL-2 only through p75.

In control experiments, anti-CD3 activated PBL, all expressing TAC, were also tested. Anti-p75 mAb did not inhibit the proliferation of anti-CD3 activated PBL to IL-2 while the combination of both anti-TAC and anti-p75 virtually eliminated the proliferative response (data not shown).

DISCUSSION

The role that different forms of the IL-2 receptor play in the activation of resting NK cells or resting T-cells has been well understood. However, the role of the intermediate affinity IL-2 receptors in the activation of NK cells or T-cells remains unclear. A recent study has shown that the interaction of IL-2 with the intermediate affinity IL-2 receptors is essential for the activation of NK cells and T-cells. This interaction is mediated by the CD56+ NK cells and the CD3+ T-cells. The role of the intermediate affinity IL-2 receptors in the activation of NK cells and T-cells has been well understood.

* S. D. Voss, R. J. Robb, G. Weil-Hillman, J. A. Hank, K. Sugamura, M. Tsuda, and P. M. Sondel. Increased expression of the IL-2 receptor β chain (p70) on CD56+ NK cells following in vivo IL-2 therapy, submitted for publication.
characterized. A short term incubation of resting NK cells in high doses of IL-2 caused an increase in cytolytic activity independently of TAC expression (11, 15-17). However, the proliferative response of resting NK cells to IL-2, resulting in generation of LAK activity (11, 14-17), was found to be dependent on TAC expression in the form of high affinity receptors. In this study, we present data indicating that NK dependent on TAC expression in the form of high affinity receptors on resting T-cells (8, 9, 12), was found to be responsible for mediation of LAK activity, as shown previously (23, 26, 27). CD3+/CD56+ T-cells isolated from posttherapy patient PBL, despite expressing the TAC receptor, neither proliferated in response to IL-2 nor mediated LAK activity (Tables 1 and 3). In vivo IL-2 therapy, sustaining IL-2 plasma levels of 30 units/ml (33), may cause suppression of T-cells as shown by nonresponsiveness to IL-2, a decrease in PHA response, and a decrease in allogeneic responses (34, 35). These T-cells may be defective in their IL-2 receptor function, expressing only low affinity TAC receptors due to down regulation of the TAC receptor or high affinity receptor, and thus may be unable to transmit the IL-2 signal as suggested in other studies (36, 37).

The cells from in vivo activated PBL depleted of the fraction of CD3+/TAC+ T-cells not only minimally expressed TAC but also maintained this low TAC expression on CD56+ cells and T-cells following further expansion of these cells in culture in IL-2. In addition, these in vivo activated PBL released only minimal levels of TAC receptor. In contrast, pretherapy resting PBL, initially depleted of TAC+ cells and activated with IL-2 in vitro for 8 days, did reexpress TAC on the surface of both CD56+ cells and T-cells at levels comparable with unseparated pretherapy PBL. Furthermore, concomitant with the increase in the percentage of TAC+ cells, these pretherapy PBL released high levels of soluble TAC into the culture supernatant during the course of the in vitro IL-2 activation (Table 2) as shown previously for resting PBL activated by IL-2 (29) or PHA or CD3 (38, 39).

During IL-2 therapy, we (29) and others (40) have noted high levels of soluble TAC receptors in the patients' serum during the infusion of IL-2, at a time of lymphopenia in the peripheral blood. This may indicate that an activation process of IL-2 responsive lymphocytes by IL-2 therapy occurred while the majority were in compartments other than peripheral blood (41). Following cessation of the IL-2 infusion, high numbers of activated lymphocytes returned to the peripheral circulation without a major change in TAC secretion (29). In the present study, we have found that following therapy most of the circulating lymphocytes expressing TAC were T-cells but these cells did not respond readily to IL-2 in vitro. The circulating CD56+ NK lymphocytes were activated and highly responsive to IL-2 but very few of them expressed or released TAC, given the limits of detection of the flow cytometry and enzyme linked immunosorbent assays. A possible explanation is that during the IL-2 infusion, the resting NK cell population migrated to lymphoid compartments as they were activated by IL-2 causing TAC expression and release ultimately leading to proliferation of the cells in an activation process parallel to that described for resting NK cells or T-cells (11–13, 15-17). Subsequently, the NK cells, released back into the circulation after IL-2 therapy, may have lost or down regulated their TAC expression and high affinity IL-2R to limit and control cell proliferation in response to lower (physiological) concentrations of IL-2. Alternatively, the in vivo activation of NK cells by IL-2 might have occurred without expression or involvement of TAC, using only the p75 receptor. In this case, the increased soluble TAC measured in the patient's serum may have been released by cells other than NK cells, possibly the T-cells which lost their responsiveness by down regulation of their IL-2R as described above. In either case, following in vivo IL-2, these circulating NK cells are highly responsive to IL-2 but maintain their TAC–phenotype with continued IL-2 exposure in vitro. All the NK clones (CD56+/CD16+/CD3−) generated in our laboratory do not express TAC and remain TAC− with further expansion in IL-2.3 These clones proliferate in response to IL-2 and maintain their LAK cytotoxic activity, probably using only the p75 receptor, similarly to the posttherapy PBL.

Furthermore, these in vivo activated NK cells require higher doses of IL-2 (>44 pm or 10 units/ml) to induce significant proliferation, suggesting that they primarily bind IL-2 through the intermediate affinity p75 IL-2R. This was confirmed by the inhibition studies with an anti-p75 mAb showing a dramatic inhibition of proliferation, while anti-TAC mAb inhibited only the poor proliferation obtained at low doses of IL-2. These data suggest that NK cells activated in vivo by IL-2 respond and are induced to proliferate to IL-2 mainly through the p75 receptor with only a minor component of cells using the high affinity IL-2R complex. This is in contrast to unstimulated pretherapy PBL which seem to respond to IL-2 stimulation through a combination of high affinity receptors and intermediate affinity p75 receptors. The involvement of p75 in the proliferative response by these cells is suggested by the partial inhibition with anti-p75 (Fig. 3A) and is in agreement with a recent study on resting NK cells (42). This differs sharply from the lack of inhibition by anti-p75 alone observed in human leukemic cell lines (31, 32) or in CD3 activated PBL (data not shown), cells that have been shown to function essentially through the high affinity receptor complex (9, 31, 32). The involvement of the high affinity IL-2 receptor complex in the proliferation of pretherapy PBL was suggested by the shift in the dose response following high doses of IL-2 caused by anti-TAC and is in agreement with results obtained using CD3 or PHA stimulated lymphocytes (8, 9) and human leukemic cell lines (31, 32). The proliferative response of human tumor infiltrating lymphocytes propagated in vitro with 200 units/ml IL-2 (predominantly T-cells) has been shown to be also inhibited by anti-TAC, regardless whether the cells expressed TAC or not, suggesting a transient TAC expression on the TAC− cells (43). This was different from the lack of inhibition by anti-TAC at 100–1000 units/ml IL-2 demonstrated by the in vivo IL-2 activated NK cells in the present study.

Expression of the p75 IL-2R has been demonstrated at a cellular (42) and molecular level (15) on resting NK cells. Phenotypic and molecular studies performed on the TAC–activated NK cells obtained following therapy with IL-2 are currently under way to characterize at the molecular level the IL-2 receptor on these cells. We (23) and others (44) have shown previously that following in vivo IL-2, the predominant population of NK cells mediating LAK activity expressed a high density of CD56 antigen. These CD56+ cells may have
acquired greater expression of p75 receptors during in vivo IL-2 activation that could render them more responsive to high concentrations of IL-2. This is consistent with recent flow cytometry analysis using the anti-p75 TU27 mAb showing a striking increase in the intensity of TU27 labeling on CD56+ cells from posttherapy PBL compared to a lower intensity of TU27 staining on pretherapy CD56+ cells. Moreover, the level of TU27 staining on CD56+ cells from posttherapy PBL was greater than that by YT cells, a NK cell line expressing a high number of p75 receptors (9). However, 129IL-2 binding assays revealed much lower levels of intermediate affinity IL-2 binding sites on posttherapy PBL than on YT cells. Further studies are currently under way to clarify this discrepancy.

Two cell lines known for expressing predominantly p75 such as YT and MLA144 (9, 31, 45–47) are convenient for molecular studies but not for functional responses to IL-2 since they are not dependent on IL-2 for their proliferation. In vivo activated patient PBL, however, represent a unique model system for studying IL-2/IL-2 receptor interaction on IL-2 dependent cells that do not express TAC and function essentially through the p75 IL-2R.

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IL-2 RECEPTORS ON IL-2 ACTIVATED PBL

Natural Killer Cells Activated by Interleukin 2 Treatment \textit{in Vivo} Respond to Interleukin 2 Primarily through the p75 Receptor and Maintain the p55 (TAC) Negative Phenotype


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