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ORIGINAL ARTICLE

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Increased generation of autologous tumor-reactive lymphocytes by anti-CD3 monoclonal antibody and prothymosin α

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Abstract Anti-CD3 monoclonal antibody (mAb) activates in vitro peripheral blood mononuclear cells (PBMC) to lyse a variety of tumor cell lines in a non-major histocompatibility-complex(MHC)-restricted manner [subsequently referred to as anti-CD3-activated killer (AAK) cytotoxicity]. Prothymosin α (ProTα) is a biological response modifier that exerts its effects primarily on mononuclear cells, especially when these cells' effector functions are impaired. In this study, we report that ProTα enhances the AAK cytotoxicity in PBMC from healthy donors. This effect was more profound with cancer patients' PBMC, which were deficient in their ability to respond with enhanced AAK cytotoxicity upon in vitro stimulation with anti-CD3. Thus, cancer patients' PBMC, activated with a combination of anti-CD3 and ProTα, exhibited increased AAK activity and efficiently lysed both autologous tumor and Daudi targets. The ProTα effect on PBMC was demonstrated to involve stimulation of adhesion molecules (CD2, CD18, CD54, CD49f) and CD25 expression, up-regulation of perforin mRNA transcription, increased numbers of perforin-positive (+) cells and elevated production of interleukin-2 (IL-2), interleukin-1β (IL-1β) and tumor necrosis factor α (TNFα). Moreover, effector $CD8^+$ and $CD56^+$ cells pretreated with anti-CD3 and ProTα contained high cytoplasmic perforin levels and increased expression of IL-1β- and TNFα-specific receptors. The induction of autologous-tumor-reactive $CD8^+$ and $CD56^+$ lymphocytes in anti-CD3-activated PBMC by ProTα provides an alternative protocol aimed at the improvement of clinical results in cellular adoptive immunotherapy of cancer.

Key words Anti-CD3 mAb · Prothymosin α · Cytotoxicity · Anticancer activity · Adoptive immunotherapy

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Introduction

Prothymosin α (ProTα) is a highly acidic polypeptide ($pI = 3.55$) of 12 500 Da, first isolated from rat thymus [34] and subsequently from several other mammalian tissues [35, 51]. Its sequence of 109 amino acids has been deduced from RNA isolated from simian-virus-40-transformed human fibroblasts and from clones isolated from a human spleen cDNA library [26]. The two sets of receptors described, with high and low affinities for ProTα on human peripheral blood lymphocytes (PBL) [22, 23], may facilitate the elucidation of its role as well as its mode of action. ProTα was demonstrated to be active in vitro by potentiating human T lymphocyte responses to various stimuli [4] and in vivo by protecting mice against infection with *Candida albicans* and against the growth of autologous leukemic cells [3]. The biological activity of ProTα is exerted by the intact polypeptide, whereas its amino-terminal fragment, thymosin α1 {ProTα (1–28) [32]}, is effective [46], but in significantly higher doses than the parental molecule [51].

When activated in vitro with interleukin-2 (IL-2), peripheral blood mononuclear cells (PBMC, referred to as lymphokine-activated killer or LAK cells) cause lysis of a wide range of tumor cells in a non-MHC-restricted manner [3]. Most of this activity is carried out by natural killer (NK) cells, although the involvement of T ($CD8^+$) lymphocytes in such "LAK-like" cytolytic processes has also been reported [13, 44, 57]. LAK cells have been used in vivo to achieve effective antitumor responses [3]. An alternative method to induce non-MHC-restricted cytotoxicity is to activate PBMC with anti-CD3 mono-

clonal antibody (mAb). Cross-linking surface CD3 with anti-CD3 mAb results in specific activation events associated with up-regulation of the IL-2-specific receptor, cytokine synthesis and secretion, cell proliferation and aquisition of antigen non-specific cytotoxicity [63]. The capacity of anti-CD3 to induce cellular cytotoxicity may be an important index of potential antitumor activity. Given the fact that ProTα is capable of inducing various types of cytotoxic responses, including those mediated by T [14] and NK [9] lymphocytes, we examined the effect of ProTα on the anti-CD3-activated killer (AAK) cytotoxicity both in healthy individuals and cancer patients.

We present here results demonstrating that ProTα is capable of enhancing this type of cytotoxicity in both groups, but its effect is more intense with cancer patients' PBMC. Moreover, ProTα induced autologous-tumor cytotoxicity amongst the anti-CD3-activated PBMC. Our data suggest that PBMC activated in vitro with a combination of anti-CD3 and ProTα may improve clinical results in cancer immunotherapy.

Materials and methods

Patients

A group of 75 patients (23 male and 52 female; age: 55–79, average: 65) with advanced (stage IV) lung ($n = 18$), colorectal ($n = 6$), ovarian ($n = 23$) and breast cancer ($n = 26$) and melanoma ($n = 2$) were included in our study. All patients had been free of any type of anticancer therapy for at least 30 days prior to blood collection. Age- and sex-matched hospital staff volunteers were included as healthy donors. All donors were apprised of the study and required to provide written informed consent. The study was approved by the Review Board of Saint Savas Cancer Hospital.

Reagents

ProTα from bovine thymus was purified according to Haritos and Horecker [34]. In brief, bovine thymus from a 15-month-old calf was cut into small pieces immediately after excision and dropped into liquid nitrogen. The frozen tissue was pulverised in a ceramic mortar chilled in solid CO_2, powder corresponding to 28 g tissue was dispersed into 400 ml boiling water and boiling continued for 5 min more in order to prevent degradation of ProTα by endogenous proteases. The suspension was homogenized in a Sorval Omni-mix blender and centrifuged, and the supernatant was acidified by mixing it with 1/10 volume of ten-times-concentrated buffer A (1 M HCOOH/0.2 M pyridine pH 2.9) and kept at −10 °C until processed. After thawing, the sample was centrifuged and the supernatant lyophilized. The syrupy residue was redissolved in buffer A and applied to a 2.7×93-cm column of Sephacryl S-200 (Pharmacia) equilibrated with buffer A. The peptides recovered from four such gel-filtration experiments were pooled and further separated by reverse-phase high-performance liquid chromatography (RP-HPLC) on a C18 column (Lichrosorb, 5 μm, HPLC Technologies, UK). Elution was performed with a linear gradient of 10%–50% acetonitrile in 0.1% trifluoroacetic acid over 60 min. The isolation procedure yielded approximately 60 μg pure peptide (as controlled by amino acid analysis)/g fresh tissue. The endotoxin level was 0.01 ng/100 μg ProTα as measured in a standard *Limulus* assay. Enzyme-linked immunosorbent assay kits specific for IL-12, tumor necrosis factor α (TNFα), interferon γ (IFNγ) and granulocyte/macrophage-colony-stimulating factor (GM-CSF) were obtained from Endogen (Boston, Mass.) and for IL-1β, IL-2, IL-6, and IL-7 from R&D Systems Europe (Abingdon, UK). Anti-CD3 (anti-CD3-ε IgG, clone 145-2C11) and anti-human IL-1β receptor (anti-IL-1βR) mAb were from Pharmingen (San Diego, Calif.). mAb specific for CD18, CD2, CD54, CD49f, CD62L, CD56, CD29 CD40L (defining adhesion molecules) and CD25 (defining the low-affinity IL-2R, p55) were obtained from Caltag (San Francisco, Calif.). Anti-(MHC class I), anti-(MHC class II), anti-CD4, anti-CD8, anti-human TNFα receptor (anti-TNFαR) and anti-perforin mAb were obtained from Pharmingen. For phenotype analyses, mAb conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used as described in the respective figures. FITC- or PE-conjugated anti-mouse IgG was obtained from Caltag and PE-conjugated streptavidin from Pharmingen. Recombinant human cytokines IL-2, TNFα and IL-1β were obtained from Endogen.

Isolation of autologous tumor cells

This was performed as described [11]. Briefly, specimens of peritoneal effusions from patients with metastatic breast ($n = 14$) and ovarian ($n = 13$) cancer and pleural effusions from patients with lung adenocarcinoma ($n = 9$) were subjected to centrifugation at 400 *g* for 5 min to sediment cells that were further placed on top of a 75% Ficoll/Hypaque gradient, overlayed on 100% Ficoll-Hypaque and spun at 700 *g* for 25 min. Tumor cells were collected from the top of the 75% Ficoll-Hypaque and cryopreserved in liquid N_2 until ready for use in the cytotoxicity assays, at which time cells were carefully thawed, slowly diluted in RPMI-1640 medium and washed. Tumor cells were assayed only if their viability was over 80%.

Cell cultures

PBMC (2×10^6 cells/ml) were activated in 25-cm^2 flasks (Costar, Cambridge, Mass.), precoated with anti-CD3 mAb, in 5 ml RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Gibco), 2 mM L-glutamine (Sigma Chemical Company, St.Louis, Mo.) and 100 μg/ml gentamycin (complete medium). Immobilization of anti-CD3 mAb was performed by coating the flasks with 5 μg/ml mAb (anti-CD3-ε) as previously described [2]. ProTα was added at the optimal concentration, i.e., 160 ng/ml, as defined from dose/response studies performed (data not shown). Following a 3-day incubation at 37 °C, 5% CO_2, cultures were harvested and spun down. Supernatants were stored at −80 °C until assayed for cytokine levels. PBMC from the same cultures were tested as effectors in the cytotoxicity assays.

Isolation of lymphocyte effectors from activated PBMC

Highly purified $CD56^+$ cells (more than 96%) were obtained using an immunomagnetic isolation procedure [8]. In brief, anti-CD3-activated PBMC (5×10^6–7×10^6 cells/ml), cultured in the presence or absence of ProTα, were incubated for 1 h at 4 °C with 0.5 μg anti-CD56 mAb followed by a further 30 min incubation at 4 °C with 2.25–2.5×10^6 Dynabeads M450 coated with sheep anti-mouse IgG (Dynal, Oslo, Norway). The $CD56^+$ cells attached to the Dynabeads were trapped by a magnet (MPC-6, Dynal). After the bead/$CD56^+$ cell suspension had been washed to remove unbound cells, the magnet was removed and the cell suspension was incubated overnight at 37 °C, allowing the beads to detach from the cells and to be trapped thereafter by the magnet. $CD4^+$ and $CD8^+$ cells (more than 98% purity) were isolated from the activated PBMC by a method similar to the one described for $CD56^+$ cells [54]. Detachment of M-450 Dynabeads from $CD8^+$ or $CD4^+$ cells was performed using the DETACHαBEAD solution (Dynal) according to the manufacturer's instructions.

Cytotoxicity assay

This was performed as described [10]. Briefly, 100-µl aliquots of effectors (2×10^6 cells/ml in fresh complete medium) were plated in 96-well microplates (Costar) and incubated overnight with 100 µl containing 5×10^3 ^{51}Cr-labeled tumor targets or as indicated in Fig. 5. Targets included autologous tumor cells, the Burkitt's lymphoma cell lines Raji and Daudi and autologous PBMC blasts as controls. The latter were prepared by 3-day incubation of 1×10^6 PBMC/ml with 10 µg/ml phytohemagglutinin (Sigma) in a total 5 ml of complete medium in 25-cm^2 flasks. Labeling was performed with 100–150 µCi sodium [^{51}Cr]chromate (The Radiochemical Centre, Amersham, UK). Target cells were also incubated in medium alone and with 2% Triton-X (Sigma) for estimations of spontaneous and maximum release respectively. Spontaneous isotope release never exceeded 13% of the maximum release. Where indicated, mAb were added to the cultures at 10 µg/ml final concentration [13] for the entire incubation period. All cultures were set up in triplicate and the percentage cytotoxicity was estimated according to the formula: 100 × (test ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release).

Phenotype analyses

Flow cytometry was conducted according to Baxevanis et al. [5, 8] on a FACScan (Becton Dickinson, Mountain View, Calif.) flow cytometer. The direct single-color immunofluorescence assay was performed with FITC- or PE-conjugated mAb as indicated in the respective figures. Expression of CD40L was analyzed by indirect immunofluorescence, using FITC-conjugated anti-mouse IgG as secondary antibody. Biotinylated anti-CD62L and anti-IL-1βR mAb were detected with PE-conjugated streptavidin. For two-color flow-cytometric analysis, cells were incubated for 30 min at 4 °C with the first mAb (FITC-labeled anti-CD4, -CD8 or -CD56) and then for a further 30 min at 4 °C with the second mAb (PE-labeled anti-CD25, anti-TNFαR or biotinylated anti-IL-1βR and PE-streptavidin). Perforin was detected intracellularly. To enhance intracellular fluorescence, protein secretion was inhibited by the addition of 1 µM brefeldin A (Sigma) [38]. Intracellular perforin staining was then analyzed as described by Jung et al. [37]. In brief, anti-CD3-activated PBMC were fixed with 4% paraformaldehyde for 10–15 min and 0.1% saponin in phosphate-buffered saline to permeabilize the cell membrane. Anti-perforin mAb was used at 1 µg/ml. To detect perforin-positive lymphocyte subsets, PBMC were stained with purified anti-perforin mAb and PE-anti-mouse IgG (both steps 30 min at 4 °C), followed by a further 30-min incubation with the subset-specific mAb (e.g. FITC-conjugated anti-CD4, -CD8 or -CD56). Flow-cytometric data were calculated using LYSYS (Becton Dickinson) software.

RNA isolation and Northern blot analysis

Total cellular RNA from 4×10^7 PBMC incubated with medium (control) or as indicated, was isolated by the method of Chomczynski and Sacchi [18]. A 20-µg sample of total RNA was size-fractionated on formaldehyde/agarose gels and hybridized overnight to a 2.9-kb ^{32}P-labeled human perforin cDNA (kindly provided by Dr.Trinchieri, The Wistar Institute, University of Philadelphia, Pa.). The filter was rehybridized with glyceraldehyde 3-[α-^{32}P]phosphate dehydrogenase cDNA (1.4 kb) from Clontech Laboratories (Palo Alto, Calif.) for normalizing RNA quantities.

Results

In previous studies, we have demonstrated that cancer patients exhibit low levels of non-MHC-restricted cell-mediated cytotoxicity [13, 8, 15, 56]. Thus, as expected, basal mean AAK cytotoxicity in cancer patients was significantly lower than healthy donors' (HD) values (17% compared to 42% for Raji and 10% compared to 25% for Daudi; $P < 0.05$ by Student's t-test; Table 1). When the same PBMC were co-incubated with anti-CD3 and 160 ng/ml of ProTα, we noticed a marked increase in AAK cytotoxicity against both Raji and Daudi targets in all patients tested (mean increase using Raji: 147% and Daudi: 150%; $P < 0.005$). In HD, who already showed high levels of AAK cytotoxicity against both tumor targets, the effect of ProTα was less pronounced (mean increase: 45% and 68% against Raji and Daudi targets respectively; $P < 0.01$). Incubation of PBMC with ProTα alone resulted in marginal levels of cytotoxicity, similar to those obtained in plain medium (less than 6% in all cases tested; data not shown).

Cytolytic activity depends on the activation, recognition and binding of the effector lymphocyte to the tumor target, and several cytokines are reported to be involved in the activation of killer cells [3]. Adhesion molecules also play a critical role in target lysis by various types of cytotoxic cells, non-MHC-restricted killer cells included [29, 42, 43, 49, 58]. Thus, to explain the enhancing effect of ProTα on the anti-CD3-induced cytotoxicity, we next examined its involvement in (i) cy-

Table 1 Prothymosin α (ProTα) restores to normal levels the deficient anti-CD3-induced activation of cancer patients' peripheral blood mononuclear cells. Cytotoxicity assays with anti-CD3-stimulated PBMC as effectors against ^{51}Cr-labeled Raji and Daudi targets were performed as described in "Materials and methods". − Effector PBMC stimulated with anti-CD3 mAb alone; + effector PBMC stimulated with anti-CD3 mAb and ProTα. Results show percentage cytotoxicity ±SD from the pooled data. The percentage increase is given by 100 × [(anti-CD3 + ProTα value) − (anti-CD3 value)]/(anti-CD3 value)

Donors	Cytotoxicity (%)					
	Raji cells			Daudi cells		
	−	+	Increase (%)	−	+	Increase (%)
Melanoma (n = 2)	22 ± 6	53 ± 12	141	10 ± 3	27 ± 6	170
Colorectal cancer (n = 6)	17 ± 5	52 ± 13	206	11 ± 2	31 ± 7	182
Lung cancer (n = 9)	12 ± 3	39 ± 7	225	9 ± 3	20 ± 6	122
Breast cancer (n = 12)	19 ± 5	35 ± 11	84	13 ± 5	29 ± 5	123
Ovarian cancer (n = 10)	18 ± 5	30 ± 10	67	7 ± 2	19 ± 6	171
Mean values for cancer (n = 39)	17 ± 3	42 ± 9	147	10 ± 2	25 ± 5	150
Healthy donors (n = 20)	42 ± 10	61 ± 9	45	25 ± 7	42 ± 10	68

Table 2 Quantification of cytokine levels in cultures with anti-CD3-stimulated PBMC in the presence or absence of ProTα. The same donors (*HD* healthy donors; *Ca* cancer patients) as those from Table 1 were tested. The percentage increase is given by 100× [(anti-CD3 + ProTα value) − (anti-CD3 value)]/(anti-CD3 value). *IL* interleukin, *TNF* tumor necrosis factor, *IFN* interferon, *GM-CSF* granulocyte/macrophage-colony-stimulating factor

Cytokines	Anti-CD3 (ng/ml)		Anti-CD3 + ProTα (ng/ml)		Increase (%)	
	HD	Ca	HD	Ca	HD	Ca
IL-1β	1.8 ± 0.4	0.7 ± 0.3	2.4 ± 0.4	1.2 ± 0.3	33	71
IL-2	5.0 ± 1.0	2.7 ± 0.9	6.4 ± 1.6	4.2 ± 1.2	28	55
IL-6	0.9 ± 0.3	0.5 ± 0.1	0.9 ± 0.2	0.6 ± 0.2	0	2
IL-7	1.3 ± 0.4	0.7 ± 0.2	1.2 ± 0.3	0.7 ± 0.3	0	0
IL-12	2.9 ± 0.6	1.7 ± 0.6	3.0 ± 0.5	1.7 ± 0.7	3	0
TNFα	1.7 ± 0.4	0.6 ± 0.2	2.5 ± 0.2	1.0 ± 0.3	47	66
IFNγ	1.3 ± 0.4	0.7 ± 0.3	1.3 ± 0.3	0.7 ± 0.1	0	0
GM-CSF	2.9 ± 0.6	1.9 ± 0.6	3.1 ± 0.5	2.0 ± 0.5	6	5

tokine production by and (ii) adhesion molecule antigen expression on anti-CD3-activated PBMC. We also assayed for CD25 antigen expression, which characterizes activated cells [19], in an effort to investigate whether the observed low levels of AAK cytotoxicity with patients' PBMC were due to insufficient activation by anti-CD3, thereby gaining an insight into the mechanism(s) by which ProTα abrogates this deficiency. In the course of our studies, we initially measured the levels of cytokines known to promote non-MHC-restricted cytotoxicity [3] in supernatants from anti-CD3-stimulated PBMC cultured in the presence or absence of ProTα. Recent data from our laboratory have shown that, upon activation with anti-CD3, PBMC from patients with advanced cancer produce smaller amounts of cytokines than do those from HD [16]. This was also the case in the group of patients studied here (Table 2; mean decrease: 47 ± 8; by Student's *t*-test $P < 0.01$ for all cytokines tested). In the presence of ProTα, a statistically significant increase in the levels of IL-1β, IL-2 and TNFα produced by the anti-CD3-stimulated PBMC was noticed, which became more evident in cancer patients than in HD (percentage increase: 71%, 55%, 66% in patients compared to 33%, 28%, 47% in HD for IL-1β, IL-2 and TNFα respectively). No enhancement in the levels of IL-6, IL-7, IL-12, IFNγ and GM-CSF was observed. The superior immunostimulatory synergistic effect of anti-CD3 plus ProTα to a single incubation of anti-CD3 with each one of these cytokines (i.e., IL-1β, IL-2 or TNFα), was recorded in the following series of experimentations. As shown in Fig. 1, none of the cytokines individually could significantly enhance killing of Daudi targets by anti-CD3-activated patients' PBMC. Only IL-2 at a high dose (1000 IU/ml) could enhance cytotoxicity, though to levels that were significantly lower than those achieved with ProTα (16% compared to 26% killing; $P < 0.05$). Similar results were also obtained against Raji targets (data not shown).

In parallel, PBMC from the same cultures were analyzed for adhesion molecule expression. As shown in Fig. 2, anti-CD3-activated PBMC co-cultured with ProTα expressed CD2, CD18, CD54 and CD49f at significantly higher levels than those cultured without

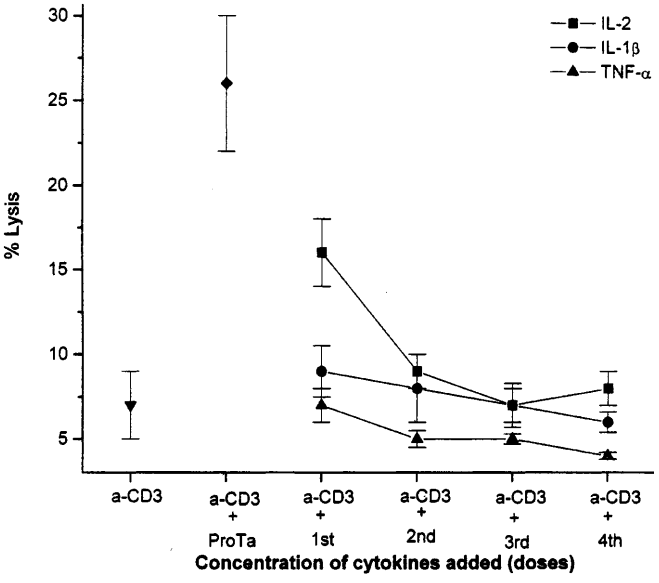


Fig. 1 Superior immunoenhancing synergistic effect of addition of anti-CD3(a-CD3) plus prothymosin α (*ProTα*) to incubation of patients' peripheral blood mononuclear cells (*PBMC*) with anti-CD3 plus each of the recombinant cytokines interleukin-2 (*IL-2*), IL-1β and tumor necrosis factor α (*TNFα*). Patients' PBMC (colorectal cancer, $n = 2$; breast cancer, $n = 4$ and ovarian cancer, $n = 7$) were incubated for 3 days with immobilized anti-CD3 mAb and 160 ng/ml ProTα or each of the recombinant cytokines and then tested for cytotoxicity against Daudi targets. First, second, third and fourth doses of each of the recombinant cytokines used were respectively: 1000, 100, 10 and 1 IU/ml for IL-2; 100, 10, 1 and 0.1 ng/ml for IL-1β; 200, 20, 2 and 0.2 ng/ml for TNFα

ProTα. Basal levels of adhesion molecule expression were almost identical amongst HD and cancer patients and these were equally enhanced in the presence of ProTα (Fig. 2). In contrast, there were no significant changes in the expression of other adhesion molecules, including CD62L, CD56, CD29 and CD40L (data not shown). There was also a statistically significant increase in the expression of the activation marker CD25, detected in almost 80% of HD PBMC cultured with anti-CD3 plus ProTα, as opposed to 50% in cultures with anti-CD3 alone (Fig. 3). The levels of CD25 expression in patients' PBMC after culture with anti-CD3 were

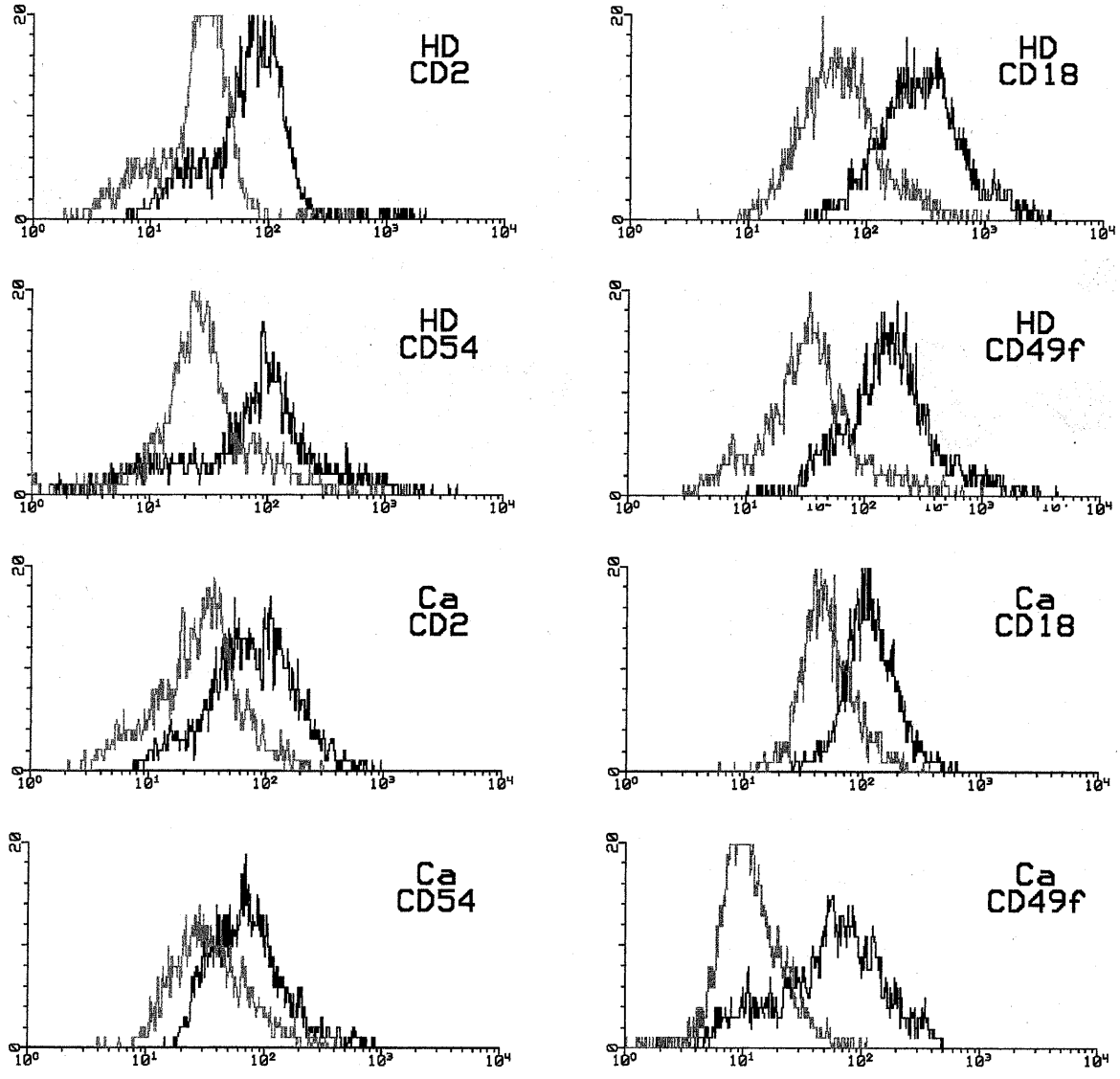


Fig. 2 ProTα enhances the expression of adhesion molecules on anti-CD3-stimulated mononuclear cells. PBMC were cultured for 3 days with solid-phase anti-CD3 mAb with or without 160 ng/ml ProTα. The cells were stained with a panel of mAb and analyzed by flow cytometry as described in Materials and methods. *Black lines* stimulation with anti-CD3 and ProTα; *gray lines* stimulation with anti-CD3 alone. Representative data of one healthy donor (*HD*) and one cancer (*Ca*) patient are shown. No significant changes in either cell intensity or number of positive cells in any of the four experiments performed with different donors were detected

much lower (32%; Fig. 3), suggesting insufficient activation, which subsequently could explain their reduced cytotoxic capacity. In accordance with this, patients' PBMC also contained less perforin-positive (+) cells (20% compared to 35% of HD). In the presence of ProTα the levels of both surface $CD25^+$ and cytoplasmic $perforin^+$ cells were restored to almost normal levels (63% and 37% respectively; Fig. 3). There was also a weaker, although significant, increase in the percentage of $perforin^+$ cells among HD PBMC (54% compared to 35% without ProTα).

The enhanced perforin protein levels resulted from the up-regulation of perforin mRNA transcription. Thus total RNA from a patient's PBMC incubated with anti-CD3 and ProTα was examined by Northern blot analysis. Basal levels of perforin mRNA could be detected in PBMC incubated in medium alone. However, culturing of PBMC in the presence of anti-CD3 and ProTα for 6 h caused a dramatic increase in the expression of the perforin gene transcription (to 24 times the control), which was significantly higher than that induced by anti-CD3 (9-fold) or ProTα alone (3-fold; Fig. 4).

Ex vivo anti-CD3-activated PBMC have been reported in some cases to cause regression of the autologous tumor, when reinfused into patients along with IL-2 [62]. Therefore, it was of interest to determine the levels of cytotoxicity against autologous tumor cells, mediated by patients' PBMC activated in vitro with anti-CD3 in the presence or absence of ProTα. In Fig. 5, we show lysis curves obtained at different effector:target (E/T) ratios using PBMC and autologous tumors from patients with lung ($n = 3$), ovarian ($n = 7$) and breast

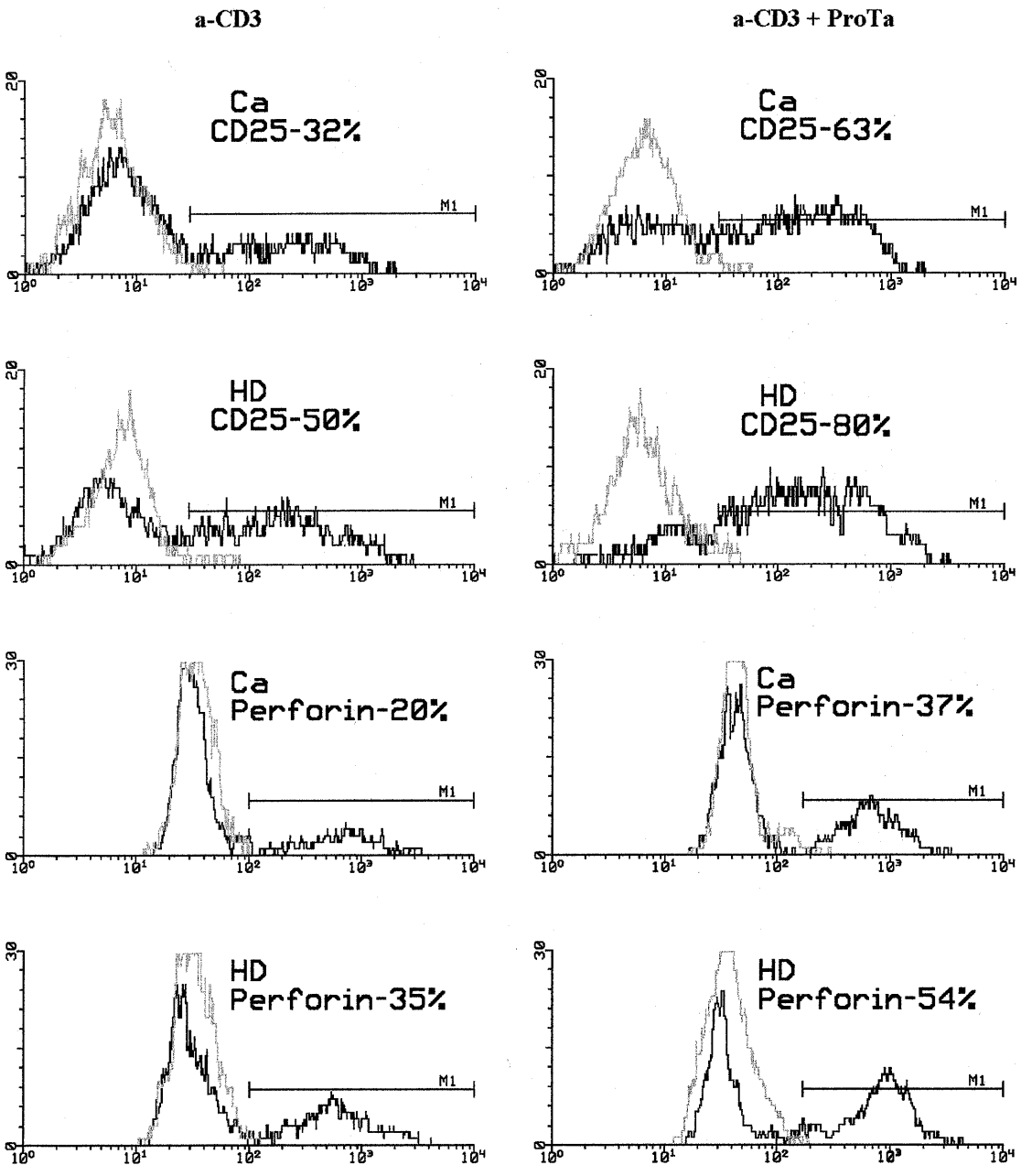


Fig. 3 ProTα positively modulates surface expression of CD25 (low-affinity IL-2 receptor) and cytoplasmic perforin levels in anti-CD3-stimulated PBMC. Flow-cytometric analysis of perforin levels was performed as described in Materials and methods. *Black lines* stimulation with anti-CD3 (*a-CD3*) and anti-CD3 plus ProTα (*a-CD3 ProTα*); *gray lines* (not stimulated) samples. Representative data of one healthy donor (*HD*) and one cancer patient (*Ca*) are shown. As in Fig. 2, no significant differences were observed in the four experiments performed in total

($n = 4$) cancer. The levels of AAK cytotoxicity against the autologous tumor targets were low in all patients tested (mean cytotoxicity values at a 40:1 E/T ratio were: 10.6% for lung cancer, 8.7% for ovarian cancer and 5.4% for breast cancer). The addition of ProTα significantly increased AAK-mediated autologous tumor killing at all ratios tested (28%, 26% and 16% at 40:1 E/T ratio for lung, ovarian and breast cancer respectively). No killing was detected (with or without ProTα) when autologous PBMC blasts were used as targets. PBMC cultures with ProTα alone exhibited only negligible levels of cytotoxicity (data not shown).

In order to characterize the tumor-reactive lymphocytes generated in cultures with anti-CD3 and ProTα, we performed cytotoxicity assays in the presence of mAb specific for crucial surface molecules, already reported to be involved in cytolytic processes mediated by various effector cell types [3, 29, 42, 43, 49, 58]. Cytotoxicity against both autologous tumor and Daudi targets was strongly inhibited by mAb against adhesion molecules CD2, CD18, CD54 and CD49f (range of inhibition: 50%–61%). In contrast, MHC-, CD25- and T-cell-sub-

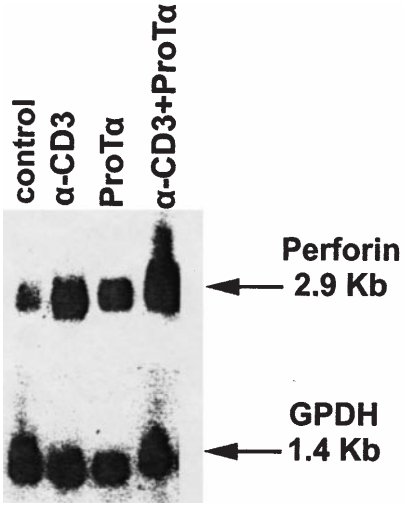


Fig. 4 Expression of perforin mRNA is up-regulated by ProTα in anti-CD3-activated patients' PBMC. PBMC (2×10^6/ml) from a patient with lung adenocarcinoma were incubated with medium (control), solid-phase anti-CD3 (5 μg/ml) alone (α-*CD3*), ProTα (160 ng/ml) alone (*ProTα*) and solid-phase anti-CD3 (5 μg/ml) plus ProTα (160 ng/ml) (α-*CD3* + *ProTα*) for 6 h at 37 °C. Total RNA was isolated and Northern blot analysis was performed on 20 μg total RNA. The filter was rehybridized with a cDNA probe for human perforin and glyceraldehyde 3-phosphate dehydrogenase (*GPDH*) was used as an internal control for equal loading

set-specific mAb did not affect the cytotoxic response (Fig. 6). These results suggest that our in vitro model triggers the generation of non-MHC-restricted effector lymphocytes involved in the cytolytic process against autologous tumor and Daudi targets.

The preceding experiments (Table 2 and Fig. 3) demonstrated that ProTα further activates patients' PBMC cultured with anti-CD3, as shown by the increase of $CD25^+$ cells, resulting in enhanced cytotoxicity, and was correlated with (i) increased number of cells containing cytoplasmic perforin molecules and (ii) increased production of IL-1β, IL-2 and TNFα. In addition, to characterize the effector-cell types that mediate this ProTα-induced enhanced cytotoxicity, we quantified the expression of surface IL-2R and cytoplasmic perforin molecules on lymphocyte subsets by dual immunofluorescence staining. Figure 7A–C shows that the expression of surface CD25 antigen (IL-2R) was enhanced in all lymphocyte subsets activated with anti-CD3 plus ProTα, as compared to those activated with anti-CD3 alone: 33% versus 19% for $CD4^+CD25^+$ (Fig. 7A), 18% versus 10% for $CD8^+CD25^+$ (Fig. 7B) and 11% versus 5% for $CD56^+CD25^+$ (Fig. 7C) cell subsets respectively. ProTα induced an enhanced expression of IL-1βR and TNFαR only in anti-CD3-activated $CD8^+$ and $CD56^+$ cells; TNFαR was expressed in 10% of both cell types, while IL-1βR was expressed in 12% of $CD8^+$ and 9% of $CD56^+$ cells. In cultures treated with anti-CD3 alone, the two receptors were expressed at equally marginal levels (2%–3%). Cytoplasmic perforin levels were also enhanced in both $CD8^+$ and $CD56^+$ lymphocytes (Fig. 7D): 10% $CD8^+$perforin$^+$ and 5% $CD56^+$perforin$^+$ cells in cultures with anti-CD3 compared to 22% $CD8^+$perforin$^+$ and 10% $CD56^+$perforin$^+$ cells in cultures with anti-CD3 plus ProTα ($P < 0.01$ in both cases). Our data suggest that ProTα further activates $CD4^+$, $CD8^+$ and $CD56^+$ cells in anti-CD3-stimulated PBMC cultures, as indicated by the expression of IL-2R (CD25). The cytolytic functional activity of $CD8^+$ and $CD56^+$ cells is also potentiated through the enhanced expression of cytoplasmic perforin levels. Both lymphocyte subsets were shown to participate almost equally in killing both autologous tumor and Daudi targets (Fig. 8).

Discussion

Anti-CD3 mAb has been reported to induce cytotoxic functions in lymphocyte populations. Sutharinan et al. [59] were the first to show that pretreatment of human alloreactive memory cells with anti-CD3 resulted in the induction of specific cytolytic and natural-killer-cell-like cytotoxicity. Immobilized anti-CD3, along with IL-2, has successfully been used for large-scale expansion of PBMC with non-MHC-restricted cytotoxicity for adoptive cellular immunotherapy [62]. Moreover, anti-CD3 along with IL-2 was demonstrated to induce autologous tumor-reactive T lymphocytes during an autologous mixed lymphocyte/tumor cell culture [59, 61]. Anti-CD3 has also been reported to stimulate killer cells to lyse acute myeloid leukemia cells [39] and to induce in vitro expansion and activation of mucin-reactive T-helper lymphocytes from patients with colorectal cancer [40]. In a pilot clinical study, tumor-infiltrating lymphocytes activated for 48 h with anti-CD3 mAb and expanded in vitro with low doses of IL-2 were successfully used in clinical trials in patients with melanoma and renal cell carcinoma [31].

In the present study, we demonstrate that patients with cancer in advanced stages show deficiencies in cytotoxic cell activities, since their PBMC fail to efficiently lyse autologous or allogeneic tumor targets upon incubation with anti-CD3. The contribution of this communication is that ProTα induces, in a non-MHC-restricted manner, killing of autologous tumor cells by anti-CD3-activated $CD8^+$ and $CD56^+$ lymphocytes from cancer patients. It should be noted that, although patients with different types of cancer were included in this study, the variability between the patients' groups in the cytotoxicity assays was low. This is best shown in Table 1, where the standard deviation of the mean values against Raji and Daudi targets is ±3 (i.e. 17%) and ±2 (i.e. 20%) respectively. When higher mean cytotoxicity values were achieved in the presence of ProTα, analogous standard deviations were determined (21% and 20%). Similarly, low interassay variation between cytotoxicity assays performed either with patients' PBMC or selected subpopulations against the same target at different times points was observed. Thus, in

Table 1 and Figs. 1, 8, mean cytotoxicity values against Daudi targets ranged from 7% to 10%, whereas those against autologous tumors were 8% (Figs. 5, 8). Futher addition of ProTα did not affect the interassay variability, and mean cytotoxicity against Daudi targets remained between 25% and 30% (Table 1 and Figs. 1, 6, 8), whereas the increased mean autologous tumor killing ranged from 23% to 26% (Figs. 5, 6, 8).

Thus far, ProTα has been shown to exert an in vitro potentiating effect on human T-cell-proliferative responses to cellular and soluble antigens, which was associated with a ProTα-induced increase in IL-2 production by the antigen-activated T cells and HLA-DR expression on monocytes [3, 4, 6]. Evidence for a role of ProTα in potentiating cytotoxic responses initially came from our previous report, in which the in vivo anticancer

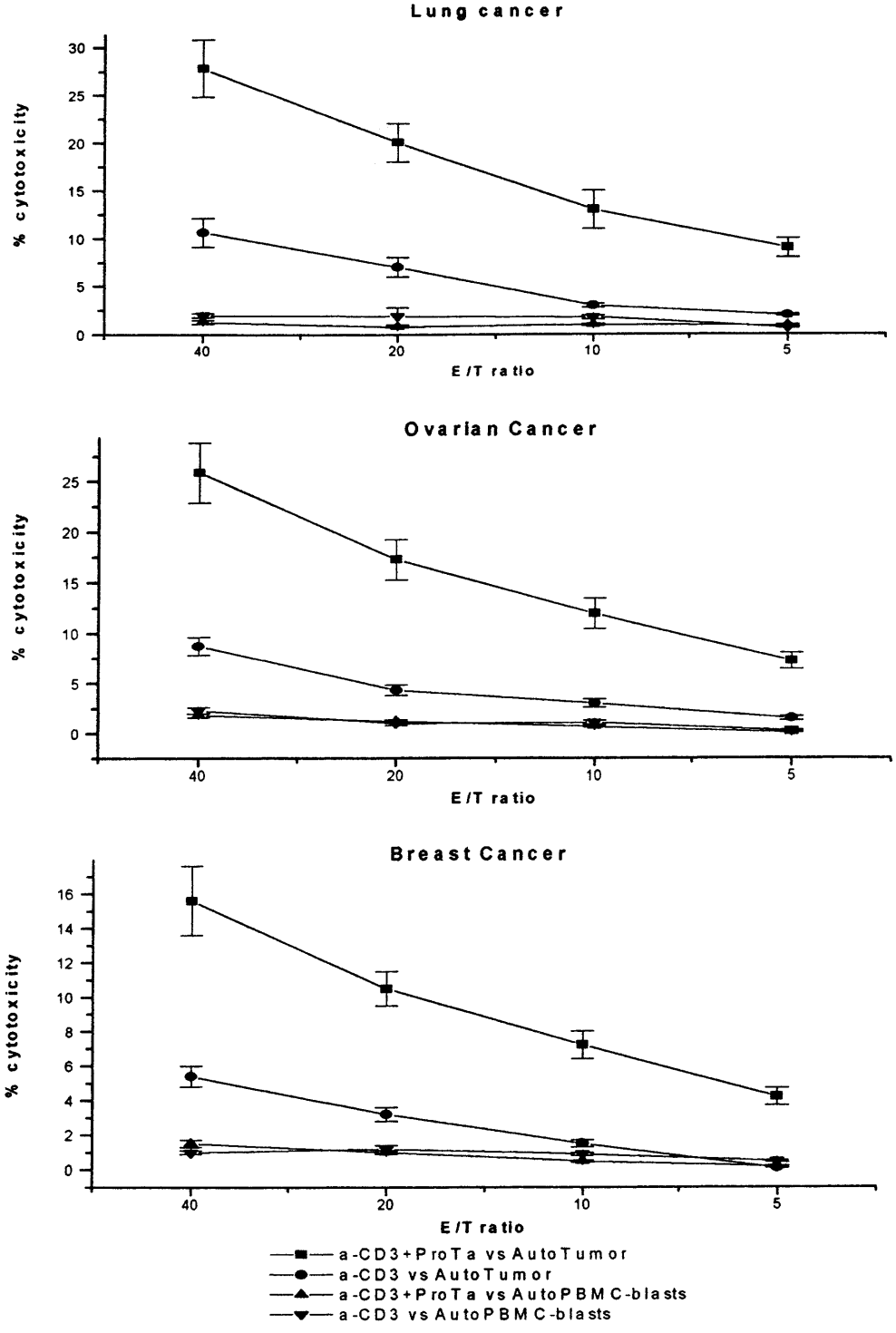


Fig. 5 ProTα enhances anti-CD3-activated cytotoxicity against autologous tumor targets. The latter were isolated from pleural ($n = 3$) and peritoneal ($n = 11$) malignant effusions from patients with lung adenocarcinoma ($n = 3$) and ovarian ($n = 7$) and breast ($n = 4$) cancer. Mean values from triplicate cultures are shown. Autologous PBMC blasts were prepared as described in Materials and methods

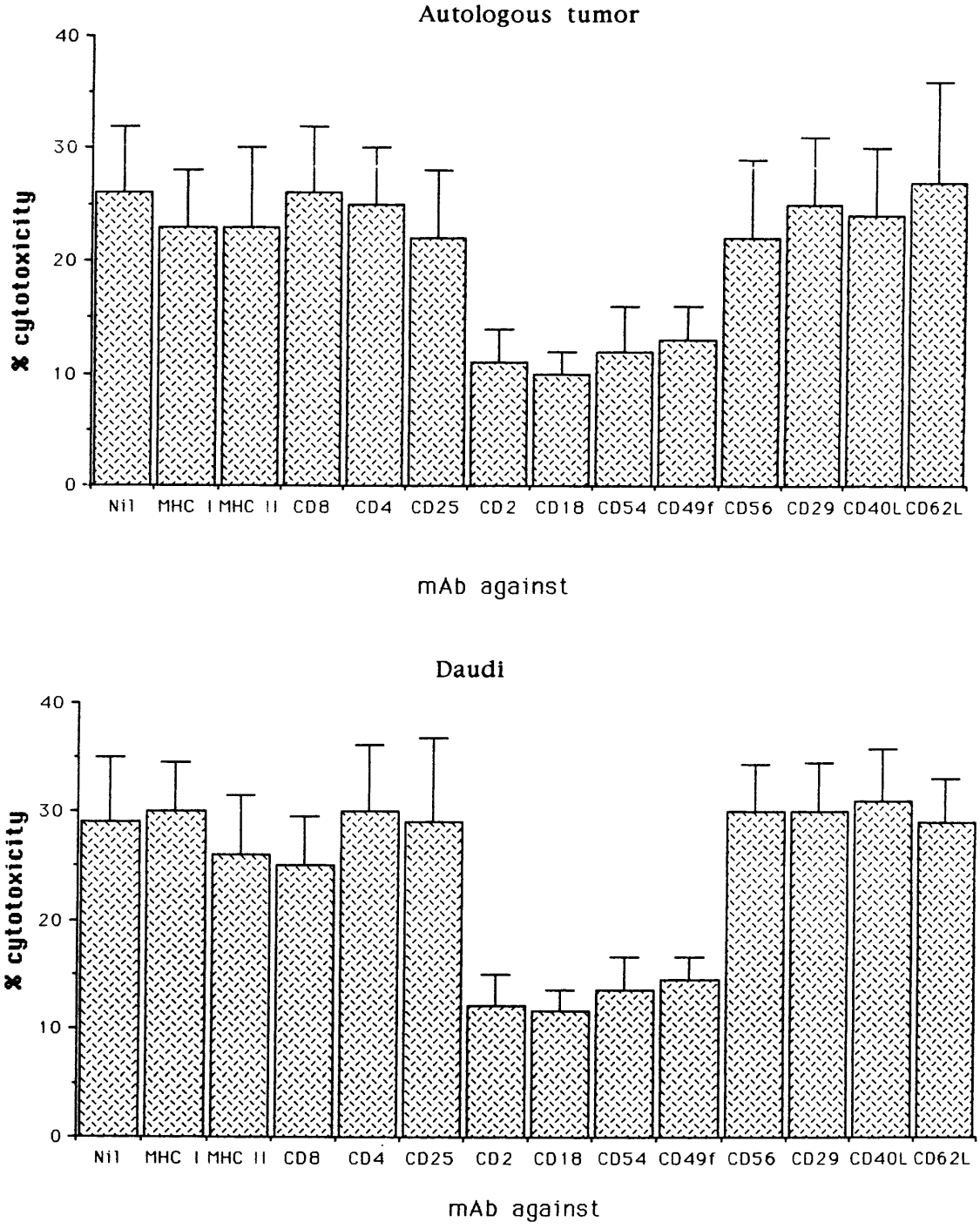
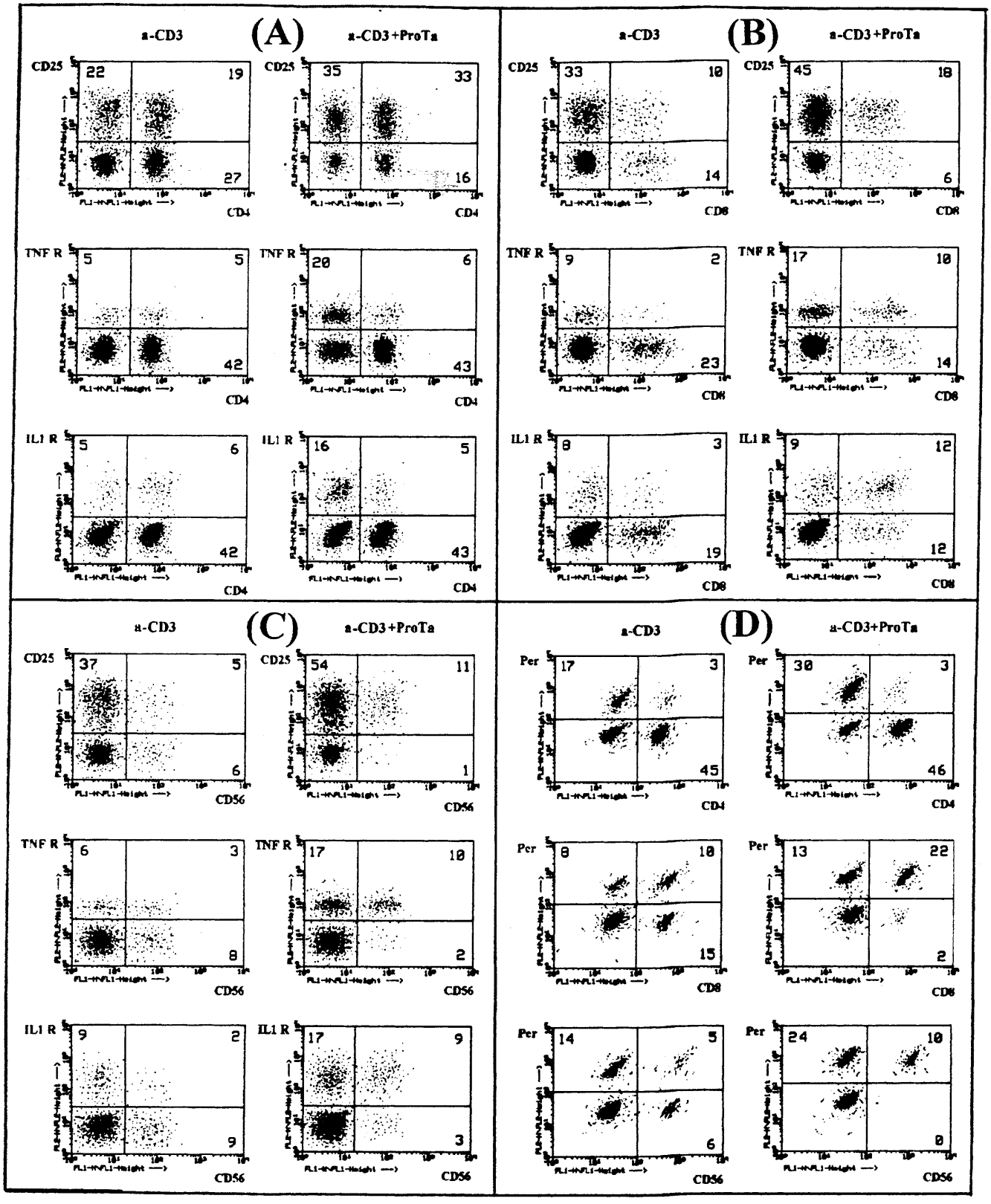


Fig. 6 mAb-specific inhibition of the ProTα-induced cytotoxicity against autologous tumor or Daudi cells by anti-CD3-stimulated PBMC. mAb (10 μg/ml final concentration) were present throughout the entire incubation period (overnight). Mean values ± SD from pooled data of 7 patients (lung cancer, $n = 2$; ovarian cancer, $n = 3$; breast cancer, $n = 2$) are shown. The same patients were tested in both types of cytotoxicity. *Nil* anti-CD3 + ProTα

activity of ProTα was shown to be, at least partly, mediated by ProTα-activated tumoricidal peritoneal exudate cells [52]. ProTα was also shown to increase several types of cytotoxicity significantly in in vivo and in vitro models [9, 12, 14, 21, 30, 33]. Such stimulatory effects were mainly attributed to the ability of ProTα to increase endogenous IL-2 production and, at the same time, to decrease prostaglandin E_2 synthesis [14]. In addition, Gruenberg et al. [33] and Eckert et al. [25] most recently demonstrated the capacity of ProTα to stimulate the immunocytotoxicity of IL-2-activated lymphocytes against colorectal carcinoma. These observations, along with data from our laboratory showing an association between decreased IL-2 levels and deficient non-MHC-restricted cytotoxicity in cancer patients



[8], suggest that ProTα may operate in the in vitro model presented herein, by a similar mechanism. Indeed, in this study we demonstrate increased production of IL-2 by patients' PBMC activated by a combination of anti-CD3 plus ProTα, which could explain their enhanced non-MHC-restricted cytotoxicity against autologous tumor and Daudi targets. This enhanced cytotoxic response could also be ascribed to the increased TNFα and IL-1β

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Fig. 7A–D Two-color cytograms of 3-day cultured cells (anti-CD3 versus anti-CD3 + ProTα) labeled with CD4-FITC (**A**), CD8-FITC (**B**), CD56-FITC (**C**) and CD25(IL-2R)-PE, TNFαR-PE, IL-1βR-biotin (+streptavidin-PE) mAb. **D** Two-color cytograms of the same cells labeled with anti-perforin-specific mAb, PE-anti-mouse IgG and FITC-conjugated CD4, CD8 and CD56 mAb as described in Materials and methods. Data are from one representative experiment (lung cancer patient) out of nine conducted. Percentages of positive cells in the two groups (*a-CD3* and *a-CD3+ ProTα*) did not significantly vary among the different patients analyzed

levels that were also measured in cultures with anti-CD3 plus ProTα. Thus, TNFα was shown to elicit cytolytic responses in the absence of exogenous IL-2 [36]. A synergism between IL-2 and TNFα in the generation of non-MHC-restricted cytotoxicity has also been reported [20]. Accordingly, such a functional interaction between IL-2 and TNFα involved induction of both TNFα and IL-2 receptors on the effector lymphocytes [3, 20, 36, 47]. IL-1 has also been demonstrated to synergize with IL-2 in the induction of cytotoxic activity and the expression of IL-1 receptors in effector cells was found to be a prerequisite for the cytolytic process [24, 28]. In agreement with this, we found increased expression of IL-2-, TNFα- and IL-1β-specific receptors on both $CD8^+$ and $CD56^+$ cells, which were shown to carry out almost all of the AAK cytotoxicity. In addition, the increased expression of the adhesion molecules CD2, CD18, CD54 and CD49f, reported to be involved in the binding of effector cytotoxic lymphocytes to the tumor

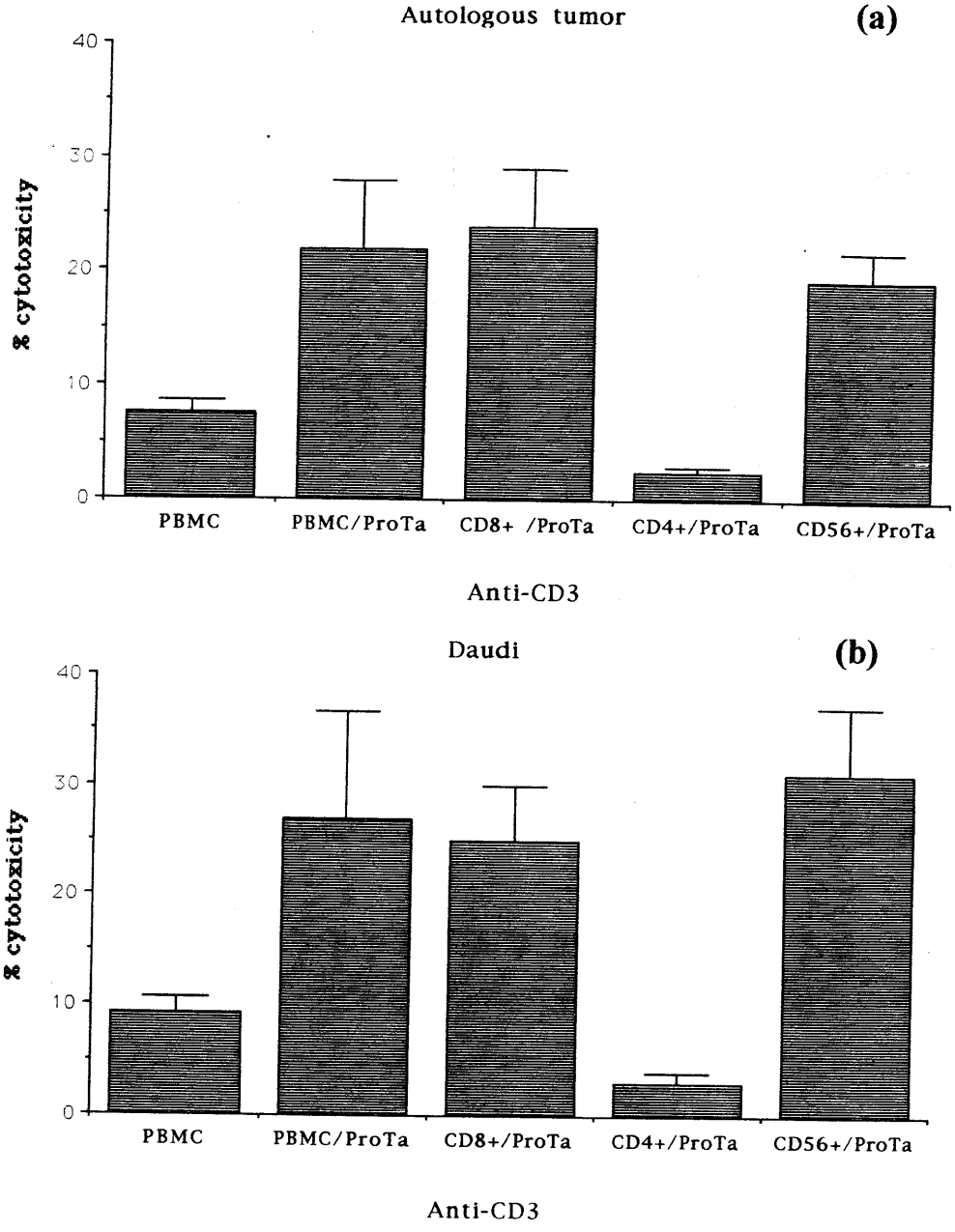

Fig. 8 a Enhancement of autologous tumor-specific cytotoxicity by ProTα in anti-CD3-stimulated PBMC is mediated by $CD8^+$ and $CD56^+$ cells. $CD8^+$, $CD4^+$ or $CD56^+$ cells isolated from stimulated (anti-CD3+ ProTα) PBMC were tested as effectors against autologous tumor targets. Mean cytotoxicity values ± SD from pooled data are shown. Patients with lung cancer ($n = 4$), ovarian cancer ($n = 3$) and breast cancer ($n = 8$) were tested. Tumor cells were collected from malignant effusions as described in Materials and methods. **b** Augmentation of LAK cytotoxicity against Daudi targets by ProTα in anti-CD3-stimulated PBMC is mediated by $CD8^+$ and $CD56^+$ cells. The same PBMC as in **a** were tested. *PBMC* stimulation of PBMC with anti-CD3 alone; *PBMC/ProTα* stimulation of PBMC with anti-CD3 plus ProTα with PBMC tested as effectors; *$CD8^+$/ProTα*, *$CD4^+$/ProTα*, *$CD56^+$/ProTα* stimulation of PBMC with anti-CD3 plus ProTα with $CD8^+$, $CD4^+$ or $CD56^+$ cells tested as effectors

targets [3, 29, 42, 43, 49, 58], may also contribute to the potentiated AAK activity. This was indirectly shown by the use of mAb specific for each of these molecules which, when separately added, abrogated a substantial level (up to 60%) of the total cytotoxic response. Whether the enhanced expression of adhesion molecules is mediated directly by ProTα or indirectly by one or more ProTα-induced cytokines (e.g. IL-2, TNFα, IL-1β) deserves further investigation.

ProTα significantly increased the percentage of anti-CD3-activated lymphocytes ($CD8^+$ and $CD56^+$) containing cytoplasmic perforin. When released from storage in the cytoplasmic granules of activated T, NK and LAK effector cells, perforin polymerizes within the target cell membrane via a Ca^{2+}-dependent pathway, causing membrane perforation and cell death [53]. So far, IL-2, IL-12 and IL-15 have been reported to augment perforin expression in NK and T cells [17, 29, 55]. Our results add yet another soluble mediator, ProTα, which can induce up-regulation of cytoplasmic perforin molecules in cancer patients' PBMC. Whether ProTα directly increases cytoplasmic perforin levels or its effect is exerted indirectly via the enhanced production of IL-2 (e.g. by the activated $CD4^+$ cells) remains at present unknown.

The ProTα-induced enhancing effect on cytokine production was more profound in cultures with cancer patients' PBMC, producing low levels of cytokines upon activation with anti-CD3. Cellular immunity in cancer patients with advanced disease has been reported to be impaired in several aspects, including T-cell activation and proliferation in response to a variety of stimuli [7, 9, 15, 33, 41, 56]. Recently, it was also shown that T cells from patients with advanced cancer have abnormal T-cell-receptor(TCR)/CD3 complex structure, in particular the absence of the CD3 ζ chain [1, 50]. The loss of CD3 ζ appears to be related to unstable expression of the TCR-CD3 complex and low T cell activation and proliferation [2, 50].

Patients' T cells were also found to have decreased levels of cell-surface and cytoplasmic CD3 ε chains [48], which are important to the assembly and expression of the entire CD3 complex [45]. Such deficiencies may contribute to insufficient activation of cancer patients' PBMC with anti-CD3. In agreement with this, our results demonstrate low percentages of patients' PBMC expressing the activation surface marker CD25 or intracellular perforin molecules upon stimulation with anti-CD3. Such an incomplete activation of PBMC with anti-CD3 may lead to a limited secretion of cytokines and reduced levels of cytotoxicity. Another factor that may contribute to the decreased cytokine production by the anti-CD3-activated patients' PBMC is the hyperproduction of IL-10. Recent data from our laboratory demonstrated high titers of IL-10 in supernatants derived from cultures of cancer patients' PBMC with anti-CD3 [16]. This cytokine has been reported to suppress the secretion of IFNγ, IL-1β, TNFα and IL-6 [27, 60, 64], and therefore could also be responsible for the overall low cytokine production in such cultures. ProTα may thus increase cytokine production by cancer patients' PBMC during activation with anti-CD3 either by normalizing the expression of CD3 ζ and/or ε chains, by reducing the production of IL-10 or both. The same mechanism(s) of action of ProTα may also be responsible, albeit to a lesser extent, for the observed enhancement of cytokine production by anti-CD3-activated HD-derived PBMC. Work is now in progress in our laboratory to clarify these aspects.

Adoptive cellular therapy with patients' activated killer cells is, at present, the most popular method of applied cancer immunotherapy. However, several serious obstacles have stood in the way of satisfactory clinical responses. One of the most serious limitations is the insufficient activation of autologous tumor-reactive lymphocytes in in vitro cultures. By selectively stimulating such cells among the anti-CD3-activated PBMC from patients with advanced cancer, ProTα may prove useful in future therapeutic interventions. This potential application of ProTα should, however, be preceded by further experimentations to characterize better the mechanism(s) underlying its effect.

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