

Alterations in Expression and Function of Signal-transducing Proteins in Tumor-associated T and Natural Killer Cells in Patients with Ovarian Carcinoma¹

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ABSTRACT

Tumor-associated lymphocytes (TALs) freshly isolated from patients with cancer usually manifest reduced proliferative and cytolytic functions. To determine whether alterations in signal transduction contribute to functional impairments seen in TALs, we purified populations of T and natural killer (NK) cells by negative selection from ascites of seven patients with ovarian carcinoma. The average purity was $84 \pm 5\%$ for CD3⁺ TALs and $77 \pm 10\%$ for CD3⁺CD56⁺CD16⁺ TALs. Expression of several signal transduction molecules, including the CD3- ϵ , CD3- ζ , and Fc ϵ RI- γ chains, p56^{lck} protein tyrosine kinase, and phospholipase C- γ 1, was studied in these cells using Western blotting. A marked decrease in expression of ζ and Fc ϵ RI- γ associated with CD3 or Fc γ RIIIA was observed in T or NK cells obtained from TALs, as compared to T or NK cells purified from normal peripheral blood. Expression of CD3- ϵ , as assessed using flow cytometry, Western blotting, or ELISA was also reduced in purified TAL-T cells relative to that in normal peripheral blood T cells. Surface expression of CD3 on T cells and Fc γ RIIIA on NK cells obtained from TALs was significantly decreased in comparison to normal peripheral blood lymphocytes (PBLs): the mean fluorescence intensity of CD3 was 277 ± 18 for TAL-T ($n = 7$) versus 349 ± 13 for PBL-T ($n = 9$) and that of CD16 was 58 ± 1 for TAL-NK ($n = 7$) versus 385 ± 55 for PBL-NK ($n = 23$) cells. These observations suggest a defect in assembly of T cell receptor and Fc γ RIIIA multicomponent transmembrane receptors, which are ζ and γ dependent. In addition to

alterations in expression, the function of these receptors was also modified, since cross-linking of CD3 on TAL-T and CD16 on TAL-NK cells with the respective monoclonal antibodies resulted in a pattern of protein phosphorylation that was distinct from that observed in normal PBLs. Expression of tyrosine kinase p56^{lck} and its kinase activity were also depressed, while expression of phospholipase C- γ 1 appeared to be normal in most preparations of the TALs tested. *In vitro* proliferation of TAL-T in response to anti-CD3 monoclonal antibody and TAL-NK cells to interleukin 2 were significantly depressed as was the ability to produce IFN- γ . In contrast, TAL-T cells were able to produce interleukin 10 at levels similar to those secreted by normal PBLs. Thus, in TALs obtained from patients with advanced ovarian cancer, alterations in expression and activity of signaling molecules were associated with reduced cellular functions such as proliferation and production of certain cytokines.

INTRODUCTION

Patients with advanced malignancies have been reported to have progressively impaired immune responses (1, 2). Cancer-related immunodeficiency may be tumor induced, appears to correlate with the tumor burden, and could contribute to the progression of the disease. Mechanisms responsible for immunosuppression observed in cancer patients are not yet resolved. Cellular unresponsiveness has been well documented for TILs³ freshly isolated from the tumor microenvironment (3-6). Several possible mechanisms have been suggested to account for the lack of immune competence of freshly isolated TILs, including deletion or selective inactivation of tumor-reactive T lymphocytes (7, 8), the presence of suppressor lymphocytes (9, 10), or macrophages (11) or production of suppressor factors by tumor cells (12-14).

Recently, Mizoguchi *et al.* (15) have demonstrated that splenocytes from tumor-bearing mice had altered expression of intracellular signal transduction molecules and depressed immune responses. In mice bearing a colon carcinoma, MCA-38, for a period longer than 26 days, CD8⁺ T cells had impaired cytotoxicity, decreased expression of tumor necrosis factor α and granzyme B genes, and decreased ability to mediate anti-tumor responses *in vivo* as compared to T cells obtained from normal mice or mice bearing tumors for shorter periods of time

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³ The abbreviations used are: TIL, tumor-infiltrating lymphocyte; mAb, monoclonal antibody; PTK, protein tyrosine kinase; TAL, tumor-associated lymphocyte; NK, natural killer; TcR, T-cell receptor; IL, interleukin; PLC, phospholipase C; ECL, enhanced chemiluminescence; GAMIG, goat antimouse immunoglobulin; PBL, peripheral blood lymphocyte; Ab, antibody; MFI, mean fluorescence intensity; IL-2R, IL-2 receptor; RCC, renal cell carcinoma.

Table 1 Diagnoses and previous treatment of the patients with ovarian carcinoma included in the study

Patient	Age (yr)	Diagnosis	Treatment
9111	72	Papillary serous adenocarcinoma	No Rx ^a
9348	50	Papillary serous adenocarcinoma	Multiple chemo Rx; end stage disease
9350	64	Endometrioid adenocarcinoma	Multiple chemo Rx; end stage disease
9531	45	Papillary serous adenocarcinoma	No Rx
9662	80	Papillary serous adenocarcinoma	No Rx
9965	65	Papillary serous adenocarcinoma	No Rx
9977	48	Endometrioid adenocarcinoma	No Rx

^a Rx, treatment.

(15, 16). No differences were detected between normal T cells and those from tumor-bearing mice in the percentage or fluorescence intensity of cells positive for the TcR- α/β heterodimer or the CD3 complex. However, in contrast to cells from normal mice, those from tumor-bearing mice had reduced Ca^{2+} mobilization when stimulated with anti-CD3 mAbs, altered basal pattern of protein tyrosine phosphorylation, and reduced expression of the src family PTK, p56^{lck} and p59^{lyn}. T cells from tumor-bearing mice expressed the unusual TcR/CD3 complexes, in which CD3- ζ was undetectable, and the ζ chain was replaced by Fc ϵ RI- γ chain. More recently, fresh TILs obtained from patients with renal cell carcinoma (17) or colorectal carcinoma (18) were also found to have a marked decrease in the expression of the TcR-associated ζ chain. Although NK cells purified from the tumor microenvironment or cancer patients' peripheral blood have not been studied for deficiencies in signaling, cytoplasmic expression of CD16- ζ , as assessed by the flow cytometry analysis of permeabilized TILs or PBLs of patients with colorectal carcinoma, was found to be reduced relative to that of CD16- ζ in PBLs of normal donors (18).

Ascitic fluids of patients with ovarian carcinoma have been shown to contain a relatively high proportion of NK cells (CD3⁻CD16⁺ or CD3⁻CD56⁺) as well as CD3⁺ T cells (19). We, therefore, were able to purify populations of tumor-associated T and NK lymphocytes from such fluids and to investigate expression and function of signal transduction proteins in these cells. In comparison to normal PBLs, tumor-associated NK and T cells isolated from ascites of patients with ovarian carcinoma demonstrated deficient expression of ζ , lower basal levels of protein tyrosine phosphorylation, altered patterns of protein phosphorylation when triggered via surface CD3 or CD16, and reduced expression and kinase activity of p56^{lck}. These deficiencies in expression and function of signaling molecules were associated with reduced proliferation and an altered profile of cytokine secretion by the NK or T cells isolated from ascites and stimulated with IL-2 or by cross-linking of surface CD3.

MATERIALS AND METHODS

Patients. Ascitic fluids and peripheral blood were collected from seven patients with ovarian carcinoma at University Hospital (Birmingham, AL) or at Magee Women's Hospital, University of Pittsburgh Medical Center (Pittsburgh, PA). The patients ranged in age from 45 to 80 years, with a mean age of 60.6 ± 13.2 years (Table 1). Five patients were diagnosed to have papillary serous adenocarcinoma and two endometrioid

adenocarcinoma. The ovary was the primary site of malignancy for all patients. Five of seven patients were untreated, and two patients received chemotherapy in the past but were end stage at the time of specimen collection and were not receiving any treatment. This study was approved by the Institutional Review Board for human use at both institutions.

Purification of T or NK Cells from Peripheral Blood.

Mononuclear cells were isolated by Ficoll-Hypaque gradient centrifugation from leukapheresed products obtained from platelet donors (Central Blood Bank of Pittsburgh) or from heparinized blood obtained from each patient at the same time as the ascitic fluid. To select for PBLs, monocytes were removed on nylon-wool columns as described previously (20). T or NK cells from normal donors were purified from PBLs by negative selection as described earlier (21). In brief, PBLs were incubated in the presence of anti-CD3, anti-CD19, and anti-CD14 mAbs to select for NK cells and anti-CD16, anti-CD19, and anti-CD14 mAbs to select for T cells (10 $\mu\text{g}/\text{ml}$; DAKO Corp., Carpinteria, CA) for 30 min at 0°C. The cells were then washed twice and incubated with magnetic beads coated with goat antimouse immunoglobulins (1 cell:30 beads; PerSeptive Diagnostics, Cambridge, MA) for 30 min at 0°C. After each of two successive incubations with magnetic beads, a magnet was used to separate beads with attached cells. Two-color flow cytometry was used to determine the purity of the negatively selected NK and T-cell populations.

Isolation of Ovarian TALs. Ascitic fluids (850–1300 ml) were obtained from seven patients with ovarian carcinoma. The fluids were centrifuged to obtain cell pellets. The cells were washed twice in RPMI 1640, placed on Ficoll-Hypaque discontinuous density gradients, and centrifuged to harvest TALs and tumor cells as described by us earlier (4). The TAL preparations were usually not contaminated by more than 5% tumor cells and were more than 90% viable, as determined by a trypan blue dye exclusion test.

Flow Cytometry. Staining and flow cytometry analyses were performed as previously described utilizing a FACScan (Becton Dickinson) for two-color analysis (22). In brief, $2\text{--}4 \times 10^5$ cells/tube in PBS containing 0.1% sodium azide were incubated with different combinations of fluorescein- or phycoerythrin-labeled mAbs for 30 min at 4°C. All mAbs were pretitrated on normal human mononuclear cells to determine optimal working dilutions. The IgG1 and IgG2a isotype controls and normal saline controls were included in all experiments.

Western Blotting. Cell preparations of PBLs, PBL-NK, and PBL-T from normal donors and TAL, TAL-NK, and TAL-T from ascites of patients with ovarian carcinoma were lysed by incubation in lysis buffer supplemented with high concentrations of protease inhibitors, which have been demonstrated to completely inhibit granulocyte proteases [(50 mM HEPES (pH 7.2), 150 mM NaCl, 5 mM EDTA, 1 mM *o*-vandate, 2.5% Triton X-100, 200 μ g/ml trypsin/chymotrypsin inhibitor (Sigma, St. Louis, MO), 200 μ g/ml chymostatin (Boehringer Mannheim, Indianapolis, IN), and 2 mM phenylmethylsulfonyl fluoride (Sigma)] for 20 min at 4°C. The lysate was centrifuged at 14,000 rpm for 10 min, and the postnuclear supernatant was harvested as well as samples for quantitation of protein concentration utilizing a Bio-Rad DC protein assay (Hercules, CA). The rest of the lysate was then mixed with an equal volume of 2× SDS-polyacrylamide gel sample buffer, boiled for 5 min, and either stored at -70°C for later use or immediately subjected to electrophoresis. Cellular lysates were electrophoresed in 12% (for CD3- ζ , CD3- ϵ or Fc ϵ RI- γ), 10% (for Lck), or 7.5% (for phosphotyrosine and PLC- γ 1) SDS gels under reducing conditions according to the method of Laemmli (23). The separated proteins were then electrophoretically transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA). Following blocking by a 3% solution of nonfat dried milk and 1% BSA for 1 h at room temperature, the membranes were blotted with anti- ζ antiserum generously provided by Dr. R. Robb (OncoTherapeutics, Cranberry, NJ), anti-Fc ϵ RI- γ (rabbit antiserum 934; a generous gift from Dr. J.-P. Kinet, National Institute of Allergy and Infectious Diseases, NIH), anti-CD3- ϵ (DAKO Corp.), or anti-p56^{lck} (Upstate Biotechnology, Inc., Lake Placid, NY), all used at a 1:1000 dilution and antiphosphotyrosine (4G10; UBI) and anti-Fc ϵ RI- γ antibodies at a 1:5000 dilution. Optimal dilutions of the antibodies were determined in titration experiments using serial antibody dilutions to blot the same cellular lysate. After washing in Tris-buffered saline with 0.05% Tween 20, the membrane was incubated with antirabbit or antimouse immunoglobulin-horse-radish peroxidase (1:5000 dilution; Amersham, Arlington Heights, IL) for 1 h. Protein bands were detected by enhanced chemiluminescence (ECL, Amersham).

Induction of Protein Tyrosine Phosphorylation. Purified T or NK cells (normal PBLs or TALs) at 2.5×10^6 cells/ml/sample were incubated with anti-CD3 mAb (10 μ g/ml; Immunotech, Westbrook, ME) or anti-CD16 (3G8; 10 μ g/ml purified from hybridoma), respectively, for 30 min at 4°C. Cells were then washed twice with cold medium and resuspended in solution containing 20 μ g/ml F(ab')₂ of GAMIg (Sigma) prewarmed to 37°C. Cells were incubated in a 37°C water bath for 2 to 10 min and then rapidly pelleted and solubilized in lysis buffer. To determine the presence of phospho- ζ after cross-linking of CD3 or CD16 on T or NK cells, respectively, cell lysates (50 μ g measured protein/sample) were precleared with rabbit antihuman immunoglobulin and protein A-Sepharose beads and then subjected to immunoprecipitation of ζ by specific rabbit antiserum (OncoTherapeutics) and protein A-Sepharose beads. Phosphotyrosine-containing proteins including phospho- ζ were detected by blotting with the antiphosphotyrosine mAb 4G10, followed by horseradish peroxidase-conjugated GAMIg and the ECL detection system.

In Vitro PTK Assay. Freshly purified PBL-T or PBL-NK cells from normal donors or TAL-T or TAL-NK cells from ovarian ascites (5×10^6 /sample) were treated with anti-CD3 or anti-CD16 mAb for 30 min on ice. Cells were washed twice with cold RPMI 1640 supplemented with 2% FCS and incubated with 20 μ g/ml F(ab')₂ fragments of GAMIg as secondary cross-linking Ab for 5 min at 37°C. Stimulated cells were lysed at 4°C for 30 min and postnuclear supernatant was harvested after centrifugation at 14,000 rpm at 4°C for 10 min. Lysates used for these studies were prepared from equal numbers of purified cells and confirmed to contain equivalent protein concentrations. Anti-p56^{lck} mAbs, at a concentration of 2 μ g/100 μ l/5 $\times 10^6$ cells, were used to immunoprecipitate the tyrosine kinase from cell lysates. The immunoprecipitates were washed with PBS/1% NP40, Tris-LiCl, and Tris-NaCl-EDTA. Kinase buffer [50 mM Tris (pH 7.4), 10 mM MnCl₂, 10 mM MgCl₂, 10 μ M ATP, and 10 μ Ci/sample of [γ -³²P]ATP (DuPont NEN Research Products, Boston, MA) were added and incubated for 20 min at room temperature. The reaction was stopped by the addition of 2× Laemmli buffer. The samples were then boiled and loaded onto a 10% SDS-PAGE followed by autoradiography.

CD3- ζ and CD3- ϵ ELISA. Microtiter plates were coated with rabbit anti-CD3 (5 μ g/ml; DAKO) during overnight incubation at 4°C and blocked with 2% nonfat dry milk in PBS for 1 to 2 h at 4°C. PBLs or purified T cells at a dose range of 0.6 – 15×10^6 cells were incubated in 65 μ l digitonin lysis buffer [100 mM HEPES (pH 7.2), 300 mM NaCl, 10 mM EDTA, 2 mM *o*-vandate, 1% digitonin, 200 μ g/ml trypsin/chymotrypsin inhibitor, 200 μ g/ml chymostatin, and 2 mM phenylmethylsulfonyl fluoride] on ice for 15 min. After addition of 560 μ l cold PBS, the lysates were centrifuged at 14,000 rpm for 5 min, and the cell lysis supernatant was added at 100 μ l/well to CD3-precoated microtiter plates. Following 1 h incubation at 4°C and three washes with PBS, the secondary antibodies, either mouse antihuman CD3- ϵ (OKT3, 7.5 μ g/ml in PBS/0.2% milk; Ortho) or anti-CD3- ζ mAb (TIA-1, 5 μ g/ml; Coulter Electronics, Hi-aleah, FL), were added at 100 μ l/well. Following additional incubation at 4°C for 30 min and four washes in PBS, the tertiary Ab antimouse immunoglobulin-alkaline phosphatase (1:500 dilution in PBS/0.2% milk; Southern Biotek) was added for additional incubation of 30 min at 4°C. The plates were then washed five times with PBS and incubated with 100 μ l alkaline phosphatase reagent (1 μ g/ml *p*-nitrophenyl phosphate in 10% diethanolamine, 240 μ M MgCl₂, pH 9.8) for 1 to 3 h at 37°C. Absorbance at 405 nm was read on an automatic microplate spectrophotometer. Each experiment included a standard curve for the presence of CD3- ϵ and CD3- ζ in frozen lysates of purified human PBL-T ranging in number from 0.6 to 15×10^6 cells. EBV-transformed lymphoblastoid cell lines were used as negative controls. The experimental values were compared to the standard curve generated in the same assay.

Proliferation Assays. Proliferation of T cells was stimulated by anti-CD3 (clone 35; AMAC) or anti-CD2 (clone 6F10.3; AMAC) mAbs (5–10 μ g/ml) immobilized to plastic surfaces of round-bottomed 96-well microtiter plates by overnight incubation in 0.05 M NaHCO₃ (pH 9.2) at 4°C and/or by IL-2 (600–1500 IU/ml; Cetus). Proliferation of NK cells was stimulated by IL-2 used at 1500–6000 IU/ml in RPMI 1640

Table 2 Phenotypic characteristics of TALs obtained from ascites of patients with ovarian carcinoma or of PBLs from normal individuals

	CD3 ⁺	CD3 ⁺ CD56 ⁺ and/or CD3 ⁺ CD16 ⁺	CD8 ⁺	CD4 ⁺	CD19 ⁺	CD14 ⁺
Patients						
PBL (<i>n</i> = 4)	52 ± 4	39 ± 5	20 ± 1	29 ± 5	4 ± 1	3 ± 2
TAL ^a (<i>n</i> = 7)	60 ± 8	28 ± 8	31 ± 7	30 ± 5	5 ± 2	10 ± 3
TAL-T ^b (<i>n</i> = 5)	84 ± 5	12 ± 4	41 ± 8	41 ± 9	1 ± 1	0
TAL-NK ^c (<i>n</i> = 3)	12 ± 7	77 ± 10	N/A	N/A	2 ± 1	4 ± 2
Normal donors						
PBL-T ^b (<i>n</i> = 10)	92 ± 1	7 ± 1	28 ± 2	63 ± 3	1 ± 1	0
PBL-NK ^c (<i>n</i> = 40)	4 ± 2	94 ± 2	N/A	N/A	1 ± 1	1 ± 0

^a TALs were separated from ascitic fluids by Ficoll-Hypaque gradient centrifugation. The data are percentage of positive cells (mean ± SD).

^b Purified TAL-T or PBL-T cells were obtained from ascites or PBLs by depletion of CD16⁺, CD14⁺, and CD19⁺ cells.

^c Purified TAL-NK or PBL-NK cells were obtained from ascites or PBLs by depletion of CD3⁺, CD14⁺, and CD19⁺ cells.

supplemented with 2 mM L-glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin, 25 mM HEPES buffer, and 10% heat-inactivated FCS (all from GIBCO, Grand Island, NY). Proliferative responses of all cell preparations cultured at a cell density of 10⁶ cells/ml were measured by [³H]thymidine uptake (1 µCi/well, 6.7 Ci/mmol; New England Nuclear) during the last 18 h of 3-day cultures.

Cytokine Production Assays. To induce cytokine production, purified T cells were incubated at a cell density of 10⁶ cells/ml for 48 h in round-bottomed microwells precoated with mAbs to CD3 or CD2 (10 µg/ml). Supernatants were collected and stored frozen (−80°C). The levels of IFN-γ or IL-10 in the supernatants were assessed using a commercially available ELISA kits (Endogen, Cambridge, MA). The assays were performed at the Pittsburgh Cancer Institute's Immunological Monitoring and Diagnostic Laboratory under strict quality control conditions, including calibration of all assays against International Cytokine Standards (Biological Response Modifier Program, Frederick, MD) and determinations of interassay and intraassay variability, as described earlier (24).

RESULTS

Recovery and Purity of Ovarian TALs. The mean number ± SE of TALs obtained from seven ascitic fluids was 248 ± 60 × 10⁶. In five of seven cases, the majority of TALs (70%) were used for purification of TAL-T and TAL-NK cells by negative selection with magnetic beads, as described in "Materials and Methods." In two cases, the number of recovered TALs was not sufficient for negative selection, and only unseparated TALs were studied. Following negative selection, the mean yield of purified T cells was 62 ± 25 × 10⁶ and that of purified NK cells was 45 ± 22 × 10⁶, with viability exceeding 95%. Using flow cytometry, the purity of TAL-T and TAL-NK preparations was 84 ± 5% and 77 ± 10, respectively, as shown in Table 2. Unseparated TALs contained mainly T cells and NK cells and a low percentage of monocytic cells (Table 2). All cell preparations contained <2% granulocytes. PBLs obtained from the patients were not separated into T or NK cells, since only a limited number of these cells was available.

Expression of Receptor-associated ζ, ε, or FcεRI-γ Chains in TALs. To examine the level of expression of signal-transducing subunits of TcR/CD3 or FcγRIIIA in TALs relative to that in normal PBLs, Western blots were performed. Using antibodies specific for the CD3-ζ, CD3-ε, or FcεRI-γ chains known to be associated with TcR or FcγRIIIA, cellular lysates of TALs were analyzed and compared to lysates of normal PBLs included as controls in every Western blot. As shown in Fig. 1, the CD3-ζ in PBL, TAL, TAL-T, or TAL-NK cells from patients with ovarian carcinoma was at a level undetectable using Western blotting, while it was usually strongly expressed in PBL, PBL-T, or PBL-NK cells obtained from various normal individuals. In contrast to the ζ chain, expression of the CD3-ε chain was detectable using Western blotting in all ovarian TALs tested. However, in several patients, its expression was reduced in comparison to that in normal PBL-T cells (Fig. 1). The presence of the CD3-ε chain in NK cells, which do not express surface CD3, is explainable by the fact that intracytoplasmic CD3 reported to exist in IL-2-activated NK cells (25, 26) and in precursors of NK cells in fetal liver (27) is also expressed in mature resting NK cells.⁴ As shown in Fig. 1C, expression of intracytoplasmic CD3-ε was variable in normal human NK cells.

We next evaluated the same cellular lysates used for Western blots in an ELISA established to measure levels of the CD3-ζ and CD3-ε chains. In each ELISA, lysates of different doses of PBL-T from healthy donors (0.6–15 × 10⁶ cells) were used to construct a standard curve for expression of CD3-ε or CD3-ζ. After subtraction of background values obtained with EBV-transformed lymphoblastoid cell lines, the expression of CD3-ε or CD3-ζ was calculated relative to the normal controls. As shown in Table 3, when an anti-CD3 mAb was used as a capture reagent, both the CD3-ζ and CD3-ε chain expressions were found to be decreased in TAL-T relative to normal PBL-T. In contrast to the results obtained using Western blotting, in an ELISA, the ζ chain was found to be expressed at the lower level than normal level rather than absent. It appears, that while

⁴ Unpublished observations.

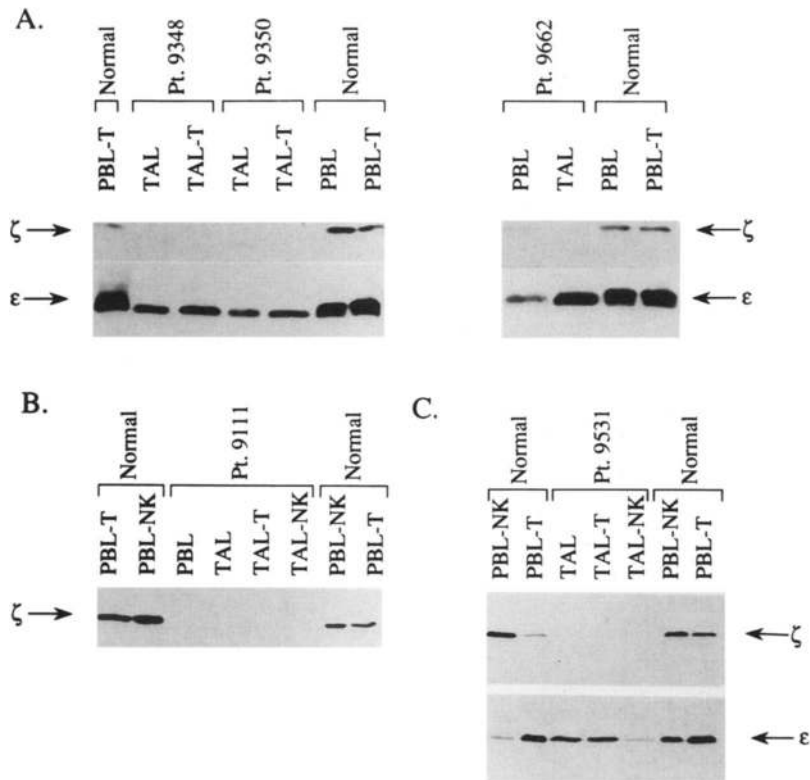


Fig. 1 Expression of CD3- ζ and CD3- ϵ in TAL, TAL-T, or TAL-NK cells isolated from ovarian ascites as well as PBLs obtained from patients with ovarian carcinoma as compared to PBL, PBL-T, or PBL-NK cells obtained from normal donors. Lysates of 2.5×10^6 cells/lane were electrophoresed in 12% SDS gels. The separated proteins were electrophoretically transferred to a nitrocellulose membrane and blotted with anti-CD3- ζ or anti-CD3- ϵ (both at 1:1000 dilution). Experiments performed with cells obtained from five of seven patients studied are shown.

Table 3 Expression of CD3- ζ and CD3- ϵ on TALs obtained from ascites of patients with ovarian carcinoma and assessed by a CD3- ϵ -capture ELISA^a

	CD3- ζ	CD3- ϵ
TAL (9348)	87 ^b	63
TAL (9350)	44	27
TAL (3880)	49	79
PBL (3880)	39	74
TAL (9111)	28	30
PBL (9111)	35	50

^a ELISA was performed as described in "Materials and Methods." All tests were performed in triplicate, and the mean absorbance at 405 nm was calculated. Lysates of EBV-transformed lymphoblastoid cell lines were used as negative controls and usually gave similar values to those obtained with medium controls. In each experiment, standard curves were generated for the presence of CD3- ϵ and CD3- ζ in lysates obtained from a predetermined number of normal human PBL-T cells. After subtraction of the background values obtained with lysates of EBV-transformed lymphoblastoid cell lines, the experimental values were calculated based on the standard curves generated in the same assay and the percentage of positive T cells in the sample as assessed by flow cytometry.

^b The data are percentages of control PBL-T cells used to generate the standard curve.

ELISA can discriminate between low-range levels of expression of the ζ chain, a certain minimal level of ζ expression is required to allow positive detection by Western blotting. The results of ELISA confirmed that expression of both the CD3- ζ and CD3- ϵ chains associated with the TcR/CD3 complex was decreased in TALs or patients' PBLs relative to that in normal PBLs. Al-

though previous studies did not report reduced expression of CD3- ϵ in TILs (17, 18), our observations of decreased expression of CD3- ϵ in TAL-T, as assessed by ELISA, have been recently corroborated using flow cytometry analysis of permeabilized colorectal carcinoma infiltrating T cells (28). It is likely that alterations in the expression of CD3- ϵ could only be detected using quantitative assays such as ELISA or measurements of fluorescence intensity, whereas Western blotting was not sufficiently quantitative. The ELISA results also exclude the possibility that in contrast to normal peripheral lymphocytes, T cells at the tumor site express altered ζ chain, which has lost the epitopes recognized by a specific antiserum. This possibility seems somewhat unlikely, since we have used two sources of antisera specific for distinct ζ epitopes and observed its reduced expression in TALs, with each reagent, utilizing either ELISA or Western blotting.

The γ subunit of FcR is an essential component of the high-affinity receptor for IgE (Fc ϵ RI) and the low-affinity receptor for IgG (Fc γ RIIA), and it has been demonstrated to associate with the TcR complex (29). The Fc ϵ RI- γ chain has been shown to substitute for CD3- ζ in gut intraepithelial lymphocytes of mice lacking the CD3- ζ gene (30), in T cells obtained from spleens of tumor-bearing mice (15), and in TIL from patients with colorectal carcinoma (18, 28). It was, therefore, expected that due to a decrease or loss in the ζ chains observed in TALs, enrichment in the expression of the Fc ϵ RI- γ chains might be detected in these lymphocytes. However, as shown in Fig. 2, expression of the Fc ϵ RI- γ chains was decreased in most purified TAL-T and TAL-NK cells, as measured using

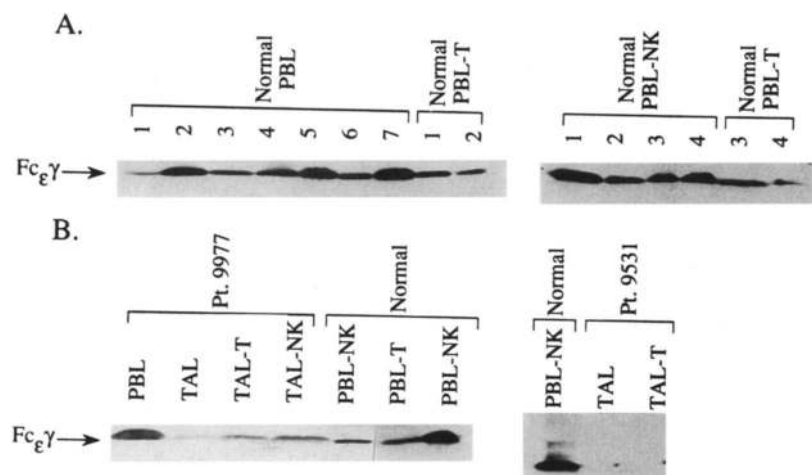


Fig. 2 Expression of FcεRI-γ in TAL, TAL-T, or TAL-NK cells isolated from ovarian ascites as compared to purified T and NK cells obtained from peripheral blood of normal donors. Lysates of 2.5×10^6 cells/lane were electrophoresed in 12% SDS gels. The separated proteins were electrophoretically transferred to a nitrocellulose membrane and blotted with anti-FcεRI-γ at 1:5000 dilution. A, results obtained with normal PBL, PBL-NK, and PBL-T cells in two different experiments. B, results with TALs from two of seven ovarian ascites studied.

Table 4 Expression of CD3 on TAL-T and CD16 on TAL-NK cells relative to that on PBLs obtained from normal donors^a

Cell/source	CD16 ⁺	CD3 ⁺
PBLs of normal donors	385 ± 55 (n = 23)	349 ± 13 (n = 9)
TALs from ovarian carcinoma ascites	58 ± 1 ^b (n = 7)	277 ± 18 ^c (n = 7)

^a Data are MFI ± SD.
^b Significantly decreased at $P < 0.0001$ (Mann-Whitney u test) relative to purified NK cells from PBLs of normal donors.
^c Significantly decreased at $P < 0.03$ (Mann-Whitney u -test) relative to purified T cells from PBLs of normal donors.

Western blotting, relative to that in normal PBL-T or PBL-NK cells, respectively.

Since the CD3-ζ and FcεRI-γ have been shown to be important signaling proteins and to play a role in the assembly of the respective surface receptors, expression of CD3 and CD16 (FcγRIIIA) proteins on the surface of TALs was next evaluated using flow cytometry. As shown in Table 4, MFI of CD16 was significantly reduced in TALs compared to that of normal PBLs ($P < 0.0001$). The MFI of CD3 was also reduced in TALs, and although significant at $P < 0.03$, this reduction in CD3 expression was not as striking as that in CD16. Fig. 3 illustrates the shifts in MFI of CD16 and CD3 observed in representative preparations of TAL-T or TAL-NK cells compared to T or NK cells purified from PBLs of normal volunteers.

Basal and Stimulated Tyrosine Phosphorylation Patterns in TALs. The reduced expression of receptor-associated proteins such as CD3-ζ, CD3-ε, or FcεRI-γ, which are critical for cellular signaling, might be expected to result in impaired signal transduction via either TcR/CD3 on T cells or FcγRIIIA on NK cells. We, therefore, examined the basal and receptor-stimulated patterns of phosphotyrosyl proteins in TAL-T and TAL-NK cells using Western blotting with antiphosphotyrosine. The basal level of proteins phosphorylated on tyrosine in freshly isolated TALs, TAL-T, or TAL-NK was reduced and altered relative to that of T or NK cells from normal PBLs (Fig. 4). Furthermore, following *in vitro* stimulation of the cells with

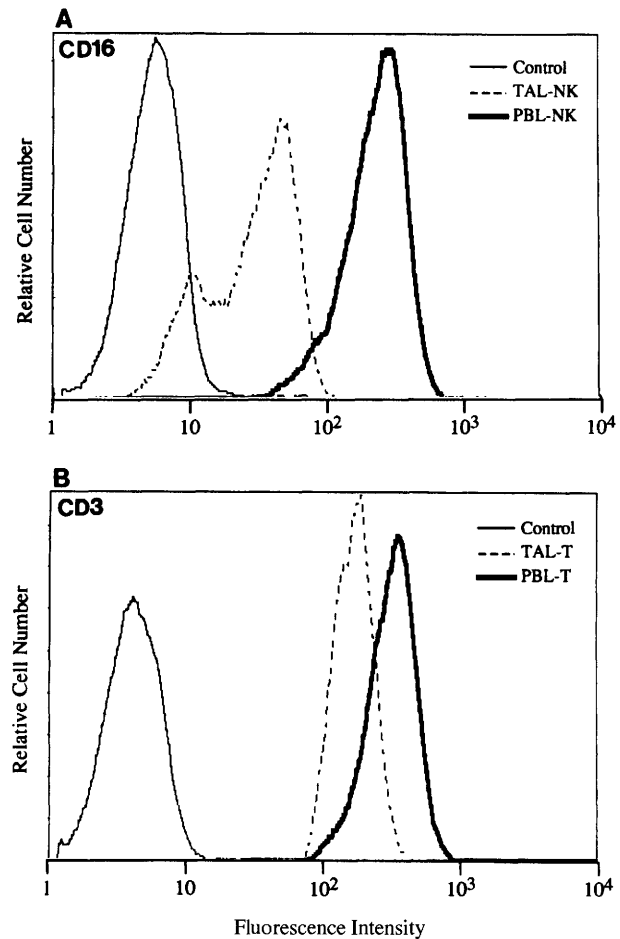


Fig. 3 Expression of CD16 (A) or CD3 (B) on the surface of TAL-NK or TAL-T cells, respectively, as compared to that on NK or T cells purified from PBLs of normal donors. Control cells were stained with FITC-conjugated IgG1 and PE-IgG_{2a}. The results shown are representative of the TAL-T and TAL-NK cell preparations tested and listed in Table 4.

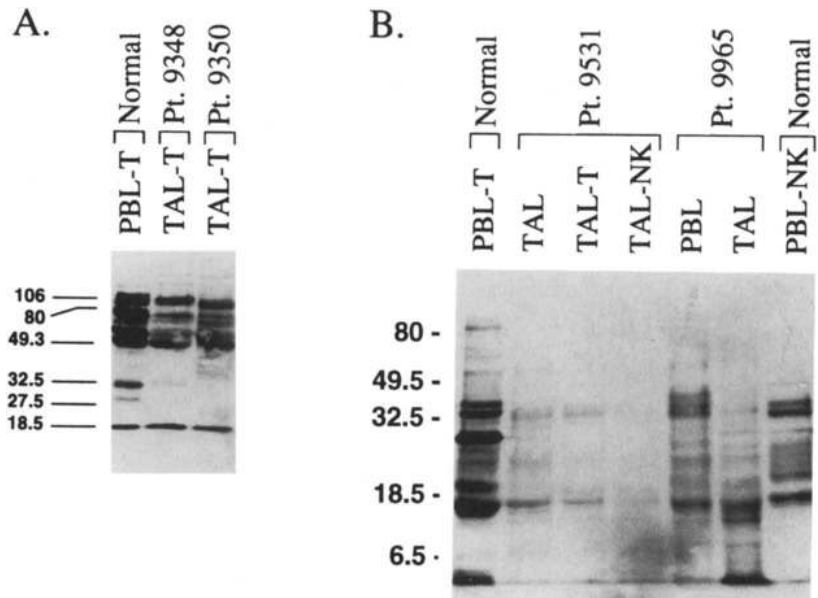
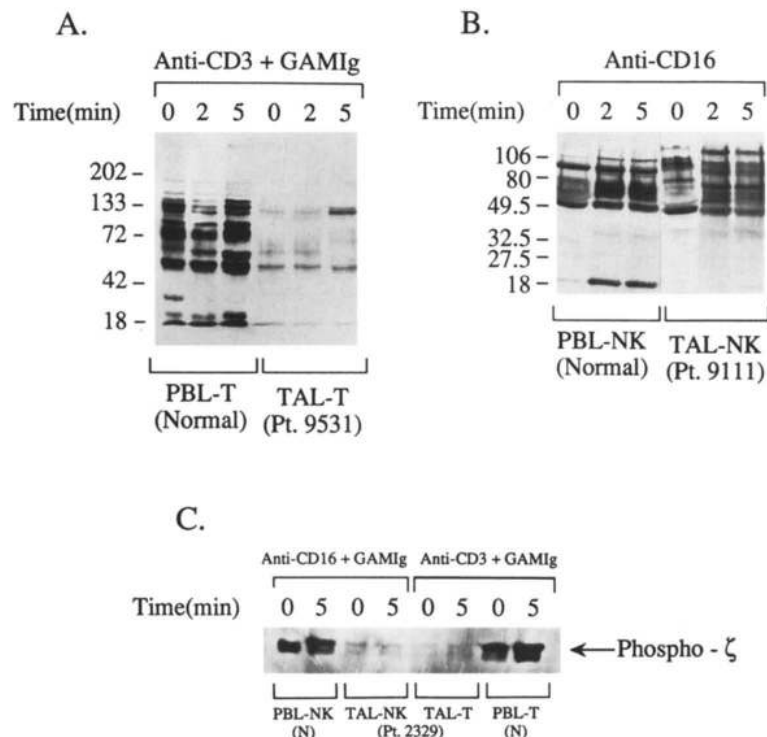


Fig. 4 Basal patterns of phosphotyrosyl proteins in TAL-T (A) or TAL-NK or PBL (B) cells from patients with ovarian carcinoma as compared to PBL-T or PBL-NK cells from normal donors. Lysates of 2.5×10^6 cells/lane were electrophoresed in 7.5% SDS gels and blotted with antiphosphotyrosine 4G10 mAb (1:5000). Representative experiments of seven performed with TAL cells, five with TAL-T cells, and three with TAL-NK cells are shown.

Fig. 5 Stimulation of protein phosphorylation on tyrosine by cross-linking of CD3 on TAL-T (A and C) and Fc γ RIIIA on TAL-NK (B and C) cells as compared to T and NK cells purified from normal PBLs. Purified T cells at 2.5×10^6 cells/ml/sample were incubated with anti-CD3 mAb (10 μ g/ml) for 30 min at 4°C. Cells were then washed twice with cold medium and resuspended in prewarmed solution containing 20 μ g/ml F(ab')₂ of GAMIg for 2 or 5 min at 37°C. NK cells at 2.5×10^6 cells/ml/sample were incubated with anti-CD16 mAb (10 μ g/ml) for 2 or 5 min at 37°C. Lysates of each sample were electrophoresed in 7.5% SDS gels, and proteins phosphorylated on tyrosine residues were detected by immunoblot analysis using antiphosphotyrosine mAbs and ECL detection reagents. Representative experiments of five performed with TAL-T cells and three with TAL-NK cells are shown. In C, lysates of CD16-stimulated TAL-NK cells and CD3-stimulated TAL-T cells as well as T and NK cells from peripheral blood of normal donor were pre-cleared with rabbit antihuman immunoglobulin and protein A-Sepharose beads and then subjected to immunoprecipitation by rabbit anti- ζ (OncoTherapeutics) and protein A-Sepharose beads. The immunoprecipitates were boiled in Laemmli buffer for 5 min, electrophoresed in 12% SDS gels, blotted with antiphosphotyrosine 4G10 mAb and detected with ECL reagent. The high level of baseline phosphorylation of ζ in PBL-NK and PBL-T cells resulted from extended exposure time done to enable detection of the low level of expression of phospho- ζ in TAL-NK and TAL-T cells.



anti-CD3 mAb or anti-CD16 mAb (for T or NK cells, respectively) and cross-linking with GAMIg for 2 or 5 min at 37°C, significantly altered patterns of phosphorylation on tyrosine were observed in TAL-T or TAL-NK cells than in control normal peripheral T or NK cells (Fig. 5A and B). Notably, reduced or undetectable tyrosine phosphorylation of a M_r

18,000–21,000 protein, corresponding to the size of CD3- ζ under reducing conditions, was observed both in TAL-T and TAL-NK cells. To further examine the generation of phospho- ζ in TAL-T or TAL-NK cells stimulated by anti-CD3 or anti-CD16, respectively, the lysates of these cells as those of stimulated control PBL-T or PBL-NK cells were subjected to ζ

immunoprecipitation followed by blotting with antiphosphotyrosine. Although enhanced expression of phospho- ζ was clearly detected in PBL-T and PBL-NK cells from normal donors, very low expression was detected in TAL-T and TAL-NK cells purified from ovarian ascites (Fig. 5C). The absence of up-regulation in phospho- ζ following CD3 or CD16 stimulation is likely to be directly linked to the reduced expression of this receptor subunit in ovarian carcinoma-associated T and NK cells. It appears that although similar levels of total cellular proteins, as assessed by Bio-Rad DC protein assay, were loaded, phospho- ζ is hardly detected since the ζ protein level is diminished.

Expression and/or Activity of Tyrosine Kinase p56^{lck} or PLC- γ 1 in TALs. Neither the TcR nor Fc γ RIIIA subunits have intrinsic PTK functions. However, following antigen recognition or activation by specific high-affinity Ab, they induce PTK activity and are themselves phosphorylated by cellular PTKs. To determine whether the observed reduced basal or stimulated tyrosine phosphorylation was associated with reduced expression or activity of PTKs, we chose to examine levels of p56^{lck} in TALs using Western blotting and its activity in a PTK assay. As shown in Fig. 6, most preparations of TAL, TAL-T, or TAL-NK cells had reduced expression of p56^{lck} in comparison to normal PBL or normal purified T or NK cells. Reduced basal level expression of p56^{lck} in TALs was observed in five of the seven patients tested. These results were further confirmed by *in vitro* immune complex kinase assays performed to determine tyrosine kinase activity of p56^{lck} stimulated in freshly purified TAL-T or normal PBL-T by cross-linking of surface CD3 and in TAL-NK or PBL-NK cells by cross-linking of CD16. Tyrosine kinase activity of p56^{lck} was substantially lower in T and NK cells from ovarian ascites as compared to T or NK cells from peripheral blood of normal donors (Fig. 7). The reduced activity of p56^{lck} in TALs may mainly reflect its reduced expression as indicated by Western blotting. These data indicated that the expression and consequently the activity of one of the common signal-transducing molecules, p56^{lck}, functionally associated with the TcR/CD3 complex in T cells, Fc γ RIIIA in NK cells, and IL-2R β in both cells is altered in TALs. However, this reduced expression appears to be selective since another enzyme implicated in TcR or Fc γ RIIIA such as PLC- γ 1 (Fig. 8) demonstrated a normal level of expression in most TAL preparations tested.

Functional Responses of TAL-T or TAL-NK Cells. To be able to correlate alterations in signaling molecules observed in TALs with *in vitro* functional response of these cells, both proliferation and cytokine production assays were performed. Purified T or NK cells from patients' PBLs or TALs were compared with normal PBL-T for the ability to proliferate in response to IL-2 or to anti-CD3 and anti-CD3 + IL-2 (for T cells only). As shown in Fig. 9, both T and NK cells purified from TALs were significantly depressed in their respective proliferative responses relative to normal T or NK cells tested in the same assays. Similarly, when production of cytokines by these TAL-derived populations was examined, the ability to produce IFN- γ after stimulation with anti-CD3 or anti-CD2 mAbs was found to be depressed in three of four TAL-T populations (Fig. 10A). On the other hand, TALs activated *in vitro* with anti-CD3 antibody produced IL-10 at the levels

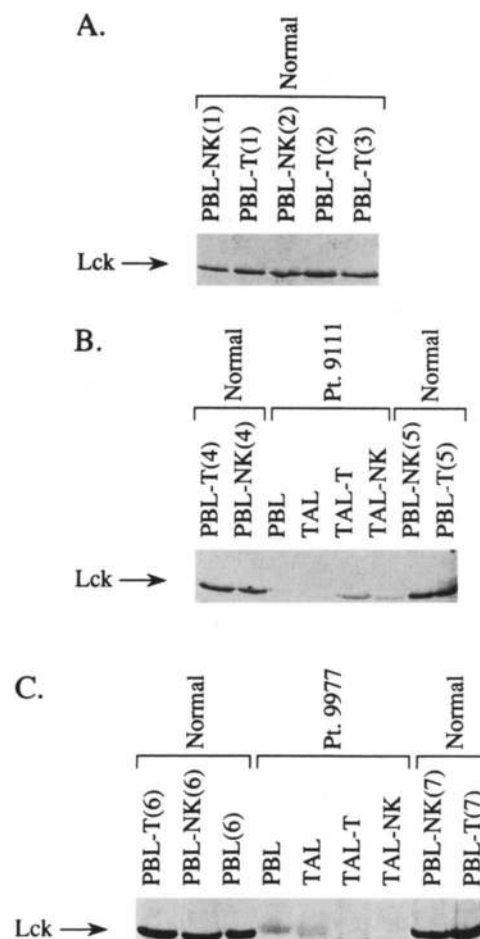


Fig. 6 Expression of p56^{lck} in TAL, TAL-T, or TAL-NK cells as compared to T and NK cells purified from peripheral blood of normal donors. Lysates of 2.5×10^6 cells/lane were electrophoresed in 10% SDS gels. The separated proteins were electrophoretically transferred to nitrocellulose membrane and blotted with anti-p56^{lck} at 1:1000 dilution. A, results obtained with normal PBL-T and normal PBL-NK. B and C, results obtained with PBL and TAL cells obtained from two of seven ovarian ascites studied.

comparable to those produced by normal PBL-T cells under the same experimental conditions (Fig. 10B).

These results indicate that signal transduction alterations observed in patients' T and NK cells purified from TALs translate into specific functional defects in these cells. These defects appear to be partial rather than complete, and they clearly affect proliferative responses and the ability to produce some cytokines.

DISCUSSION

Lymphoid cells freshly isolated from a variety of human solid tumors have been extensively characterized for functional responses in the last several years (3–6, 31, 32). Combined experience from many laboratories clearly indicates that lymphoid cells derived from the tumor microenvironment are immunosuppressed, *i.e.*, they show a variable but significant de-

Fig. 7 *In vitro* PTK assays for p56^{lck} in ovarian carcinoma patient's PBLs and ascites TAL or TAL-T cells stimulated by cross-linking of surface CD3 or ascites TAL-NK cells stimulated by cross-linking of surface CD16. PBL, PBL-T, or PBL-NK cells from normal donors serve as controls. T or NK cells at 5×10^6 /sample were treated with anti-CD3 or anti-CD16 mAbs (10 μ g/ml), respectively, for 30 min on ice. Cells were washed twice with cold medium and incubated with 20 μ g/ml F(ab')₂ fragments of GAMIg as secondary cross-linking Ab at 37°C for 5 min. Following lysis, Lck was immunoprecipitated by rabbit antihuman Lck (N-terminal) Ab and protein A-Sepharose beads. The kinase reaction was carried out in the presence of 1 μ M ATP and 10 μ Ci [γ -³²P]ATP. Reaction products were resolved by electrophoresis, and dried gels were subjected to autoradiography and ³²P quantitation by PhosphorImager.

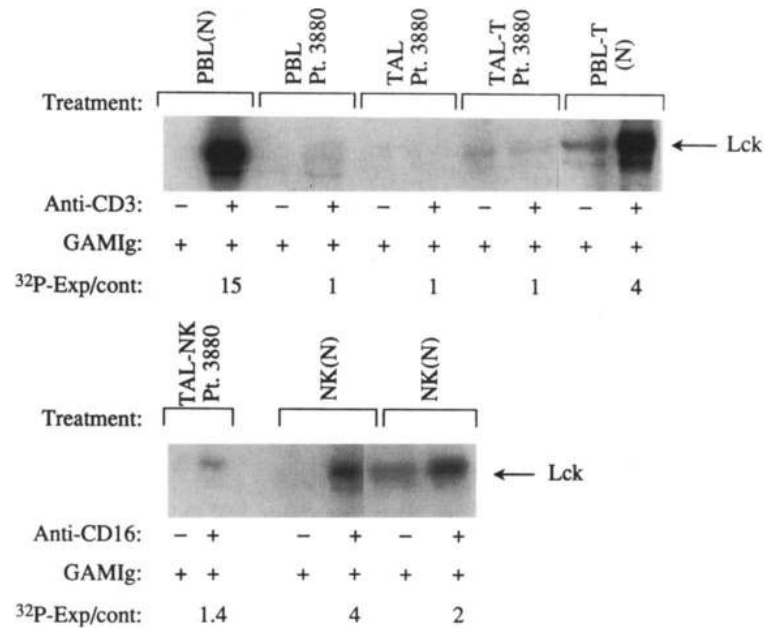
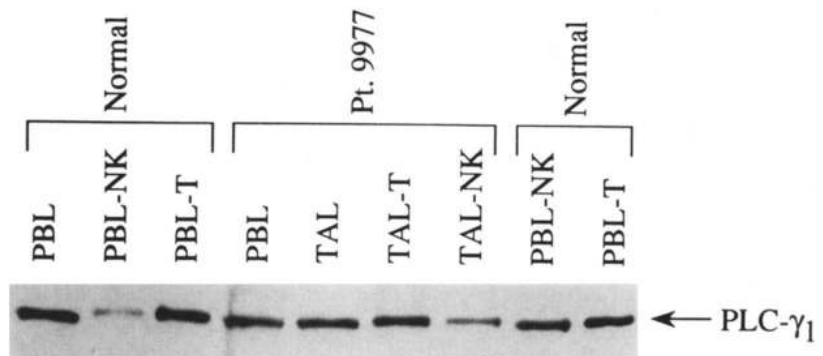


Fig. 8 Expression of PLC- γ 1 in TAL, TAL-T, or TAL-NK cells as compared to that of T and NK cells purified from peripheral blood of normal donors. Lysates of 2.5×10^6 cells/lane were electrophoresed in 7.5% SDS gels. The separated proteins were electrophoretically transferred to a nitrocellulose membrane and blotted with anti-PLC- γ 1 antibodies at 1:1000 dilution. Representative results obtained with PBLs and TALs from one patient are shown.



crease in proliferative responses at the population as well as at the single-cell level (3, 31) in cytolytic function (5), in migration (33), and in the ability to produce certain cytokines (34, 35). The reason for functional impairments observed in TILs remains unknown, but the possibility that the tumor contributes to it by down-modulating responses of lymphoid cells has been considered and may be one of the plausible explanations for these impairments (13, 14, 32).

Several recent studies performed in murine models of tumor growth have indicated that T cells obtained from tumor-bearing animals had alterations in the expression of signal-transducing molecules and were defective in cytolytic function, had decreased expression of tumor necrosis factor α and granzyme B genes and were unable to mediate antitumor effects *in vivo* after their adoptive transfer to syngeneic mice with established hepatic metastases (15, 16). More recently, decreased expression of the NF- κ B/Rel family of transcription factors was reported in T cells obtained from mice bearing Renca, a murine

renal cell carcinoma (36). The results of these murine studies have provided a possible explanation for immunological defects observed in T cells obtained from tumor-bearing hosts and led to a series of molecular studies with human TILs and PBLs from patients with cancer. Thus, Finke *et al.* (17) confirmed the presence of major alterations in the expression of the ζ chain and p56^{lck} protein in TILs of patients with RCC. The group at Karolinska Institute reported that TILs obtained from patients with colon carcinoma showed reduced expression of the ζ chain in both CD3⁺ T cells and CD16⁺ NK cells, as determined by flow cytometry (18).

Based on these initial studies with murine T cells from tumor-bearing animals or human TILs or PBLs from patients with RCC and colon carcinoma, it appeared that alterations in signal transduction might be indeed responsible for poorer immunological responses of TILs relative to the patients' PBLs or normal PBLs (15–18). It was not clear from these studies, however, to what extent different subsets of immune effector

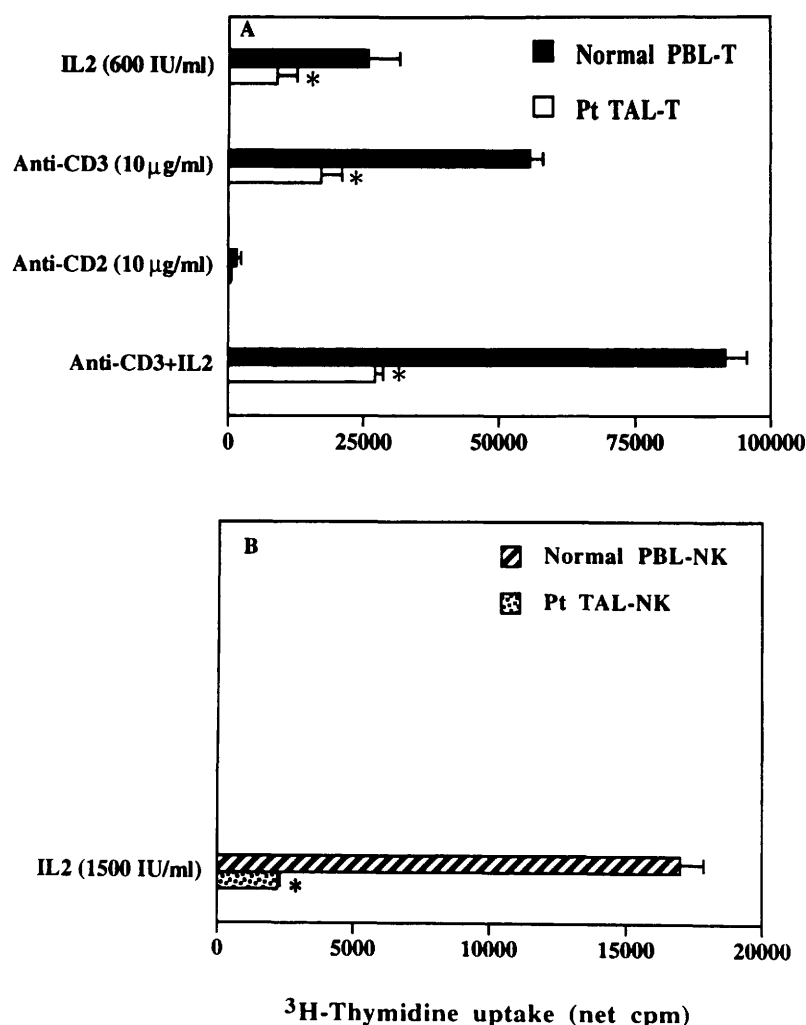


Fig. 9 Proliferative response of ovarian ascites TAL-T (A) and TAL-NK (B) as compared to T or NK cells purified from normal PBLs. [³H]thymidine uptake was determined after a 3-day culture in the presence of IL-2 and/or anti-CD3 or anti-CD2 mAbs immobilized to the plastic surface at a concentration of 10 μg/ml. Anti-CD2 from a single clone (6F10.3) served as a negative control. Results shown are mean ± SE of cells obtained from four patients and four normal donors. *, significantly reduced proliferation ($P < 0.001$) of TAL-T or TAL-NK cells as compared to that of normal T or NK cells.

cells were affected by the alterations in signal transduction or whether such alterations could be reliably correlated to functional deficits demonstrable in these cells. To address these issues, we selected to use ascitic fluids from patients with advanced ovarian carcinoma. The ability to recover $>200 \times 10^6$ TALs from such fluids allowed us to purify from TALs both T cells and NK cells in numbers sufficient for concurrent phenotypic, signal transduction, and functional assays. Using highly enriched cells, we demonstrated that a variety of alterations in the signal-transducing molecules were present not only in T but also in NK cells in patients with ovarian carcinoma. Although these alterations were most pronounced in T and NK cells obtained from ascites, they were also detectable in PBLs of these patients. Furthermore, they were also detectable in T cells isolated from the solid tumor microenvironment. In experiments comparing TALs from ovarian ascites to TILs separated from tissue of ovarian carcinoma, we found a similar reduction in expression of ζ and p56^{lck} ovarian carcinoma-infiltrating lymphocytes, as assessed by Western blotting (results not shown).

Since the ζ chain has been shown to play a prominent role in TcR expression and antigen-driven activation, it is reasonable

to assume that deficiency in expression of this protein may be directly related to the cellular dysfunction observed in tumor-bearing hosts. However, several studies (37) have demonstrated that the TcR/CD3 complex can utilize two autonomous signaling modules: through the TcR- ζ chain or through the CD3- γ , δ , and ϵ subunits. Therefore, it might be possible that only selective signaling pathways are impaired or altered as a result of deficiency in ζ . Consistent with this possibility are our findings of a normal level of IL-10 secretion by CD3-stimulated TAL-T. These results indicate that not all effector functions activated via TcR/CD3 are indiscriminately impaired in the tumor microenvironment. Indeed, recent studies have shown that depending on the exact chemical nature of the ligand, the TcR can produce a spectrum of cellular responses, ranging from complete activation to partial activation, anergy, or profound inhibition (38–40). Furthermore, TcR ligand-induced hyporesponsiveness in T cells was associated with alterations in phospho- ζ , which did not bind Zap70 (38, 39). Since ζ -deficient TcR appears to be partly functional but mediates selective cellular inhibition, it is possible to speculate that in the absence of ζ , T or NK cell stimulation may evoke a negative cellular regulatory mechanism.

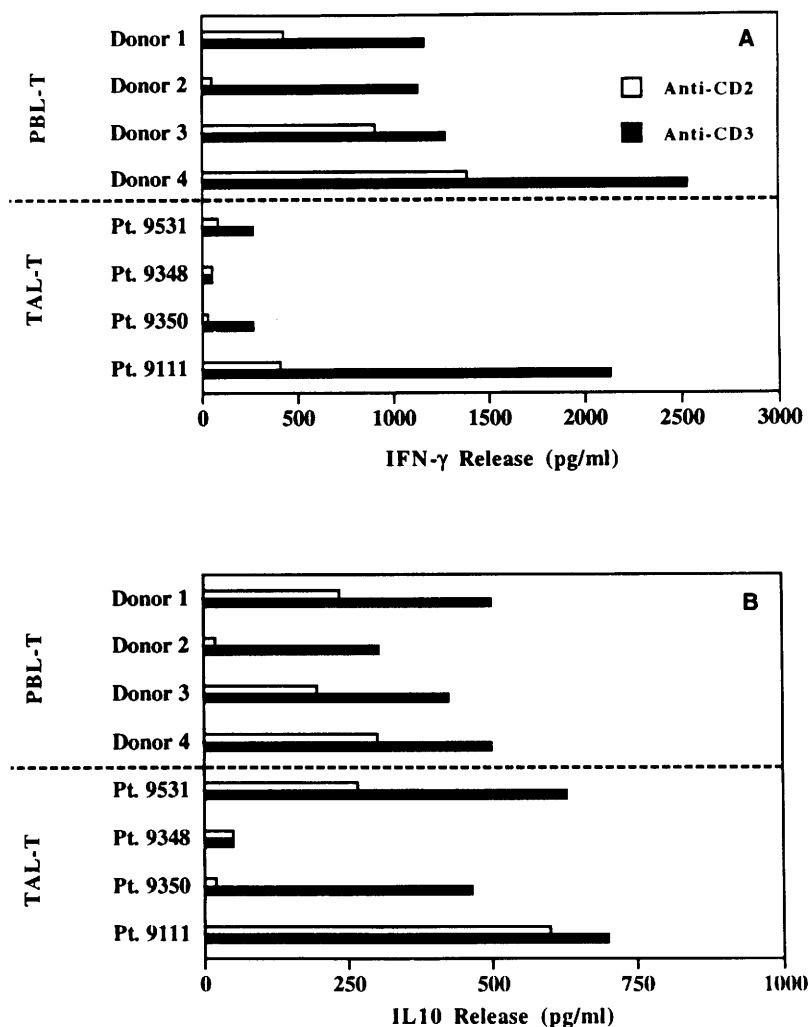


Fig. 10 Production of IFN- γ (A) or IL-10 (B) by TAL-T cells as compared to purified T cells from normal donors in response to CD3 or CD2 triggering. Levels of IFN- γ or IL-10 were determined by commercially available ELISA in supernatants of T cells cultured for 3 days in the presence of anti-CD3 or anti-CD2 mAbs immobilized to the plastic surface at the concentration of 10 μ g/ml.

Both ζ and Fc ϵ RI- γ chains have been demonstrated to play a role in the assembly of the receptor complexes and their translocation to the cellular membrane. Thus, the observed reduced expression of CD16 on NK cells and CD3 on T cells may result from the reduced availability of these proteins. However, it appears that the level of deficiency in ζ and Fc ϵ RI- γ chains is more pronounced than the reduction in expression of CD16 or CD3. It is, therefore, possible that the loss of ζ and Fc ϵ RI- γ chains could take place after assembly of the receptors at the cell surface, resulting in a decreased stability of the receptors and their reduced expression on the cell surface.

Several PTKs including Lck, Fyn, Zap-70, Syk (41), Shc (42), and PI 3-kinase (43) have been implicated in TcR signaling. Lck is not generally directly associated with the TcR but interacts with the coreceptors CD4 and CD8, which colocalize with the TcR during antigen recognition (44). Studies of Lck-deficient cells have indicated that p56^{lck} is essential for TcR signal transduction (45, 46). p56^{lck} is also activated and coimmunoprecipitated with Fc γ RIIIA in NK cells following cross-linking by specific mAb or immune complexes (45, 46). Therefore, the reduced expression and/or activity of p56^{lck} in CD3-stimulated TAL-T or CD16-stimulated TAL-NK, as compared

to their counterparts in normal peripheral blood, may contribute further to the modification in the transduction of signals via a ζ -deficient receptor. In addition to its involvement in TcR activation of T cells and Fc γ RIIIA activation of NK cells, p56^{lck} has been shown to be involved in signal transduction via IL-2R β upon binding of IL-2. Thus, the down-regulation in proliferation of TAL-T or TAL-NK in response to IL-2 might be related to the deficiency in p56^{lck} in these cells. It appears that the impairments in functional activation via distinct signaling pathways triggered via distinct surface receptors such as TcR/CD3 on T cells, Fc γ RIIIA on NK cells, and IL-2R on both cells may relate to deficiencies in commonly recruited and utilized intracellular kinases and substrates.

The recent observations of altered expression of components of the NF- κ B complex in mice-bearing RCC (36) and in TILs from RCC patients (47) lend support to the possibility that multiple signal transduction pathways are impaired in TALs. Any stimulus that results in activation of T cells appears to activate NF- κ B, including treatments with antigen, anti-CD3, anti-CD2, anti-CD28, calcium ionophores, phorbol esters, lectins, and cytokines (48). Therefore, modifications in expression and function of this family of transcription factors suggest that

alterations in signal transduction mechanisms may not be restricted to those induced via the TcR in T cells, or FcR in NK cells, but may more broadly affect signal transduction mechanisms in lymphocytes of tumor-bearing hosts.

The reduced expression and altered function of intracellular signaling proteins may serve as a mechanism of induction of anergy at the tumor site. Although the molecular mechanisms which induce anergy are not clear, some of the molecular changes in anergic T cells which prevent their response to antigen restimulation has been demonstrated to be strikingly similar to those found recently in immunosuppressed TILs. Anergic T cells express constitutively reduced amounts of p56^{lck} (49), have altered phospho- ζ , and lack of Zap-70 recruitment (38, 39). Thus, it appears that T-cell anergy may be sustained, at least in part, by alterations in signaling events. It has been recently shown that *in vitro* activation by phorbol ester phorbol 12-myristate 13-acetate or concanavalin A induces partial proteolysis of the ζ chain in T lymphocytes (50). Furthermore, lymphocyte activation through the TcR or basophil activation through FcεRI has been shown to result in ubiquitination of ζ and FcεRI- γ (51, 52). Once ligated by the polypeptide ubiquitin, proteins will be selectively degraded by specific protease complexes that act on ubiquitinated proteins (53). It is therefore possible that ubiquitin-mediated proteolysis of signaling subunits of multicomponent receptors such as TcR and FcR may serve as a mechanism of desensitization of these receptors as observed in anergic T cells as well as in immunosuppressed tumor-associated T and NK cells. Tumor-mediated chronic activation or as yet unknown mechanisms may induce selective intracellular proteolysis of signaling proteins at the tumor site.

The observations we and others have reported regarding signal transduction defects in TILs or TALs have important clinical implications. Assuming that these defects are tumor induced, there is a good rationale for attempting to reverse this immunosuppression *in vivo* by therapeutic administration of cytokines or other biological response modifiers. Indeed, a variety of therapeutic strategies, including gene therapy with genetically engineered tumor or other autologous cells, are currently available for modification of the tumor microenvironment (54). IL-2 gene therapy on tumor-induced alterations in signal transduction in T cells was recently studied in mice-bearing IL-2-transduced fibrosarcoma (55). Reduced levels of TcR- ζ , p56^{lck}, and p59^{lyn} as well as calcium mobilization in response to CD3 cross-linking observed in spleen T cells obtained from mice bearing the parental tumor were not seen in mice bearing IL-2-secreting tumors. Thus, the presence of IL-2 at the tumor site appeared to prevent the onset of hyporesponsiveness. Unless the tumor microenvironment is modified to make it less immunoinhibitory, adoptive transfer of *in vitro*-activated effector cells is likely to be ineffective. It is possible that the limited therapeutic effects of adoptive immunotherapy with lymphokine-activated killer, TIL, and other effector cells observed thus far might be, at least in part, due to immunosuppressive influences of the tumor microenvironment. Additional studies aimed at unraveling the mechanisms involved in or responsible for immunosuppression in tumor-bearing hosts are both important and necessary.

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