Differential Expression of CD3ζ Message and Protein in Tumor Infiltrating Lymphocytes from Solid Tumor Specimens and Malignant Ascites from Patients with Ovarian Carcinoma

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Abstract. *The expression of the CD3ζ subunit was investigated in fresh (uncultured) tumor-infiltrating lymphocytes (TILs) isolated from either solid tumor (ST) specimens or ascites (ASC) from patients with epithelial ovarian carcinoma (EOC). Western blot analysis of CD3ζ immunoprecipitates using anti-CD3ζ rabbit serum revealed that in 6 out of 6 patients with EOC, the CD3ζ protein was absent from ST-TILs. Immunoprecipitation with anti-phosphotyrosine monoclonal antibody (anti-PY20) from ST-TILs from one patient revealed bands co-migrating with the phosphorylated CD3ζ. CD3ζ protein was found to be expressed in only 1 out of 7 ST-TILs from patients with EOC. ASC-TILs were available in 5 of these patients and immunoprecipitation/Western blotting experiments using anti-CD3ζ rabbit serum revealed that CD3ζ protein was expressed in all 5. In addition, CD3ζ protein was expressed in 3 additional ASC-TIL specimens for which ST-TILs were not available. Therefore, the CD3ζ protein was expressed in ASC-TIL isolated from 8 out of 8 patients with EOC. CD3ζ protein was also expressed on peripheral blood mononuclear cells (PBMCs) from patients with EOC and from normal donors. RT-PCR studies of fresh ST-TIL specimens, using CD3ζ-specific primers, revealed that CD3ζ transcripts were absent from 13 out of 21 patients with EOC, down-regulated in 4 patients and present at levels comparable to those found in PBMCs in 4 other patients. In contrast, CD3δ transcripts were present at comparable levels in all specimens. Treatment with recombinant interleukin-2 (rIL-2) (600 IU/ml) restored the expression of CD3ζ protein and transcripts in cultured ST-TILs, whereas fresh ST-TILs did not express CD3ζ, in contrast to fresh ASC-TILs. These results demonstrate differential expression of CD3ζ in ST-TILs versus ASC-TILs in patients with EOC. CD3ζ transcripts and protein were found to be absent from most ST-TILs from patients with EOC, whereas they were expressed in ASC-TILs and PBMCs from such patients.*

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Most tumors in humans and animals are infiltrated by lymphocytes that have been designated tumor-infiltrating lymphocytes (TILs). TILs may represent an immune response by the host to the tumor and in certain tumors, their presence in increased numbers has been associated with improved prognosis and increased survival (reviewed in 1). TILs are comprised primarily of CD3+ T lymphocytes (1). We (2-8) and others (9-13) have shown that T-cell lines exhibiting cytolytic activity and/or cytokine production, primarily restricted to autologous tumor cells, can be developed *in vitro* using recombinant interleukin 2 (rIL-2) added to TILs from patients with malignant melanoma, ovarian carcinoma and other tumors (reviewed in 1). The development of these T-cell lines does not require repeated stimulation by autologous tumor cells, suggesting that TILs contain T-cells that have already been activated and have proliferated *in vivo* in response to particular antigen(s). Cytolytic activity of these T-cell lines and clones can be blocked at the effector cell level by monoclonal antibodies (mabs) to the CD3 antigen and to the T-cell antigen receptor (TCR) and at the target cell level by mabs to HLA class I (2-8). These TIL-derived T-cell lines can be expanded *in vitro* by a few thousand-fold (11-16) and have been used for adoptive immunotherapy trials in patients with melanoma (reviewed in 11-13) and ovarian carcinoma (15, 16).

The first step of an immune response involves recognition by the TCR of an antigenic epitope, which in the case of the vast majority of αβ TCR$^+$ T-cells is a peptide:MHC complex. A signal for T-cell activation is initiated by the α- and β-chain TCR, which are recognition (ligand- binding) subunits and it is transduced through the CD3 proteins (gamma (γ), delta (δ), epsilon (ε) and zeta (ζ)) to structures inside the cell (17, 18). One of the earliest events in T-cell activation resulting from the engagement of the TCR with antigen is the phosphorylation of the CD3ζ protein, that leads to the activation of ZAP-70 protein tyrosine kinase (19), which in turn activates phospholipase C γ1. An increase in intracellular Ca^{++} and activation of protein kinase C follows. These internal signals lead to the proliferation and activation of the responding T-cell(s) and production of several cytokines, including interleukin-2 (IL-2).

Several studies have shown that TILs exhibit impaired immune responses *in vitro* to polyclonal activators (reviewed in 1). This impairment has been attributed to the down-regulation of the CD3ζ protein at either the protein or the transcript level. CD3ζ expression is absent or down-regulated in TILs from a large number of tumor types including MCA-38 murine colon carcinoma transplantable tumor (20), CMS-5 murine fibrosarcoma (21), renal cell carcinoma (22), colorectal carcinoma (23), Hodgkin's disease (24), malignant melanoma (25), nasopharyngeal carcinoma (26) and breast cancer (27). Several laboratories have investigated the down-regulation of CD3ζ in T-cells from malignant ascites (ASC) and T-cells from solid tumor (ST) specimens from patients with epithelial ovarian carcinoma (EOC), with conflicting results (28-31). Down-regulation of CD3ζ in T-cells from ASC from patients with EOC has been reported by Lai *et al*. (28). In contrast, Lockhart *et al*. (31) reported that ASC T-cells from patients with EOC express CD3ζ, which is absent from ST specimens from patients with EOC.

We report here the differential expression of CD3ζ transcript and protein in TILs from ST specimens and malignant ASC from patients with EOC.

Patients and Methods

Patients. ST specimens from 22 patients with EOC who had undergone surgery at the hospitals of the Temple University Health System were used in this study. The patients at the time of surgery were aged in the range of 45 to 81 years and were untreated previous to surgery (Table I). ASC specimens were provided from 8 patients with EOC and were collected under sterile conditions with heparin added to 1 unit/ml of ASC. Peripheral blood was provided from some of these patients and from normal donors and was collected in heparinized collection tubes (Becton-Dickinson, Franklin Lakes, NJ, USA). These studies have been approved by the Institutional Review Board (IRB) of Temple University Hospital.

Preparation of single-cell suspensions.

ST specimens from patients with EOC: ST specimens, devoid of fatty tissue, normal and necrotic tissue from patients with EOC, were divided into portions and were either minced to ~1 mm in 10 ml of AIM V medium (Gibco-BRL, Grand Island, NY, USA) in a sterile petri-dish (Fisher Scientific, Pittsburgh, PA, USA) and used immediately for the preparation of single cell suspensions, or they were frozen in Tissue-tek® optimal cutting temperature (OCT) compound (Miles Inc., Elkhart, IN, USA) and used at a later time for the preparation of RNA. Single-cell suspensions were prepared as previously described (14) by digestion with 3% collagenase Type I (Sigma, St. Louis, MO, USA) in AIM V medium and 0.02% DNAse I (Sigma) in AIM V, overnight at 37˚C. The cells were washed twice with Hanks Balanced Salt Solution (HBSS; Cellgro, Herndon, VA, USA), layered onto Ficoll-Hypaque (Pharmacia, Piscataway, NJ, USA) density cushion and centrifuged at 650 ×g for 30 min at room temperature. The mononuclear cell layer was washed twice with HBSS and resuspended in AIM V. Cells were counted and viability was determined with trypan blue dye. The mononuclear cells were separated into aliquots, with one aliquot resuspended in AIM V with 10% fetal calf serum (FCS), (Gibco-BRL) and gentamycin (100 mg/ml), and the other aliquot cryopreserved in liquid nitrogen in 95% FCS and 5% dimethyl sulfoxide (DMSO). These cells have been designated herein after as solid tumor-derived (ST)-TIL.

Ascites from patients with EOC: Ascitic fluid from patients with EOC was centrifuged at 800 ×g for 10 minutes and the single cell suspension was washed twice with HBSS and resuspended in HBSS to a density of 1-5×10^6 cells/ml. The ASC-derived cells were then

Table I. *Ovarian carcinoma patient profile*.

Patient	Age* (Years)	Stage	Grade$^+$	Carcinoma type$^+$
OV1	▽	IIIb	Good 1	Papillary adenocarcinoma
OV2	70	IIIc	Poor 3	Papillary adenocarcinoma
OV3	46	IV	Mod 2	Adenocarcinoma
OV4	▽	IV	Poor 3	Papillary serous carcinoma
OV5	55	IIIb	Mod 2	Mucinous adenocarcinoma
OV6	45	IIIc	Mod 2	Mucinous adenocarcinoma
OV9	51	IIIb	Poor 3	Papillary /clear cell carcinoma
OV16	80	IIIc	Mod 2	Papillary serous carcinoma
OV22	61	IIIc	Mod 2	Papillary serous carcinoma
OV23	75	IIIc	Mod 2	▽
OV24	72	IIIc	Poor 3	Papillary serous carcinoma
OV25	61	Ic	Mod 2	▽
OV26	81	IIIa	Poor 3	Papillary serous carcinoma
OV27	61	Ia	Poor 3	Papillary serous carcinoma
OV28	60	IIIc	Poor 3	Endometrial sinus tumor
OV29	▽	IIIc	Good 1	Mucinous cystadenocarcinoma
OV31	72	IIIc	Mod 2	Papillary serous carcinoma
OV32	67	Ia	Well 1	Low malignant, potential tumor
OV33	53	IIIc	Poor 3	Papillary serous carcinoma
OV35	73	IIIc	Poor 3	Adenocarcinoma
OV36	75	IIIc	Mod 2	Papillary serous carcinoma
OV37	77	IIIc	Poor 3	Papillary serous carcinoma
OV38	▽	IIIc	Poor 3	Mucinous adenocarcinoma

*At time of operation; $^+$according to the World Health Organization (WHO) and the International Federation of Gynecology and Obstetrics (FIGO) unified classification of common epithelial tumors; ▽ no information available.

layered onto a Ficoll-Hypaque cushion and centrifuged at 650 ×g for 30 minutes at room temperature. The mononuclear cell layer was washed twice with HBSS and resuspended in AIM V. Cells were counted and viability was measured with trypan blue dye. These cells have been designated herein after as ASC-TILs.

Peripheral blood mononuclear cells (PBMCs): Peripheral blood from normal donors and ovarian carcinoma patients were diluted 2:1 with HBSS and layered onto Ficoll-Hypaque cushion and centrifuged at 650 ×g for 30 minutes at room temperature. The mononuclear cell layer was washed twice with HBSS and resuspended in AIM V. Cells were counted and viability was measured with trypan blue dye. Isolated PBMCs were cryopreserved in liquid nitrogen in 95% FCS and 5% DMSO for use in protein and transcript assays.

rIL-2-derived T-cell lines. ST-TILs, ASC-TILs or PBMCs from patients with EOC were cultured at a density of 1×10^6 cells/ml in AIM V medium supplemented with 10% FCS (Gibco-BRL) and 600 IU/ml rIL-2 (Chiron-Cetus, Emeryville, CA, USA) as previously described (14). ST-TIL-, ASC-TIL- and PBMC-derived T-cell lines and fresh ST-TILs, ASC-TILs and PBMCs were frozen in 10% FCS/10% DMSO/AIM V.

Antibodies. OKT3 (CD3ε) monoclonal antibody (IgG2a) was purchased from Ortho Diagnostics (Raritan, NJ, USA). The isotype control was an IgG2a myeloma protein. PY20 mab (anti-phosphotyrosine mab) was obtained from ICN Biomedicals. An anti-CD3ζ rabbit serum was raised against a glutathione *S*-transferase (GST) fused cytosolic portion of murine CD3ζ protein (amino acids 63 to 164), tested and found to recognize human CD3ζ.

Immunoprecipitation/Western blot analysis. Cells were lysed in 0.5 ml of ice-cold TNE (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% NP-40) buffer, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 µg/ml of aprotinin/leupeptin mixture. The cell suspension was vortexed to achieve a homogeneous suspension and left for 20 min on ice. Supernatant, obtained by 10 min centrifugation at 13,000 ×g, was used for immunoprecipitation. CD3ζ was immunoprecipitated by addition of 1 µl of anti-CD3ζ rabbit serum to 0.5 ml of sample. Preimmune normal rabbit serum (NRS) was used as the negative control. After two hours of incubation at 4˚C, 30 µl of 50% proteinA-agarose (Sigma) slurry or 50 µl of washed Pansorbin (Calbiochem, La Jolla, CA, USA) were added and samples were incubated for 2 hours at 4˚C with constant mixing. Pellets were collected by centrifugation, washed 3 times with TNE buffer, and adsorbed proteins were eluted by 5 min heating at 95˚C in 30 µl of Laemmli sample buffer (32). Eluted material was applied to a 13% polyacrylamide gel. Electrophoresis was performed according to Laemmli in a BioRad Miniprotean gel system. CD3ζ was detected by immunoblotting after transfer to nitrocellulose with the same anti-CD3ζ serum at 1/400 dilution, followed by ^{125}I-labeled Protein A. The Western blot was exposed to autoradiograph film (Fuji) at –70˚C.

RNA isolation. RNA was prepared from solid tumors specimens from patients with EOC using RNAzol B (Tel-Test, Friendswood, TX, USA). In short, 2 ml of RNAzol B per 0.1 g of tissue were used and after homogenization 0.1 v/v of chloroform was added to the homogenate. After agitation and cooling on ice for 15 min, the homogenate was centrifuged at 12,000 ×g for 15 min at 4˚C. The aqueous layer was removed and a second RNAzol B extraction was performed. An equal volume of isopropanol was added to the aqueous layer and after cooling on ice for 15 min, the precipitation mixture was centrifuged at 12,000 ×g at 4˚C for 15 min. The pellet was washed twice with 75% ethanol. RNA from PBMCs and all TIL single-cell suspensions was prepared by the same method. The cells were resuspended in 0.2 ml RNAzol B per 10^6 cells for RNA extraction.

Table II. *Primers used in PCR and hybridization*.

Primer	Primer sequence (5'to 3')
CD3δ-5'	CTGGACCTGGGAAAACGCATC
CD3δ-3'	GTACTGAGCATCATCTCGATC
CD3ζ-5'	ACAGAGCTTTGGCCTGCTGGATC
CD3ζ-3'	TAGGTGTCCTTGGTGGCTGTACT
GAPDH-5'	GACAACAGCCTCAAGATCATCAGC
GAPDH-3'	AAGTCAGAGGAGACCACCTGGTGC

Synthesis of cDNA. Five µg of total RNA were used in a reverse transcription reaction with 0.5 µg oligo dT primer and Superscript II reverse transcriptase (RT) (Gibco-BRL). Briefly, the RNA and primers were heated to 70˚C for 10 min and subsequently put on ice. dNTP, DTT, and RT buffer (preheated to 42˚C) were added to the annealed RNA/primer mix and RT was added. The RT reaction was incubated at 42˚C for 50 min and then heat inactivated at 90˚C. A dilution of the RT was used in PCR reactions. Genomic DNA contamination was checked for by performing PCR on the RNA before reverse transcription using the conditions below.

PCR. A dilution of the cDNA was used for 35 cycles of amplification. After initial denaturation at 94˚C, each cycle consisted of 45 s at 94˚C, 45 s at 57˚C and 60 s at 72˚C. A final extension of 6 minutes at 72˚C was carried out. For the CD3ζ amplification, an annealing temperature of 59˚C was used.

Quantification by MIMIC PCR. The MIMICs (Clontech, Palo Alto, CA, USA) were constructed according to the manufacturer's established protocol, as described elsewhere (33, 34). Briefly, composite primers were constructed that included sequence from a nonhomologous piece of DNA and sequence from the target transcript. After amplification, the MIMIC DNA constructed was quantitated and used as a template for the semi-quantitative analysis of transcripts using the transcript-specific primers. A serial dilution of the MIMIC was combined with cDNA template until a titration was resolved using a 1.2% agarose gel. The PCR primers used are shown in Table II. A Scanjet 4L (Hewlett Packard, Hercules, CA, USA) was used to digitize the agarose gel pictures and invert them. The inverted picture was analyzed using the SigmaGel (Jandel Scientific, San Rafael, CA, USA) program allowing density measurements to be carried out. A standard curve was constructed from measuring the MIMIC dilution densities and the transcript levels from the samples were calculated from the standard curve, as previously described (33, 34).

Results

Tyrosine phosphorylation state of ovarian carcinoma TILs. To examine if there were differences in the phosphorylation of proteins with molecular weight close to that of CD3ζ in TILs from patients with ovarian carcinoma, Western blotting

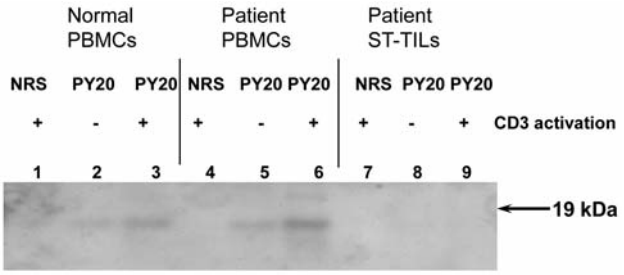

Figure 1. *Tyrosine phosphorylation state of tumor-infiltrating lymphocytes (TILs) from a patient with epithelial ovarian carcinoma (patient OV9). Peripheral blood mononuclear cells (PBMCs) from a normal donor, PBMCs from patient OV9 with EOC and solid tumor (ST)-TILs from the same patient were stimulated with anti-CD3 mab or media alone for 3 minutes. The cells were lysed, analyzed by SDS-PAGE (12.5%) under reducing conditions and probed with an anti-phosphotyrosine mab.*

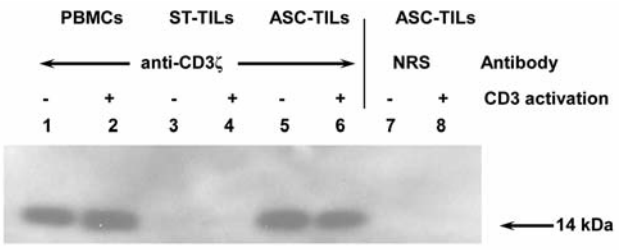

Figure 2. *Level of CD3ζ polypeptide chain in EOC TILs from patient OV16. PBMCs, ascites-derived (ASC)-TILs and solid tumor-derived (ST)-TILs from a patient with ovarian carcinoma were lysed and immunoprecipitated with either normal rabbit serum (NRS) or anti-CD3ζ serum. The lysates were analyzed by SDS-PAGE (12.5%) under reducing conditions and probed with an anti-CD3ζ rabbit serum.*

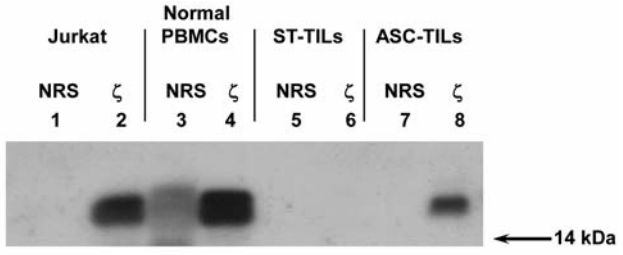

Figure 3. *Level of CD3ζ polypeptide chain in EOC TILs from patient OV16. Normal PBMCs, and PBMCs, ASC-TILs and ST-TILs from a patient with EOC were immunoprecipitated with NRS or anti-CD3ζ serum. The lysates were analyzed by SDS-PAGE (12.5%) under reducing conditions and probed with an anti-CD3ζ rabbit serum.*

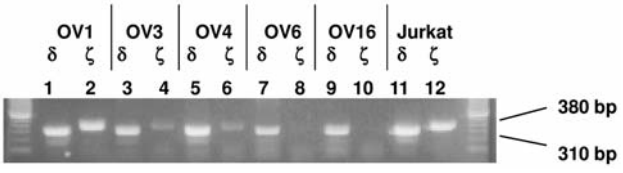

Figure 4. *Levels of CD3δ and CD3ζ transcripts in patients with EOC. Total RNA was isolated from ST-TILs (patients OV1, OV3, OV4, OV6, and OV16) and Jurkat T-cell line and RT was performed using oligo-dT. PCR for CD3δ and CD3ζ was carried out on the first-strand cDNA and run on a 1.2% agarose gel.*

analysis was performed. Representative results are shown in Figure 1. Using an anti-phosphotyrosine mab, the state of tyrosine phosphorylated proteins, before and after stimulation with a CD3 mab, was compared between ST-TILs of a patient with EOC and PBMCs from the same patient. PBMCs from a normal donor were used as a positive control and preimmune serum for immunoprecipitation was used as a negative control. PBMCs from the normal donor and the patient with EOC exhibited a phosphorylated protein at approximately 18 kDa (Figure 1, lanes 2 and 5). After stimulation with the anti-CD3 mab, the 18 kDa band was significantly increased in intensity and a 21 kDa band appeared (lanes 3 and 6). The expected molecular masses of these bands are equal to those of the phosphorylated forms of CD3ζ subunit. In contrast, neither ST-TILs with nor those without anti-CD3 stimulation exhibited the phosphorylated proteins (lanes 8 and 9). These results suggest that in ST-TILs there is either a defect in tyrosine phosphorylation or that the CD3ζ protein is absent. Both may result in impaired T-cell responses to the tumor cells.

The CD3ζ polypeptide is absent from or down-regulated in ST-TILs, but it is expressed on ASC-TILs from patients with EOC. To determine whether the lack of tyrosine phosphorylation proteins at 18 kDa and 21 kDa were because of defective tyrosine phosphorylation in ovarian ST-TILs, or the absence of the CD3ζ protein, immunoprecipitation/Western-blotting experiments were carried out using anti-CD3ζ serum. Lysates from ST-TILs, ASC-TILs, PBMCs from a normal donor or Jurkat cells were immunoprecipitated with either NRS or anti-CD3ζ antibody and analyzed by SDS-PAGE under reducing conditions. The proteins were transferred to nitrocellulose and identified using the same anti-CD3ζ rabbit serum. Representative results are shown in Figures 2 and 3. In Figure 2, samples of single cell suspensions from ST-TILs, ASC-TILs and PBMCs from the same patient with EOC (OV16) were stimulated with either anti-CD3 mab or medium, and then subjected to immunoprecipitation/Western blotting analysis using anti-CD3ζ serum. Both PBMCs (lanes 1 and 2) and ASC-TILs (Lanes 5 and 6) expressed the CD3ζ polypeptide, whereas ST-TIL did not (lanes 3 and 4). Stimulation with anti-CD3 mab had no effect on the expression of the CD3ζ polypeptide.

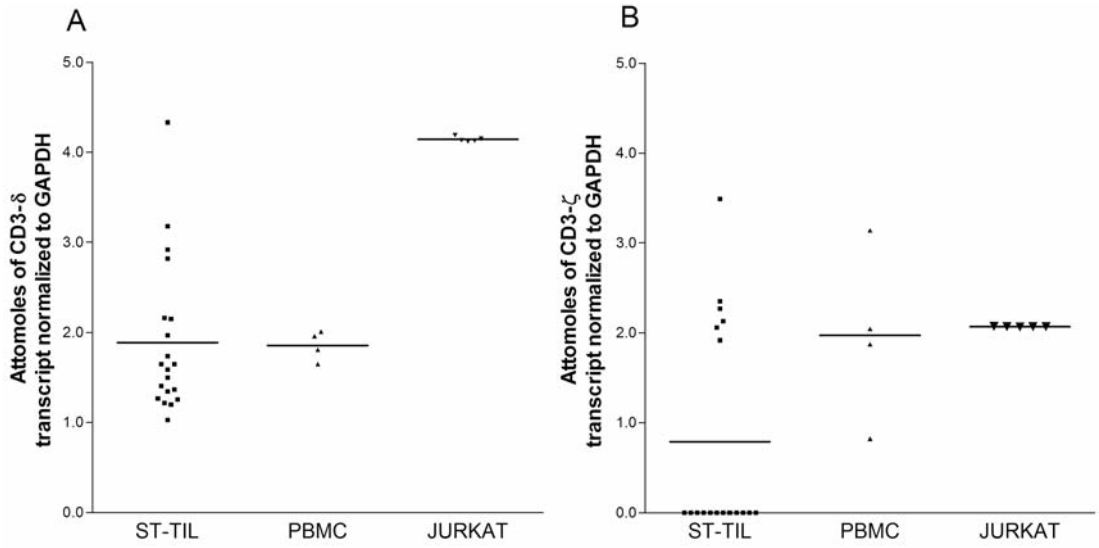

Figure 5. *Semi-quantitative analysis by competitive PCR of CD3δ (A) and CD3ζ (B) transcripts normalized to GAPDH in patients with EOC. Total RNA was isolated from ST-TILs, PBMCs and Jurkat T-cell line and RT was performed using oligo-dT. Semi-quantitative analysis using the MIMIC competitive PCR method for CD3δ, CD3ζ and GAPDH was carried out on the first-strand cDNA. The CD3δ and CD3ζ levels were normalized to GAPDH for each specimen.*

Results from another representative experiment are shown in Figure 3. CD3ζ protein was expressed in ASC-TILs (lane 8), but not in ST-TILs (lane 6) from patient OV22. Normal PBMCs (lane 4) and cells of the Jurkat T-cell line (lane 2) expressed CD3ζ.

ST-TILs from six patients with EOC (OV16, OV24, OV25, OV31, OV37, OV22) were examined for the presence of the CD3ζ protein and it was determined that none of these six patients expressed CD3ζ protein (Table III). Immunoprecipitation with the anti-PY20 mab (anti-phosphotyrosine) from ST-TILs from patient OV9, revealed bands co-migrating with the phosphorylated CD3ζ (data not shown). ST-TILs from patient OV9 exhibited down-regulated levels of CD3ζ transcripts (see below; Table III). Therefore, CD3ζ protein was expressed only in ST-TILs from 1 out of 7 patients examined.

ASC-TILs were available from 5 of these 7 patients and all 5 (OV16, OV24, OV25, OV31, OV37) expressed CD3ζ protein (Table III). The CD3ζ protein was also expressed in ASC-TILs from 3 additional patients (OV23, OV26 and OV29) with EOC (Table III). ST-TILs from these 3 patients were not available. Therefore, the CD3ζ protein was expressed in ASC-TILs from 8 out of 8 patients (OV16, OV24, OV25, OV31, OV37, OV23, OV26 and OV29) examined, and in ST-TILs from only 1 out of 7 patients examined.

Additionally, CD3ζ protein was expressed in 8 out of 8 PBMC specimens from patients with EOC, in 2 out of 2 PBMCs from normal donors and in cells of the Jurkat lymphoblastoid T-cell line. To confirm that the absence of the CD3ζ subunit was not due to the process of digesting the solid tumor specimens to single cell suspension, ASC-TIL were subjected to the same enzymatic treatment. Because ASC-TILs are already in a single cell suspension, their enzymatic treatment may be more rigorous than that of ST-TILs. Nevertheless, immunoprecipitation/Western blotting analysis demonstrated that the expression of CD3ζ polypeptide by TIL was not affected by the digestive conditions (results not shown).

CD3ζ transcripts are absent from or are down-regulated in ovarian carcinoma ST-TILs. The finding that the CD3ζ polypeptide was not expressed in ST-TIL prompted us to investigate whether CD3ζ transcripts were expressed in these specimens. Similar studies were not carried out with ASC-TIL, because immunoprecipitation experiments demonstrated the expression of the CD3ζ protein in 8 of 8 patients with EOC (see above and Table III). RNA was isolated from PBMC and ST-TIL, cDNA was synthesized as a template for CD3-δ, CD3ζ and GAPDH specific PCR (representative results are shown in Figure 4). Semi-quantitative analysis was carried out through competitive RT-PCR. The δ and ζ transcripts were normalized to GAPDH and the levels of gene expression in the specimens were calculated. Although CD3δ transcripts were present in all specimens examined, expression of CD3ζ transcripts fell into three categories: no expression (lanes 8 and 10), low expression (lanes 4 and 6) and normal expression (lanes 2 and 12). All results on CD3ζ transcripts

Table III. *Summary of CD3 ζ polypeptide chain expression and levels of CD3δ and CD3ζ transcripts in patients with ovarian carcinoma.*

Patient	CD3ζ protein		ST CD3δ transcript	ST CD3ζ transcript
	ST	ASC		
OV 16	–	+	+	–
OV 24	–	+	+	–
OV 25	–	+	+	–
OV 31	–	+	+	–
OV 37	–	+	+	–
OV 22	–	n/d	+	–
OV 23	n/d	+	+	–
OV 26	n/d	+	+	–
OV 29	n/d	+	n/d	n/d
OV 5	n/d	n/d	+	–
OV 6	n/d	n/d	+	–
OV 27	n/d	n/d	+	–
OV 32	n/d	n/d	+	–
OV 33	n/d	n/d	+	–
OV 9	+*	n/d	+	+/–
OV 3	n/d	n/d	+	+/–
OV 4	n/d	n/d	+	+/–
OV 30	n/d	n/d	+	+/–
OV 1	n/d	n/d	+	+
OV 2	n/d	n/d	+	+
OV 36	n/d	n/d	+	+
OV 38	n/d	n/d	+	+

n/d, Not done. +, Expressed; –, not expressed; +/–, down-regulated; *Immunoprecipitation with the anti-PY20 mab (anti-phosphotyrosine) revealed bands co-migrating with the phosphorylated CD3ζ. Immunoprecipitation/Western blotting experiments with anti-CD3ζ were not carried out.

are summarized in Table III. In every specimen where Western blot analysis did not demonstrate a CD3ζ polypeptide, there was also no expression of CD3ζ transcript. Out of all specimens examined, 13 out of 21 fresh ST-TILs did not express any CD3ζ transcript, an additional 4 had low expression and 4 had normal expression. PBMCs from four patients with EOC expressed CD3ζ transcripts (Figure 5).

In vitro treatment of ovarian carcinoma ST-TIL with rIL-2 restores expression of CD3ζ transcript and protein. We examined whether *in vitro* treatment with rIL-2 would restore CD3ζ expression in the ST-TIL of two patients with EOC. Figure 6A demonstrates the absence of CD3ζ polypeptide in fresh ST-TILs (lane 2) and the presence of CD3ζ polypeptide in fresh ASC-TILs (lane 5) from patient OV37, respectively. After 1 week *in vitro* in culture of ST-TILs with rIL-2 (600 IU/ml), the expression of the CD3ζ polypeptide was restored (lane 3). ASC-TILs cultured for 1 week with rIL-2 (600 IU/ml) continued to express CD3ζ polypeptide (lane 6). Culture of ST-TILs from patient OV31 with rIL-2 (600 IU/ml) also demonstrated restoration of the expression of the CD3ζ polypeptide (data not shown).

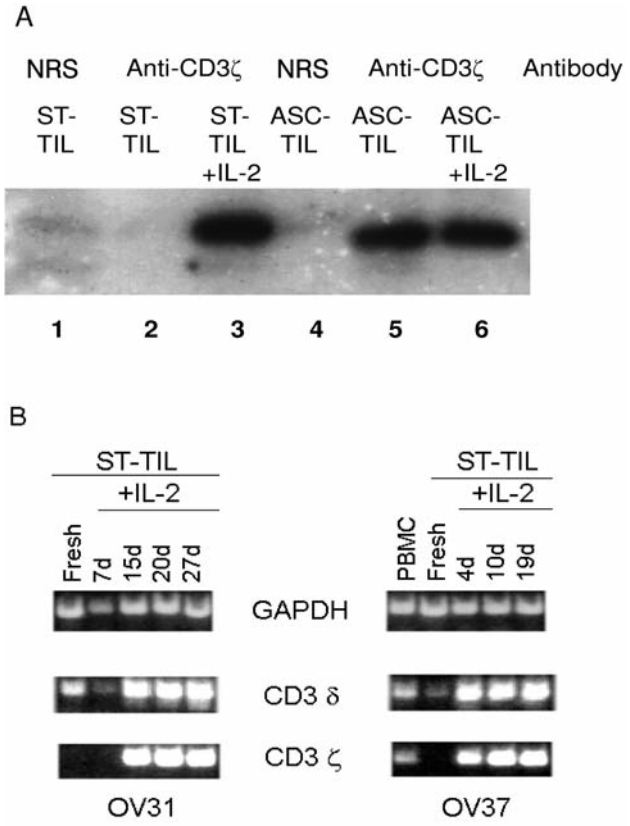

Figure 6. *In vitro treatment of ST-TILs with rIL-2 restores expression of CD3ζ transcripts and protein. A, ST-TILs, ST-TILs cultured for 1 week with rIL-2 (600 IU/ml), ASC-TILs and ASC-TILs cultured for 1 week with rIL-2 (600 IU/ml) were immunoprecipitated with either normal rabbit serum (NRS) (lanes 1 and 4) or anti-CD3ζ antibody (lanes 2, 3, 5 and 6). The lysates were analyzed by SDS-PAGE (12.5%) under reducing conditions and probed with an anti-CD3ζ rabbit serum, as described in Materials and Methods. B: Fresh ST-TILs from patients OV31 and OV37 did express CD3δ transcripts but not CD3ζ transcripts. PBMCs from patient OV37 expressed both CD3ζ and CD3δ transcripts. Culture of ST-TILs from patient OV31 with rIL-2 (600 IU/ml) for 15 days and from patient OV37 with rIL-2 (600 IU/ml) for 4 days resulted in restoration of the CD3ζ expression. Total RNA was isolated and RT was performed using oligo-dT, as described in Materials and Methods. PCR for GAPDH, CD3δ and CD3ζ were carried out on the first-strand cDNA and the results were analyzed using 1.2% agarose gels, as described in Materials and Methods.*

Fresh ST-TILs from patients OV31 and OV37 did not express CD3ζ transcripts, although the expression of CD3δ transcripts was evident (Figure 6B). PBMCs from patient OV37 expressed both CD3ζ and CD3δ transcripts (Figure 6B). Culturing of ST-TILs from patient OV31 with rIL-2 (600 IU/ml) for 15 days and from patient OV37 with rIL-2 (600 IU/ml) for 4 days resulted in restoration of the CD3ζ expression in ST-TILs. CD3ζ expression in ST-TILs from patients with EOC can be restored *in vitro* by treatment with rIL-2 (Figure 6B).

Discussion

We report here the differential expression of CD3ζ message and protein in ST-TILs and ASC-TILs from patients with EOC. The CD3ζ protein was not expressed on ST-TILs from all six patients with EOC examined. CD3ζ transcripts were absent from ST-TILs from 13 out of 21 patients with EOC. ST-TILs from 4 patients exhibited low levels of CD3ζ transcripts and ST-TILs from 4 additional patients expressed normal levels of CD3ζ transcripts in comparison to the controls. CD3δ transcripts were found in substantial amounts in ST-TILs and ASC-TILs from all patients examined. While CD3ζ protein was found to be expressed in all 8 out of 8 ASC-TIL samples from patients with EOC, it was expressed only in 1 out of 7 ST-TILs specimens from patients with EOC. These results demonstrate a differential expression of CD3ζ in ASC-TILs *versus* ST-TILs from patients with EOC. Our findings are in agreement with those of Lockhart *et al*. (31) who reported that ASC T-cells from patients with EOC expressed CD3ζ, which was absent from ST specimens. In contrast, Lai *et al*. (28) reported that CD3ζ was absent or down-regulated in ASC T-cells from patients with EOC.

Our research group has reported differential expression of cytokine transcripts (IL-2 and interferon (IFN)-γ) in ST-TILs and ASC-TILs from patients with EOC. These transcripts are expressed at a statistically significant higher frequency in ASC-TILs than in ST-TILs (34). Merogi *et al*. (35) reported that ASC-TILs from patients with EOC had lower transcript and protein expression of IFN-γ, IL-2 and IL-4, whereas there was an increase in IL-10 expression in comparison to PBMCs from the same patients. These results suggest that the microenvironment of ASC-TILs and ST-TILs is different and that the influences that they are under may also be different. For example, the ratio of tumor cells to lymphocytes is generally higher in ST specimens than in ASC. In addition, the ST-TILs are present in solid tissue and should have closer interactions with the tumor cells as compared to the ASC-TILs, which are almost in single-cell suspensions. It has been demonstrated in colorectal carcinoma that as lymphocytes were further away from the tumor, the levels of CD3ζ expression on TILs increased (36). Additionally, a correlation has been reported between apoptosis and CD3ζ levels in ASC-TIL from patients with EOC (37).

CD3ζ subunits are necessary for the proper assembly and surface expression of the TCR complex as in the absence of CD3ζ, partially assembled complexes, are targeted for degradation (38). Furthermore, the absence of expression of CD3ζ transcripts and protein in ST-TILs, in contrast to certain ASC-TIL specimens, suggests the possible involvement of transcriptional factors. The ETS family member Elf-1 has been demonstrated to have a role in transactivation of the CD3ζ gene (39). Elf-1 has also been shown to play a role in the expression of several other T lymphocyte-specific genes such as IL-2 (40).

TILs are believed to represent the immune response of the host to the tumor. They fulfill many criteria of T-cells that have been activated *in vivo* in an antigen-specific manner: (a) They express early, intermediate and late activation antigens (41, 42), which indicate the presence of an ongoing immune response. (b) TILs from patients with malignant melanoma or EOC can be expanded *in vitro* in low concentrations of IL-2 to a few thousand-fold (reviewed in 1) and often exhibit cytotoxicity or cytokine production, primarily restricted to autologous tumor cells only. (c) Sequence analysis of β-chain TCR transcript of TILs revealed the presence of substantial proportions of identical β-chain TCR transcripts in EOC (43) and several other tumor types (reviewed in 1), suggesting the presence of oligoclonal populations of T-cells. Moreover, we have shown the presence of clonally expanded γδ TCR^+ T-cells in TILs and the peripheral blood of patients with EOC (44). These clonally expanded αβ TCR^+ and γδ TCR^+ T-cells underwent proliferation and clonal expansion *in vivo* at the site of the tumor in response to tumor antigens which, in the case ovarian carcinoma, have not yet been fully identified.

All these results taken together, as well as the identification of tumor antigens in several tumor types, demonstrate the presence of antitumor T-cell responses, which reside, among others, in TILs. However, it appears that as the tumor progresses, these T-cells in TILs become progressively anergic and ineffective in controlling the tumor, by a variety of mechanisms including CD3ζ down-regulation and the following: (a) Downregulation of HLA class I expression on tumor cells. TILs cannot be expanded *in vitro* from EOC tumor specimens with reduced levels of HLA class I expression (45). Increased HLA class I expression on EOC tumor cells correlates with T-cell infiltration *in vivo* and T-cell expansion *in vitro*, in low concentrations of rIL-2. (b) Tregs ($CD4^+CD25^+FOXP3^+$) suppress specific tumor T-cell immunity in human cancer and promote the growth of human tumors *in vivo* (46). In ovarian cancer, Tregs are associated with high death risk and reduced survival (46). (c) Production of IL-10 and TGF-β. Both molecules have extensive immunosuppressive properties. TGF–β is produced in high concentration by malignant cells and by certain normal cells (47). IL-10 may be produced by tumor cells and normal cells (reviewed in 1). HLA-DR-negative, IL-10-producing monocytes from the malignant ASC of patients with EOC produce both IL-10 and TGF-β (48). Thus, tumor cells as well as non-malignant T-cells may be actively inhibiting the immune response by production of cytokines that directly affect the T-cell response. (d) Monocyte/macrophages with suppressor activity (49, 50). (e) Tumor cells expressing FasL can eliminate T-cells expressing Fas by apoptosis in the same way certain T-cells can eliminate tumor cells, using the Fas/FasL system (reviewed in 51). It appears that neutralization of tumor escape and immunosuppressive

mechanisms may be required for the development of clinically effective immunotherapy approaches, including tumor vaccines and adoptive immunotherapy (52).

Absence or down-regulation of the CD3ζ protein has been associated with anergy, immunological unresponsiveness and chronic inflammation. In addition to CD3ζ absence or down-regulation in TILs from patients with cancer, CD3ζ is also down-regulated in certain autoimmune diseases, viral infections and other cases of chronic inflammation. Absence of expression or down-regulation of CD3ζ protein has been reported in synovial T-cells and PBMCs from patients with rheumatoid arthritis (53, 54), T-cells infiltrating the synovial membrane of patients with osteoarthritis (55), T-cells from patients with pulmonary tuberculosis (56), in circulating CD8 lymphocytes from patients with HIV infection (57) and others. Many autoimmune diseases in humans may be specific antigen-driven T-cell diseases (58). The down-regulation of CD3ζ in autoimmune diseases may be a regulatory feedback mechanism that may play a role in the pathogenesis of these diseases.

The CD3 T-cell differentiation antigens play a critical role in T-cell functions. We and others have demonstrated that the CD3 complex is involved in proliferative T-cell responses and in specific T-cell-mediated cytotoxicity (59). Anti-CD3 mabs block a post-adhesion/post recognition stage of the cytolytic T-cell process (60). We have demonstrated that anti-CD3 mabs induce the production of IFN-γ by human PBMCs (61). We have also demonstrated that anti-CD3 mabs induce suppressor cells of the $CD4^+CD8^-$ and $CD4^-CD8^+$ phenotypes (62, 63).

The loss or down-regulation of CD3ζ is reversible by rIL-2 treatment *in vitro* or *in vivo* in TILs from metastatic melanoma, colorectal hepatic metastases and renal cell carcinoma (64-66). However, as we mentioned earlier, neutralization of tumor escape and immunosuppressive mechanisms additional to the down-regulation of CD3ζ must be accomplished to permit the development of clinically effective immunotherapy approaches. This may be required for both tumor vaccines and adoptive immunotherapy methods (52).

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