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CD8+ Tumor-Infiltrating Lymphocytes Are Primed for Fas-Mediated Activation-Induced Cell Death But Are Not Apoptotic In Situ

Sasa Radoja,*† Masanao Saio,*‡ and Alan B. Frey2*

Induction of Fas-mediated activation-induced cell death in antitumor T cells has been hypothesized to permit tumor escape from immune destruction. Several laboratories have proposed that expression of Fas ligand (L) by tumor is the basis for this form of T cell tolerance. In this study, we characterized murine tumor-infiltrating lymphocytes (TIL) for activation status, cell cycle status, level of apoptosis, cytokine secretion, and proliferative capacity. TILs express multiple activation markers (circa CD69, CD95L, CD122, and LFA-1) and contain IL-2 and IFN-γ mRNAs, but are neither cycling nor apoptotic in situ. In addition, TIL are dramatically suppressed in proliferative response and do not secrete IL-2 and IFN-γ. However, upon purification and activation in vitro, TIL secrete high levels of IL-2 and IFN-γ, enter S phase, and then die by Fas-mediated apoptosis. Activation by injection of anti-TCR Ab or IL-2 into tumor-bearing mice induced TIL entrance into S phase preceding apoptosis, showing that TIL have functional TCR-mediated signal transduction in situ. Our data demonstrate that TIL, not tumor, express both Fas and FasL, are arrested in G1, do not secrete cytokine in situ, and, upon activation in vitro and in vivo, rapidly die by activation-induced cell death. The Journal of Immunology, 2001, 166: 6074–6083.

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in proliferative response and effector phase function, TIL TCR-mediated signal transduction is intact in situ. In addition, our results show that the Fas-mediated pathway of apoptosis is activated by TCR signaling in TIL and is dominant over survival.

Materials and Methods

Mice

C3H/HeN and C57BL/6 male mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were housed four per cage in a barrier facility and maintained on a 12-h light, 12-h dark cycle (0700–1900 h) with ad libitum access to food and water. A sentinel program revealed that the mice were mouse hepatitis virus negative, and the tumor cell lines are mouse hepatitis virus negative as assessed by mouse assessment profile service testing. Experiments involving animals were conducted with the approval of the New York University School of Medicine Committee on Animal Research.

Tumors

The 6-1 tumor was created by expression of plasmids encoding activated murine Ha-ras plus p53 genes in primary C3H/HeN embryonic fibroblasts. The properties of this tumor have been described previously (21). MCA-38 adenocarcinoma was the gift of Y. Liu (Ohio State University, Columbus, OH). These tumors do not contain mRNA encoding FasL (data not shown). Tumor cell lines were removed from tissue culture plastic by incubation in HBSS containing 2 mM EDTA and washed three times in HBSS. The viability of cell lines was determined by trypan blue dye exclusion, and 2 × 10^6 cells were injected s.c. in a volume of 0.1 ml of HBSS for tumor induction.

Tissue culture

RPMI 1640 medium (BioWhittaker, Walkersville, MD) was used for isolation and culture of macrophages and T cells and was supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.002 mg/ml -glutamine, and 10% FBS (Intergen, Purchase, NY). DMEM was used for culture of tumor cell lines. All tissue culture supplements were supplied by Life Technologies (Grand Island, NY).

Cytotoxicity

For single-color analysis, splenocytes (10^6), from control or tumor-bearing mice or TIL (2–3 × 10^6) were washed once with FACS buffer (HBSS without phenol red, 1% BSA (Sigma, St. Louis, MO), and 1% sodium azide. Cells were incubated for 45 min on ice with 0.0005 mg/ml of fluoro-chrome-conjugated Abs in a volume of 0.1 ml including 0.01 mg human IgG (Baxter Scientific Products, Chicago, IL) and 0.002 mg anti-murine cd16/32 to block nonspecific binding (clone CT-CD4; CalTag Laboratories); CD4 (clone CT-CD4; CalTag Laboratories); CD8 (clone CT-CD8b; CalTag Laboratories); CD3 (clone 500-A2; CalTag Laboratories); CD25 (clone PC61.5.3; CalTag Laboratories); CD28 (clone CT-CD28; CalTag Laboratories); CD95 (clone Jo2; BD PharMingen); IgG (Baxter Scientific Products, Chicago, IL) and 0.002 mg anti-murine CD69 (clone H1.2F3; CalTag Laboratories); CD95 (clone Jo2; BD PharMingen); IgG (Baxter Scientific Products, Chicago, IL) and 0.002 mg anti-murine CD62L (clone MEL 14; CalTag Laboratories); CD19 (clone H57-597; BD PharMingen); CD20 (clone H57-597; BD PharMingen); IgG (Baxter Scientific Products, Chicago, IL) and 0.002 mg anti-murine CD40L (clone MR1; Southern Biotechnologies Associates, Birmingham, AL); CD7 (clone IM7; CalTag Laboratories); CD45RB (clone 16A; CalTag Laboratories); CD45RA (clone 14.8; CalTag Laboratories); CD62L (clone ME14; CalTag Laboratories); CD69 (clone H1.2F3; CalTag Laboratories); CD95 (clone Jo2; BD PharMingen), San Jose, CA); CD95L (clone MFL3; BD PharMingen); CD122 (clone TM-β1; BD PharMingen); F4/80 (clone CL-A3-1; CalTag Laboratories); Ly6C (clone MK1.4; Southern Biotechnology Associates); granulocytes (clone RB6-8C5; BD PharMingen); TCR-β (clone H57-597; CalTag Laboratories); CTLA4 (clone VC10-F410–11; BD PharMingen); and LFA-1 (clone 2D7; BD PharMingen).

Isolation of TIL

Tumors were dissected and chopped into small pieces using a razor blade before incubation (1 g/10 ml) with a mixture of enzymes dissolved in HBSS (collagenase type I 0.05 mg/ml), collagenase type IV 0.05 mg/ml, hyaluronidase 0.025 mg/ml, all from Sigma, DNDase 1 0.01 mg/ml, and soybean trypsin inhibitor 0.2 trypsin inhibitor units/ml), both from Boehringer Mannheim, Indianapolis, IN) for 15 min at 37°C. Cells were recovered by centrifugation and resuspended in a fresh aliquot of enzymes for a second 15-min incubation at 37°C. Undigested material was settled for 2 min at 0 × g, and liberated cells were recovered and washed by centrifugation in complete medium. T cells were isolated using immuno-magnetic separation using type MS® or VS® columns and anti-CD4- or anti-CD8-conjugated magnetic beads according to the manufacturer’s instructions (Miltenyi Biotec, Bergish-Gladbach, Germany). Briefly, 0.01 ml of the suspension was diluted to 0.01 ml in ice cold HBSS (containing 0.5% BSA). After incubation for 15 min on ice, cells were washed and passed through the separation column. After washing of the column with cold buffer and removal from the magnet, cells were eluted and repurified on a second column. In each experiment, aliquots of isolated cells were analyzed for cell surface expression of various markers by flow cytometry and were routinely >95% CD3+ T cells purified thourly from splenocytes of control mice do not express activation Ags (CD25 and CD69), do not transcribe IL-2 mRNA, and do not incorporate triflated thymidine in proliferation assay unless stimulated in vitro.

Proliferation assay

Single-cell splenocyte suspensions were prepared by grinding spleens between the ends of microscope slides, and viability was assessed by trypan blue exclusion. Splenocytes or purified T cells were plated in 96-well plates (Flow Laboratories, McLean, VA) in complete medium; plates were coated with purified anti-TCR-β mAb (H57-597; 0.01 mg/ml for 60 min at 37°C). In some experiments, purified anti-CD28 Ab (CalTag Laboratories) was used at the same concentration to stimulate cells in conjunction with anti-TCR Ab. Cultures were pulsed at 48 h with 0.5 μCi [3H]thymidine (2 Ci/mM; ICN Pharmaceuticals, Costa Mesa, CA), harvested 18–24 h later using an automated cell harvester (Wallac, Gaithersburg, MD), and incorporation of radiolabel was determined by liquid scintillation counting (Microbeta 1450; Wallac). Data are expressed as mean triplicate or quadruplicate determinations ± SE.

Chromium release assay

CTL activity of TIL was determined in standard 51Cr release assays. In brief, 10^5 target cells, cognate tumor cells, or syngeneic but non cross-reactive MC57G tumor cells were incubated with 0.2 mCi Na[ 51 ]CrO 4 in RPMI 1640 medium for 60 min at 37°C. Cells were washed twice with complete medium and transferred to round-bottom 96-well plates at 5 × 10^3 cells/well. Effector cells were prepared by in vitro culture of TIL in complete RPMI 1640 medium overnight in the presence of 100 U/ml rIL-2. Cells were added to target cells at varying numbers in a final volume of 0.2 ml to give the E:T ratios as indicated in the figure legends. After a 4-h incubation at 37°C, 0.1 ml of supernatants were harvested, and released radiolabel was determined by scintillation counting. Maximal release from target cells was determined by treatment of cells with 1% Triton X-100, spontaneous release was determined from cultures of labeled target cells incubated with medium only, and the formula used for determination of specific lysis was: [(experimental release – spontaneous release)/(maximal release – spontaneous release)] × 100.

Cell cycle analysis

CD8+ TIL or spleen cells were isolated by magnetic immunobead labeling as described above. A total of 10^6 cells were permeablized with cold 70% ethanol (5 min at −20°C). After being washed with PBS, the cells were incubated with 50 U/ml RNase A (Boehringer Mannheim) for 15 min at 37°C. The cells where then stained with 0.100 mg/ml propidium iodide (PI) for a minimum of 1 h at room temperature (RT) and analyzed by flow cytometry.

RNA isolation, reverse transcription, and PCR amplification

Total cellular RNA was isolated from T cells without in vitro stimulation, used to prepare cDNA, and used to program PCR amplification as described previously (22).

Immunocytochemistry

Tumors were removed and embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen, and stored at −80°C. Four-micron cryosections were cut, air-dried, and fixed with cold acetone for 10 min. Specimens were treated with 0.1% hydrogen peroxide, 0.01% trimun azide in PBS to block endogenous peroxidase activity. After being washed in PBS, stained with biotinylated anti-CD4 or -CD8 Abs (0.01 mg/ml for 1 h at RT), washed with PBS, and reacted for 40 min at RT with strepavidin ABC (avidin/biotin complex) alkaline phosphatase (Dako, Carpinteria, CA). Reactions were visualized by development with fuchsin (Dako). Following staining for T cells, slides were treated with avidin/ biotin blocking reagent (Vector Laboratories, Burlingame, CA) according to the manufacturer’s instructions, stained with 0.01 mg/ml biotinylated anti-F4/80 for 1 h at RT, washed with PBS, and stained with VECAStain Elite Abc kit (Vector Laboratories) for 40 min at RT. Reactions were
visualized by development in 0.03% hydrogen peroxide, 0.03% 3,3′-diaminobenzidine (Sigma) (in 0.05 M Tris-HCl (pH 7.6)). Slides were fixed with 4% paraformaldehyde in PBS for 10 min, counterstained with 0.5% methyl green (in 0.1 M sodium acetate (pH 4.0) for 10 min), and examined.

**In situ analysis of apoptosis (TUNEL assay)**

Subcutaneous tumor was isolated, embedded in Tissue-Tek OCT compound (Sakura Finetek), frozen in liquid nitrogen, and stored at −80°C until sectioning. Four-micrometer cryosections were prepared using a model CM1900 cryostat (Leica, Bannockburn, IL). Slides were air-dried at RT before fixation with cold acetone for 10 min (~20°C). Specimens were reacted with 0.01 mg/ml biotinylated anti-mouse CD8 or CD4 Abs (BD PharMingen) for 1 h at RT, followed by streptavidin ABC alkaline phosphatase (Dako) for 40 min RT. Reactions were developed using a Dako fuchsin substrate-chromogen system before fixation with 4% paraformaldehyde in PBS (10 min at RT). Between each step, specimens were washed with PBS for 30 min at RT. After T cell staining, slides were analyzed by TUNEL assay using the Apoptag Plus Peroxidase In Situ Apoptosis Detection kit (Intergen). Specimens were pretreated with 10% normal sheep serum in PBS before reaction with peroxidase-conjugated sheep polyclonal anti-digoxigenin according to the manufacturer’s instructions. Slides were counterstained with 0.5% (w/v) methyl green (in 0.1 M sodium acetate (pH 4.0) for 10 min) before photography. Controls for TUNEL assay included omission of TdT enzyme before immunocytochemistry, which revealed no reaction product.

**Cell labeling with 5-bromo-2′-deoxyuridine (BrdU)**

Tumor-bearing or control mice were fed BrdU (0.8 mg/ml; Sigma) in drinking water. Mice were injected with either purified control hamster IgG, anti-TCR Ab, or IL-2 at the dosages indicated in the figures. CD8+ TIL or spleen cells were isolated and analyzed by flow cytometry after labeling with FITC-conjugated anti-CD8 and PE-conjugated anti-BrdU (BD PharMingen) as described (23).

**Results**

**Characterization of immune cell tumor infiltrates and activation status**

Single-cell suspensions of tumors were prepared by enzymatic digestion after excision of any necrotic portion and characterized for the nature and extent of immune cell infiltration by flow cytometry. For these analyses, we studied two tumors, a fibrosarcoma made by expression of cDNAs encoding dominant-negative p53 plus activated Ha-ras genes in primary embryonic fibroblasts termed “6-1” (21, 22), and, separately, a chemically induced adenocarcinoma, MCA-38 (24). For all experiments, data achieved with either tumor were highly similar, if not identical, and representative data is shown. Tumors that have grown for ≤3 wk have undetectable infiltration of neutrophils, NK cells, or B cells. Tumors at this stage are infiltrated with low levels of both CD4+ and CD8+ T cells at approximately the same level (1–2%). In contrast to the low level of T cell infiltration, F4/80+ macrophages are comparatively abundant, comprising ~25% of tumors at 3 wk of growth. The number of infiltrating macrophages also increases as a function of tumor size and reaches ~40% of tumors at 4–5 wk of growth (~2.5 cm3).

To visualize the distribution of T cells and macrophages within the tumor bed, we performed immunocytochemistry on frozen, thin sections of tumor. Relatively rare T cells were found dispersed throughout the tumor bed in apposition with both macrophages and tumor. Clusters of T cells were almost never seen; neither were close apposition of CD4+ and CD8+ TIL (data not shown).

CD8+ T cells were purified from tumor cell suspensions by magnetic immunobead isolation and characterized for expression of a variety of T cell surface markers by Ab labeling and flow cytometry (Fig. 1). Purified TIL express uniform levels of the TCR and CD3ζ, showing that TIL are not contaminated with non-CD8+ T cells. The state of activation of purified TIL was similarly determined by flow cytometry and can be summarized as demonstrating a mixed phenotype characteristic of both activated and memory cells: CD11a+, CD25−, CD40L−, CD44+, CD45RAlow, CD45RBhigh, CD62Llow, CD69+, CD95L−, CD122+, Ly6Chigh.

Because TIL have a mixed phenotype in terms of expression of cell surface activation markers, we examined their activation status in more detail by analysis of expression of mRNAs that are typically expressed in T cells following Ag recognition. RNA was isolated from freshly purified CD4+ and CD8+ TIL (i.e., not activated in vitro) and used to program RT-PCR using primers specific for IL-2 and IFN-γ (Fig. 2), granzymes A and B, and perforin...
TCR recognition of cognate Ag.

TIL contain mRNA-encoding molecules whose transcription is initiated by anti-TCR Ab for 48 h before isolation of RNA. This analysis shows that RNA samples were prepared by stimulating spleen-derived T cells with 1\textsuperscript{incorporation of thymidine into both CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells. However, 1\textsuperscript{incorporation of thymidine when nonimmune hamster IgG is used instead of anti-TCR Ab, and both CD4\textsuperscript{+} and CD8\textsuperscript{+} TIL expressed mRNAs encoding perforin and granzymes A and B, showing that TIL are activated in situ, because these RNAs are not transcribed in nonactivated T cells.

**Characterization of purified TIL**

The proliferative capacity of purified TIL was assessed by measurement of titrated thymidine incorporation after stimulation with plate-bound anti-TCR Ab. Control CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells were purified and tested for proliferation under identical conditions as for TIL (isolation from spleens using enzymatic digestion and magnetic immunobeads). Control T cells and TIL do not incorporate thymidine when nonimmune hamster IgG is used instead of anti-TCR Ab, and both CD4\textsuperscript{+} and CD8\textsuperscript{+} control T cells incorporate thymidine upon activation in vitro (Fig. 3A). However, incorporation of thymidine into both CD4\textsuperscript{+} and CD8\textsuperscript{+} TIL is dramatically reduced compared with an equivalent number of control T cells.

Because of concern that the inflammatory milieu of the tumor microenvironment may attract T cell infiltration that does not represent recruitment of antitumor T cells, TIL were tested for Ag-specific lysis against syngeneic tumor cells that do not immunize mice to resist challenge injections of MCA-38 and therefore do not express cross-reactive tumor Ags. (Freshly purified TIL are not lytic in standard 4-h lysis assays; however, if TIL are cultured briefly in vitro, substantial lytic activity is detected.) One such non-cross-reactive tumor is MC57G, which is not appreciably lysed by anti-MCA-38 TIL, demonstrating that TIL contain Ag-specific T cells (Fig. 3B). In addition, freshly isolated TIL were tested for tumor-specific release of IFN-γ (Fig. 3C), again showing that cognate tumor cells, but not non-cross-reactive tumor, stimulate release of cytokine in vitro.

Visualization of TIL cultures during the proliferation assay by microscopy showed that, in comparison to control T cells, TIL did not blast and appeared greatly reduced in number (data not shown). This was surprising because cell surface activation Ags were strongly expressed by TIL (Fig. 1), and we anticipated that TIL would vigorously proliferate. Therefore, we considered the possibility that TIL were dying upon activation in vitro. To directly
assess this possibility, TIL were isolated and stimulated with plate-bound anti-TCR Ab for increasing time and cell recovery, determined by enumeration of viable cells after staining with PI (Fig. 4A). (CD4+ TIL behaved identically with CD8+ TIL in subsequent experiments; for simplicity, only CD8+ TIL data is shown). The number of control T cells increased dramatically under these activating conditions. In contrast, the number of TIL activated with anti-TCR Ab rapidly declines such that, compared with control T cells, by 72 h, <10% of cells are present. The kinetics of the decrease in cell recovery suggests that the basis for reduced incorporation of tritiated thymidine into TIL activated in vitro is the dramatic reduction in cell number.

The basis for TIL death upon activation in vitro was investigated by asking whether TIL expressed cell surface phosphatidylserine (Fig. 4B). Both control T cells and freshly isolated TIL do not express significant levels of annexin V binding (2–4%), implying that TIL are not apoptotic in situ. However, CD8+ TIL, but not control CD8+ spleen T cells, expressed significant levels of annexin V binding upon in vitro activation. The kinetics of phosphatidylserine expression after activation in vitro suggests that TIL become annexin V+ preceding the decline in cell recovery.

The phenotype of TIL after activation was further examined by analysis of cytokine production in vitro. TIL were isolated, activated with anti-TCR Ab, and the levels of IL-2 and IFN-γ secreted were determined by ELISA of conditioned medium (Fig. 4C). Freshly isolated TIL do not secrete detectable cytokine in the absence of stimulation, although mRNA encoding both IL-2 and IFN-γ is present. Secretion of IFN-γ was detected after ~4 h in vitro and, by 6 h, the level produced was substantial (~2000 U/10^6 cells in 6 h) and almost the same as that secreted by activated primary MLR CD8+ T cells (data not shown). Control spleen CD8+ T cells activated identically do not produce detectable IL-2 or IFN-γ (data not shown). In addition, corroborating the ELISA findings, intracellular flow cytometric analysis of freshly isolated TIL did not detect the presence of cytokine proteins (Fig. 4D). Intracellular staining after TIL isolation and activation in the presence of monensin showed that a portion of TIL secrete cytokines after activation in vitro; without activation there was no detectable cytokine protein. This analysis shows that, upon activation, substantial TIL cytokine synthesis and secretion occurs preceding AICD.

Expression of cell surface phosphatidylserine after TIL activation in vitro suggested that TIL die by induction of apoptosis. Because freshly isolated TIL express both cell surface Fas and FasL and become apoptotic upon activation in vitro, TIL appear to die by activation-induced cell death. The role of Fas and FasL in this process was examined by inclusion of anti-FasL Ab (Fig. 4E) in the in vitro proliferation assay. Inclusion of isotype-matched

![Image](http://www.jimmunol.org/)

**FIGURE 4.** A. Recovery of CD8+ TIL after activation in vitro. CD8+ TIL or control spleen CD8+ T cells were isolated by magnetic immunobead as described in Materials and Methods. Cells were plated in triplicate in 96-well plates in the presence of saturating amounts of plate-bound anti-TCR Ab (0.1 ml at 0.01 mg/ml). Wells were harvested at the times indicated, and viable cells were enumerated by staining with PI and flow cytometry. This analysis shows that recovery of viable CD8+ TIL decreases dramatically following ligation of the TCR in vitro. B. CD8+ TIL become annexin V+ after TCR ligation in vitro. TIL or control spleen CD8+ T cells were isolated by magnetic immunobead as described in Materials and Methods. Cells were plated in 24-well plates in the presence of saturating amounts of plate-bound anti-TCR Ab (0.1 ml at 0.01 mg/ml). Wells were harvested at the times indicated and analyzed by flow cytometry after staining with FITC-conjugated annexin V. This analysis shows that freshly isolated TIL do not bind annexin V but express cell surface phosphatidylserine after activation in vitro. C. TIL cytokine ELISA. TIL were isolated, plated in vitro in the presence or absence of plate-bound anti-TCR Ab for the indicated times (2 × 10^3 cells/well), and conditioned medium was collected and analyzed by ELISA for the presence of IL-2 or IFN-γ as described (40). Standard curves made using recombinant cytokines were linear to ~50 pg/ml/D. Flow cytometric analysis of IL-2 and IFN-γ expression. TIL were isolated and incubated in medium containing 3 μM monensin for 5 h with or without activation with plate-bound anti-TCR Ab before intracellular cytokine staining with fluorochrome-conjugated anti-IL-2 or IFN-γ. This experiment shows that TIL do not express IL-2 or IFN-γ unless activated, and only a small percentage of TIL are secreting. E. Proliferative deficit of CD8+ TIL after TCR ligation in vitro is reversed by blocking Fas-FasL interaction. TIL were isolated by magnetic immunobead as described in Materials and Methods. Proliferation of purified T cells was determined by incubation in 96-well plates (5 × 10^5 cells/well) that were precoated with saturating amounts of anti-TCR or control Ab (not stimulated). Wells contained either control of FasL as FasL interaction is blocked.
control Ab had no effect on thymidine incorporation into CD8\(^+\) TIL because TIL proliferation, as noted previously, was depressed relative to control spleen-derived T cells. In contrast, inclusion of anti-FasL Ab restored proliferation close to the level of control T cells. Anti-FasL Ab also restored recovery of TIL to levels seen with control T cells (data not shown). Collectively, the experiments shown in Fig. 4 show that TIL are primed for AICD upon in vitro ligation of the TCR.

**TIL cell cycle status**

TIL cell cycle status was assessed by flow cytometry after staining with PI. Despite expression of a variety of cell surface activation markers (Fig. 1), freshly isolated TIL are not cycling because only 6–8% of cells are in S phase (compared with 4–6% of control spleen T cells; data not shown). Upon stimulation with anti-TCR Ab in vitro for increasing time, the same percentage of TIL enter S phase as do control T cells; within 24 h of activation, ~20% of cells are in S phase, and the percentage of cells in S phase increases to >50% at 48 h (data not shown). The number of TIL available for cell cycle analysis at any given time after activation in vitro decreases dramatically (Fig. 4A), but, of those cells remaining in culture, the percentage in S phase is equivalent to that of control cells. This suggests that, although TIL can be stimulated through the TCR and progress with normal kinetics into S phase, the Fas-mediated pathway of apoptosis is activated and dominant over survival.

**TIL are not apoptotic in situ**

Several laboratories have reported that expression of FasL by tumors induces death of Fas\(^+\) antitumor T cells, thereby contributing to tumor escape from immune destruction (20). This notion has been called into question by the rigorous demonstration that human melanoma do not express FasL (19); however, the possibility that other tumor types may express FasL that contributes to antitumor T cell inactivation remains open. In addition, because TIL express both Fas and FasL, the possibility exists that TIL may die upon contact in vitro by fratricide or suicide. In consideration of this point, viable TIL that do not express cell surface phosphatidyserine can be isolated, strongly implying that TIL are not apoptotic. However, we considered that apoptotic TIL may be rapidly cleared from the tumor and may therefore be unable to be recovered by magnetic immunobeading, a possibility enhanced by the abundance of macrophage in tumors. To directly assess whether TIL are apoptotic, we performed in situ immunohistological characterization of apoptosis in tumors. Tumor cryosections were analyzed by TUNEL assay and immunocytochemical labeling for either CD4 or CD8. We found that, with the exception of the necrotic portion of the tumor, there is very little apoptosis of any cell type in the tumor bed and that only 3–8% of TIL were TUNEL\(^-\) (data not shown). TUNEL\(^+\) TIL were in the early phase of apoptosis by several criteria: cells had intact plasma membrane with no apparent surface blebs, HRP immunocytochemical reaction product was localized only to nuclei, and TIL were not condensed (i.e., they contained abundant cytoplasm that was unstained). We analyzed multiple individual tumors at different stages of growth and viewed over 2200 individual microscopic fields in this determination (summarized in Table I).

**TIL are hyperresponsive to activation stimuli in vitro**

TIL have an activated phenotype in situ, evidenced by expression of cell surface activation markers and stable cytokine RNA but are not cycling nor apoptotic. However, upon activation in vitro, TIL are readily induced to AICD. Therefore, we considered the possibility that TIL in situ are not apoptotic because they receive sub-optimal activation stimuli that is insufficient to drive them out of G\(_1\) phase and into subsequent apoptosis. Alternatively, the activation threshold may be higher for TIL than for control cells. These possibilities were tested in the next sets of experiments.

We first asked whether purified TIL would proliferate under suboptimal activation conditions in vitro. Control T cells and purified TIL were stimulated with increasing amounts of anti-TCR Ab before measurement of incorporation of tritiated thymidine. We found that, under suboptimal activation conditions, TIL incorporate identical levels of thymidine as control T cells (Fig. 5A). At the lower concentrations of anti-TCR Ab, there was a dose-dependent increase in thymidine incorporation into TIL whose magnitude was identical with control T cells. However, as has been noted above, at saturating anti-TCR Ab concentration (10 \(\mu\)g/ml), incorporation of thymidine into TIL is dramatically depressed relative to control T cells.

The recovery of TIL after in vitro activation under suboptimal TCR ligation was also determined (Fig. 5B). Recovery of control T cells showed a strict dependence on concentration of activating plate-bound anti-TCR Ab; 1 \(\mu\)g/ml maintained the number of T cells at input levels over the 3 days of the experiment. Because tritiated thymidine was incorporated into control cells stimulated at this level (reflecting cell division), the number of cells probably reflects a balance between cell death and expansion. At saturating anti-TCR Ab concentration, control T cells rapidly divide and expand in culture; after 1–2 days of stimulation, cultures begin to increase and, by day 3, the density has tripled. In contrast, TIL show a rapid decline in the number of viable cells, which is inversely proportional to the concentration of activating anti-TCR Ab, and, by day 3 of culture, the density is dramatically reduced.

Suboptimal stimulation in vitro resulted in tritiated thymidine incorporation into TIL at the level of control T cells and also decreased recovery of TIL at later time points, suggesting that quiescent TIL were highly sensitive to TCR ligation resulting in AICD in vitro. We measured the effect of suboptimal activation upon cell cycle progression (Fig. 5C). After 24 h of activation using the lowest concentration of activating Ab (0.1 \(\mu\)g/ml anti-TCR), control T cells remain in G\(_1\) and, at 1 \(\mu\)g/ml, control T cells are slightly stimulated to enter S phase. In contrast, a high percentage of TIL enter S phase at the lowest concentration of stimulating Ab used, and the number of cycling cells increases in a dose-dependent manner. This experiment showed that TIL rapidly enter S phase under conditions of TCR ligation wherein control T cells are unaffected and remain in G\(_1\).
Induction of apoptosis at suboptimal conditions of TCR ligation was examined because the recovery of TIL is dramatically reduced under these conditions of activation in vitro (Fig. 5D). Control T cells, although slightly induced to incorporate thymidine and enter S phase under suboptimal conditions, do not become apoptotic. In contrast, TIL cell surface phosphatidylserine expression is high after stimulation with the lowest anti-TCR Ab concentration tested, and, at the intermediate concentration, cells are highly apoptotic. Collectively, the experiments shown in Fig. 5 suggest that, relative to control T cells, TIL are hypersensitive to TCR ligation and enter S phase before becoming apoptotic.

Injection of tumor-bearing mice with anti-TCR Ab or rIL-2 activates TIL in situ and induces TIL apoptosis

Activation of purified T cells by TCR ligation in vitro is widely accepted to reflect Ag recognition in terms of activation of signal transduction. However, we considered the possibility that, within the tumor microenvironment, the presence of tumor cells, stroma, or infiltrating macrophage may alter TIL TCR responsiveness. Therefore, we asked whether ligation of TIL TCR in situ could induce either TIL activation or apoptosis. Tumor-bearing mice were injected with anti-TCR Ab, control hamster IgG, or rIL-2. Twelve hours later, TIL were isolated and plated in the presence of radiolabeled thymidine to measure DNA synthesis (Fig. 6A). TIL isolated from mice injected with anti-TCR Ab incorporated thymidine without further stimulation in vitro, whereas if mice were injected with control IgG, little proliferation was seen. Spleen CD8+ T cells were also activated by injection of anti-TCR Ab, showing that activation of T cells in vivo by anti-TCR Ab was not restricted to TIL. As was seen for injection of anti-TCR Ab, TIL are activated by IL-2 administration such that they incorporate thymidine in vitro without additional activation in vitro. In contrast to activation by injection of anti-TCR Ab, spleen T cells from the same tumor-bearing mice are not activated by IL-2, suggesting that TIL are primed in situ.

Anti-TCR Ab activation of TIL in situ was confirmed by measurement of BrdU incorporation into TIL in tumor-bearing mice (Fig. 6B). Tumor-bearing mice were fed BrdU at the same time as they were injected with anti-TCR Ab. Twenty hours later, TIL were isolated and analyzed for BrdU incorporation by staining with anti-BrdU Ab followed by flow cytometry. Incorporation of BrdU into TIL was dramatically increased by activation with anti-TCR Ab in situ (15 vs 4%), showing that TIL are activated by anti-TCR Ab administration. Because anti-TCR Ab does not cause...
cells were isolated and plated at 5×10^4 cells/well in triplicate in 96-well plates. Tumor-bearing mice were separately injected with control hamster IgG (0.01 or 0.05 mg/injection; control), and non-tumor-bearing mice were injected with either anti-TCR, control hamster IgG, or rIL-2 before isolation of CD8^+ spleen cells and TIL by magnetic immunobeading. A. Activation of TIL in situ induces proliferation. Tumor-bearing mice were injected i.v. with 0.01 mg anti-TCR, 0.05 mg control IgG, or rIL-2 (2000 U/mouse). Twelve hours later, cells were isolated and plated at 5×10^6 cells/well in triplicate in 96-well plates. Wells were pulsed with tritiated thymidine at the time of cell plating and were harvested for scintillation counting 16 h later. This experiment shows that TIL are activated in situ by injection of either anti-TCR Ab or IL-2 such that, after isolation, they incorporate tritiated thymidine in vitro without further activation in vitro. B. Activation in situ induces increased BrdU labeling of TIL. Tumor-bearing mice were fed BrdU in water at the same time as injection with 0.01 mg anti-TCR or control IgG. Twenty hours later, TIL were isolated and stained with PE-conjugated isotype-matched control or PE-conjugated anti-BrdU Ab before analysis by flow cytometry. This experiment shows that TIL are activated by injection of anti-TCR Ab such that they incorporate BrdU in situ to a greater extent than mice injected with control IgG.

FIGURE 6. Injection of tumor-bearing mice with anti-TCR Ab activates TIL. Tumor-bearing mice were injected i.p. with either anti-TCR IgG at the doses indicated or with rIL-2 (2000 U/mouse). Tumor-bearing mice were separately injected with control hamster IgG (0.01 or 0.05 mg/injection; control), and non-tumor-bearing mice were injected with either anti-TCR, control hamster IgG, or rIL-2 before isolation of CD8^+ spleen cells and TIL by magnetic immunobeading. A. Activation of TIL in situ induces proliferation. Tumor-bearing mice were injected i.v. with 0.01 mg anti-TCR, 0.05 mg control IgG, or rIL-2 (2000 U/mouse). Twelve hours later, cells were isolated and plated at 5×10^6 cells/well in triplicate in 96-well plates. Wells were pulsed with tritiated thymidine at the time of cell plating and were harvested for scintillation counting 16 h later. This experiment shows that TIL are activated in situ by injection of either anti-TCR Ab or IL-2 such that, after injection, they incorporate tritiated thymidine in vitro without further activation in vitro. B. Activation in situ induces increased BrdU labeling of TIL. Tumor-bearing mice were fed BrdU in water at the same time as injection with 0.01 mg anti-TCR or control IgG. Twenty hours later, TIL were isolated and stained with PE-conjugated isotype-matched control or PE-conjugated anti-BrdU Ab before analysis by flow cytometry. This experiment shows that TIL are activated by injection of anti-TCR Ab such that they incorporate BrdU in situ to a greater extent than mice injected with control IgG.

an increase in the number of TIL recovered compared with control tumor-bearing mice, which would indicate an activation-dependent change in body distribution of antitumor T cells (data not shown), the observation that only a portion of total TIL is labeled by BrdU in tumor-bearing mice that were not injected with anti-TCR Ab means that rarely are TIL dividing at the site of tumor; only after anti-TCR Ab injection do TIL proliferate.

The previous two experiments are interpreted to mean that TIL are able to be activated in situ to enter S phase. However, we have also shown that TIL are hypersensitive to AICD in vitro. Therefore, we asked whether activation in situ induces AICD by analyzing TIL for annexin V reactivity after activation in situ (Fig. 7). Tumor-bearing mice were injected with control IgG, anti-TCR Ab, or rIL-2 before isolation and flow cytometric analysis after annexin V labeling. Control IgG injection induced modest TIL phosphatidylserine expression in TIL and spleen T cells of tumor-bearing mice but not in the control non-tumor-bearing mice (~9% of TIL vs 3% of control spleen CD8^+ T cells). In contrast, after anti-TCR Ab injection into tumor-bearing mice, significant phosphatidylserine expression was induced in both TIL and, to a lesser extent, spleen T cells. In addition, administration of rIL-2 to tumor-bearing mice caused a rapid increase in TIL annexin V reactivity. Contrary to induction of proliferation in spleen T cells in mice receiving anti-TCR Ab, significant AICD was not induced in spleen T cells after IL-2 injection. The susceptibility of TIL to IL-2–induced AICD suggests that, in accordance with the data of Refaeli and colleagues (who showed that repeatedly activated T cells, but not nonactivated T cells, are susceptible to IL-2–induced AICD (1)), TIL are previously activated.

Spleen T cells of tumor-bearing mice have recently been shown to be activated in situ in that effector phase cytokine mRNAs are expressed at high levels (22). Therefore, we suggest that the increase in annexin V binding to spleen T cells of tumor-bearing mice reflects their activated phenotype in situ. In support of this contention, annexin V binding to spleen cells of control mice was minimal. Expression of phosphatidylserine is substantial in TIL after in situ activation such that almost 50% of TIL bind annexin V.

This experiment illustrates two additional important points. First, the fact that annexin V^+ TIL can be isolated in high numbers shows that apoptotic TIL are isolable from tumor digests, and, therefore, previous experiments that quantified apoptotic TIL were not likely to reflect false-negative results due to a potential inability to recover apoptotic TIL. Second, because the level of TUNEL^+ macrophages in tumor cryosections is very low and the TUNEL reaction detects labeling of apoptotic T-cell DNA after phagocytosis of T cells by macrophage in vitro (data not shown), annexin V^+ apoptotic TIL are not immediately phagocytosed in situ, which would also lead to underestimation of the extent of TIL apoptosis in situ.

Discussion
Apoptosis of infiltrating T cells in several human cancers has been reported for several tumor types (17, 25–28), and FasL expression by tumor cells has been postulated to be the basis for TIL apoptosis because of the observations that primary tumor cells can express FasL (20). However, tumor expression of FasL has recently been refuted for human melanoma (29). Therefore, the conclusion that tumor cell expression of FasL is the basis for the enhanced apoptosis of TIL remains unsettled. Irrespective of whether tumors express FasL, some TIL are undoubtedly apoptotic, although the extent of TIL apoptosis is variable and likely depends upon multiple factors including extent of necrosis and tumor stage and size.
memory cells, except TIL are CD69^{high}, whereas memory cells are TIL express many cell surface markers that are characteristic of the tumor microenvironment on T cells in situ. We believe that the observed differences reflect the influence of the tumor microenvironment on T cells in vivo. We propose that the observed differences reflect the influence of the tumor microenvironment on T cells in vivo.

Although TIL express cell surface activation Ags and contain cytokine mRNAs indicative of activation, TIL are not actively proliferating or engaged in effector phase function in situ. This conclusion is reached by several observations. First, antitumor T cells do not incorporate BrdU after arrival into tumor tissue. This contention is supported by the findings that, immediately upon isolation, a low percentage of TIL are in S phase, and isolated TIL do not incorporate tritiated thymidine in vitro. Furthermore, although TIL secrete high levels of IFN-γ upon activation in vitro, cytokine secretion in situ is not detected, implying that they are incompletely activated. Finally, the very low level of apoptosis of tumor cells (as revealed by TUNEL assay of tumor cryosections) demonstrates that TIL are nonlytic in situ. This conclusion is further strengthened by our observation that freshly purified TIL are nonlytic in vitro (data not shown).

TIL are nonfunctional in situ but are Ag-specific. This conclusion is based upon our observation that, in addition to not proliferating and secreting cytokine in situ as shown above and although TIL are not lytic upon isolation, after purification and a short period of recovery in vitro, tumor-specific lytic function is recovered. Freshly isolated TIL secrete IFN-γ upon stimulation with cognate tumor, showing that effector phase functions of TIL are differentially inhibited. This contention is also supported by the work of others using transgenic murine models expressing TCR that recognize cognate tumor Ags wherein TIL are shown to accumulate in tumor tissue but are nonlytic in situ or in vitro (33). In addition, as shown above, TIL are partially activated in situ (expression of cytokine mRNAs) and express cell surface markers characteristic of memory/effector T cells. Collectively, our data show that, instead of actively responding to activation by cognate tumor Ag in situ, TIL are quiescent and nonresponsive. There have been many reports of use of TIL in antitumor adoptive transfer experiments both in rodent models (34) and experimental human immunotherapy (35). For that purpose, TIL are isolated and cultured in the presence of high concentrations of IL-2 to expand sufficiently for therapy. Our data is consistent with those publications. If TIL are purified and cultured briefly in the presence of IL-2, they enter the cell cycle and regain tumor-specific lytic function. Tumor-draining lymph node (LN) cells are also used in adoptive immunotherapy protocols (36). LN T cells have a different phenotype in terms of responses to activation with anti-TCR Ab, demonstrating a fundamental difference compared with TIL. LN cells are not primed for AICD, nor are they apoptotic in vivo. We believe that the observed differences reflect the influence of the tumor microenvironment on T cells in situ.

With regard to potential similarity of TIL to memory T cells, TIL express many cell surface markers that are characteristic of memory cells, except TIL are CD69^{high}, whereas memory cells are CD69^{low}. In addition, TIL share one property that is characteristic of CD8^{+} memory cells as defined in two recent studies: rapid secretion of IFN-γ upon TCR ligation in vitro (37, 38). Furthermore, TIL share another characteristic of memory T cells in that the kinetics of activation and the activation threshold are lower than naive T cells; memory T cells rapidly proliferate under suboptimal activating conditions that do not activate naive T cells, although this point is controversial. In contrast to memory cells, TIL die by apoptosis under weaker activation conditions compared with control or memory T cells. Another difference between TIL and memory CD8^{+} T cells is the ability to induce CTL activity; memory CTL require 24 h of ex vivo activation to develop lytic activity (37), whereas, as is seen upon assay of proliferative capacity, when stimulated by TCR ligation in vitro, TIL are nonlytic and undergo apoptotic death. Therefore, although TIL have cell surface markers of memory cells, in distinction to memory cells, TIL are nonproliferative and are primed for AICD.

TIL AICD in vitro is mediated by Fas-FasL interaction, as shown by inhibition of cell death upon inclusion of blocking anti-FasL Ab. Fas-mediated TIL death may occur in situ, as suggested by the observation that TIL appear as isolated cells within tumor. Based upon our in vitro studies, if TIL were closely apposed in situ, Fas-mediated AICD may occur and these T cells would be eliminated. However, this contention may be a peculiarity of the murine tumors studied because of two considerations. First, tumor growth in transgenic mice expressing TCR reactive with cognate tumor Ag accumulate significant TIL populations that are also not apoptotic in situ (S. Radoja, unpublished observation). Second, human tumors frequently have high levels of infiltrating nonapoptotic T cells (39). Therefore, we consider the observation that FasL^{+} TIL are not apoptotic in situ to have an unknown basis. Perhaps, because AICD requires TCR-mediated signal transduction in addition to Fas-FasL interaction, it is possible that TCR-mediated signal transduction is blocked in TIL such that the requisite TCR signal is unable to be transmitted, thereby preventing AICD. Another consideration is that TIL express FasL in situ, which may be an inherent characteristic of TIL, and cognate Ag expression by tumor cells in situ is deficient such that TIL are not being stimulated by Ag recognition. Our data wherein TIL were activated in situ in tumor-bearing mice, resulting in increased TIL apoptosis, supports this contention. These two possibilities are currently being further tested.

The biochemical basis for induction of TIL unresponsiveness within the tumor is not yet understood but has several consequences. On one hand, being nonresponsive, TIL cannot kill antigenic tumors, which confers a growth advantage to tumors. On the other hand, if, upon recognition of cognate tumor Ag, TIL were fully responsive, induction of AICD would likely result and TIL would then be eliminated by apoptosis. There are several reports of apoptotic T cells in primary human tumors, although an accurate assessment of the extent of AICD in situ remains to be determined. Therefore, understanding the factors that influence induction of TIL nonresponsiveness in situ, which includes defining how some TIL can avoid induction of AICD and maintain effector phase functions, is a major objective for tumor immunology.

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References


