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ORIGINAL ARTICLE

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Cytotoxic T cells infiltrating a glioma express an aberrant phenotype that is associated with decreased function and apoptosis

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Abstract In this study, we report on novel alterations found in rat intracranial (i.c.) tumor-infiltrating T lymphocytes (TIL) that are indicative of T cell defects and death. FACS analysis showed that the cytotoxic T cells (CTL) infiltrating rat T9.F gliomas were CD3ε^+, $\alpha\beta$TCR$^+$, CD8α^+, but CD8β^-. These lymphocytes also stained positive for the B cell-specific marker, CD45RA, as well as Annexin-V, signifying apoptotic changes. Functional and biochemical analyses were performed to assess whether the aberrant phenotype was linked to other defects. When CD8α^+ TIL were purified and stimulated in vitro, their proliferative capacity was markedly diminished in comparison with CD3$^+$CD8α^+CD8β^+ T cells isolated from the spleens of naive, non tumor-bearing rats. Furthermore, the mean fluorescence intensity of surface CD3ε was dramatically reduced in the CD3$^+$CD8α^+CD8β^- TIL population as compared with CD3$^+$CD8α^+CD8β^+ TIL from the same tumor-bearing animal. Biochemical studies revealed that the expression of TCRζ and LAT were reduced in lysates generated from CD8α-purified TIL with respect to CD8α-purified T cells from naive spleen. We believe that these degenerative changes are reflective of chronic T cell receptor ligation, because in vitro culture of rat splenocytes or purified T cells with ConA or anti-CD3 mAb induced the same alterations. In vitro, the downregulation of CD8β could be inhibited by the caspase inhibitor, z-VAD. These results suggest that the aberrant CTL phenotype found in the TIL of glioma-bearing rats may be novel signals for their impending death and degenerating anti-tumor immune function.

Key words CTL · Tumor immunity · Apoptosis · Glioma

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Introduction

Gliomas are malignant central nervous system (CNS) tumors derived from the glial lineage. Effective cellular, anti-tumor immunity for a glioma, like for all syngeneic tumors, is dependent upon the efficient activation, clonal proliferation and subsequent lysis of tumor cells by glioma-specific T cells. While it was widely believed that the CNS is an "immune privileged" site [10], it has become increasingly clear that T lymphocytes can enter the CNS in a variety of diseased states [5, 13]. Yet, even when it has been documented that large populations of lymphocytes do breech the blood-brain barrier (BBB) to enter CNS tumors, lysis of glioma cells and eradication of the neoplasm does not occur [29].

Cytotoxic T cells (CTL) constitute the principal immune effector cell-type responsible for the recognition and subsequent destruction of a syngeneic tumor. Numerous tumor-specific CTL epitopes have been identified [1] and evidence suggests that productive CTL responses can induce tumor immunity [3, 9]. Yet, even though glioma-specific markers have been discovered [14, 17, 19], functional responses by glioma epitope-specific CTL have not been shown. Successful generation of peripheral tumor and glioma-specific CTL can be induced by cytokine gene therapy [3, 7, 8, 11, 36], dendritic cell immunotherapy [18, 24], as well

as viral vector immunization strategies [40]; this suggests that tumor-specific antigens can stimulate CTL if the epitopes are presented in a favorable environment. However, the immune response to an untransduced intracranial neoplasm is inadequate and fails to abrogate tumor progression. Failure of glioma-specific CTL to control tumor growth is often thought to result from multiple factors, such as the production of soluble immunosuppressive factors and fas ligand, which can inhibit T cell responses or induce apoptosis, respectively (reviewed in [6]). Thus, the ultimate result of a tumor's immune evasion strategies is an insufficient tumor-specific T cell response to the progressively growing neoplasm.

The purpose of this study was to phenotypically characterize the TIL population infiltrating a rapidly progressing i.c. T9.F glioma and detect changes that might signify degenerative anti-tumor immune function. We believe that the phenotypic changes in the tumor infiltrating CTL population may be a link to the functional T cell defects and promiscuous T cell activation induced cell death (AICD) found in tumor-bearing patients [22, 30, 31, 37]. These findings will hopefully lead to a more comprehensive understanding of the complex interactions between TIL and tumor cells, as well as lead to more promising anti-tumor immunotherapy regimens.

Materials and methods

Animals

Inbred female Fischer 344 rats weighing 120–140 grams were obtained from Harlan Sprague Dawley (Indianapolis, Ind.). Animals were housed in a climate controlled, AAALAC approved vivarium, and were provided free access to food and water. All experimental animal procedures were approved by the Institutional Animal Care and Use Committee. Principles of laboratory animal care (NIH publication No. 85–23, revised 1985) were followed, as well as specific national laws, where applicable.

Cell lines and cell culture

All tumor cell lines were cultured in complete medium (CM) consisting of DMEM (Gibco BRL, Grand Island, N.Y.) supplemented with 10% FBS (Gibco BRL) and non-essential amino acids. Tumor cell lines were maintained as adherent monolayers in culture flasks, incubated at 37 °C in an atmosphere of 5% CO_2, 95% air, and passed biweekly, using 0.5% trypsin. The T9 glioblastoma cell line was provided by Dr. Gale A. Granger, Department of Molecular Biology and Biochemistry, University of California, Irvine, CA. Clone T9.F was isolated from the T9 cell line in a clonogenic assay [8]. Tumor cells were routinely monitored for contamination by mycoplasma, bacteria and fungus. Spleen cell cultures, as well as purified T cells, were cultured in RPMI-1640 supplemented with 10% FBS, L-glutamine, antibiotics, HEPES and 2-ME (all from Gibco BRL).

Tumor implantation

T9.F tumor cells were used for i.c. implantation, as has been previously described [8]. Briefly, animals were anesthetized, the scalp hair was shaved, wiped with betadine and an incision was made over the cranial midline. Animals were placed in a stereotactic apparatus, bregma was located and used as a reference point for injections. A hand-held Dremel drill was used to create a shallow depression 4 mm to the right of the sagittal suture and 1 mm posterior to the coronal suture and 5 µl of the tumor cell suspension (1×10^5 cells) were injected into the posterior parietal lobe of the brain at a depth of 3.5 mm using a Hamilton syringe and a 26 gauge needle secured to the arm of the stereotactic apparatus. The needle track was sealed with bone wax and the incision was closed with surgical staples.

FACS analysis

Single cell suspensions containing lymphocyte populations were routinely generated from surgically removed tissues (i.e. spleens, lymph nodes, gliomas). In general, tissue was aseptically excised and forced through a 70 µm nylon cell strainer (Fischer Scientific, Raleigh, N.C.) using the butt of a 3 cc syringe. The resulting cell suspensions were then separated on a Ficoll density gradient (Histopaque 1077, Sigma, St. Louis, Mo.) and the mononuclear cells located at the interface were collected. Cell suspensions were washed three times with cold 5% FBS-PBS and viable mononuclear cells were enumerated using a hemocytometer and trypan blue exclusion. The resultant cell populations were subjected to a battery of fluorescently-conjugated monoclonal antibodies (mAbs) for FACS analysis, so that the various lymphocyte populations could be investigated. Typically, 1×10^6 cells were stained in V-bottomed, 96-well microplates (Costar Brand, Fisher Scientific, Raleigh, N.C.) in a volume of 50 µl 5% FBS-PBS. Cells were suspended in a cocktail containing three mAbs coupled to FITC, PE or biotin and incubated in the dark for 30 min on ice. The mAbs used were: IgG isotype, CD3ε, CD4, CD8α, CD8β, CD45RA, NKR1.1, $\alpha\beta$TCR and $\gamma\delta$TCR (all from Pharmingen, San Diego, Calif.). After the primary antibody incubation, the cells were washed twice and incubated with streptavidin-PerCP (Becton Dickinson Immunocytometry Systems, San Jose, Calif.), using a 1:10 dilution, for 30 min on ice. Finally, the cells were washed twice, fixed in 1% paraformaldehyde and stored at 4 °C until analysis. Three-color FACS analysis was performed using a Coulter Epics XL-MCL Flow Cytometer (Miami, Fla.).

T cell purification

T cell purification was performed using magnetic bead positive selection (anti-CD8α or anti-PanT Cell-labeled beads, Miltenyi Biotech, Auburn, Calif.). Briefly, for CD8α^+ TIL purification, tumor infiltrating mononuclear cell suspensions were made as detailed above. The lymphocyte fraction was then enriched on a Ficoll density gradient, followed by adherence to tissue culture dishes for one hour, to remove contaminating monocytes or microglia. Ten million splenocytes, or TIL, were then resuspended at 10^7 cells/80 µl, followed by the addition of the magnetic bead-conjugated antibody (anti-CD8α or PanT; 20 µl/10^7 cells). Positively-labeled cells were collected on columns and either used for in vitro studies or lysed for immunoblotting. Purity was assessed by FACS analysis of selected T cell populations and routinely generated between 80 and 90% purity.

Proliferation assays

Purified T cells were cultured at a density of 1×10^6 cells/ml in a total volume of 200 µl on anti-CD3 mAb-coated 96-well plates (mouse anti-rat CD3ε, 10ug/ml) or with 5 µg/ml Concanavalin A (ConA, Sigma). For ConA stimulation, purified T cells were co-cultured with irradiated spleen cells (2500 rad, 1:10 ratio) from naive animals. Cells were cultured for 3 days and pulsed with 1 µCi/ml [^{3}H]thymidine (^{3}H-TdR) for the last 12–18 h. Cells were then harvested on a 96-well plate harvester (Packard, Meriden, Conn.) and ^{3}H-TdR incorporation measured using a beta plate reader (Packard).

Western blotting

Preparation of cell lysates

Equal numbers of purified T cells (typically 3–6x10^6 cells) were lysed in ice-cold lysis buffer containing 50 mM Tris-HCl, 300 mM NaCl, 1% Triton X-100, 2 mM EDTA and a protease inhibitor cocktail (2.5 mM Aprotinin and Pepstatin, 1 μM PMSF, 0.4 mM Na_3VO_4). Insoluble materials were removed by centrifugation for 20 min at 12,000 × g. The protein concentration of lysates was quantified using a micro BCA protein assay kit (Pierce Chemicals, Rockford, Ill.).

Western blotting

10–15 μg of lysate were run on 4–12% NuPAGE MES gels (Novex, San Diego, Calif.) and transferred to nitrocellulose. Immunoblots were blocked with 3% non-fat dry milk, 2% goat serum and 0.1% Tween-20 in PBS for 1°h and then incubated overnight with mAbs to p56^{Lck} (Lck, 1 μg/ml, Upstate Biotechnology, Lake Placid, N.Y.), T Cell Receptor-zeta (TCRζ, diluted 1/100, Santa Cruz Biotechnology, Santa Cruz, Calif.) TCR zeta associated protein-70 (ZAP-70, dil. 1/5000, Transduction Labs, Lexington, Ky.), or Linker for Activation of T cells (LAT, diluted 1/250, Transduction Labs) at 4°C with rotation. The immunoblots were washed three times with PBS containing 0.1% Tween-20, incubated with an HRP-conjugated secondary mAb (goat anti-mouse IgG, diluted 1/3000, Upstate Biotechnology) for 1°h at room temperature and positive bands developed using chemiluminescence (SuperSignal, Pierce). Densitometric analysis was performed using a Molecular Dynamics Personal Densitometer and ImageQuant software.

In vitro activation of splenocyte cultures and assessment of apoptosis

Spleens were aseptically removed from age-matched animals and single-cell suspensions were made. The lymphocyte fraction was then enriched using a Ficoll density gradient. Lymphocytes were cultured in 6-well plates at 1 × 10^6 cells/ml for 4 days, with ConA added at a concentration of 5.0 μg/ml. When purified T cells were used, 2×10^5 PanT-purified T cells were added to each well on anti-CD3 mAb-coated 96-well plates and incubated for 4 days before examination. For inhibition of apoptosis, the caspase inhibitor, z-VAD (Enzyme Systems, Dublin, Calif.), was used at 50 μM. After 4 days, the cells were collected, counted and subsequently stained for the various T cell surface markers. Assessment of apoptotic cells was made using propidium iodide (PI) and Annexin-V labeling (Pharmingen). Briefly, after labeling 1 × 10^6 cells with CD8α^{PerCP} and CD45RA^{PE}, the cells were washed in binding buffer and incubated with Annexin-V^{FITC} (1:5 dilution) for 20 min in the dark at room temperature. Dual labeling of Annexin-V and propidium iodide was also used to distinguish early apoptotic cells from dead cells. The cells were immediately analyzed by FACS.

Statistics

The statistical analysis was performed using the two-tailed, Student's paired *t* test. Differences were considered significant when the calculated *P* value was less than 0.05 ($P < 0.05$).

Results

Phenotypic analysis of T cells infiltrating the intracranial T9.F glioma

We have previously described a highly tumorigenic subclone of the rat T9 glioma cell line and designated it T9.F [8]. Intracranial implantation of 1×10^5 T9.F glioma cells reliably induces large tumor growth, with the consequence that the animal must be euthanized between 20 and 24 days post-implantation. Flow cytometric analysis of T cells isolated from lymphoid organs (spleen and lymph node) of non-tumor bearing rats was initially performed to characterize the T cell surface marker profiles of normal T cells (Fig. 1). Typical CD8$^+$ T cells stain positively for the $\alpha\beta$TCR, CD3ε, as well as the CD8α and CD8β coreceptor heterodimer. In order to examine the phenotype of CTL infiltrating a rapidly progressing i.c. T9.F glioma, TIL were isolated and stained when the animals displayed terminal symptoms from tumor growth. FACS analysis was performed on the TIL, with gating based on the lymphocyte population from the forward and side scatter characteristics of control thymocytes or lymph node cells. The results revealed a large population of TIL that were $\alpha\beta$TCR$^+$, CD3ε^+ and CD8α^{low}, but showed very low, to

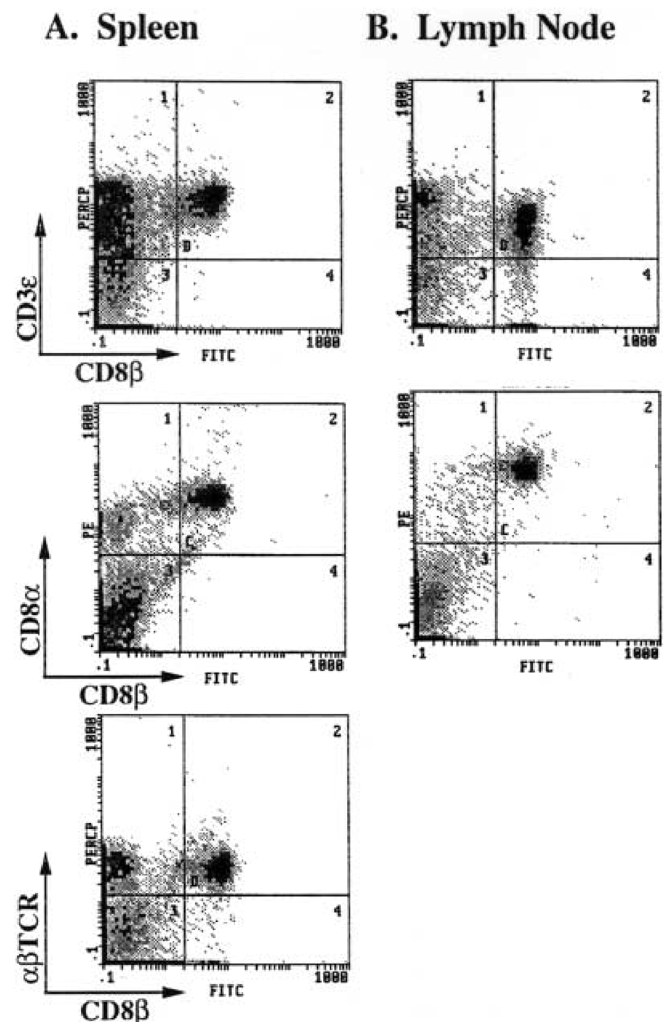

Fig. 1A–B FACS staining of naive, non tumor-bearing CD8$^+$ T cells. Lymphocytes were isolated from the **A** spleen and **B** lymph nodes of naive, non-tumor-bearing rats and stained for: CD3ε and CD8β; CD8α and CD8β; $\alpha\beta$TCR and CD8β

nonexistent levels of CD8β expression (Fig. 2). Natural killer (NK) cells and $\gamma\delta$ T cells are known to express homodimers of CD8α; however, the TIL did not stain positively using mAbs to these surface markers. Furthermore, a large fraction of these TIL stained positive for Annexin-V and misexpressed CD45RA (rat homologue to the murine B220), which is upregulated on dying or defective T cells [12, 28]. The misexpression of CD45RA and Annexin-V positive staining was confined to the TIL showing increased side scatter (Fig. 3). Importantly, the aberrant CTL phenotype was also confined to the TIL population, reflecting changes induced at the site of the tumor, and not typically seen in the peripheral lymphoid tissues (Table 1). These results suggested that the T cells isolated from i.c. gliomas were a phenotypically aberrant and apoptotic, thymus-derived CTL population.

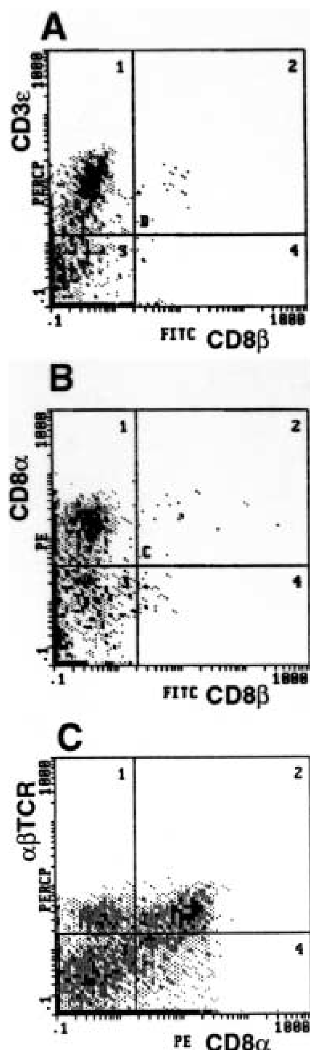

Fig. 2A–B Aberrant CTL phenotypes isolated from i.c. T9.F gliomas. FACS analysis of TIL with staining for **A** CD3ε and CD8β, **B** CD8α and CD8β, and **C** $\alpha\beta$TCR and CD8α, showing a CD3$\varepsilon^{+}\alpha\beta$TCR^{+}CD8$\alpha^{dull}$, but CD8$\beta^{-}$ CTL population. These results are representative of at least five independent experiments

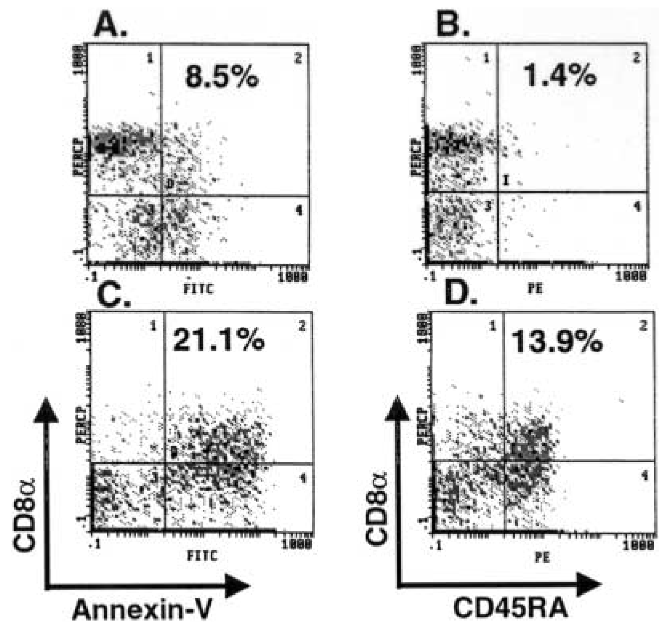

Fig. 3A–D Novel apoptosis markers for CTL in T9.F gliomas. Gating on the population with low side scatter shows relatively few **A** CD8α^{+}Annexin-V^{+} and **B** CD8α^{+}CD45RA^{+} T cells, whereas gating from the population with increased side scatter shows a large increase in the double labeling of both **C** CD8α^{+}Annexin-V^{+} and **D** CD8α^{+}CD45RA^{+} T cells. Annotated numbers reflect the percentage of positively labeled cells from the selected gates. The data are representative of at least three independent experiments

T9.F glioma-infiltrating CTL harbor proliferative and signal transduction defects

To understand whether the aberrant phenotype of the CTL infiltrating the i.c. T9.F glioma could also be linked to impaired effector functions, we purified CD8α^{+} TIL and examined their proliferative abilities. FACS analysis of TIL has consistently demonstrated that greater than 90% of the CTL are CD8α^{+}CD8β^{-} (data not shown); therefore, by purifying CD8α^{+} T cells from the i.c. T9.F TIL, we could generate pure populations of the aberrant TIL population. In response to both ConA and anti-CD3 mAb stimulation, CD8α^{+} TIL demonstrated decreased proliferation in comparison with CD8α^{+} T cells isolated from age-matched, non-tumor bearing spleens (Fig. 4). These CD8β^{-} TIL also exhibited other common characteristics of defective T cells. As shown in Table 1, the mean fluorescence intensity (MFI) of surface CD3ε was uniformly decreased in CD8α^{+}CD8β^{-} TIL (in comparison with CD8α^{+}CD8β^{+} TIL from the same animal; $P < 0.00001$). The decreased expression of surface CD3ε in tumor-associated T cells has been reported previously and linked to T cell death [26]. Furthermore, using lysates made from CD8α-purified TIL, Western blotting showed marked decreases in the expression of the T cell signaling proteins TCRζ and LAT, whereas reductions in Lck and ZAP-70 were not as dramatic (Fig. 5). Thus, the aberrant CTL phenotype does translate to functional deficits and biochemical

Table 1 Decreased expression of CD3ε on CD8α^{+}CD8β^{-} TIL

Animal	MFI[a] CD3ε (CD8α^{+}CD8β^{+})	MFI[a] CD3ε (CD8α^{+}CD8β^{-})	% CD3ε^{+b} CD8α^{+}CD8β^{-}	Mean ± SD % CD3^{+} CD8α^{+}CDβ^{-}
Naive spleen[c]	12.2	10.7	9.4	10.3 ± 1.6
T9.F#1 spleen[c]	10.4	7.4	11.1	
T9.F#1 TIL[d]	6.92	4.66	43.4	44.6 ± 19.1
T9.F#2 TIL	7.48	4.74	71.9	
T9.F#3 TIL	10.8	7.59	30.2	
T9.F#4 TIL	11.6	5.21	32.7	
T9.F#5 TIL[d]	13.1	7.3	42.7	66.6 ± 17.0
T9.F#6 TIL	11.5	7.13	66.4	
T9.F#7 TIL	13.0	7.49	79.7	
T9.F#8 TIL	11.9	6.28	77.7	
T9.F#9 TIL[d]	13.0	7.53	71.2	66.7 ± 6.1
T9.F#10 TIL	11.6	7.44	62.7	
T9.F#11 TIL	14.4	7.02	72.5	
T9.F#12 TIL	16.6	8.74	60.2	

[a] Mean fluorescence intensity
[b] Data refer to the percentage of CD3^{+}CD8α^{+}CD8β^{-} T cells in relation to the total percentage of CD3^{+} T cells in the lymphocyte gate
[c] Data from control and i.c. T9.F-bearing spleens are shown to illustrate that CD8β is not significantly downregulated in peripheral CTL
[d] Data from three independent experiments (n = 4/experiment), in which TIL were analyzed for the expression of CD3ε on CD8α^{+}CD8β$^{+/-}$CTL. Differences between each experimental group were statistically significant; $P < 0.00001$

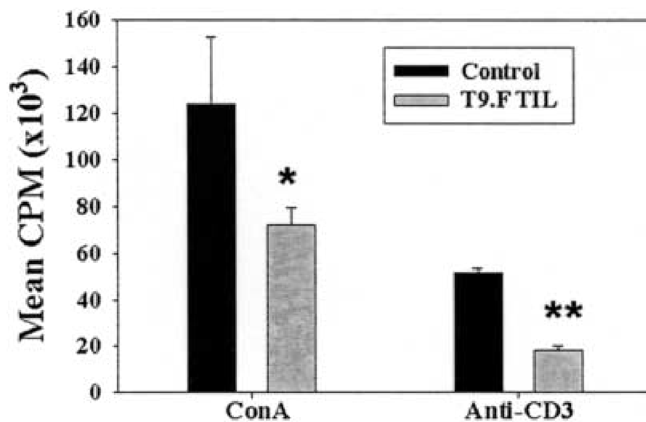

Fig. 4 Aberrant CD3^{+}CD8α^{+}CD8β^{-} TIL display proliferative impairments in response to polyclonal mitogens. CD8α^{+} T cells were purified from i.c. T9.F gliomas and age-matched, non tumor-bearing spleens. Purified T cells were then stimulated, in vitro, with either ConA or anti-CD3 for three days and proliferation assessed with 3H-TdR incorporation. The results shown are representative of two independent experiments. *$P < 0.05$; **$P < 0.03$

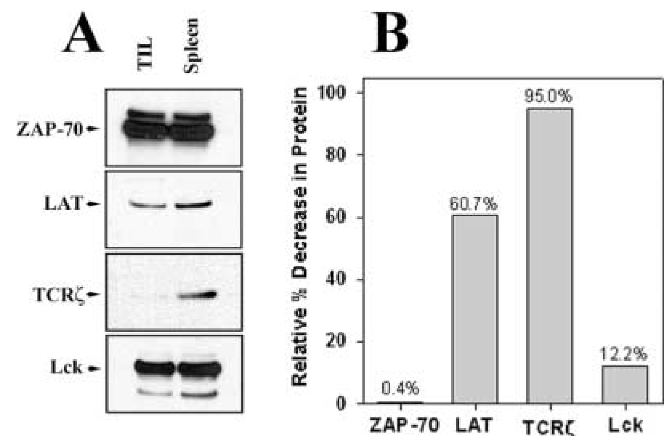

Fig. 5A–B CD8α-purified TIL show decreased expression of T cell signaling proteins. **A** CD8α-purified CTL were isolated from i.c. T9.F gliomas (*left lanes*) and naive spleen (*right lanes*), lysed, and immunoblotted with mAbs specific for ZAP-70, LAT, TCRz or Lck. **B** Data from these blots were analyzed by densitometry and the relative percent decrease (in comparison to control CTL lysates) in the levels of specific signal transduction proteins is shown

alterations previously shown to be characteristic of defective and/or dying T cells. Therefore, we believe that this unique phenotype represents a verifiable population of defective, apoptotic CTL infiltrating an i.c. glioma.

Activation of naive, splenic T cells induces the downregulation of CD8β, misexpression of CD45RA and Annexin-V positivity through a Caspase-dependent pathway

The apoptosis of activated T cells has been reported in several systems [16, 34, 42] and the down-regulation of T cell coreceptors has also been shown both in vitro [12, 28] and in vivo [35]. To determine if the phenotypic and apoptotic alterations seen in the CD8^{+} TIL population were associated with AICD and restricted to CTL infiltrating i.c. gliomas, we cultured normal CD8^{+} T cells with polyclonal mitogens, for extended periods of time, to induce AICD and examined whether these cells exhibited similar phenotypic and apoptotic changes observed in vivo. We found that a significant proportion of activated, splenic CD8^{+} T cells downregulated the expression of CD8β after 4 days of culture with ConA as compared with non-treated CD8^{+} T cells (Fig. 6). The CD3^{+}CD8α^{+}CD8β^{+} population was replaced with

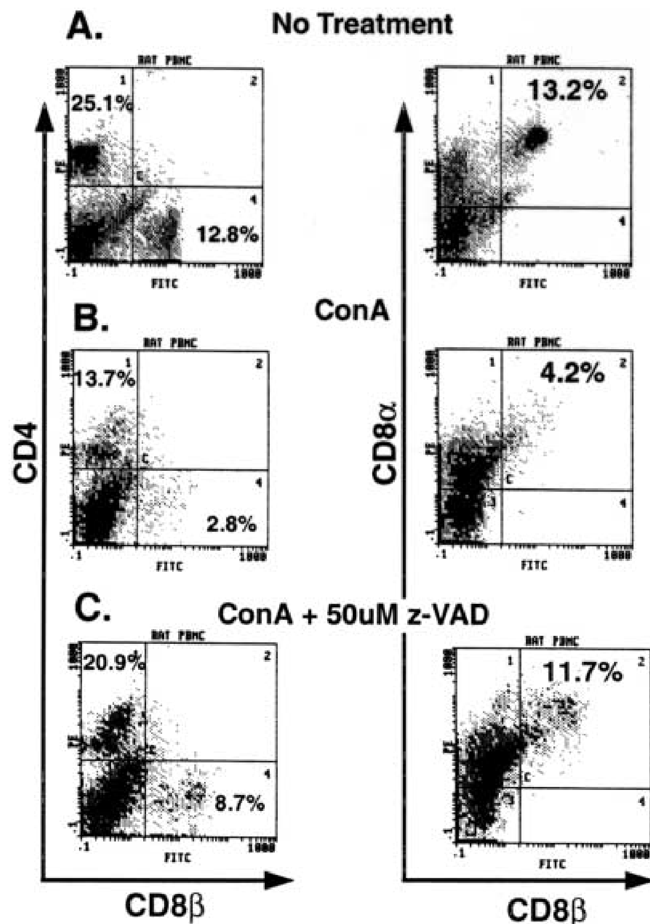

Fig. 6A–C Aberrant CTL phenotype is linked to an apoptotic signaling cascade. Naive spleen cells were cultured with **A** media alone, **B** 5.0 μg/ml ConA, or **C** 5.0 μg/ml ConA + 50 μM z-VAD for 4 days and assayed for their T cell coreceptor expression. Annotated numbers reflect the percentage of positively labeled cells in the lymphocyte gate after 4 days in culture. The results shown are representative of at least three independent experiments

a reciprocal population of $CD3^{+}CD8\alpha^{+}CD8\beta^{-}$ $CD45RA^{+}$Annexin-V^{+} T cells, mirroring the observations of TIL isolated from i.c. gliomas (data not shown). To show that the downregulation of CD8β was not dependent on or influenced by other non-T cell populations, $CD3^{+}$ splenic T cells were purified and activated with anti-CD3 mAb in culture and the results were identical to what was seen in Ficoll-purified, ConA-activated splenocyte cultures. Further data, which suggested that these T cell changes were part of an apoptotic cascade, were provided by the addition of the caspase inhibitor, z-VAD. The addition of z-VAD to the ConA-activated spleen cell cultures was able to prevent and/or rescue the aberrant $CD8^{+}$ T cell phenotype and decreased the loss of CD8β and T cell misexpression of CD45RA (Figs. 6B and 6C). These findings suggested that the loss of CD8β, misexpression of CD45RA and positive staining for Annexin-V may represent novel changes signaling apoptosis for $CD8^{+}$ T cells in the rat.

Discussion

In this study, we present findings that indicate that the CTL population infiltrating the rapidly progressing i.c. T9.F glioma possess phenotypic abnormalities characteristic of cells undergoing apoptosis. These changes may be a result of influences other than the microenvironment of the i.c. glioma, because the same alterations can be induced by in vitro AICD of T cells after long-term ConA or anti-CD3 mAb stimulation. The phenotypic changes of the CTL appear to be reflective of the early stages of apoptosis because this T cell population stains positively for Annexin-V, but is PI negative. The alterations can be inhibited by co-incubation with the caspase inhibitor, z-VAD, which implies that caspase-dependent, apoptotic signaling pathways play a role in the observed T cell alterations. These CTL, when purified and put into culture, can be induced to proliferate, suggesting that they may be rescued from apoptotic death. However, their proliferative abilities are significantly impaired with respect to purified $CD8^{+}$ T cells from non-tumor-bearing animals. Thus, these findings are important because they correlate phenotypic alterations associated with the apoptotic death of T9.F glioma-infiltrating CTL with diminishing CTL function and anti-tumor activity.

In this study, we have shown that $CD8\alpha^{+}$ TIL exhibit decreased expression of the T cell signaling molecules CD3ε, TCRζ and LAT. We have not been able to demonstrate marked decreases in Lck, whose protein levels have been shown recently to be reduced in peripheral T cells from brain tumor patients [23]. However, it has been shown that tumor infiltrating T cells can possess normal protein levels of the relevant T cell signaling molecules, but are impaired in their tyrosine phosphorylation and activity [38]. Interestingly, it has also been shown recently that the critical adapter molecule, LAT, cannot associate with CD8α in the absence of CD8β [2]. It is tempting to speculate that the loss of CD8β in TIL might severely inhibit the ability of the CTL to interact with the major histocompatibility complex (MHC) class I on glioma cells, as well as disrupt the signaling cascade and potentially predispose the T cells towards an autoreactive, apoptotic pathway. On the other hand, CD8β may serve as a survival or signaling factor, whereby its downregulation initiates an apoptotic path.

The mechanisms by which progressively growing tumors evade the host immune response are varied. Selective outgrowth of malignant cells that have reduced expression of the MHC [20], or defects in antigen processing, has been reported [32, 33]. Recently, it has also been reported that the efficiency of tumor surveillance [25], or accessibility of advanced tumors to functional T cell infiltration [9], is compromised in tumor-bearing animals. T cells infiltrating in and around tumors have been shown to progressively develop functional defects related to changes in the expression and/or localization

of proteins involved in signaling through the TCR [4, 21, 23, 38]. The extent of these T cell signaling defects has even been shown to correlate with advanced tumor progression [15, 27, 39]. We believe that the alterations we have found constitute T cell defects related to apoptosis and chronic signaling through the TCR. The chronic state of activation that the TIL experience by long-term exposure to tumor antigens may make them more susceptible to AICD, which has been observed in peripheral blood T cells from i.c. glioma-bearing patients [22] and patients with other peripherally-located tumors [30, 31, 37]. It is possible that continuous activation of glioma-specific CTL may occur because of the abundance of immunogenic T9.F glioma cells located in the confined space of the CNS. The continuous interaction of CTL with glioma-specific peptides may result in chronic ligation and signaling through the $\alpha\beta$TCR/CD3 complex, similar to the in vitro effects of anti-CD3 mAb or ConA. Presumably, the activated CTL express functional Fas and FasL, which subsequently induces autoreactive AICD through an apoptotic pathway [41, 42]. This promiscuous AICD that occurs at the i.c. glioma site may signal the failure of an effective, anti-tumor T cell response and represent a method of tumor evasion. Thus, the population of aberrant, apoptotic CTL at the site of a progressive i.c. tumor is not likely to be contributing to meaningful anti-tumor immune function and could possibly hinder it.

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