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Characterization of Defective CD4−CD8− T Cells in Murine Tumors Generated Independent of Antigen Specificity

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Immune-based therapy confers limited benefits to hosts bearing late-stage tumors. Mounting evidence points to local suppression of T cell function as the most substantial barrier to effective antitumor immunity in hosts with large tumor burdens. Despite this, events responsible for locally defective T cells and immune suppression in tumors remain unclear. We describe in this study a predominant T cell population localized within two murine tumors that is characterized by expression of apoptotic markers and TCRαβ/CD3, but not CD4, CD8, or NK-associated markers. These defective cells resembled double negative (DN) T cells in lpr mice, harbored defects in the expression of T cell signaling molecules, and produced the anti-inflammatory cytokine, IL-10.

Conditions known to increase or decrease the accumulation of lpr DN T cells had corresponding effects on local DN tumor infiltrating lymphocyte (TIL) levels and inversely impacted host survival. Adoptive transfer into s.c. tumors demonstrated that naive CD8+ T cells were highly susceptible to becoming DN TIL, and local supplementation of tumors with nontumor Ag-bearing MHC class I-expressing fibroblasts decreased both this susceptibility and endogenous DN TIL levels. These findings identify a major defective T cell population with suppressive potential within tumors. The data also suggest that local T cell defectiveness is controlled by the tumor environment independent of cognate Ag specificity per se. Decreasing defective DN TIL levels by increasing noncognate peptide MHC class I availability, or modulating TCR or cytokine signaling may facilitate host survival by bolstering endogenous immunity to late-stage tumors, and may help improve therapeutic tumor vaccines. The Journal of Immunology, 2004, 172: 1602–1611.

T lymphocyte-mediated immune surveillance is thought to be one of the principal host defense mechanisms that prevent tumor formation (1). In many rodent tumor models, the mobilization of T cell responses is also sufficient to eradicate tumors early during their establishment (2–4). However, such mobilization is clearly less effective when it occurs late during tumor progression, in both rodent tumor models and human cancer patients (5–7).

Recent studies suggest an inability of functionally normal T cells to infiltrate or persist in late stage solid tumors because of physical barriers and/or emigration from the tumor site (5, 8, 9). Nevertheless, substantial lymphocytic infiltrates including T cells are associated with the vast majority of solid tumors. Despite this, tumor-infiltrating T lymphocytes (TIL)5 typically fail to provide effective antitumor immunity, indicating that functionally defective T cells predominate within tumors. This functional defectiveness has been attributed to various mechanisms, including inadequate Ag-presenting and/or costimulatory capacity of tumor cells leading to T cell ignorance or anergy, respectively (10, 11), and the production of immune suppressive factors by the tumor (12, 13).

A variety of localized defects in T cell signaling molecules have been reported in murine tumor models and human cancer patients. These defects include decreased levels of surface CD3ε, TCRγδ, phospholipase Cγ1, p56lck, p59fyn, and decreased tyrosine phosphorylation of ZAP-70 and phospholipase Cγ1 (14–16). Increases in spontaneous T cell apoptosis associated with intracellular signaling defects have also been described (17–19). Functional and molecular TIL defects alike have been linked to apoptotic cell death induced by ligation of fas on T cells (20, 21). The precise events initiating cell death induction and T cell defect acquisition, however, remain unknown. Defects in tumor-associated T cells represent one of the few immune parameters that can correlate with human tumor outcome (16, 22–24). Therefore, understanding the events giving rise to TIL defects and their potential to exacerbate tumor progression is critical to evaluate the role of endogenous immunity in tumor progression. In addition, such knowledge is critical to determine how to optimally enhance immunity for clinically relevant therapeutic effects.

Because tumor-specific T cells are enriched in tumors, and because tumor-associated T cell defects are often localized within tumors, it is generally assumed that TIL defect generation involves recognition of and/or responsiveness to tumor-associated Ags. This assumption, however, is supported by little direct evidence. This lack of evidence can be traced to the difficulty of accurately quantifying decreases in relatively broadly expressed signaling molecules within an inherently fragile, dying subpopulation of T cells. Defining discrete cellular phenotypes harboring particular signaling molecule defects might provide clues as to how TIL...
defects arise and how to prevent their accumulation, and would allow ready quantification of TIL defects under experimental conditions to directly assess both their derivation and relevance to tumor progression. We describe in this study a defective tumor-associated T cell population that harbors an unusual TCRαβ+CD3ε−CD4−CD8− B220+ double negative (DN) TIL phenotype, whose presence is associated with tumor progression. The lack of coreceptor expression, expression of the B220 isoform of CD45, and positive annexin V staining indicated that these cells were apoptotic. Moreover, DN TIL were deficient in TCRγδ and CD3ε expression, two hallmark defects in murine and human TIL, and they expressed the anti-inflammatory cytokine IL-10, whereas CD4+ and CD8+ TIL and peripheral T cells from tumor-bearing hosts did not exhibit these characteristics. Using this phenotype to quantify locally defective T cells in tumors, we show that the presence of DN TIL is impacted by CD8+ T cells and MHC class I expression, and requires expression of the fyn tyrosine kinase in T cells. Finally, DN TIL presence is enhanced in fas-deficient lpr mice, but diminished by local introduction of nonanitgenic MHC class I, provided it is competent to co-engage TCR and CD8. Thus, the presence of defective and potentially immunosuppressive T cells in tumors follows the same rules establishing the formation of Ag nonspecific CD4−CD8− T cells under generally MHC class I-deficient conditions, and may be reduced without knowledge of tumor-associated Ag identity.

Materials and Methods

Animal specifications

C57BL/6, lpr, and B6.PL mice were obtained from The Jackson Laboratory (Bar Harbor, ME), whereas fynT−/− mice were obtained from R. Perlmutter and M.C. Miceli (Merck, Whitehouse Station, NJ and University of California, Los Angeles, CA, respectively). All were housed in a pathogen-free vivarium. Females aged 6–8 wk were used for tumor implantation, except in experiments involving fynT−/− mice, in which female C57BL/6 control mice were gender- and age-matched (within 1 mo) to the former. Average ages for depicted experiments were 8 ± 0 mo for C57BL/6 and 9.33 ± 1 mo for fynT−/− mice. C57BL/6 and fynT−/− mice both exhibited slightly decreased tumor survival with increasing age in this range.

Tumor cell preparation and injection

Cultured murine GL26 glioma cells were harvested by trypsinization, viable cells counted, and resuspended in 1% methylcellulose before intracranial (i.c.) injection. B16-F10 melanoma cells were similarly harvested and resuspended in 100 µL PBS for s.c. injection. A total of 5000 GL26 tumor cells in 2 µL of 1% methylcellulose were implanted i.c. using a stereotactic rodent frame, with injection 1 mm posterior and 2.5 mm lateral to the junction of the coronal and sagittal sutures (bregma), at a depth of 2 mm. GL26 tumors typically ranged from 15 to 65 mg, but smaller tumors were analyzed in immunohistochemical analyses. For s.c. implantation, 10,000–50,000 tumor cells were inoculated s.c. in 100 µL PBS. B16 tumors typically ranged from 500 to 1200 mg. We have observed identical proportions of DN and other tumor populations within TIL in similarly sized B16 tumors arising from initial injection of 500, 5000, or 50,000 tumor cells (data not shown).

Adoptive transfer of naive CD8 T cells

Splenic CD8 T cells from B6.PL mice were purified using anti-CD8 immunobeads (Miltenyi Biotec, Sunnyvale, CA). CD8 T cells were incubated with a mixture of Abs, FITC anti-CD8a (53-6.7) plus PE anti-CD44 (BD Pharmingen, San Diego, CA), prior to isolation of naive CD8 T cells (CD8+CD44low) by cell sorting using a FACScan flow cytometer (BD Biosciences, San Jose, CA). Nine days after tumor implantation, 106 naive CD8 T cells in 20 µL were adoptively transferred into the established s.c. B16 tumors. Mice were sacrificed the following day and tumors were removed and processed for TIL analysis.

Immunohistochemistry

Frozen glioma and melanoma sections (7 µm) were fixed with acetone, and endogenous peroxidase activity eliminated with 0.3% H2O2/PBS before staining. Nonspecific binding sites were blocked with 5% normal goat serum (Zymed Laboratories, San Francisco, CA). Sections were incubated with a mixture of rat anti-mouse CD4 (GK1.5; BioDesign International, Saco, ME) with rat anti-mouse CD8a (K1T5; BioSource International, Camarillo, CA) followed by a secondary alkaline phosphatase goat anti-rat IgG (Vector, Burlingame, CA) followed by a biotinylated secondary Ab broad spectrum (Zymed). Slides were then reacted with enzyme conjugated HRP-streptavidin (Zymed). CD3-positive cells were revealed by incubating sections with aminooethyl carbazole substrate solution (Zymed). Lack of colocalization of CD3 with CD4 or CD8 demonstrates the presence of DN TIL in situ. Individual staining for CD4 and CD8 was performed as positive controls and normal rat IgG (isotype-matched, BD Pharmingen) or normal rabbit serum (Accurate Chemical and Scientific, Westbury, NY) were used as a negative control.

Coinjection of IL-2-transduced cells with wild-type tumor cells

Control vector- and IL-2-transduced B16 tumor cells (gifts of D. Pardoll, Johns Hopkins University, Baltimore, MD) were admixed in ratios of 100:1, 10:1, 1:1, and 1:10 (control to IL-2). Flanks of mice were injected with 50,000 vector-transduced B16 cells, with one of these ratios of control to IL-2-transduced B16 cells (50,000 cells total), or with 50,000 IL-2-transduced B16 cells alone in 100 µL PBS, and tumors harvested when they attained a size of 1 cm3. Tumor sizes were statistically indistinguishable between groups exhibiting DN TIL differences (p = 0.49 for wild-type vs IL-2-secreting tumors with decreased DN TIL; p = 0.64 for IL-2-secreting tumors with decreased DN TIL vs other IL-2-secreting tumors, in two-tailed t-tests). Tumors were processed and TIL prepared for flow cytometric analysis.

TIL preparation and staining

Tumors were removed from mice, minced with a scalpel, and physically dissociated by passage through progressively smaller diameter hypodermic needles (18–27 gauge), and single-cell suspensions generated. Resident lymphocytes (TIL) were counted, and 500,000 TIL incubated with mixtures of fluorochrome- or biotin-conjugated mAbs recognizing TCRγδ (GL3), TCRβ (H57-597), CD3ε (145-2C11), CD4 (RM4-5), CD8α (53-6.7), NK1.1 (PK136), CD45R/B220 (RA3-6B2), and/or CD2 (RM2 ) , and with Streptavidin-PerCP secondary (all BD Pharmingen). TIL were distinguished from tumor cells based on forward/side light scatter gate established from healthy and/or autochthonous host spleen cell preparations. Apoptosis was detected by incubating 106 cells (2 × 10^6/µl) in PBS plus 2% serum for 10–15 min, 25°C with Annexin V-FITC (BD Pharmingen) plus anti-CD3ε, and B220, followed by incubation with secondary, washing, and immediate analysis. Parallel stains with propidium iodide were performed to verify specificity. L-Kb+ and L-Kb+227 were stained with mAb recognizing H-2Kb (20-8-4S) or control Ab, washed, and analyzed. For the intracellular IL-10 staining, TIL (1 × 10^6 in 50 µl FACS buffer = 1 × PBS, 2% FCS) were stained with optimal concentrations of anti-CD3-PE, anti-CD4-bio and anti-CD8-bio mAbs 30 min on ice, washed, and incubated with Streptavidin-PerCP to label T cell surface molecules. Cells were then washed twice and permeablized by incubation in fixation/permeablization buffer 20 min on ice without inhibition of protein transport. Permeablized cells were washed and resuspended in permeablized cell washing buffer (PermWash; BD Pharmingen) and incubated with anti-IL-10-FITC (JES3-9D7) or isotype-matched control mAb-FITC diluted 1/50 in PermWash for 30 min on ice. Cells were washed twice and resuspended in PermWash and three-color flow cytometry performed. All comparative data were analyzed on a FACSCalibur flow cytometer with equivalent gating and voltage.

Results

DN TIL phenotype

We initially characterized TIL from two distinct tumors, i.e., GL26 gliomas and s.c. B16 melanomas growing in syngeneic C57BL/6 mice, using FACS analysis. As shown, 14–84% of TIL from i.c. GL26 (Fig. 1a) and s.c. B16 (Fig. 1b) tumors expressed CD3ε+, but were devoid of CD8 and CD4. CD3ε+CD4−CD8− (DN) proportions within TIL (%DN TIL) varied broadly across this 14–84% range, with roughly equal proportions of animals exhibiting a percentage of DN TIL cells in total TIL (GL26 or B16) falling into each of four quartiles: 14–31%; 32–49%; 50–67%; and 68–84%. The
FIGURE 1. Characterization of CD4⁺CD8⁻ DN TIL in murine tumors. Flow cytometric analysis of TIL in i.c. GL26 (a) and s.c. B16 (b) tumors. Fresh TIL were stained for CD4, CD8, CD3e, TCRαβ, TCRγδ, NK1.1, CD2, B220, and annexin V. Plots represent data from single tumor-bearing mice, with the exception of the annexin V plots and the B220/TCR plots for the B16 tumor, which were obtained from separate individual tumors and were derived from identical electronic gating and voltage. Variation in the percentage of B220⁻ (DN) T cells from mouse to mouse accounts for the discrepancy in proportions of B220⁻ cells in the TCRγδ relative to other plots. The TCRγδ⁺ B220⁻ population from 13 GL26 and B16 tumors exhibited from 3 to 18% variation relative to TCRαβ⁺ B220⁻ cells from the same individual mice, with the lower (3–6%) variation observed exclusively when TCRαβ and TCRγδ were analyzed together in the same sample. Although some CD4⁺ and/or CD8⁺ (single positive) TIL as well as CD4⁺CD8⁻ DN TIL were present outside of the gates used for analyses, DN TIL never occupied a lower proportion of T cells than in the gates depicted. Data depicted are representative of at least four independent experiments, with analogous findings in over 50 tumor-bearing mice.

most consistent finding with respect to the CD3e⁺CD4⁻CD8⁻ population, however, was that they comprised a much greater proportion (85–99%) of infiltrating T cells (rather than total TIL) in almost all (80–90%) GL26 and B16 tumors from mice with terminal tumor symptoms (Fig. 1, a and b and data not shown). DN TIL outside of the depicted forward/side scatter gates consistently represented equal or greater proportions of total CD3e⁺ cells as those depicted within these gates, refuting the possibility that we were observing a nonrepresentative fraction of T cells. Further characterization revealed that CD3e⁺ CD4⁺CD8⁻ cells expressed TCRαβ, but not TCRγδ on their surface and were therefore TCRαβ lineage, CD4⁺CD8⁻ T cells (Fig. 1, a and b). CD4, CD8 DN TIL did not express markers of NK cells, such as NK1.1 (Fig. 1, a and b), or DX5 (data not shown). Greater than 90% of CD3e⁺ CD4⁺CD8⁻ TIL also expressed B220, whereas non-DN tumor-infiltrating T cells did not express B220, a marker usually present on B cells, as well as on T cells undergoing apoptosis (25). Confirming their apoptotic status, DN TIL bound annexin V (Fig. 1, a and b).

The DN TIL phenotype represented the vast majority of tumor infiltrating T cells undergoing apoptosis in both i.c. GL26 (Fig. 1a) and s.c. B16 tumors (Fig. 1b). The s.c. B16 tumors generally harbored increased DN TIL compared with i.c. GL26, when their proportions were considered within total infiltrating lymphocytes (Fig. 1, a and b).

Immunohistochemical identification of DN TIL in tumors

To verify that the CD3e⁺CD4⁺CD8⁻ DN TIL phenotype was evident in situ, we examined tumor tissue by immunohistochemistry. T cell infiltration into i.c. GL26 gliomas was observed throughout the tumor tissue. Few T cells were present in the adjacent normal tissue. The i.c. GL26 tumor sections harbored sizable proportions of CD3e⁺ cells (Fig. 2, d–i), and the proportion of these cells expressing CD4 or CD8 decreased with increasing tumor size (Fig. 2, d–i); 90–95% in the smallest tumor (Fig. 2, d and g), 50–65% in the intermediate sized tumor (Fig. 2, e and h), and <30% in the largest tumor (Fig. 2, f and i). Double staining of CD4 plus CD8 (blue) and CD3e⁺ (red) confirmed the presence of CD3e⁺CD4⁺CD8⁻ T cells in situ (Fig. 2, d–i). This population was not an artifact of Ab cross-blocking or failure of one of the coreceptor Abs (Fig. 2, a and b). Together these data qualitatively verify flow cytometry results, confirming the presence of a CD3e⁺CD4⁺CD8⁻ T cell population within murine gliomas in situ. A similar population was also observed within B16 tumors (data not shown). Evidently, the formation of these dying T cells
is accelerated, their emigration impaired, or their clearance delayed, within tumor tissue.

**Signaling defects in DN TIL**

Tumor infiltrating T cells have been shown to exhibit defects in the expression of multiple signaling molecules. The accumulation of these T cell signaling molecule defects has been correlated with tumor stage in various human cancers (22–24) and with tumor size in malignant glioma patients (16). Because T cell signaling molecule defects have been associated with T cell apoptosis, and because apoptotic DN TIL represented the vast majority of murine tumor-infiltrating T cells, we hypothesized that DN TIL might account for some of these defects. To test this, we examined specific molecules known to be down-regulated in defective tumor-associated T cells. We focused on molecules whose expression levels could be easily quantified in conjunction with surface phenotypic markers in fresh TIL, to avoid extensive manipulation of potentially fragile cells that could compromise quantification.

Decreased TCRζ expression and surface CD3ε intensity, as well as decreased Bcl-2 expression are recognized characteristics of defective tumor-associated T cells (20, 26–28). Although some GL26 gliomas and s.c. B16 melanomas exhibited decreased TCRζ expression, surface CD3ε intensity, and Bcl-2 expression relative to peripheral CD4/CD8+ T cells from the same mice (Fig. 3a). CD4/CD8+ TIL exhibited higher levels of TCRζ and surface CD3ε relative to DN TIL, comparable to those of peripheral T cells in the same mice (Fig. 3a). Although not all CD4/CD8+ T cells expressed high TCRζ levels, their pattern of expression was similar to that of peripheral cells (Fig. 3a). Similarly, CD4/CD8+ TIL from GL26 tumors exhibited Bcl-2 levels comparable to those of peripheral CD4/CD8+ T cells from the same mice, but CD4/CD8+ TIL from B16 tumors exhibited little Bcl-2 (Fig. 3a). Although the basis for this latter discrepancy is unknown, it is consistent with the demonstration in Fig. 1 that all B16-infiltrating T cells are annexin V+, whereas some GL26-infiltrating T cells appear to be annexin V−, which is independent of their CD4/CD8+ TIL proportions (5.08% of total TIL in B16; 1.34% of total TIL in GL26 tumors depicted). Taken together, these data indicate that DN TIL uniquely represent tumor-associated T cells harboring multiple molecular signaling defects similar to those associated with fas-mediated T cell death in human cancers. Whereas other tumor-infiltrating T cells may have initiated cell death pathways, as evidenced by decreased Bcl-2 expression and potential apoptosis (Fig. 3a), they do not harbor the same spectrum of signaling defects as DN TIL.

Local IL-2 production by tumors is known to prevent tumor-associated T cell defects, as well as modulate the function of lpr DN T cells (29–31). To further validate that DN TIL uniquely represent tumor-infiltrating defective T cells, we assessed the impact of local IL-2 production by B16 tumor cells on DN TIL presence. IL-2 production was not studied in GL26 tumors due to the marked i.c. toxicity of this cytokine that results from vascular leakage (32, 33). Because IL-2-transduced B16 cells do not form tumors (2), we compared the time to palpable tumor formation (roughly 1 cm3) induced by the injection of various ratios of IL-2-transduced B16 cells (B16/IL-2) with vector-transduced B16 cells (B16/wild-type). Local low IL-2 secretion (1:100, B16/IL-2 to B16/wild-type) induced the same rapid tumor formation and DN TIL accumulation as found in B16/wild-type tumors (Fig. 3b). Progressively higher local IL-2 secretion induced statistically distinct times to tumor formation. Moreover, some animals with higher ratios of B16/IL-2 tumor cells failed to develop tumors entirely (Fig. 3b). Associated with the delayed tumor formation was a progressive reduction in DN TIL accumulation in animals that received increasing ratios of B16/IL-2 tumors (Fig. 3b). Thus, the proportion of tumor-infiltrating T cells that exhibited the DN TIL phenotype was significantly decreased in B16/IL2 plus B16/wild-type-implanted animals relative to mice implanted with B16 tumor alone (Fig. 3b). Delayed tumor formation and DN TIL accumulation was not due to reduced numbers of wild-type B16 tumor cells implanted, because all control animals receiving similar amounts of wild-type B16 tumor cells alone developed tumors with unchanged DN TIL proportions (Fig. 3b). In addition, TIL numbers per milligram tumor were not significantly different among any tumor groups (wild-type B16 alone, 2.7 ± 1.2 × 105 TIL/mg tumor; 1:100 B16/IL-2 to B16/wild-type, 1.2 ± 0.65 × 105 TIL/mg tumor; 1:10 B16/IL-2 to B16/wild-type, 2.6 ± 0.93 × 105 TIL/mg tumor; 1:1 B16/IL-2 to B16/wild-type, 1.9 ± 0.79 × 105 TIL/mg tumor; 1:10 B16/IL-2 to B16/wild-type, 3.0 ± 2.9 × 105 TIL/mg tumor). Therefore, dilution of an unchanging number of DN TIL by much greater numbers of proliferating non-DN T cells cannot easily account for decreased proportions of DN TIL in B16/IL-2-containing tumors. These findings support the similarity of DN TIL to previously characterized tumor-associated signaling molecule-defective T cells, whose levels are also diminished by IL-2 (30, 31).

The extent of T cell defects can predict more aggressive behavior of tumors, suggesting that they impede beneficial antitumor T cell function. The question of whether T cell defects passively or actively impede antitumor immunity, however, is unresolved. The prevention of substantial DN TIL presence by IL-2 suggests that type 2 T cell polarization may be involved in tumor-associated T cell defects. In this context, PBMC from certain cancer patients exhibit coordinate modulation of defects such as decreased TCRζ chain expression together with production of the type 2 cytokine.
IL-10 (34). IL-10 is traditionally a cytokine secreted by Th2 cells. However, CD8+ T cells can be induced to secrete IL-10 after incubation with IL-4 (35), which can subsequently induce the down-regulation of CD8 expression and inhibit cytotoxicity. Thus, it was particularly intriguing that DN TIL expressed low but detectable levels of intracytoplasmic IL-10, whereas other T cells in tumor-bearing hosts did not (Fig. 3c). Moreover, intracytoplasmic IL-10 could not be detected in non-TIL from GL26 or B16 tumor cell suspensions, suggesting that DN TIL are the major source of IL-10 in both these tumors (data not shown). IL-10 is known to dominantly inhibit T cell function in general, and has been demonstrated to mediate the specific immune suppressive and tumor-promoting properties of tumor/TIL suspensions in the s.c. B16 tumor model (36). The identification of DN TIL as major producers of IL-10 in GL26 and B16 tumors therefore strongly suggests that DN TIL actively inhibit local antitumor T cell activity and could promote tumor progression. Thus, DN TIL presence may constitute an important mechanism of immune escape deployed at the local level.

**Correlation of DN TIL proportion with tumor size and host survival**

Solitary rather than metastatic tumor models are expected to better reflect the impact of local tumor phenomena in general on disease progression. GL26 gliomas are localized and nonmetastatic, whereas B16 melanomas are highly disseminated. In this context, DN TIL levels were directly and precisely proportional to tumor size in GL26-bearing mice, and inversely proportional to both phenotypically normal CD4/CD8+ T cells and host survival (Figs. 2 and 3d). Thus, the putative impact of DN TIL presence on tumor growth is evident in GL26-bearing mice. Moreover, the extent of systemic T cell signaling defects parallels tumor size in malignant glioma patients (16). Therefore, these data also validate that murine DN TIL harbor tumor-associated signaling defects similar to those in analogous human tumors. The increase in DN TIL proportions with increasing GL26 tumor size mimicked the apparent decrease in all CD3+ cells seen by immunohistochemistry in progressively larger GL26 tumors, which may represent clearance of an increasing proportion of apoptotic T cells in ever larger tumors.
Although there was no significant correlation between size and DN TIL levels in B16 tumors (data not shown), this also raised the question of whether extrinsic environmental parameters such as hypoxia in large tumors, or intrinsic T cell properties accounted for aspects of DN TIL presence. In part to address this issue, we examined DN TIL presence in mice with intrinsic T cell defects.

Regulation of DN TIL levels by fas and the TCR-linked kinase, Fyn

Due to their inherently apoptotic and fragile nature, large numbers of DN TIL could not be purified to directly assess their role in tumor progression. Therefore, we sought to modulate their levels in vivo to determine their relevance to tumor progression, as well as to further examine the factors influencing their derivation. Given their similarity to lpr DN T cells and unique possession of T cell signaling molecule defects, we hypothesized that DN TIL levels might be modulated by conditions known to affect lpr DN T cells in general.

Absence of CD2 expression, a molecule involved in T cell activation that is present on normal murine T cells, established that DN TIL were phenotypically identical to the largest and most impaired population of CD4+CD8+ T cells accumulating in autoimmune-prone lpr mice. Consistent with functional similarity to lpr CD4+CD8+ T cells, DN TIL presence was significantly enhanced in tumors growing in lpr mice relative to simultaneously implanted size-matched tumors growing in wild-type C57BL/6 mice (Fig. 4a). These findings suggest that fas either mediates the clearance or inhibits the formation of DN TIL.

Crossing lpr mice with those harboring a targeted disruption of the fyn gene reduces lpr DN T cell accumulation and markedly improves host survival (37, 38). We asked whether fyn-deficiency in T cells altered DN TIL levels and host survival in the i.c. GL26 tumor model. Fyn−/− mice exhibit a T cell-specific deficiency in fyn expression that is manifested as a substantial TCR signaling deficit in thymocytes, but not in peripheral T cells (39). Fyn−/− mice harboring i.c. GL26 gliomas displayed significantly fewer DN TIL than simultaneously implanted age-matched wild-type mice (p = 0.014; Fig. 4b). The proportion of CD4/CD8+ TIL in i.c. GL26 gliomas was not, however, significantly altered in tumors growing in fynT−/− mice relative to simultaneously implanted size-matched tumors growing in wild-type C57BL/6 mice (Fig. 4a). These findings suggest that fas either mediates the clearance or inhibits the formation of DN TIL.

Mechanisms giving rise to DN TIL could reveal a predominant pathway of death-dependent T cell defectiveness in tumors (20, 21), and elucidating such mechanisms could reveal potential targets for enhancement of antitumor immunity. To gain further insight into the mechanisms of DN TIL formation, it was necessary to identify T cell populations susceptible to becoming DN TIL. Similarities between DN TIL and DN lymphocytes arising from lpr mice prompted us to ask whether DN TIL might originate from the CD8+ lineage, as do DN T cells in lpr mice (40). We were unable to significantly induce the DN T cell phenotype in vitro after incubation of T cells with anti-Fas mAb in the presence or absence of tumor supernatant or with tumor supernatant alone (data not shown). Therefore, to address the issue of CD8+ T cell susceptibility to becoming DN TIL we implanted GL26 tumors in β2-microglobulin knockout (β2m−/−) mice and compared the percentages of DN TIL per milligram tumor to those from wild-type C57BL/6 mice. β2m−/− mice have substantially reduced numbers of CD8+ T cells (41) and when implanted with i.c. GL26 tumors,
were implanted into wild-type and \( \beta_{2m}^{-/-} \) mice. Values of DN TIL per milligram tumor are represented for wild-type (○) mice \((n = 12)\) and for \( \beta_{2m}^{-/-} \) (●) mice \((n = 12)\). Average percentages of infiltrating DN T cells (heavy bar) per milligram tumor were 0.55% and 0.19% for wild-type and \( \beta_{2m}^{-/-} \) mice, respectively \((***, p < 0.001, \text{two-tailed} \ t \ \text{test})\). Tumor sizes were \(122.5 \pm 36.6\) mg and \(190.2 \pm 22.4\) mg for wild-type and \( \beta_{2m}^{-/-} \) mice, respectively \((p = 0.06, \text{single-tailed} \ t \ \text{test})\). b, Adoptive transfer of CD8\(^{+}\)CD44\(^{low}\) T cells into B16 tumors. CD8\(^{+}\) T cells from B6.PL mice were immunomagnetically purified and then cell sorted for low CD44 expression. Purity was assessed by FACS analysis. Naive status of cells was confirmed by staining for CD8, CD4, CD3\(^{-}\), CD62L, and CD45RB. c, Phenotype of CD8\(^{+}\) T cells after transfer. Purified CD8\(^{+}\)CD44\(^{low}\) T cells \((10^6 \ \text{cells})\) were adoptively transferred into B16 tumors growing in wild-type mice. Thy1.1\(^{+}\) donor cells were analyzed. Transferred cells had lost CD8 expression by 48 h after transfer. Data are representative of more than three experiments.

To corroborate these findings, we adoptively transferred Thy-1 congenic, naive (CD44\(^{high}\)) CD8\(^{+}\) T cells into B16 tumors growing in wild-type mice. We used only s.c. B16 tumors due to the ready visualization and targeting of tumor tissue afforded by this model, as well as their relatively high DN TIL levels. Donor cells were CD62L\(^{+}\) and CD45RB\(^{+}\) confirming their naive status (Fig. 5b), and within 48 h, >99% of them were devoid of CD4 or CD8 (Fig. 5c). This indicated that the local tumor environment is sufficient to confer the DN TIL phenotype to any naive wild-type CD8\(^{+}\) T cell. Although preferential migration into tumors may be afforded to Ag-specific T cells, this suggests that defectiveness of T cells within tumors, per se, is not necessarily contingent upon their Ag specificity or prior Ag reactivity.

**Regulation of DN TIL accumulation by syngeneic MHC class I**

Given the enhanced presence of DN TIL in lpr mice, mechanisms thought to underlie coreceptor down-regulation in lpr T cells might also be relevant to DN TIL. In addition, decreased DN TIL levels in the context of T cell-specific fyn deficiency suggested that receptor signaling in T cells also influenced DN TIL levels. Moreover, acquisition of the DN TIL phenotype by adoptively transferred naive CD8\(^{+}\) T cells into tumors suggested that DN TIL formation was not necessarily dependent on cognate Ag specificity. DN T cells can arise in wild-type or TCR transgenic mice under MHC class I-deficient conditions or those in which TCR and CD8 cannot co-engage noncognate MHC class I ligands that signal T cell survival (42). CD8\(^{+}\) T cells from lpr mice are particularly susceptible to CD8 loss under such conditions (42). Lack of TCR plus CD8 co-engagement of MHC class I results in extinguished CD8 expression, up-regulation of both fas and fas ligand, and consequent triggering of apoptosis. GL26 and B16 tumor cells exhibit
relatively low MHC class I expression, and the level of MHC class I expressed correlated inversely with the extent of DN TIL presence in these tumors (Fig. 6a and data not shown). The inability of TCR and CD8 on tumor infiltrating T cells to co-engage noncognate MHC class I (due to local MHC class I deficiency) was therefore an attractive candidate to account for DN TIL presence in i.c. GL26 gliomas and s.c. B16 melanomas. To test the validity of this, we coinfected murine fibroblasts (L cells) transfected with constructs encoding self-type MHC class I (H-2K\(^a\)), or mutated MHC class I (H-2K\(^{b/A227}\)) incapable of engaging TCR-MHC class I-CD8 coreceptor complexes (43), together with GL26 or B16 tumor cells into syngeneic mice. Both L cell transfecants express identical allogeneic MHC class I and differ only in H-2K\(^b\) expression, which is syngeneic with respect to the tumor host mice. Moreover, these L cell transfecants are not professional APC, cannot cross-present tumor Ags to specific tumor-infiltrating T cells, and are in this respect nonantigenic. The level of H-2K\(^b\) expression on L cell transfecants (H-2K\(^b\) or H-2K\(^{b/A227}\)) was also similar, and substantially exceeded H-2K\(^b\) levels on GL26 or B16 tumor cells (Fig. 6a).

Mice coinfected with tumor cells plus H-2K\(^b\)-transfected L cells displayed significantly decreased DN TIL presence compared with mice injected with tumor alone, and most tumor infiltrating T cells were CD4/CD8\(^-\), under these conditions (Fig. 6b). Similarly, mice coinfected with tumor cells plus H-2K\(^b\)-transfected L cells displayed significantly decreased DN TIL presence relative to those coinfected with H-2K\(^{b/A227}\)-transfected L cells (Fig. 6c). The effect of H-2K\(^b\) in decreasing DN TIL levels was more pronounced in GL26 tumors that express higher endogenous levels of MHC class I relative to B16 tumors (Fig. 6a), suggesting that MHC class I on tumor cells may also influence DN TIL levels. Similar to tumor-bearing fyn\(^{-/-}\) mice, C57BL/6 mice that received H-2K\(^b\)-transfected L cell tumor cell coinjections displayed a greater than 50% long-term survival, but this could not be distinguished from an effect of local allogeneic L cells alone on host survival (data not shown). In addition, coinjection of H-2K\(^b\)-transfected, but not H-2K\(^{b/A227}\)-transfected L cells plus Thy-1-congenic naive CD8\(^+\) T cells into established s.c. B16 tumors led to retention of increased levels of normal CD4/CD8\(^-\) T cells (Thy1.1/ B220\(^-\)) of both donor and recipient strains (Fig. 7). This indicates that noncognate MHC class I acts directly on CD8\(^-\) T cells to prevent their acquisition of the DN TIL phenotype, and further suggests that endogenous DN TIL are predominantly derived from MHC class I-reactive (i.e., CD8\(^+\)) T cells.

**Discussion**

In this study, we have shown that the predominant population of T cells infiltrating two distinct types of tumors (i.e. GL26 glioma and s.c. B16 melanomas) is phenotypically aberrant and apoptotic (annexin V\(^+\)), expressing CD3\(^e\), TCR\(\beta\), and B220, but not CD4 nor CD8. The apoptotic characteristics led us to first test whether these DN TIL were similar to the apoptotic, signaling-defective T cells seen in tumor-bearing animals and patients (17–20, 28). We found that DN TIL were unique among tumor infiltrating T cells in their down-modulation of TCR\(\zeta\) and surface CD3\(^e\), two hallmarks of TIL defectiveness in tumors, whereas distinct tumor-infiltrating T cells could also exhibit Bcl-2 down-modulation and/or apoptosis in some tumors. The accumulation of DN TIL also correlated precisely with the size of GL26 gliomas, similar to the correlation between tumor size and T cell signaling defects in malignant glioma patients (16). Long-term animal survival and proportions of DN TIL in tumors responded to the local secretion of IL-2, suggesting that the extent of defective T cells can predict the aggressiveness of the tumor. Formation of defective CTL at the tumor site could theoretically impede an existing antitumor response through passive or active means. As such, it was interesting that the DN TIL population expressed IL-10, further consistent with this population’s contribution to an immunosuppressive local tumor microenvironment that actively favors tumor growth over T cell activation. Together, these results suggest that DN TIL represent a discrete phenotype marking a defective T cell population within murine tumors. The identification of a surrogate for defective T cells allowed for further examination of parameters that modulate their formation and presence within tumors.

To better understand the relevance of DN TIL levels to tumor progression, as well as parameters important for their formation, we tested whether we could modulate DN TIL levels in vivo. As previously mentioned, the local secretion of IL-2 decreased the accumulation of DN TIL and the survival of these animals directly correlated with levels of DN TIL. IL-2 has previously been shown to ameliorate the extent of tumor-associated T cell defects (30, 31), as well as modulate the function of lpr DN T cells (29). Because of the similarity of the DN TIL phenotype to lpr DN T cells, as well as their enhanced presence in tumors growing in lpr mice, we asked whether mechanisms involved in coreceptor down-regulation in lpr T cells might also be relevant to DN TIL.

In this context, we first tested whether fyn signaling could regulate DN TIL accumulation. FynT\(^{-/-}\) mice harboring i.c. GL26 gliomas not only had significantly reduced levels of DN TIL, but their survival was also significantly enhanced, with at least 50% of the animals failing to develop tumors. These findings suggest that T cell signaling through fyn is critical for the coordinated formation of DN TIL together with the promotion of tumor formation. Next, we tested whether the availability of MHC class I, which exacerbates DN T cell accumulation in lpr mice, also influenced
the accumulation of DN TIL. We found that \( \beta_{m}m^{-1} \) mice implanted with i.c. GL26 gliomas harbored significantly lower percentages of DN TIL when normalized for tumor mass relative to wild-type mice. DN TIL modulation in \( \text{fyn}\gamma^{-/-} \) or \( \beta_{m}m^{-1} \) hosts was evident either in the absence of any difference in tumor size (\( \text{fyn}\gamma^{-/-} \)) or after factoring out tumor size differences (\( \beta_{m}m^{-1} \)), indicating the independence of such modulation from tumor size and associated hypoxia. Finally, we demonstrated that the availability of syngeneic MHC class I at the tumor site could prevent the accumulation of DN TIL and enhance survival, and that this prevention was dependent on the ability of MHC class I to coordinate bind TCR and CD8. Hypoxia due to increased tumor size did not play any role in decreased DN TIL following introduction of class I-transfected L cells into tumors, because this short-term manipulation did not impact tumor size in any way.

Thus, at least four independent methods established that DN TIL are T cells in general and MHC class I-restricted (i.e., CD8\(^{+}\) ) T cells in particular: surface marker expression, behavioral similarity to well-defined and phenotypically similar T cell populations, modulation in mice genetically deficient for T cell-specific molecules and those involved in T lineage development, and responsiveness to local MHC class I. These data also suggest that tumor-associated T cell defectiveness is a function of local deficiency in self-MHC class I in general, can be independent of tumor Ag specificity, and can be substantially counteracted without knowledge of specific Ag responsiveness in two distinct murine tumors. Because the tumors chosen for analysis expressed low to nearly absent levels of MHC class I, we cannot exclude the possibility that additional mechanisms may operate to prevent tumor rejection in tumors with relatively high MHC class I expression or those otherwise dissimilar to B16 or GL26. The function of noncognate MHC class I in B16 and GL26 tumors may be to superefy fyn-dependent triggering of apoptosis in infiltrating CD8\(^{+}\) T cells. Taken together, our findings suggest that similar mechanisms underlie the formation of DN T cells in autoimmune, as well as in the formation of defective DN TIL in cancer. In this light, both phenomena appear to be dependent on noncognate MHC class I capable of coordinately binding TCR and CD8 as well as on IL-2, two parameters associated with T cell survival (30, 31, 42, 44).

This helps justify that decreasing DN TIL or manipulating mechanistic parameters controlling their levels, such as fyn signaling, MHC class I availability, coreceptor activity, or perhaps T cell survival signaling, may be useful in augmenting endogenous or induced antitumor immunity to effectively treat tumors.

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