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Tumor-Infiltrating Lymphocytes Exhibiting High Ex Vivo Cytolytic Activity Fail to Prevent Murine Melanoma Tumor Growth In Vivo¹

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The identification of tumor-associated Ags recognized by CD8⁺ CTL and prevention of tumor outgrowth by adoptive transfer of these CTL demonstrates that CD8⁺ T cells play a major role in antitumor immunity. We have generated B16.F10 melanoma cells that express the glycoprotein epitope amino acid 33–41 (GP33) of the lymphocytic choriomeningitis virus (LCMV) to examine antitumor CD8⁺ T cell response in C57BL/6 mice immune to LCMV and in mice transgenic for the LCMV GP33-specific P14 TCR (P14 TCR mice). We find that B16.F10_{GP33} tumor cells grew in syngeneic C57BL/6 mice without inducing T cell tolerance. LCMV infection or adoptive transfer of LCMV-specific effector T cells delayed but did not prevent growth of preestablished tumors in these mice. However, B16.F10_{GP33} tumor cells were rejected in mice immune to LCMV and in mice treated with LCMV-specific effector T cells on the same day as the tumor. Surprisingly, B16.F10_{GP33} tumor cells grew in P14 TCR transgenic mice despite an abundance of tumor-associated Ag-specific CD8⁺ T cells. In these mice, freshly isolated tumor-infiltrating lymphocytes exhibited an activated phenotype and displayed high GP33-specific cytolytic activity when assessed ex vivo. Thus, B16.F10_{GP33} melanoma cells are able to initiate, but not to sustain, a GP33-specific CTL response sufficient to clear the tumor enduringly. *The Journal of Immunology*, 1998, 161: 2187–2194.

The lack of efficient immune responses to control tumor growth may be due to a variety of reasons. The loss of or decreased expression of MHC molecules at the tumor cell surface may reduce the immunogenicity of the tumor (1, 2). Efficient T cell activation requires both a signal delivered through the Ag-specific TCR and a costimulatory signal triggered by direct interaction between accessory molecules at the T cell surface and their respective ligands expressed on APCs. The lack of costimulatory molecules on the surface of most tumor cells may result in partial activation of tumor-reactive T cells and lead to their deletion or unresponsiveness (3). The production of immunosuppressive molecules such as TGF- β by tumor cells may also contribute to the escape of tumors from immunologic control (4, 5). Alternatively, defective lymphocyte homing into the tumor may account for the failure of the immune system to mediate an efficient CTL activity at the tumor site (6). Fas ligand expression on the surface of tumor cells, such as melanomas, may induce apoptosis of T cells entering the site of tumor growth and thus explain the immune privilege of these tumors (7, 8). A low precursor frequency of tumor Ag-specific T cells may result in a lower rate of expansion of the reactive T cells in comparison with tumor growth and pre-

vent tumor eradication. Finally, tumor-specific T cell responses may prevent tumor cell growth, but may select for tumor Ag-negative variants in vivo (9).

It is established that an antitumor immune response occurs in various malignancies. Different cell types, including NK cells, dendritic cells, macrophages, helper T cells, and CTL can cooperate to generate an immune response. Several tumor-associated Ags recognized by CD8⁺ T lymphocytes have been identified (reviewed in Ref. 10). Long-term T cell lines derived from the tumor mass, expanded and differentiated in the presence of IL-2, have been shown to specifically recognize tumor Ags and to exhibit MHC-restricted lysis specific for autologous tumor cells (11–13). Consistent with the predominant role of CD8⁺ T cells in antitumor immunity, adoptive transfer of tumor-specific CTL has been shown to prevent tumor outgrowth or to result in marked regression of established tumors (14, 15). It is likely that the CTL activity of T cell lines or clones derived from tumor-infiltrating lymphocytes (TIL)³ is due to the addition of high doses of IL-2 for their maintenance. Antitumor CTL responses have also been generated from PBMC, spleen cells, and tumor-draining lymph nodes for the development of immunotherapies for cancer treatment (16). In vitro stimulation of these cells with tumor Ag in the presence of exogenous cytokines was required to detect a significant CTL activity.

Many attempts have been performed to analyze TIL obtained directly from fresh tumors. However, functional studies using ex vivo-isolated TIL are difficult because of the low yield of the lymphocytes recovered. In the present report, we describe a melanoma model with a defined tumor-associated Ag to study tumor-specific

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³ Abbreviations used in this paper: TIL, tumor infiltrating lymphocytes; LCMV, lymphocytic choriomeningitis virus; B6, C57BL/6 mice; P14 TCR mice, LCMV GP33-specific TCR transgenic mice; aa, amino acid.

CD8⁺ T cells freshly isolated from a tumor mass. B16.F10 melanoma cells were transfected with a minigene encoding the sequence of residues 33 to 41 (GP33 peptide) of the glycoprotein from the lymphocytic choriomeningitis virus (LCMV). These B16.F10_{GP33} melanoma cells, which expressed the H-2D^b-restricted GP33 CTL epitope, were transferred s.c. into syngeneic C57BL/6 (B6) mice and into B6 mice transgenic for the LCMV GP33-specific P14 TCR (P14 TCR mice) (17). Using this new model we addressed the following questions: 1) Are there specific CD8⁺ T cells infiltrating the tumor mass? 2) Do these TIL exhibit specific cytolytic activity or are they tolerated by the tumor cells? 3) Can TIL induce tumor regression? 4) does the CTL response select for Ag-loss variants in vivo? and 5) do GP33-specific T cells induced by a LCMV infection in vivo mediate antitumor activity on preestablished tumors?

Materials and Methods

Mice

B6 mice were obtained from our breeding colony or from Harlan Winkelmann (Borchen, Germany). The P14 TCR mice, line 318 and line 327, expressing a V α 2/V β 8 TCR specific for amino acids (aa) 33–41 (GP33 peptide, KAVYNFATM) of the LCMV glycoprotein in association with the H-2D^b molecule, have been described previously (17, 18). Line 318 expresses the transgenic TCR on 50 to 60% of CD8⁺ T cells and has a normal CD4 to CD8 ratio, whereas line 327 expresses the transgenic TCR on 85 to 95% of CD8⁺ T cells and has a highly skewed CD4 to CD8 ratio. Line 327 back-crossed onto the D^b mutant mouse strain B6.C-H-2^{bm13} (K^b, D^{bm13}) has been described previously (19). Male and female mice of between 8 and 16 wk of age were used. Mice were bred and kept in a conventional mouse house facility.

Cell lines

The murine melanoma cell subline B16.F10, originally established by Prof. I. Fidler (20), was provided by Dr. H. Pahl (Klinik für Tumorbologie, Freiburg, Germany). B6.F10_{GP33} tumor cells were derived from parental B16.F10 cells by gene transfection using transfectam (Promega, Mannheim, Germany). The LCMV GP33 minigene was generated by cloning primers Kbg146 (5'-CC GGT GCC ACC ATG AAA GCT GTG TAC AAT TTC GCC ACC TGT TGA G-3') and Kbg147 (5'-GAT CC TCA ACA GGT GGC GAA ATT GTA CAC AGC TTT CAT GGT GGC A-3') corresponding to the LCMV GP33 epitope (M-KAVYNFATM) in the *PinAI* and *BamHI* restriction sites of the β -actin-driven expression vector pActin-IRES-TK-Neo (NTS) (21). The resulting transfectants were cloned and screened for GP33 expression by CTL assays. The B6.F10_{GP33} melanoma line (clone 20) used in this study was kept in culture under G418 (200 μ g/ml) (Life Technologies, Gaithersburg, MD) selection. EL-4 (H-2^b), a thymoma cell line, was used as target cells. Cells were cultured in DMEM high glucose, supplemented with 10% heat-inactivated FCS, non-essential amino acids, glutamine, streptomycin, and penicillin (all from Life Technologies).

Tumor cell inoculation

Mice were injected s.c. into the right flank with 10⁶ B16.F10 or B16.F10_{GP33} tumor cells in 100 μ l PBS. Mice were checked for the presence of a palpable tumor, and tumor growth was measured every third day with a caliper. Tumor size was calculated as the product of bisecting tumor diameters. Mice bearing a tumor with a diameter >15 mm were killed according to animal resegmentation. Experimental groups consisted of three to five mice.

Virus

The LCMV-WE isolate used in this study was originally obtained from Dr. Lehmann-Grube (Heinrich Pette Institut, Hamburg, Germany). It was then propagated on L929 fibroblast cells and quantified by virus plaque assay as described (22). Mice were infected i.v. with 200 plaque-forming units of LCMV-WE.

TIL isolation

TIL were purified from tumors (100–150 mm³) grown in B6 or in P14 TCR transgenic mice. After mincing fresh tumors, TIL were isolated by centrifugation over a Ficoll gradient (Pharmacia, Uppsala, Sweden). T cells were purified using immunomagnetic separation (Macs, Miltenyi Biotec,

Bergisch-Gladbach, Germany) on RS⁺ columns (Miltenyi). Magnetic beads conjugated with anti-Thy-1.2 mAb were used as recommended by the manufacturer (Miltenyi). From 1 to 3 \times 10⁵ TIL were recovered per B6 mouse infected with LCMV or per P14 TCR transgenic mouse bearing B16.F10_{GP33} tumors. The isolated cells consisted of 40 to 60% of CD8⁺ T cells, as judged by anti-CD8 Ab staining.

Flow cytometry

Staining was performed in PBS containing 2% FCS, 0.1% NaN₃. For detection of TCR transgenic T cells, splenocytes and TIL were incubated with FITC-conjugated anti-CD8 mAb (clone 53; Life Technologies), phycoerythrin-conjugated anti-TCRV α 2 mAb (PharMingen, San Diego, CA) and biotinylated anti-TCRV β 8 mAb (PharMingen), followed by second-step Tricolor-streptavidin (Caltag, South San Francisco, CA). For the characterization of surface markers, TIL and spleen cells were stained with FITC-labeled anti-CD8, and biotinylated anti-CD62L, or anti-Fas mAb (all from PharMingen), followed by phycoerythrin-streptavidin (Caltag). Cells were analyzed on a FACSort flow cytometer (Becton Dickinson, Mountain View, CA) using CELLquest software.

Generation of primary CTL response in vitro

Responder spleen cells (5 \times 10⁶) from P14 TCR mice (line 318) were cultured with B6 stimulator spleen cells (2 \times 10⁶) coated with 10⁻⁶ M GP33 peptide in 1.5 ml of Iscove's modified Dulbecco's medium containing 10% FCS supplemented with antibiotics and β -mercaptoethanol in 24-well culture plates at 37°C. After 3 days, cells were harvested and CTL activity was determined.

Cytotoxicity assay

Cytolytic activity of spleen cells as well as freshly isolated TIL was assayed in a standard ⁵¹Cr-release assay as previously described (23). Briefly, EL-4, B16.F10, and B16.F10_{GP33} were used as target cells. EL-4 and B16.F10 target cells were coated with GP33 peptide or with the control D^b-binding adenovirus peptide 234–243 (E1A peptide, SGPSNTPEI) at a concentration of 10⁻⁶ M and were labeled simultaneously with 250 μ Ci ⁵¹Cr for 2 h at 37°C. Afterward, they were washed three times in RPMI 1640 medium. Spleen cells and TIL used as effector cells were resuspended in DMEM/10% FCS and were incubated in 96-well round-bottom plates with target cells at various ratios in a final volume of 200 μ l. After a 5-h to 15-h incubation period at 37°C in 5% CO₂, 70- μ l supernatants were harvested and counted. The ratio of CD8⁺ T cells to target cells was calculated from flow cytometric analysis of effector cells. CTL activity of in vitro-stimulated spleen cells against B16.F10 tumor cells was measured in a 6-h ⁵¹Cr-release assay. Activated spleen cells were resuspended at various concentrations to give final effector CD8⁺ T cells to target cell ratios ranging from 100:1 to 0.02:1. Ex vivo CTL activity of spleen cells and TIL against EL-4 target cells was measured in a 15-h assay. Results were expressed as percentage of specific lysis = (experimental release (cpm) – spontaneous release (cpm))/(maximum release (cpm) – spontaneous release (cpm)) \times 100.

Adoptive transfer of LCMV-immune cells

Spleen cells (5 \times 10⁷) containing 40 to 55% CD8⁺ T cells were collected from LCMV-immune B6 mice 8 days after LCMV infection and were injected i.v. in a volume of 0.5 ml medium without FCS into B6 mice bearing small, palpable, or no tumors.

Results

Expression of the LCMV GP33 T cell epitope on transfected B16.F10 melanoma cells

We have used the immunodominant CD8⁺ T cell epitope of LCMV (glycoprotein aa 33–41 = GP33) in H-2^b mice as a tumor-associated model Ag. B16.F10 melanoma (H-2^b) cells were transfected with a minigene encoding the H-2D^b-restricted LCMV GP33 epitope and expression was tested by ⁵¹Cr release assays using GP33-specific CTL. As shown in Figure 1a, GP33-transfected B16.F10 melanoma cells (B16.F10_{GP33}) were efficiently lysed by day 8 LCMV-immune effector cells from B6 mice and also by in vitro-activated spleen cells from P14 TCR mice, which express GP33-reactive TCR. The CTL activity of effector cells was GP33 specific, since these cells also lysed B16.F10 target cells loaded with the GP33 peptide (Fig. 1b), but did not recognize these

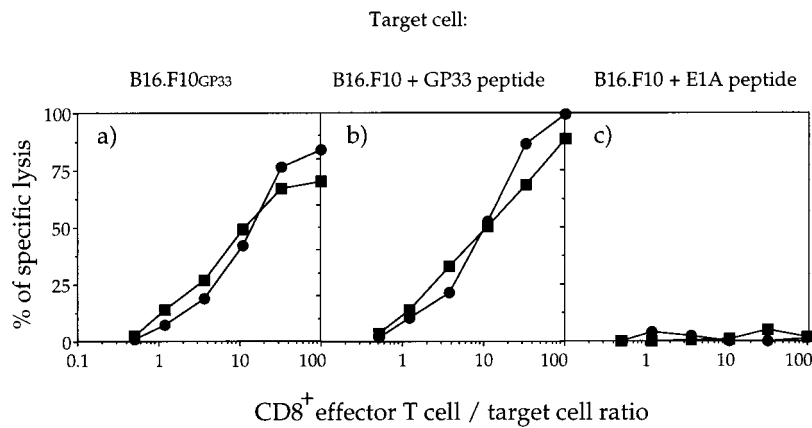


FIGURE 1. Expression of the LCMV GP33 minigene in transfected B16.F10_{GP33} melanoma cells. B6 mice were infected with LCMV. Eight days later, spleen cells were used as effector cells (closed circles). Spleen cells from P14 TCR mice were stimulated in vitro with GP33-coated B6 spleen cells. Three days later, these cells were used as effectors (closed squares). The cytolytic activity of these effector cells was tested against B16.F10_{GP33} target cells (a) and against B16.F10 target cells coated with GP33 peptide (b) or with the adenovirus E1A peptide (c) in a 6-h ⁵¹Cr release assay at various effector CD8⁺ T cell to target cell ratios. Spontaneous release in all assays was <20%. These results are representative of three independent experiments.

target cells coated with an irrelevant D^b-binding peptide from the adenovirus (Fig. 1c). Thus, the B16.F10_{GP33} transfectant used in this study was efficiently recognized both by polyclonal LCMV-specific CD8⁺ T cells from B6 mice and by monoclonal CD8⁺ T cells from P14 TCR mice.

B16.F10_{GP33} melanoma grows in naive B6 and in P14 TCR mice but not in LCMV-immune B6 mice

To assess the effect of the GP33 expression on B16.F10 tumor growth, naive and LCMV-immune B6 mice were inoculated s.c. with parental and GP33-transfected melanoma cells. As shown in Figure 2, a and b, both parental and GP33-transfected melanoma cells grew at a similar rate in naive B6 mice. In contrast, LCMV-immune B6 mice that had been infected with LCMV 6 to 8 wk before tumor cell injection were completely protected against tumor growth of B16.F10_{GP33} but not of parental B16.F10 melanoma cells (Fig. 2, c and d).

To examine whether a high number of GP33-specific CD8⁺ T cells can prevent B16.F10_{GP33} tumor establishment in vivo, parental and GP33-transfected melanoma cells were injected into P14 TCR mice (line 318), which express the transgenic GP33-specific TCR on 50 to 60% of CD8⁺ T cells. Surprisingly, B16.F10_{GP33} tumor cells grew in P14 TCR mice (Fig. 2f). The day on which palpable B16.F10_{GP33} tumors could be detected varied; B16.F10_{GP33} tumors were detected 1 to 3 wk later in the P14 TCR mice, when compared with the B16.F10 tumor growth (Fig. 2e). However, once the tumor was established, B16.F10_{GP33} cells grew as quickly in P14 TCR mice as in naive B6 mice. Similar results were obtained when P14 TCR mice from line 327 (90% of CD8⁺ T cells express the transgenic TCR) were inoculated with B16.F10 and B16.F10_{GP33} tumor cells (Fig. 2, g and h).

To test whether the delay in tumor take in P14 TCR mice was due to direct presentation of the GP33 peptide by B16.F10_{GP33} melanoma cells or due to cross-priming involving host APC, P14

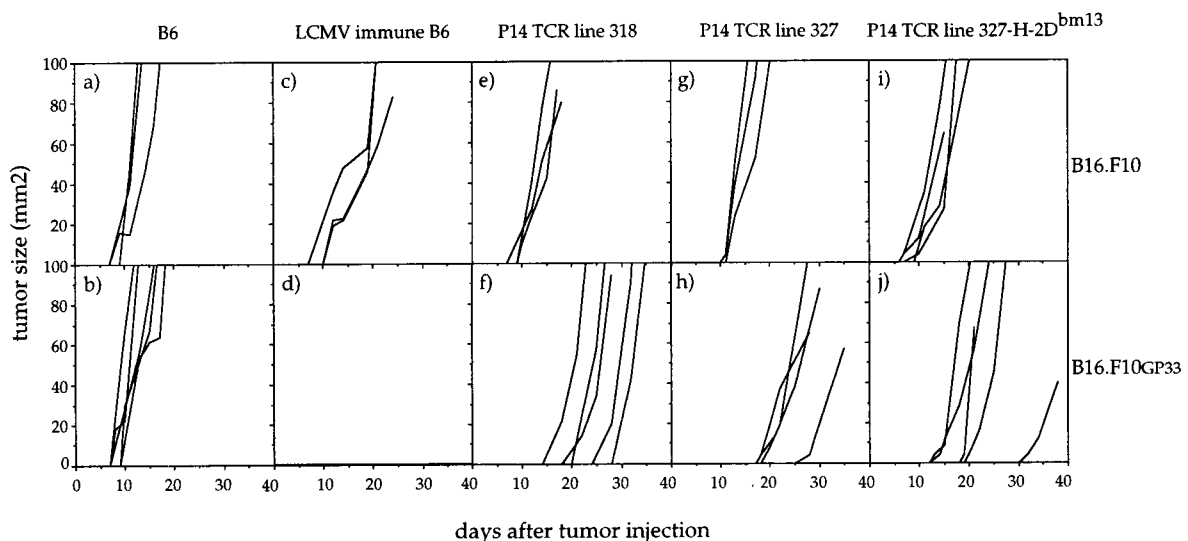


FIGURE 2. B16.F10_{GP33} tumor cells grow in naive B6 and in P14 TCR mice but not in LCMV-immune B6 mice. B16.F10 (a, c, e, g, and i) or B16.F10_{GP33} (b, d, f, h, and j) tumor cells (10⁶) were injected s.c. into the right flank of age-matched B6 mice (a and b), LCMV-immune B6 mice (>60 days after LCMV infection) (c and d), P14 TCR mouse line 318 (e and f), P14 TCR mouse line 327 (g and h), and P14 TCR mouse line 327-D^{bm13} (i and j). Three to five mice per group were used. After the detection of a palpable tumor, tumor growth was measured every third day and mice bearing a tumor with a diameter >15 mm were killed.

TCR mice (line 327) exhibiting a mutation in their D^b molecule (H-2D^{bm13}) were analyzed. The mutant D^{bm13} molecule cannot present the GP33 peptide but can positively select the GP33-specific transgenic TCR (19). Appearance of palpable B16.F10_{GP33} tumors in P14 TCR D^{bm13} mice was delayed when compared with parental B16.F10 tumors (Fig. 2, *i* and *j*), suggesting direct presentation of the GP33 peptide by B16.F10_{GP33} tumor cells in vivo.

To exclude the possibility that tumor cells that grow in P14 TCR mice represent Ag loss variants, B16.F10_{GP33} tumors were isolated from P14 TCR mice and tested in vitro for GP33 expression by ⁵¹Cr release assays using GP33-specific CTL. A total of 15 out of 16 freshly isolated B16.F10_{GP33} tumors were efficiently recognized by GP33-reactive T cells, indicating that B16.F10_{GP33} tumors from P14 TCR mice are not Ag loss variants (not shown). Taken together, these data show that despite the high frequencies of GP33-specific T cells in P14 TCR mice and the ability of activated TCR transgenic cells to lyse B16.F10_{GP33} tumor cells, B16.F10_{GP33} tumors grew progressively in P14 TCR mice. In striking contrast, B16.F10_{GP33} tumors did not grow in LCMV-immune B6 mice that exhibit 100-fold lower frequency of GP33-specific CTLp which, however, have been primed by LCMV infection.

TIL but not peripheral T cells from tumor-bearing P14 TCR mice exhibit ex vivo GP33-specific cytolytic activity

One possibility to explain B16.F10_{GP33} tumor growth in P14 TCR mice may be the inefficiency of in vivo activation of GP33-specific CD8⁺ T cells by B16.F10_{GP33} melanoma cells, which lack costimulatory molecules. To determine whether B16.F10_{GP33} melanoma cells were able to activate GP33-specific T cells in vivo, ex vivo cytolytic activity of spleen cells and of TIL directly isolated from the fresh tumors of P14 TCR mice was determined. As shown in Figure 3, *a* and *b*, spleen cells from tumor-bearing P14 TCR (line 318 and line 327-D^{bm13}) did not lyse target cells pulsed with GP33 peptide. Similar results were obtained from inguinal lymph nodes draining the tumor (data not shown). In contrast, TIL isolated from these mice exhibited high GP33-specific lytic activity (Fig. 3, *c* and *d*). The ex vivo cytolytic activity of TIL from tumor-bearing P14 TCR mice was comparable with that of LCMV-specific CTL from spleens of day 8 LCMV-immune B6 mice on a cell per cell basis (Fig. 3, *e* and *f*). No cytolytic activity of TIL from P14 TCR mice, which bore parental B16.10 tumors, could be detected (not shown). The finding that TIL isolated from tumor-bearing P14 TCR H-2D^{bm13} mice exhibit GP33-specific lytic activity further supports the view that B16.F10_{GP33} melanoma cells directly activated TCR transgenic T cells without cross-priming in vivo.

Cell surface markers of TIL from B16.F10_{GP33} tumor-bearing P14 TCR mice were analyzed by flow cytometry. As shown in Figure 4 (*right*), CD8⁺ T cells isolated from TIL displayed an activated phenotype with increased forward scatter, down-regulated TCR and CD62L, and up-regulated Fas/CD95 expression. In contrast, peripheral T cells isolated from spleen (Fig. 4, *left*) and inguinal lymph nodes (not shown) from these tumor-bearing P14 TCR mice exhibited a naive phenotype. Furthermore, 28% of CD8⁺ T cells isolated from TIL of a B16.F10_{GP33} tumor-bearing P14 TCR mouse expressed CD25 (IL-2R) whereas only 4 to 5% of the CD8⁺ T cells from spleen or lymph node of the same mouse expressed this marker.

LCMV infection of B6 mice bearing a palpable B16.F10_{GP33} tumor delays but does not prevent tumor growth

LCMV infection of B6 mice induces a strong GP33-specific CTL response. It was therefore of interest to examine whether this ef-

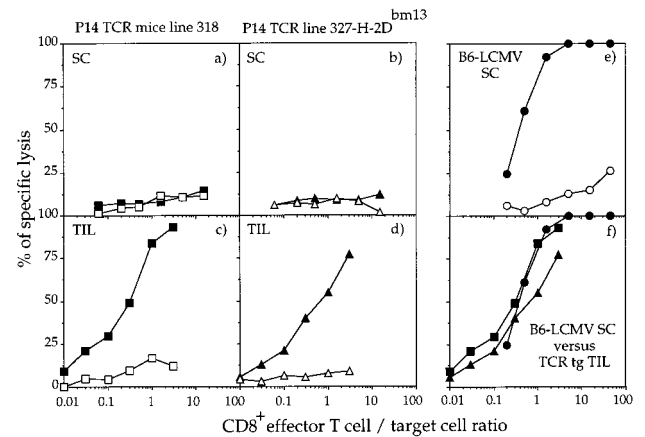


FIGURE 3. TIL, but not peripheral T cells from P14 TCR mice bearing B16.F10_{GP33} tumor, display ex vivo cytolytic activity against GP33-target cells. B16.F10_{GP33} tumor cells (10⁶) were injected s.c. into P14 TCR transgenic mice: line 318 (*a* and *c*) and line 327-D^{bm13} (*b* and *d*). Cytolytic activity of spleen cells (*a* and *b*) and TIL (*c* and *d*) isolated from the P14 TCR mice was tested against EL-4 target cells loaded with the GP33 peptide (closed squares and triangles) or with the adenovirus E1A peptide (open squares and triangles) in a 15-h ⁵¹Cr release assay at the CD8⁺ E:T ratios indicated. The TIL were collected from a pool of three B16.F10_{GP33} tumors for both P14 TCR transgenic lines. The cytolytic activity of day 8 LCMV-immune effector cells from B6 mice (*e*) against the same target cells loaded with the GP33 peptide (closed circles) or with the adenovirus E1A peptide (open circles) was assessed under the same conditions. In *f*, GP33-specific cytolytic activity of TIL from P14 TCR mice and of spleen cells from day 8 LCMV-immune B6 mice was compared. Spontaneous release in all assays was <20%. All experiments were performed three times with similar results.

ficient CTL activity was able to control preestablished B16.F10_{GP33} tumors. Parental and GP33-transfected B16.F10 tumor cells were injected s.c. into naive B6 mice. After detection of palpable tumors (day 9–10 after tumor cell injection), mice were infected with LCMV and tumor growth was measured. As shown in Figure 5*a*, two out of four parental B16.F10 tumors grew more slowly in B6 mice after the LCMV infection when compared with uninfected mice. Such a result could be explained by bystander effects of the viral infection (e.g., NK cells induced by INF- γ). The data from Figure 5*b* shows that the delay of B16.F10_{GP33} tumor growth was evident very early (in contrast to Fig. 5*a*) after LCMV infection in all mice analyzed (5/5). However, 1 to 2 wk after LCMV infection, tumor growth of B16.F10_{GP33} cells was comparable in uninfected and LCMV-infected B6 mice. To test the possibility that the tumor cells that grow in LCMV-infected mice represent Ag loss variants, B16.F10_{GP33} tumors were isolated and tested. As shown in Figure 5*c*, all four B16.F10_{GP33} tumors, freshly isolated from LCMV-infected B6 mice, were efficiently recognized by LCMV effector cells from B6 mice, indicating that B16.F10_{GP33} tumors in LCMV-infected B6 mice are not Ag loss variants.

Failure of LCMV-specific CTL to control preestablished B16.F10_{GP33} tumor growth is not due to tolerance induction

It has been suggested that tumor cells tolerize tumor-specific T cells. We therefore examined whether GP33-specific CTL could be induced in B16.F10_{GP33} tumor-bearing B6 mice by LCMV infection. Control B6 mice and B6 mice bearing palpable B16.F10 or B16.F10_{GP33} tumors were infected with LCMV, and 10 days later the cytolytic activity of spleen cells and TIL was assessed by ⁵¹Cr

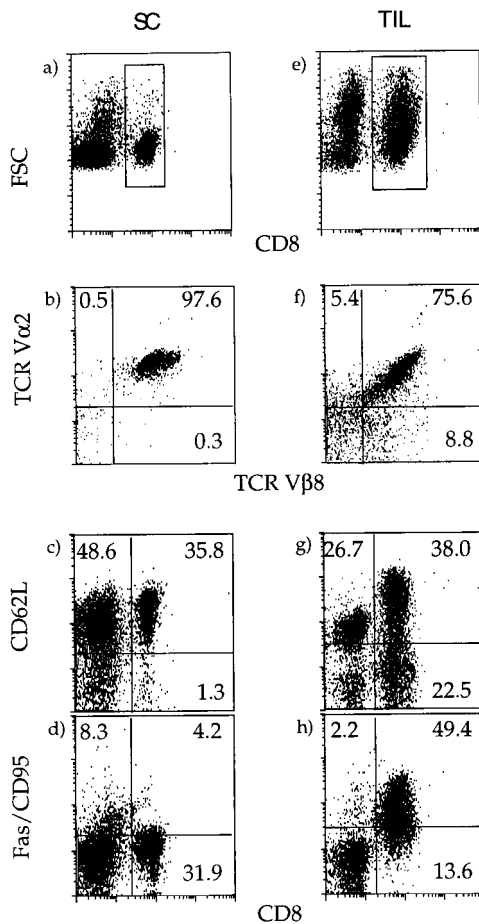


FIGURE 4. Cell surface phenotype of peripheral T cells and of TIL from B16.F10_{GP33} tumor-bearing P14 TCR mice. B16.F10_{GP33} tumor cells (10⁶) were injected s.c. into the flank of P14 TCR mice (line 327-H-2D^{bm1.3}). Fifteen days after tumor cell transfer, mice were killed and spleen cells (*a–d*) and TIL (*e–h*) were isolated and analyzed by flow cytometry. *a* and *e*, Forward scatter (FSC) vs CD8 expression; *b* and *f*, transgenic TCR Vα2/Vβ8 expression gated on CD8⁺ T cells; *c* and *g*, CD62L (L-selectin) vs CD8 expression; and *d* and *h*, CD95(Fas) vs CD8 expression.

release assay. As shown in Figure 6, spleen cells from LCMV-infected B16.F10_{GP33} tumor-bearing mice (Fig. 6c) exhibited high GP33-specific CTL activity comparable with that of control B6

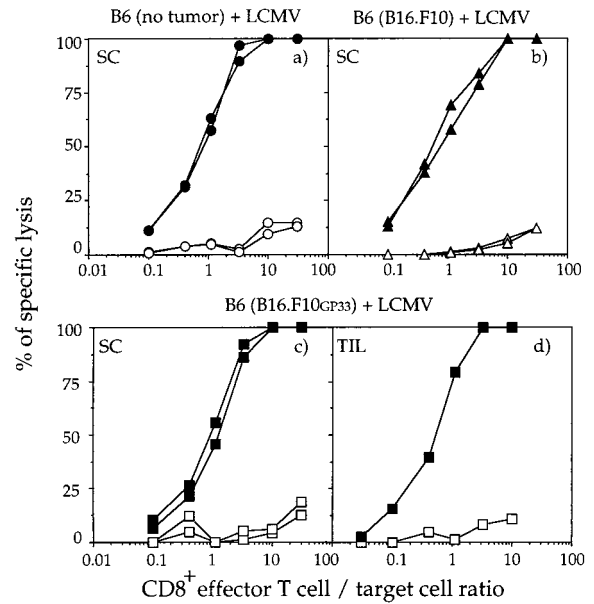


FIGURE 6. GP33-specific CTL activity at the tumor site after LCMV infection. B16.F10 and B16.F10_{GP33} tumor cells were injected s.c. into B6 mice. Control B6 mice (*a*) and mice with palpable B16.F10 (*b*) and B16.F10_{GP33} tumors (*c* and *d*) were infected with LCMV, and after 10 days, the cytolytic activity of spleen cells (*a*, *b*, and *c*) and TIL (*d*) was tested against EL-4 target cells pulsed with the GP33 peptide (closed triangles and squares) or with the control E1A peptide (open triangles and squares) in a 15-h ⁵¹Cr release assay. Spontaneous release in all assays was <25%. All experiments were performed three times.

(Fig. 6a) and B16.F10 tumor-bearing mice (Fig. 6b). Similarly, TIL isolated from B16.F10_{GP33} tumors of LCMV-infected B6 mice were highly cytolytic against GP33-coated target cells (Fig. 6d). Thus, these data show that GP33-specific CD8⁺ T cells were not tolerized by the B16.F10_{GP33} tumor and that highly active GP33-specific CTL were found at the tumor site.

Tumor cell elimination by adoptive transfer of LCMV-specific CTL

Next, we investigated whether the failure of the LCMV infection to prevent growth of preestablished B16.F10_{GP33} tumors was due to the lag time (6–8 days) between LCMV infection and induction of CTL response. Therefore, day 8 LCMV-immune effector T cells

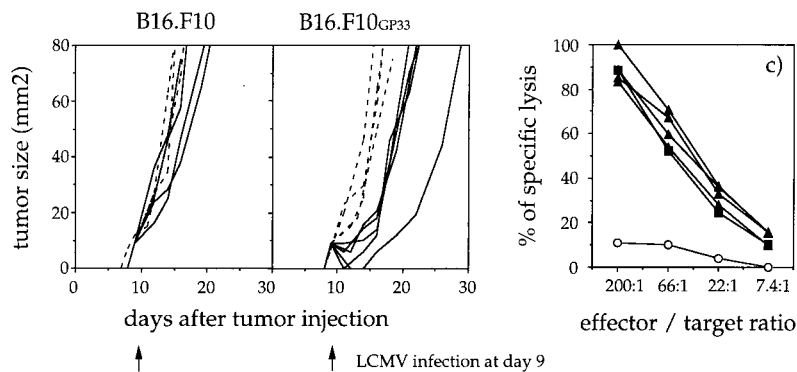


FIGURE 5. LCMV infection delays but does not prevent B16.F10_{GP33} tumor growth. B16.F10 (*a*) or B16.F10_{GP33} (*b*) tumor cells (10⁶) were injected s.c. into the flank of B6 mice. Mice with palpable tumors were either left as controls (dotted line) or were infected with LCMV (solid line). Tumor growth was measured every third day and mice bearing a tumor with a diameter of 15 mm were killed. In *c*, B16.F10_{GP33} tumor cells (triangles) isolated from these mice were tested in a 5-h ⁵¹Cr-release assay using effector cells from the spleens of day 8 LCMV-immune B6 mice. B16.F10_{GP33} tumor cells (squares) kept in vitro culture and parental B16.F10 cells (circles) were included as controls. Spontaneous release in all assays was <20%.

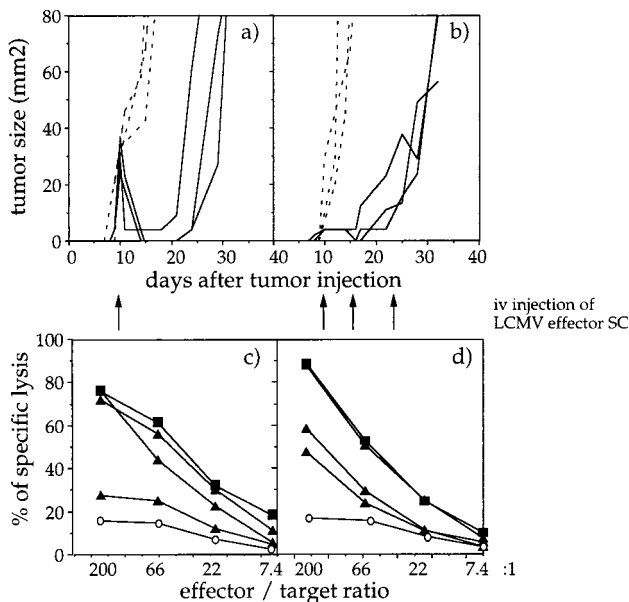


FIGURE 7. Transient B16.F10_{GP33} tumor elimination by adoptive transfer of LCMV-specific CTL. B16.F10_{GP33} tumor cells (10^6) were injected s.c. into the flank of B6 mice. In *a*, mice with small B16.F10_{GP33} tumors (20–40 mm²) were either left untreated (dotted line) or were injected with 5×10^7 effector spleen cells from day 8 LCMV-immune B6 mice (solid line). LCMV-specific effector spleen cells (5×10^7) were adoptively transferred into B16.F10_{GP33} tumor-bearing B6 recipient mice on days 10, 16, and 23 after tumor inoculation (*b*). Tumor growth was measured every third day. Mice bearing a tumor with a diameter of >15 mm were killed. B16.F10_{GP33} tumor cells (triangles) isolated from mice after one (*c*) or three (*d*) adoptive transfers were tested in a 5-h ⁵¹Cr release assay using effector cells from day 8 LCMV-immune B6 mice. B16.F10_{GP33} tumor cells (squares) kept in *in vitro* culture and parental B16.F10 cells (circles) were included as controls. Spontaneous release in all assays was <20%.

(5×10^7) were transferred into B6 mice bearing preestablished B16.F10_{GP33} tumors (20–40 mm²). As shown in Figure 7*a*, adoptive transfer of GP33-specific cells leads to dramatic decrease of tumor size; in two out of three mice, no palpable tumor could be detected 2 to 3 days after transfer. However, after a delay of 6 to 7 days without significant tumor growth, B16.F10_{GP33} tumors grew in these mice at a similar rate as in control B6 mice. LCMV-specific effector CTL injected repetitively (three times) at 6- to 7-day intervals were also not able to permanently eliminate preestablished B16.F10_{GP33} tumors (Fig. 7*b*). Most of the B16.F10_{GP33} tumors isolated from these mice were not Ag loss variants, since five out of six tumors were readily recognized by LCMV-specific effector cells *in vitro* (Fig. 7, *c* and *d*). Finally, we examined whether the day 8 LCMV immune effector T cells can prevent the growth of B16.F10_{GP33} tumors when transferred into B6 mice on the same day as the tumor. As shown in Figure 8, adoptive transfer of LCMV-specific cells prevented B16.F10_{GP33} tumor growth, but did not affect the growth of parental B16.F10 tumor cells.

Discussion

Our data show for the first time a strong cytolytic activity of *ex vivo* isolated murine TIL without *in vitro* stimulation. Nevertheless, these TIL fail to prevent melanoma tumor growth. In the new model that we describe here, B16.F10 melanoma cells have been genetically modified to express the immunodominant GP33 epitope of LCMV, which is recognized in association with the

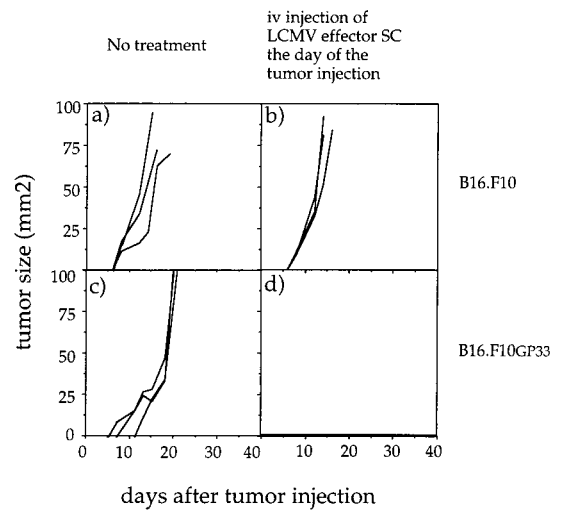


FIGURE 8. Prevention of B16.F10_{GP33} tumor growth by adoptive transfer of LCMV-specific CTL. B16.F10 (*a* and *b*) and B16.F10_{GP33} (*c* and *d*) tumor cells (10^6) were injected s.c. into the flanks of B6 mice. After tumor cell injection, mice were left untreated (*a* and *c*) or were injected with 5×10^7 effector spleen cells from day 8 LCMV-immune B6 mice on the same day (*b* and *d*). Tumor growth was measured every third day. Mice bearing a tumor with a diameter of >15 mm were killed.

H-2D^b molecule. This model was designed to investigate antitumor CTL response both in normal mice and in transgenic mice expressing a TCR specific for the GP33 peptide. Surprisingly, the B16.F10_{GP33}-transfected tumor cells grew in P14 TCR transgenic mice. This result is compatible with recent data on the establishment of a tumor expressing the L^d Ag in anti-L^d TCR transgenic mice (24), suggesting that a high precursor frequency of CTL specific for a tumor Ag may not be sufficient to prevent tumor growth. It is generally accepted that optimal T cell activation requires two distinct signaling events; signal one ensures the specificity of the interaction, signal two requires soluble factors such as IL-2 or the direct interaction of adhesion or cosignaling molecules on the T cell surface with their ligands on the APC. T cells that recognize the Ag in the absence of costimulatory signals may become anergic or be deleted (25). Consistent with the two-signal theory, K1735 melanoma cells transfected with B7 elicited more effective antitumor immunity than nontransfected tumor cells (26, 27). Surprisingly, despite the lack of B7-1 and B7-2 molecules and the low expression level of MHC class I molecules on B16.F10 tumor cells, we found that B16.F10_{GP33} tumor cells induced specific T cell activation in P14 TCR mice. It is likely that the high GP33-specific CTL activity of the TIL freshly isolated from the tumor mass accounted for the delay of B16.F10_{GP33} tumor growth in P14 TCR mice and in B6 mice in which CTL were transferred. However, it is important to note that the CTL response induced was not sufficient to prevent tumor growth. The finding that TIL isolated from tumor-bearing P14 TCR H-2D^bm13 mice exhibit GP33-specific lytic activity indicates that B16.F10_{GP33} melanoma cells directly activated P14 TCR transgenic T cells without cross-priming *in vivo*. This result is different from that obtained with the tumor model reported by Huang et al. (28) in which tumor Ag presentation occurred by host bone marrow-derived cells. Our finding is also distinct from the results of Kündig et al. (29), who demonstrated that fibroblasts can function as potent APC when Ag presentation occurs in lymphoid organs. In our model, we were able to detect CTL activity only within the tumor mass, not in the spleens or in draining lymph nodes (not shown) of tumor-bearing mice. The direct sustained presentation of GP33 peptide by the

genetically modified tumor may have overcome the requirement of the second signal *in vivo*. Such a view would be compatible with the recent demonstration of the induction of a functional T cell response through repeated Ag injections, in the absence of a costimulatory signal (30). Lucas et al. (31) showed that a primary Ag-specific response can be induced in CD28-deficient mice, but that CD28 is required for IL-2 production and sustained T cell proliferation. The lack of CD28/B7-mediated costimulation could explain the failure of the initially induced anti-tumor specific CTL response to definitively prevent the B16.F10_{GP33} tumor growth in our model.

The selection of tumor Ag loss variants would easily explain the failure of the immune system to control the B16.F10_{GP33} tumor growth. The role of a tumor-specific CTL response in the *in vivo* selection of Ag loss variants has been suggested after analyzing Ags expressed on human melanoma cell lines derived from metastases appearing at intervals of several years (9, 32). In our system, most of the freshly isolated B16.F10_{GP33} tumors from P14 TCR mice (15/16) or from LCMV-infected or adoptively transferred B6 mice (9/10) still expressed GP33, since they were efficiently recognized by GP33-reactive CTL *in vitro*. Thus, despite a high GP33-specific CTL activity at the tumor site, only few GP33 loss variants were selected *in vivo* in the tumor model analyzed here.

Immunosuppressive activity through production of immunoregulatory cytokines, such as TGF- β and IL-10, by tumor cells may paralyze the immune system in tumor-bearing hosts. In addition to this latter mechanism, recent data correlated the immunosuppressive activity of NK1.1⁺ T cells derived from the TIL of B16 melanoma with their production of TGF- β (33). The release of IL-10 by CD8⁺ T cells from murine fibrosarcoma-draining lymph nodes was also associated with a suppressed anti-tumor response *in vivo* (34). We found that TIL from LCMV-infected B16.F10_{GP33} tumor-bearing B6 mice elicited strong GP33-specific CTL activity, comparable with that of spleen cells from the same mice. This result argues against a GP33-specific immunosuppression at the tumor site. Type 1-like lymphokine production (IL-2, INF- γ , granulocyte-macrophage-CSF, and TNF- α) was shown to play a role in the regression of murine tumors by promoting tumor Ag recognition (35). The production of such lymphokines by GP33-specific TIL may overcome immunosuppression mediated by putative immunosuppressive factors. The lymphokines secreted by TIL in our model are currently under investigation. We cannot exclude the possibility that defective cytokine production resulting from partial activation of the tumor reactive T cells may explain B16.F10_{GP33} tumor establishment in our system, as recently reported in human melanoma (36).

Adoptive transfer of tumor-reactive T lymphocytes has been shown to prevent tumor growth or to result in regression of established tumors (37). Thus, Bloom et al. (38) demonstrated that a CTL line raised from splenocytes by repeated *in vitro* stimulation with the TRP-2 peptide specific for the B16 melanoma was therapeutic against 3-day-old established pulmonary metastases. In contrast, our data indicate that adoptive transfer of LCMV-immune effector cells or LCMV infection delayed, but did not prevent, the growth of established B16.F10_{GP33} tumors. This result fits in well with recent data from a double transgenic model, which demonstrated that LCMV-activated CD8⁺ T cells destroyed GP33-expressing pancreatic tumor cells and reduced tumor mass, without, however, definitive tumor clearance (39). It is interesting to note that in our system GP33-specific T cells were able to prevent the growth of the B16.F10_{GP33} tumor when CTL and tumor cells were injected at the same time point. LCMV induces a strong immune response dominated by LCMV-specific cytotoxic CD8⁺ T cells,

which results in solid and long-term protective CTL memory *in vivo* (40). Surprisingly, these CD8⁺ T cells were not efficient in long-term protection against preestablished B16.F10_{GP33} tumor growth. FasL expressed on tumor cells may contribute to the immune privilege of tumors, and recent data suggest that B16.F10 tumor cells may counterattack anti-tumor activity by inducing apoptosis of T cells via the CD95L (8). Freshly isolated CD8⁺ TIL from P14 TCR mice bearing B16.F10_{GP33} tumors expressed high levels of Fas when compared with CD8⁺ T cells from spleen or inguinal lymph nodes. The high level expression of Fas at the TIL surface may correlate with a high sensitivity to CD95-mediated apoptosis.

It is striking that the B16.F10_{GP33} tumor grew in P14 TCR mice (GP33-specific CTLp: 1/1 to 1/2 of CD8) but did not grow in LCMV memory B6 mice (GP33-specific CTLp: 1/100 of CD8). This illustrates the importance of the activation state of the responding T cell population. In contrast to naive GP33-specific CD8⁺ T cells from P14 TCR mice, GP33-specific CD8⁺ memory T cells may immediately prevent tumor growth by direct cell lysis and/or increased cytokine production, since LCMV-specific memory T cells display *ex vivo* anti-GP33 CTL activity (41, 42) and secrete higher amounts of cytokines than their naive counterparts (our unpublished observations). Additionally, memory T cells may be less dependent on costimulation than naive T cells for full-blown activation, including clonal expansion and effector cell differentiation.

We conclude that despite the strong *ex vivo* cytolytic activity of TIL, these cells fail to prevent tumor establishment. Furthermore, LCMV infection or adoptive transfer of LCMV-immune effector cells only delayed the growth of an established tumor. These results point out the difficulty of maintaining efficient long-term anti-tumor activity *in vivo*. The challenge now is to elucidate the mechanism of tumor escape in this model and to maintain and improve TIL activity *in vivo*.

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