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IL-6 Secretion by a Rat T9 Glioma Clone Induces a Neutrophil-Dependent Antitumor Response with Resultant Cellular, Antiglioma Immunity

Martin R. Graf, Robert M. Prins, and Randall E. Merchant

Previously, we reported that IL-6 transduction attenuates tumor formation of a rat T9 glioma clone (termed T9.F). This study focuses on the mechanisms of the antitumor response elicited by IL-6 and the generation of glioma immunity. Ten days post s.c. inoculation of T9.F- or IL-6-secreting T9.F cells (T9.F/IL6/hi), tumor nodules were removed and their leukocytic infiltrate was analyzed by FACS with Ab markers for T cells, B cells, granulocytes, and monocytes. T9.F/IL6/hi tumors showed a marked increase in granulocytes as compared with parental T9.F tumors, and histological examination revealed that the granulocytes were neutrophils. Animals made neutropenic failed to reject T9.F/IL6/hi tumors. FACS analysis of 17-day T9.F/IL6/hi regressing tumors and T9.F progressing tumors did not reveal any significant differences in the leukocytic infiltrates. Tumor-specific effector cells were detected in the spleens harvested from animals bearing 17-day, regressing, T9.F/IL6/hi tumors. In vitro, these effector cells lysed T9.F cells, proliferated in response to T9.F stimulator cells, and produced Th1 cytokines (IL-2 and IFN-γ) but not the Th2 cytokine, IL-4, when cocultured with T9.F stimulator cells. Rats that had rejected s.c. T9.F/IL6/hi tumors displayed a delayed-type hypersensitivity response when injected with viable T9.F cells in the contralateral flank. Passive transfer of spleen cells from these animals transferred glioma immunity to naive recipients and depletion of CD3+ T cells, before transfer, completely abolished immunity, whereas depletion of CD8+ T cells had moderate inhibitory effects on the transfer of immunity. The Journal of Immunology, 2001, 166: 121–129.

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§ Abbreviations used in this paper: GBM, glioblastoma multiforme; i.c., intracranial; 51Cr, 51-chromium; TdR, tritiated thymidine; DTH, delayed-type hypersensitivity.ophosphatase, and the median for survival is 18 months after primary diagnosis. Unfortunately, traditional treatments such as surgical tumor resection, chemotherapy, and external beam irradiation have done little to alter the progression of this deadly disease. There has been little advancement in the treatment of GBM; hence, there continues to be a need for the development of effective therapies for the treatment of patients with this disease (see Refs. 2–5 for review).

Novel approaches using immunotherapy are currently being explored for the treatment of GBM as well as other brain and peripheral solid tumors (see Refs. 6–9 for review). In these studies, the tumor cells are genetically engineered to produce immune-enhancing cytokines. The presence of these cytokines, in most cases, is believed to counteract the immunosuppressive microenvironment of the tumor, resulting in the activation local immune cells and the initiation of an antitumor response when the cytokine-secreting tumor cells are implanted into a host animal. In many studies, the genetically modified tumor cells are destroyed, and in some cases, the animal develops tumor-specific immunity against the nonmodified, parental tumor.

Pioneering studies demonstrating that tumor cells genetically altered to secrete cytokines can induce an antitumor response were reported in 1989 by Teppet et al. with IL-4-secreting plasmacytoma cells and by Fearon et al. in 1990 with IL-2-producing colon carcinoma cells (10, 11). Since these initial reports, numerous additional studies have shown similar results using different murine tumors engineered to secrete a variety of cytokines, for example 1) TNF-α-secreting sarcoma cells in C57BL/6 mice (12) and plasmacytoma cells in BALB/c mice (13); 2) IL-4-secreting renal cell carcinoma cells and colon carcinoma cells in BALB/c mice (14); 3) IL-2-secreting mammary adenocarcinoma (15), renal cell carcinoma (16), and melanoma (16, 17), all performed in BALB/c mice, and fibrosarcoma in C57BL/6 mice (18); 4) IL-7-secreting fibrosarcoma in C3H/Sed mice (19); 5) IL-1-secreting fibrosarcoma in BALB/c mice (20); and 6) GM-CSF-secreting melanoma cells in C57BL/6 mice (21) and colon adenocarcinoma in BALB/c mice (22).

Reports of cytokine-secreting glioma cells are fewer, and the results are not as extensive as murine studies with other types of tumors. Ram et al. (23) demonstrated that IL-2 secreted from transduced 9L rat glioma cells resulted in reduced s.c. tumor formation, but was completely lethal and conferred no survival advantage when implanted in the brain. Tjuvajev et al. (24) reported similar results with RG-2 rat glioma cells also genetically altered to secrete IL-2 or IFN-γ. In this regard, both the IL-2- and the IFN-γ-secreting RG-2 cells demonstrated attenuated s.c. tumor growth, although no s.c. tumor regression was observed, and no increase in survival was noted when the IL-2- and IFN-γ-secreting RG-2 cells...
were implanted in the central nervous system. Another group reported that intratumoral transfection and expression of the TNF-α gene resulted in s.c. growth inhibition of a human glioma in nude mice (25). Thus, cytokine gene therapy of intracranial (i.c.) gliomas presents a formidable endeavor that may be complicated by the immunologically privileged location.

These studies have begun to reveal the complexity of the mechanisms of the induced antitumor responses and how the primary antitumor response may ultimately result in tumor-specific immunity. However, investigations into the mechanisms are often obscured by such variables as the etiology of the tumor transfected with the cytokine gene, the specific cytokine used, the level of cytokine produced by the tumor cells, and the phenotypic makeup of the cellular infiltrate at the implantation site of the cytokine-secreting tumor cells. The early phase of an antitumor response induced by cytokine-secreting tumor cells may involve an influx of nonspecific immune effectors cells, such as macrophages or granulocytes, into the implantation site (13, 14, 19, 21, 26) followed by lymphocytic infiltrate at later stages (14, 21, 26) or can be directly dependent upon T lymphocytes (11). For example, it was shown that IL-4–secreting plasmacytoma tumor cells induce massive eosinophilic infiltration into the growing tumor nodules; this results in rapid and apparently nonspecific destruction of the tumor, and lymphocytes are not involved (10, 27). Golumbeck et al. reported that the early infiltration of murine renal cell carcinoma cells also genetically engineered to secrete IL-4 was composed of macrophages and eosinophils. T cells appeared to migrate into the rejecting tumor site during the second week, which subsequently induced tumor-specific immunity dependent upon CD8+ T cells (14). The primary antitumor responses in these two reports conform to the danger model of immunity proposed by Matzinger et al. in which an inflammatory environment is needed for the induction of antitumor immunity (28, 29). In contrast, Fearon et al. demonstrated that the antitumor response induced by IL-2–secreting colon carcinoma cells was largely dependent upon CD8+ T cells, indicating that a proinflammatory environment may not always be required (11). Dranoff et al. demonstrated, in studies involving murine melanoma cells that have been transduced with the GM-CSF gene, that the recruitment and differentiation of APCs at the tumor site is crucial in the elicited antitumor response, and Huang et al. showed that the generation of tumor-specific immunity is dependent on both CD4+ and CD8+ T cells in the GM-CSF-secreting tumor model (21, 30).

We have previously reported, in a modified rat T9 glioblastoma model, that T9 glioblastoma cells genetically altered to express IL-6 are less tumorigenic when implanted in the brain of syngeneic animals (31). When the IL-6–secreting glioma cells are injected s.c., they are rejected and the animals are protected against a subsequent i.c. challenge with the parental, noncytokine-secreting T9 glioma but not against a mammary adenocarcinoma. In this study, we investigate the cellular mechanisms of the IL-6–induced anti-glioma response, subsequent activation of tumor-specific effector cells, and their involvement in glioma immunity.

Materials and Methods

Animals

Inbred female Fischer 344 rats weighing 140–160 g and ranging in age from 4–6 mo were obtained from Harlan Breeders (Indianapolis, IN). Animals were housed in a climate-controlled, American Association of Laboratory Animal Care-approved vivarium and were provided free access to rat chow and water. All experimental animal procedures have been approved by members of the Institutional Animal Care and Use Committee, respectively. The T9 glioblastoma tumor was originally induced by the repeated i.v. injection of N-nitrosomethyleurea in a Fischer 344 rat (32). It has been reported that T9 glioma cells are derived from the 9L glioma cell line (33). The MadB106 mammary adenocarcinoma is a selected pulmonary metastases produced by the i.v. injection of the MadB100 parental adenocarcinoma (34), which was induced in a Fischer rat given an oral dose of 9,10-dimethyl-1,2-benzanthracene by Dr. Saburo Sone (35). The Fischer rat-derived RT-2 glioma was induced by the avian sarcoma virus, and the glioma cell line was provided by Dr. Yancy Gillespie (University of Alabama, Birmingham, AL) (36). Clone T9.F was isolated from the T9 glioblastoma cell line in a clonogenic assay. T9.F glioma cells were transplanted with the LXSN retroviral expression vector containing the human IL-6 cDNA and a T9.F subclone, which secreted a high level of IL-6, and IL-6 was identified and termed T9.F/IL6hi. In culture, T9.F/IL6hi constitutively produced IL-6 at a level of 35 ng/106 cells/48 h (31). Tumor cells were routinely monitored for contamination by mycoplasma, bacteria, and fungus.

Subcutaneous tumor implantation

Tumor cells for s.c. implantation were trypsinized, counted on a hemacytometer, and checked for viability by trypan blue exclusion. Cells were washed twice in PBS, and a final suspension of viable cells was made in PBS. The injection site of recipient animals was shaved and wiped with 70% ethanol. Tumors were induced by injecting a suspension of 1 × 106 viable cells in 100 μl of PBS, and tumor growth was monitored by measuring tumor diameter using calipers.

Intracranial tumor implantation

Tumor cells for i.c. implantation were prepared as previously mentioned for s.c. inoculation, and a final suspension of 2 × 106 viable cells per 100 μl of PBS was made. Animals were anesthetized by an i.p. injection of ketamine-HCl (87 mg/kg) and xylazine (6.5 mg/kg). The scalp hair was shaved and wiped with betadine, and an incision was made over the cranial midline. Animals were placed in a stereotactic apparatus, and 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. Five microliters of the tumor cell suspension (1 × 105 cells) was 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 to prevent tumor cell extravasation, and the incision was closed with surgical staples.

FACS analysis of tumor-infiltrating leukocytes

Animals were implanted s.c. with 1 × 106 tumor cells or i.c. with 1 × 105 cells. Ten or 17 days post inoculation, parental T9.F or T9.F/IL6hi tumor nodules were surgically excised and forced through a 70-μm pore strainer to generate a single cell suspension. The resulting cell suspension was washed twice with cold 5% FBS-PBS, and the viable leukocytes were enumerated using a hemocytometer and trypan blue exclusion. The concentration of leukocytes was adjusted to 1 × 107/100 μl and stored on ice.

Tumor-infiltrating leukocytes were analyzed by FACS using Ab markers for T cells (CD3, CD4, CD8), B cells (CD45RA), granulocytes (HIS48) (all obtained from PharMingen, San Diego, CA), and monocytes (ED1; obtained from SeroTec, Raleigh, NC), mAbs were either directly conjugated to fluorescent markers (FITC or PE) or were biotinylated and were subsequently detected with a peroxidase-conjugated secondary Ab. Cell surface staining was performed using standard methodology.

Briefly, 1 × 106 leukocytes were stained in a V-bottom 96-well microplate in a volume of 50 μl of 5% FBS-PBS containing a mixture of three different mAbs (0.5 μg of each purified mAb) for 30 min on ice. Cells were then washed twice with 200 μl of 5% FBS-PBS and, in the case of mAbs directly conjugated to fluorescent markers, were resuspended in 200 μl of 5% FBS-PBS containing 1% paraformaldehyde and stored in the dark at 4°C. In the case of biotinylated mAbs, cells were then incubated on ice for 30 min in a 50-μl volume of 5% FBS-PBS with streptavidin-peridinin
chlorophyll protein conjugate (Becton Dickinson Immunocytometry Systems, San Jose, CA) used at a 1:10 dilution. Cells were then washed twice with 250 μl of 5% FBS-PBS and were resuspended in 200 μl of 5% FBS-PBS containing 1% paraformaldehyde and stored in the dark at 4°C. Three-color FACS analysis was performed using a Coulter Epics XL-MCL Flow Cytometer (Miami, FL).

**Histology**

Subcutaneous tumors were carefully excised from sacrificed animals and were preserved in a 10% neutral buffered formaldehyde solution. Tissue samples were next embedded in paraffin, sectioned into 5-μm slices, mounted on glass slides, and stained with hematoxylin and eosin for microscopic analysis and photography.

**Induction of neutropenia**

Rats were made neutropenic by i.p. injection of methotrexate (2 mg/ml PBS, pH 7.5, Sigma) for 5 consecutive days (37, 38). The next day, animals were injected s.c. with 1 × 10^9 T9.F/IL6/hi cells. Rats received additional injections of methotrexate on days 5, 6, 12, 13, 19, and 20 post-T9.F/IL6/hi inoculation. The spleens of sentinel animals were analyzed by FACS using a granulocyte marker (HS85) on the day of T9.F/IL6/hi injection and after the first and second weeks to confirm the duration of neutropenia.

**In vitro tumor-specific effector cell responses**

Spleens were aseptically removed from animals with 17-day-regressing T9.F/IL6/hi s.c. tumors or age-matched control rats and forced through a 70-μm nylon cell strainer to generate single cell suspensions. Erythrocytes were lysed with Tris-buffered ammonium chloride (5 ml/spleen); spleen cells were washed twice with PBS and counted on a hemacytometer, then viability was assessed by trypan blue exclusion. Spleen cells were cultured at a concentration of 1 × 10^6 cells/ml in RPMI 1640 (Life Technologies) supplemented with 10% FBS, 0.05 mM 2-ME, and 50 U/ml of recombinant human IL-2 (Chiron Therapeutics, Emovryville, CA) in the presence of irradiated T9.F, RT-2, or MadB106 stimulator cells adjusted to 50:1 splenocyte/stimulator ratio and were used in several immune assays to detect the presence and functions of tumor-specific effector cells. Spleen cells from naive or T9.F/IL6/hi-primed animals that were cultured for 4 days with T9.F stimulator cells were used in a 5-h cytotoxicity assay with 3^11Cr-labeled T9.F, RT-2, or MadB106 viable target cells at various E:T ratios. Briefly, 1 × 10^6 viable target cells were incubated with 100 μCi of Na_2CrO_4 (Amersham Pharmacia Biotech, Piscataway, NJ) in 200 μl of PBS supplemented with 20% FBS for 3 h at 37°C, washed twice with PBS, and resuspended to a final concentration of 1 × 10^6 cells/ml in RPMI 1640 supplemented with 10% FBS, 0.05 mM 2-ME, and 10 mM HEPES (Sigma, St. Louis, MO). Five thousand target cells were added to each well of a 96-well V-bottom microplate. Spleen cells were suspended in the same medium and were added to the wells to achieve E:T ratios of 100:1, 50:1, and 25:1 in a final volume of 200 μl/well. The microplates were briefly centrifuged to initiate cell contacts and were incubated at 37°C. At the end of 5 h, the microplates were centrifuged, 100 μl of the cell-free supernatants were harvested, and the amount of 51 Cr (experimen-

**Detection of delayed-type hypersensitivity (DTH) responses**

For the assessment of a DTH response, 5 wk after animals had rejected an initial s.c. injection of 1 × 10^9 T9.F/IL6/hi cells, rats were injected in the contralateral flank with 1 × 10^6 viable T9.F parental cells. In the case of foot pad injections, the DTH response was monitored by daily measurements of foot pad swelling with a thickness gauge micrometer. The swelling response was calculated by subtracting the thickness of the noninjected hind foot pad from that of the experimental foot pad to yield a corrected foot pad swelling value.

**Depletion of CD3+ or CD8+ T cells and passive transfer of tumor immunity**

Spleens were harvested from T9.F/IL6/hi-primed animals 10 days after injection of parental T9.F cells in the contralateral flank and the appearance of a tumor reaction. The generation of single cell suspensions and the lysis of erythrocytes were performed as described above. The concentration of spleen cells was adjusted to 1 × 10^7 cells/ml PBS. For the depletion of CD8+ T cells, 1 × 10^6 spleen cells were added to individual tubes in a volume of 1 ml PBS, then 50 μg of purified anti-rat CD8α IgG2 (clone G28; PharMingen) was added to each tube and incubated for 45 min on ice. The cells used for complement controls did not receive the addition of mAbs. Cells were then washed once and resuspended in 5 ml of PBS. Cedarlane Low-Tox-M rabbit complement (Accurate Chemical & Scientific, Westbury, NY) was added to the spleen cells at a 1:10 dilution and incubated at 37°C for 1 h. Cells were then washed once and resuspended in 1 ml of PBS. For the depletion of CD3+ T cells, the same procedure was followed using 67 μg of purified anti-rat CD3 IgM (clone 1F4; Serotec). Depletion of CD8+ T cells was verified by FACS, and depletion of CD3+ T cells was verified by hemacytometer and trypan blue exclusion counts. Naive animals were injected with 1 × 10^6 complement control spleen cells, spleen cells depleted of CD8+ or CD3+ T cells. The next day, animals were implanted i.c. with 1 × 10^6 parental T9.F cells.

**Statistics**

Statistical analysis was performed using the Student paired t test, and differences with a p value <0.05 were considered significant. In the case of survival plots, results were analyzed using the LIFETEST procedure (SAS Institute, Cary, NC) and significance was determined using the Log-Rank test.

**Results**

Analysis of the of primary antitumor response elicited by T9.F/IL6/hi cells

To understand the potential cellular mechanisms of the antitumor response elicited by IL-6-secreting T9.F glioma cells, we studied the cellular infiltrate of s.c. and i.c. tumor nodules at an early (day 10) and a late (day 17) time point. The leukocytic tumor infiltrate was analyzed by FACS using mAb markers for T cells (CD3, CD4, and CD8β), B cells (CD45RA), granulocytes (HIS48), and monocytes (ED1).

For the study of the s.c. antitumor response, animals were implanted with 1 × 10^6 parental T9.F or T9.F/IL6/hi cells (three rats per group), and the tumor nodules were excised 10 days postinjection. Representative dot plots of the leukocytic infiltrate of T9.F and T9.F/IL6/hi tumors stained with mAbs for granulocytes and monocytes are shown in Fig. 1 and illustrate a significant increase in the number of granulocytes present in the T9.F/IL6/hi tumor as compared with the non-IL6-secreting T9.F parental tumor (p = 0.013). At this time point, there were no significant differences in the infiltration of CD4+ or CD8+ T cells, B cells, and monocytes/macrophages between the IL-6-secreting T9.F gliomas and the parental T9.F gliomas (data not shown). Histological examination of the parental T9.F and T9.F/IL6/hi day 10 tumors, also shown in
Fig. 1, revealed that neutrophils were the predominant subset of granulocytes infiltrating the T9.F/IL6/hi tumors. Neutrophils were noticeably absent in the parental T9.F tumors. FACS analysis was also performed on the leukocytic infiltrate of 17-day s.c. tumors. At this time point, parental T9.F tumors are progressing (tumor diameter; 10 mm), whereas T9.F/IL6/hi tumors are rapidly regressing (tumor diameter; 5 mm) (32). The granulocytic infiltration of T9.F/IL6/hi tumors that had been observed at day 10 was absent and appeared to be replaced by a T cell infiltrate (Fig. 2). Day 17 parental T9.F s.c. gliomas were also void of granulocytes and only modestly infiltrated with T cells (data not shown).

The leukocytic infiltrate of i.c. tumors 10 and 17 days post implantation is shown in Fig. 3. At day 10, there was a modest degree of infiltration by granulocytes in the IL-6-secreting tumor, which increased considerably by day 17. This is in contrast to s.c. T9.F/IL6/hi tumors, where the granulocytic infiltrate had subsided by day 17. The difference in the time period of the granulocytic infiltrate may be explained by the observation that immune reactions tend to be delayed in the brain. Interestingly, we have shown that the survival of animals implanted in the brain with T9.F/IL6/hi cells was significantly extended, although the animals ultimately succumbed to their i.c. tumor whereas 70% of the animals implanted s.c. with T9.F/IL6/hi cells completely rejected their tumors (32).
The antitumor response elicited by T9.F/IL6/hi cells is neutrophil dependent

To provide support for a functional role for the presence of neutrophils in the IL-6-induced antitumor response, rats were depleted of neutrophils with methotrexate treatment for 5 consecutive days before the s.c. implantation of T9.F/IL6/hi cells. Animals received two additional treatments of methotrexate per week for the duration of the study. Control animals did not receive methotrexate injections. Tumor development and growth were monitored by daily measurements of tumor diameter. The results of a 3-wk study are shown in Fig. 4. At the conclusion of the experiment, 100% (5/5) of the neutropenic had progressively growing T9.F/IL6/hi tumors at the injection site (mean tumor diameter = 10.9 ± 2.2 mm SD). Over the same period, all but one of the control animals (5/6) had completely rejected their T9.F/IL6/hi tumors.

Detection of tumor-specific effector cells in the spleens of T9.F/IL6/hi-rejecting animals

The results from the previous experiments suggested that neutrophils may play a role in the antitumor response elicited by T9.F/IL6/hi cells; however, animals ultimately develop long-lasting tumor-specific immunity. We used several in vitro immune assays to detect the presence of activated tumor-specific effector cells in the circulation 17 days after s.c. inoculation of T9.F/IL6/hi cells. Spleen cells were obtained from naive or T9.F/IL6/hi-primed rats and were cocultured with several syngeneic, irradiated stimulator cells (T9.F glioma, RT-2 glioma, or MadB106 adenocarcinoma) at a 50:1 (splenocytes/stimulator) ratio, and tumor-specific effector functions were evaluated in terms of cytotoxicity, cytokine secretion, and proliferation.

The supernatants from 3-day cocultures were assayed by ELISAs specific for the following rat cytokines: IL-2 and IFN-γ (Th1 associated) and IL-4 (Th2 associated). Fig. 5 illustrates that T9.F/IL6/hi-primed spleen cells secrete significant levels of IL-2 and IFN-γ in response to T9.F stimulator cells. IL-4 was not detected in the coculture medium. T9.F/IL6/hi-primed spleen cells cocultured with T9.F stimulator cells for 4 days were stained for FACS analysis using mAbs (anti-CD4 and CD8α) for phenotypic identification, followed by intracellular staining for IFN-γ using a mouse anti-rat IFN-γ mAb. The results shown in Fig. 5 indicate that both CD4+ helper T cells and CD8+ CTLs produce IFN-γ when stimulated by T9.F glioma cells. The results of a proliferation assay in which spleen cells were cocultured with several stimulator cells for 4 days and pulsed with [3 H]TdR for the last 15 h demonstrated that spleen cells from T9.F/IL6/hi-primed rats specifically proliferate in response to T9.F stimulator cells (Fig. 6).
Naive and T9.F/IL6/hi spleen cells that were cultured for 4 days with T9.F stimulator cells were used in 5-h cytotoxicity assays with different 51Cr-labeled target cells. These experiments showed that T9.F/IL6/hi-primed spleen cells specifically killed T9.F glioma cells and that naive spleen cells were not cytotoxic to T9.F target cells (Fig. 7).


Five weeks after the initial implantation of T9.F/IL6/hi cells, animals were challenged in the contralateral flank or hind foot pad with $1 \times 10^7$ viable parental T9.F cells. Naive animals developed a tumor at the injection site that grew progressively. In contrast, T9.F/IL6/hi-primed rats exhibited a DTH reaction at the site of injection. In animals that were challenged in the contralateral flank, induration and erythema were detectable within 24 h after the challenge and had reached peak intensity between days 2 and 3. Fig. 8 shows the evaluation of the DTH response by measuring the foot pad swelling in response to the T9.F challenge in the hind foot pad on the opposite side in which T9.F/IL6/hi cells were initially implanted. Mean foot pad swelling from the inflammatory reaction was the greatest between the first 24 h and the 4th day. Swelling began to subside 5 days after the challenge and by day 10, the DTH response was barely detectable. The appearance of a DTH response in T9.F/IL6/hi-primed rats indicated the presence of a tumor-specific, cell-mediated immune memory response.

Passive transfer of T9.F glioma immunity

Passive transfer of $1 \times 10^8$ spleen cells harvested from T9.F/IL6/hi-primed animals 11 ± 1 days after injection of parental T9.F cells in the contralateral flank and the appearance of a DTH reaction into naive recipients resulted in immunity to i.c. challenge with $1 \times 10^4$ T9.F parental cells (7/7 survivors) as shown in Fig. 9. However, transfer of $1 \times 10^8$ spleen cells from the T9.F/IL6/hi-primed rats depleted of T cells by anti-CD3 Ab and complement completely abrogated the transfer of immunity (0/6 survivors), and the depletion of CD8+ T cells only had moderate effects on the transfer of T9.F immunity in that 29% of the animals succumbed to the i.c. T9.F challenge (5/7 survivors). These results suggested that the induced T9.F immune memory was partially dependent upon CD8+ T cells while being completely CD3+ T cell dependent.

Discussion

In an earlier study, we reported that IL-6 secretion by a highly tumorigenic T9 clone (termed T9.F/IL6/hi) elicited an effective antitumor response that resulted in a significant extension of survival when implanted in the brain of syngeneic rats. When implanted s.c., ~70% of the animals rejected the IL-6-secreting T9.F glioma, and tumor rejection was significantly blocked by the addition of neutralizing anti-IL-6 Abs to the tumor inoculum (31). In this study, we demonstrate that the early antitumor response invoked by T9.F/IL6/hi cells may be mediated by neutrophils and that glioma-specific T cells are activated and in circulation 17 days after the s.c. injection of T9.F/IL6/hi cells. Moreover, animals that
alter the transfer of immunity, rental T9.F glioma cells. Depletion of CD8 T cells. The next day, rats were challenged i.c. with a lethal dose of pa-

tional and may be mediators of the non-T cell antitumor re-

therefore, in the above immunocompromised animal models,

that mice that reject the s.c. IL-6-secreting tumors were resis-

dependent antitumor response, which is supported by the fact

irradiated mice. These results implicate an alternative, T cell-

as other effector cells, were involved in the IL-6-elicited anti-

lyzed T9.F glioma cells, the granulocytic infiltrate was moder-

ted by 1 wk, suggesting that the immune-privileged environ-

ment of the brain may delay the influx of granulocytes. We have

reported that animals implanted in the brain with the IL-6-secre-

ting T9.F glioma cells have a significant extension of survival but

ultimately succumb to i.c. glioma burden (31).

Although it appears that neutrophils may be responsible for the

initial antitumor response elicited by T9.F/IL6/hi cells, the animals

ultimately develop long-lasting, tumor-specific immunity that is T

cell dependent. Therefore, we used several in vitro immune assays

to detect the presence and assay the effector functions of activated,

circulating, tumor-specific lymphocytes 17 days after s.c. inocula-

tion of T9.F/IL6/hi cells. In a proliferation assay, when spleen cells

from T9.F/IL6/hi-primed animals were cocultured with irradiated

T9.F glioma, RT-2 glioma, or MadB106 adenocarcinoma cells, the

splenocytes specifically proliferated only in the presence of T9.F

cells. ELISA results demonstrated that T9.F/IL6/hi-primed spleen

cells secreted IL-2 and IFN-γ (Th1 cytokines), but not the Th2
cytokine IL-4, when cocultured with T9.F cells, whereas intracel-

lar FACS of the same spleen cells showed that CD4+ helper T

cells and CD8+ T cells were positive for IFN-γ (4.9 and 6.9% positive,

respectively). When one considers that in a rat, ~35% of total

spleen cells are CD3+ and that ~1 in 1 × 10⁶ circulating T

cells in a naive animal or 1 in ~10⁴ T cells in a fully immunized

animal is specific for a particular Ag, the low percentages of CD4+

and CD8+ T cells staining positive for intracellular IFN-γ can be

justified (43). Lastly, primed spleen cells selectively lysed T9.F
glioma cells in chromium release assays, which indicates the gen-

eration of glioma-specific CTLs. In a previous study, we reported

that spleen cells obtained from rats primed with T9 glioma cells

expressing the membrane-associated isoform of macrophage-CSF

proliferated in culture when restimulated with T9 glioma cells but

failed to lyse T9 target cells in killing assays (44). Recently, Sepulveda et al. reported a novel, CD28- and IL-2-independent

pathway in which naive CD8+ T cells could be activated by TCR

ligation and costimulatory signals from IL-6 and TNF-α and that the

activated T cells were strongly cytolytic toward Ag-specific

targets (45). It is enticing to consider that the secreted IL-6 from

the T9.F/IL6/hi tumors may play a costimulatory role in the acti-

vation of glioma-specific CTLs via this alternative pathway.

ceived an i.p. injection of total spleen cells harvested from T9.F/IL6/hi-
primed rats (n = 7) or were depleted of CD3+ (n = 6) or CD8+ (n = 7) T

cells. The next day, rats were challenged i.c. with a lethal dose of pa-
eneral T9.F glioma cells. Depletion of CD8+ T cells did not significantly

alter the transfer of immunity, p = 0.14.


Other researchers have also reported that IL-6 expression by

tumor cells can result in reduced tumorigenicity. Dougherty et

al. demonstrated that murine fibrosarcoma cells genetically

modified to express human IL-6 produced fewer lung metastases

when injected i.v. and attenuated tumor formation when injected s.c. in syngeneic mice (39). They noted a significant

infiltration of macrophages in the IL-6-secreting fibrosarcoma growing s.c., suggesting an important role for IL-6 in the re-

ruitment and proliferation of tumor-associated macrophages.

In an earlier study, Porgador et al. reported that expression of human IL-6 by Lewis lung carcinoma cells also resulted in de-

creased s.c. tumor formation and suppressed lung metastasis

when injected i.v. in syngeneic mice (40). However, when these

IL-6-secreting carcinoma cells were injected in nude mice, the

c.s. tumor growth was reduced but there was no suppression of

lung metastasis. This observation suggested that T cells, as well as other effector cells, were involved in the IL-6-elicited anti-
tumor response. Interestingly, in the case of murine fibrosar-
coma cells transduced with the murine IL-6 gene, Mullen et al.

(41) reported a reduction of pulmonary nodule development in

an experimental metastasis model in normal, nude, and suble-

thally irradiated mice, which also suggests a non-T cell-medi-

ated antitumor mechanism. However, when the IL-6-secreting

fibrosarcoma cells were injected s.c., reduced tumorigenicity

was observed in normal mice but not in nude or sublethally

irradiated mice. These results implicate an alternative, T cell-
dependent antitumor response, which is supported by the fact

that mice that reject the s.c. IL-6-secreting tumors were resis-
tant to a subsequent challenge with wild-type tumor. It is note-

worthy that nude mice are void of T cells, and sublethally irra-
diation of mice primarily affects circulating lymphocytes;

therefore, in the above immunocompromised animal models,
nonlymphocyte immune cells, such as granulocytes, are func-
tional and may be mediators of the non-T cell antitumor re-

response. Current data suggest that nonspecific immune cells play

an integral role in the IL-6-mediated response; however, we

believe that we are the first to report a functional role for neu-

trophils in the IL-6-induced antitumor response.

In the T9.F/IL6/hi glioma model, s.c. tumors progress in size for

~12 days postinjection at which point a majority of the tumors

begin to regress, as detected by measurement of the tumor diam-

eter (31). FACS and cytopathological analysis of the leukocytic infiltrate of T9.F/IL6/hi 10-day s.c. gliomas revealed that the IL-6-secreting

gliomas were significantly infiltrated by neutrophils. Rats made

neutropenic failed to reject T9.F/IL6/hi s.c. tumors, suggesting a

functional role for neutrophils in the antitumor response. Although

neutrophils are the major leukocytic population targeted by meth-

otrexate, one must consider that there are marginal side effects on

other cells of the immune system (42); therefore, the lack of tumor

protection in methotrexate-treated animals injected with T9.F/

IL6/hi cells cannot unequivocally be attributed to neutropenia. In

17-day regressing T9.F/IL6/hi s.c. tumors, the granulocytic infiltrate

was not as pronounced as observed in 10-day tumors, whereas

the number of both CD4+ and CD8+ T cells was increased. Similar

FACS analysis was performed on i.c. T9.F/IL6/hi gliomas. In

contrast to s.c. T9.F/IL6/hi gliomas, the granulocytic infiltrate was

moderate in day 10 i.c. gliomas and was most pronounced at day

17, demonstrating that an antitumor response can be elicited in the

brain by the IL-6-secreting glioma cells, but it appears to be de-

layed by ~1 wk, suggesting that the immune-privileged environ-

ment of the brain may delay the influx of granulocytes. We have

reported that animals implanted in the brain with the IL-6-secret-

ting T9.F glioma cells have a significant extension of survival but

ultimately succumb to i.c. glioma burden (31).

Although it appears that neutrophils may be responsible for the

initial antitumor response elicited by T9.F/IL6/hi cells, the animals

ultimately develop long-lasting, tumor-specific immunity that is T

cell dependent. Therefore, we used several in vitro immune assays

to detect the presence and assay the effector functions of activated,

circulating, tumor-specific lymphocytes 17 days after s.c. inocula-

tion of T9.F/IL6/hi cells. In a proliferation assay, when spleen cells

from T9.F/IL6/hi-primed animals were cocultured with irradiated

T9.F glioma, RT-2 glioma, or MadB106 adenocarcinoma cells, the

splenocytes specifically proliferated only in the presence of T9.F

cells. ELISA results demonstrated that T9.F/IL6/hi-primed spleen

cells secreted IL-2 and IFN-γ (Th1 cytokines), but not the Th2
cytokine IL-4, when cocultured with T9.F cells, whereas intracel-

ular FACS of the same spleen cells showed that CD4+ helper T

cells and CD8+ T cells were positive for IFN-γ (4.9 and 6.9% positive,

respectively). When one considers that in a rat, ~35% of total

spleen cells are CD3+ and that ~1 in 1 × 10⁶ circulating T

cells in a naive animal or 1 in ~10⁴ T cells in a fully immunized

animal is specific for a particular Ag, the low percentages of CD4+

and CD8+ T cells staining positive for intracellular IFN-γ can be

justified (43). Lastly, primed spleen cells selectively lysed T9.F
glioma cells in chromium release assays, which indicates the gen-

eration of glioma-specific CTLs. In a previous study, we reported

that spleen cells obtained from rats primed with T9 glioma cells

expressing the membrane-associated isoform of macrophage-CSF

proliferated in culture when restimulated with T9 glioma cells but

failed to lyse T9 target cells in killing assays (44). Recently, Sepulveda et al. reported a novel, CD28- and IL-2-independent

pathway in which naive CD8+ T cells could be activated by TCR

ligation and costimulatory signals from IL-6 and TNF-α and that the

activated T cells were strongly cytolytic toward Ag-specific

targets (45). It is enticing to consider that the secreted IL-6 from

the T9.F/IL6/hi tumors may play a costimulatory role in the acti-

vation of glioma-specific CTLs via this alternative pathway.
Subcutaneous injection of viable, parental T9.F glioma cells in the contralateral flank from which T9.F/IL6hi tumors were rejected induced a DTH reaction, and therefore points to the generation of T9.F glioma-specific, cellular immunity. To identify lymphocyte populations responsible for cellular memory, we conducted a series of passive transfer experiments in which spleen cells obtained from T9.F/IL6hi-immunized rats were depleted of specific T cell subsets and were injected to naive recipients. The recipients were then implanted i.c. with a lethal dose of parental T9.F glioma cells. The results of these experiments indicated that the induced T9.F cellular memory is partially dependent upon CD8+ T cells and is completely CD3+ T cell dependent.

It is tempting to hypothesize that at the early stage of the IL-6-induced antitumor response, neutrophils infiltrating the s.c. T9.F/IL6hi tumors become activated and initiate tumor destruction by their effector functions, i.e., respiratory burst, degradative enzyme release, and TNF-α secretion. We are currently investigating the potential cytolytic activity of neutrophils on T9.F/IL6hi cells. APCs may then phagocytize tumor cell debris, migrate to the tumor-draining lymph node, and present tumor-specific Ags to naïve T cells. Activated T cells then traffic to the tumor site and lyse the remaining tumor cells. Activation of glioma-specific T cells and their subsequent effector functions may also be enhanced due to the presence of IL-6. A fraction of the glioma-specific T cells become memory cells and are responsible for the maintenance of glioma immunity.

We believe that successful treatment of the GBM will encompass the formation of a cellular immune response consisting primarily of glioma-specific T cells. We have demonstrated in a modified rat T9 glioblastoma model that glioma cells genetically engineered to secrete IL-6 invoke an effective, antitumor response in which the early stages may be mediated by neutrophils. During the later phase of the antitumor response, in which the T9.F/IL6hi tumor is beginning to regress, activated glioma-specific T cells are present in the circulation and proceed to generate glioma-specific T cell-dependent immunity. These findings may be important in the clinical application of IL-6-transduced glioma cells for the treatment of malignant brain tumors, where such vaccines could be used to prevent tumor recurrence after initial surgical resection.

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References


