Journal of Neuro-Oncology
DOI 10.1007/s11060-007-9442-z

Laboratory Investigation

Tumor infiltration by myeloid suppressor cells in response to T cell activation in rat gliomas

Martin R. Graf [1], Jeremy T. Sauer[2] and Randall E. Merchant [1,2]
[1]*Department of Neurosurgery, Virginia Commonwealth University Medical Center, Richmond, VA, USA;*
[2]*Department of Anatomy and Neurobiology, Virginia Commonwealth University Medical Center, Richmond, VA, USA*

Key words: brain tumor-associated immunosuppression, gliomas, glioma-vaccine, myeloid suppressor cells, neuroimmunology

Summary

We have recently reported that activation of tumor-specific T cells by subcutaneous vaccination with irradiated T9 glioma cells of syngeneic rats with a pre-existing, intracranial (i.c.) T9 glioma (T9 + vaccination) promotes the mobilization of myeloid suppressor cells (MSC) which inhibit T cell function resulting in unregulated tumor progression. The current study investigated if this immunological paradigm could be recapitulated in T cell deficient rats, in other rat glioma models or using a dendritic cell (DC) vaccine. When nude rats were used in the T9 + vaccination model, the level of MSC tumor infiltration remained low in vaccinated and control groups and there was no significant difference in tumor size between the groups. Increased tumor infiltration by MSC after vaccination with respective irradiated tumor cells was observed in the 9L, F98 and D74 gliomas. RT-2 tumors were markedly infiltrated with MSC regardless of vaccination. Enhanced tumor progression in response to immunization and T cell activation was observed in rats bearing F98 and D74 gliomas, although less pronounced than in the T9 model, and there was a trend for increased tumor size in the 9L glioma model. Increased MSC infiltrate and augmented T9 glioma growth were observed when DC pulsed with T9 cell lysate was used as a vaccine. These results suggest that MSC infiltration and unregulated tumor growth in response to vaccination is T cell-dependent; is not unique to the T9 glioma; and can be recapitulated with an alternate immunization approach.

Introduction

Malignant gliomas represent a significant class of central nervous system (CNS) tumors derived from the astrocyte lineage. Glioblastoma multiforme (GBM), a grade IV astrocytoma, is the most common form of these CNS neoplasms in adults and is extremely lethal. The average 5-year survival for GBM patients is less than 2% and the median survival after diagnosis is 18 months [1]. The traditional treatment for this disease is surgical resection accompanied by post-operative, external beam radiation. However, because of the highly invasive nature of GBM, cryptic foci of neoplastic cells in the adjacent parenchyma invariably give rise to tumor recurrence, rendering these treatments palliative. Many brain tumor researchers believe that the development of a glioma vaccine, which can activate glioma-specific T cells, would be an effective complement to surgery and radiation, and could be used to destroy residual tumor cells and prevent recurrence.

Immunization strategies to generate tumor-specific T cells include tumor cells that have been genetically altered to secrete immune enhancing cytokines; dendritic cells (DC) that have been manipulated to present tumor antigens; and active immunization with recombinant cancer peptides (reviewed [2–5]). These approaches have also been investigated in a few experimental models of glioma in both prophylactic and therapeutic studies and have yielded promising results, providing the foundation for clinical trials (reviewed [6,7]). For example, vaccination with DC pulsed with acid-eluted tumor antigens from 9L glioma cells was an effective treatment for rats with a pre-existing, intracranial (i.c.) 9L glioma [8]. This study resulted in a subsequent phase I trial in which malignant glioma patients were immunized with autologous DC that were pulsed with autologous glioma antigens [9]. It is interesting to note that there was no significant survival of the patients as compared to the non-vaccinated, control group, although DC vaccination induced the activation of systemic, tumor-specific T cells in almost half of the patients [9]. In patients that underwent a subsequent operation, an increased infiltrate of CTL and memory T cells was detected [9]. We observed a similar situation in a rat T9 glioma + vaccination model. In this regard, s.c. vaccination with irradiated T9 cells of rats with a pre-existing, i.c. T9 glioma, induced the generation of tumor-specific T cells which could be detected in the spleen, and also induced a significant number of $CD4^+$ and $CD8^+$ T cells to infiltrate the i.c. glioma; however, there was no extension of survival and, unexpectedly, survival was reduced [10,11]. Analysis of the tumor infiltrate revealed a population of immature myeloid cells which expressed both monocyte (CD11b/c) and granulocyte (HIS48)-associated lineage markers that co-localized with the activated T cells in the tumor in vaccinated animals. When purified, the immature myeloid cells suppressed the proliferative response of

tumor–specific T cells to tumor antigens (Ags) and could enhance glioma growth when passively transferred to rats bearing an i.c. T9 glioma [10].

An analogous myeloid cell population has been more intensely studied in mice and is referred to as myeloid suppressor cells (MSC). MSC can inhibit T cell effector functions such as proliferation, cytotoxicity and the production of interferon-γ, and phenotypically, MSC express surface markers characteristic of both granulocyte and monocyte lineages, such as Gr-1 and CD11b, respectively, in the mouse [12–16], reviewed in [17]. MSC mobilization has been demonstrated in various non-glial, murine tumors such as the CT26.WT colon adenocarcinoma model and the TS/A mammary carcinoma model, in addition, MSC generation is reported to be induced by the spontaneous secretion of granulocyte-monocyte colony stimulating factor (GM-CSF) by these tumors [17]. The presence of MSC is not restricted to tumor models and has been reported in other immunological conditions such as graft-vs-host disease, bacterial infections, immunization with super-Ags or vaccinia virus encoding the β-galactosidase Ag and IL-2 [18–21].

In the T9 + vaccination paradigm, we believe that s.c. vaccination and activation of T cells induce the mobilization of MSC which localize in the tumor site and suppress T cell effector functions, thereby permitting the tumor to rapidly progress. We believe that immunoregulatory MSC represent another method in which gliomas can evade tumor reactive T cells, and MSC may play a role in brain tumor-related immunosuppression. In the present study, we used nude rats in the T9 + vaccination model to assess the role of T cells in MSC mobilization; analyzed MSC levels in other syngeneic rat glioma models; and utilized an alternate immunization approach to induce MSC tumor infiltration.

Materials and methods

Animals, cell lines and culture

Inbred female Fischer 344 rats weighing 120–140 g were obtained from Harlan Sprague Dawley (Indianapolis, IN) or the National Cancer Institute (Fredrick, MD). Animals were housed in a climate controlled, AAALAC approved vivarium and were provided free access to rat chow and water. Female nude (rnu/rnu) rats, 6–7 weeks in age, were obtained from the National Cancer Institute and housed under sterile conditions. All experimental animal procedures were approved by members of the Institutional Animal Care and Use Committee. Cells were cultured in DMEM supplemented with 10% FBS and non-essential amino acids, as adherent monolayers at 37 °C, and passed biweekly with trypsin in the absence of antibiotics. Cell lines were routinely screened for mycoplasma contamination (MycoTect, Gibco BRL). All tissue culture reagents and supplements were obtained from Gibco BRL (Grand Island, NY) unless noted otherwise. The T9 glioma was a gift from Dr J. Yoshida (Nagoya University, Japan) and the 9L glioma was generously provided by Dr C. Kruse (University of Colorado Health Science Center, CO). The T9 glioblastoma tumor was originally induced by *N*-nitrosomethylurea injection [22] and has been reported to be derived from the 9L glioma cell line [23]. The RT-2 glioma was induced by the avian sarcoma virus and the glioma cell line was kindly provided by Dr Yancy Gillespi (University of Alabama, Birmingham, AL) [24]. The F98 and D74 gliomas were derived from neoplasms induced by the i.v. administration of nitrosourea [25,26] and were obtained from the American Type Culture Collection (Manassas, VA). All cell lines are syngeneic to the Fischer F344 rat.

Tumor implantation, vaccination and experimental design

Monolayers of tumor cells were trypsinized, counted on a hemacytometer and viability was assessed by trypan blue exclusion. Cells were washed twice in phosphate buffered saline (PBS) and the concentration was adjusted appropriately. The intracerebral implantation of tumor cells has been previously described [27]. In brief, anesthetized animals were placed in a stereotactic apparatus; a shallow depression 4 mm to the right of the sagittal suture and 1 mm posterior to the coronal suture was made; and 5 µl of a given tumor cell suspension was injected at a depth of 3.5 mm. Preliminary studies were performed to identify the optimal number of 9L, F98, D74 or RT-2 cells to implant so that tumors from control rats were comparable in size at day 15 to that of control T9 gliomas (~35 mg) and were determined to be 5×10^5 9L cells; 5×10^3 F98 or D74 cells; 5×10^2 RT-2 cells and 5×10^4 T9 cells. Rats were vaccinated s.c., in the hind flank, with 5×10^6 irradiated (50 Gy) tumor cells from the respective tumor as previously described [11]. Immunizations were performed 5 days after i.c. tumor inoculation. Rats were euthanized 15 days after i.c. tumor inoculation; gliomas were carefully removed, weighed and processed for analysis.

Analysis of MSC and $CD3^+$ lymphocytes in glioma infiltrates and flow cytometry

Tumors were carefully excised; forced through a 70 µm cell strainer and washed with PBS. Erythrocytes were lysed with ammonium chloride buffer when it was deemed that tumor cell preparations were excessively contaminated with erythrocytes. Viable mononuclear cells were enumerated using a hemacytometer and trypan blue exclusion. Cell surface staining and FACS analysis has been previously described [11]. Briefly, 1×10^6 cells were stained in a volume of 50 µl of 5% FBS/PBS containing a cocktail of three different mAbs for 30 min on ice. The mAb from the HIS48 hybridoma is specific for rat granulocytes and anti-CD11b/c mAb (OX-42) is specific for cells of the monocytic lineage. Cells that stained positive with both $HIS48^{high}$ and CD11b/c were identified as MSC. The anti-CD3ε mAb (G4.18) was used to identify $CD3^+$ lymphocytes. Antibodies were either directly conjugated to a fluorochrome or were biotinylated and streptavidin–Peridinin

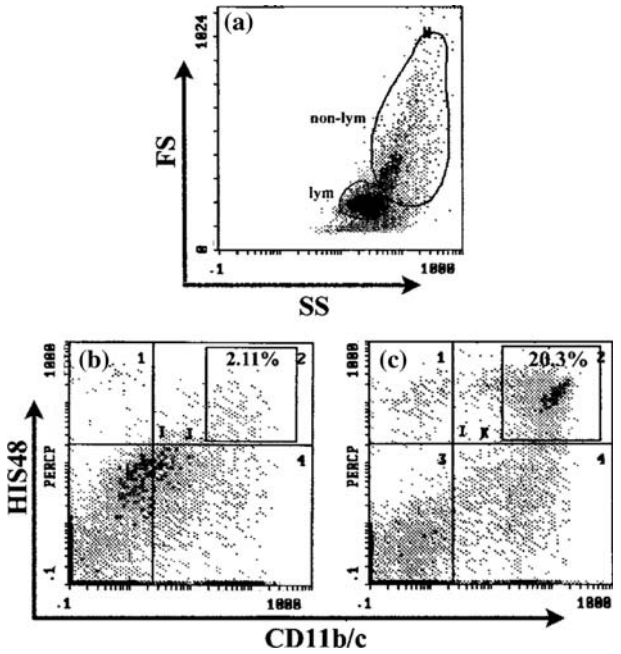

Figure 1. Tumor infiltrating MSC express granulocyte (HIS48) and monocyte (CD11b/c) associated surface antigens. (a) Forward scatter and side scatter profile of the glioma infiltrate from a T9 + vaccination rat used to identify lymphocyte (lym) and non-lymphocyte (non-lym) populations. MSC are located in the non-lym gate. (b) T9 glioma infiltrate of a non-vaccinated and (c) vaccinated rat analyzed 15 days post-implantation. MSC are HIS48^{high} and CD11b/c^{+} and annotated numbers in the histograms reflect the percentage of cells in the square gates.

chlorophyll protein (PerCP) was used as a secondary reagent for detection. Antibodies and streptavidin–PerCP were obtained from BD Pharmingen (San Diego, CA). For the analysis of CD3^{+} cells, gating was set on the lymphocyte population which was identified by forward and side scatter characteristics. For MSC analysis, gating included both the lymphocyte and myeloid cell populations unless otherwise indicated. Representative histograms and gating of the cellular infiltrate from T9 gliomas are shown in Figure 1. Additional mAbs used for FACS analysis of cultured DC include CD11b (WT.5), CD86 (24F), and RT1B (OX-6), all from BD Pharmingen.

Dendritic cell vaccine

Dendritic cells were generated from rat bone marrow cells using a modified method described by Grauer et al. [28]. Bone marrow was obtained from femurs of Fisher rats and flushed through a 70 μm screen. Erythrocytes were lysed with ammonium chloride buffer and macrophages were removed by plastic adherence. Cells were resuspended in RPMI-1640 containing 10% FBS, 0.05 mM 2-mercaptoethanol, HEPES, antibiotics/antimycotic (complete RPMI) and supplemented with recombinant rat IL-4 (10 ng/ml, R&D Systems, Minneapolis, MN) and GM-CSF (10 ng/ml, BD Pharmingen) and were cultured in 6-well plates at a density of 1×10^6/ml and 3 ml/well. After 4 days, ~80% of the media was removed and replaced with 2 ml of complete RPMI supplemented with 10 ng/ml IL-4 and GM-CSF. After three additional days of culture, all non-adherent and loosely adherent cells were collected by forceful washing with Hanks balanced salt solution (HBSS) and a brief exposure to trypsin, leaving stromal cells firmly attached to the plate. The concentration of cells was adjusted to 2×10^6/ml of complete RPMI supplemented with GM-CSF (2.5 ng/ml) and seeded into new 6-well plates, 5×10^6/well for final maturation. After 48 h, cultures were moderately agitated and washed with HBSS to harvest floating and loosely adherent cells and concentration was adjusted to 2×10^6/ml complete-RPMI and pulsed with T9 cell lysate. To generate cell lysates, T9 cells were collected, the concentration was adjusted to 6×10^6/ml in serum free RPMI, then divided into 1 ml aliquots which were subjected to eight rapid cycles of freezing (liquid nitrogen) and thawing (37 °C). Macroparticles were removed from the lysates by centrifugation at $300 \times g$ for 10 min and aliquots (6×10^6 cell equivalents in 1 ml) were stored at −80 °C. For pulsing, cell lysates were added to DC at a ratio of 3 tumor cells eqivalents:1 DC (1 aliquot of cell lysate:2×10^6 DC) in complete-RPMI supplemented with GM-CSF (2.5 ng/ml) and seeded into new 6-well plates, 4×10^6 DC-lysate/well and cultured for ~20 h. Floating and loosely adherent pulsed DC were harvested by gentle washing with HBSS, washed twice with PBS and the concentration was adjusted to 2×10^7 cells/ml PBS. Rats were vaccinated s.c. with 100 μl of pulsed DC, which equates to 2×10^6 DC pulsed with 6×10^6 T9 cell equivalents.

Statistics

Statistical analysis was performed using the student *t*-test and differences with a P-value < 0.05 were considered significant. Error bars in figures indicate SEM.

Results

T cells are required for MSC mobilization

In our T9 glioma model, tumor bearing rats are immunized s.c. with irradiated T9 cells and the activated T cells traffic to the i.c. T9 glioma. The number of CD4^{+}

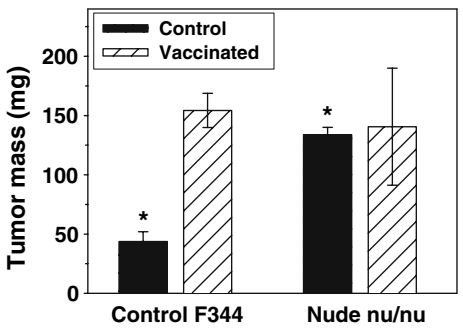

Figure 2. Mobilization of MSC and increased tumor growth after vaccination is T cell dependent. Nude or control F344 rats were implanted with 5×10^4 T9 cells and were immunized with 5×10^6 irradiated T9 cells ($n = 3$). Tumor mass was assessed 15 days post-implantation. The experiment was performed twice and yielded similar results. *$P < 0.0005$.

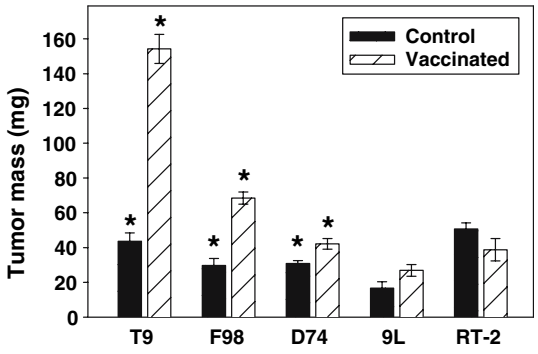

Figure 3. Tumor growth after vaccination in other glioma models. Rats were implanted i.c. with T9 (5×10^4 cells); F98, D74 (5×10^3 cells); 9L (5×10^5 cells); or RT-2 (5×10^2 cells) glioma cells and were vaccinated 5 days later with 5×10^6 irradiated cells of the respective glioma ($n = 3$). Tumor sizes of the control and vaccinated rats were compared 15 days post-implantation. This experiment was repeated once more with similar results. $^*P < 0.05$.

T cells and $CD8^+$ T cells per mg of tumor weight is ~10× greater in vaccinated rats than that of non-vaccinated rats, which are poorly infiltrated by immune cells [11]. We believe that the activation of T cells is an essential step for the mobilization of MSC; therefore, we used T cell deficient rats in our T9 + vaccination model to assess the role of T cells in this immunological paradigm. Nude rats were implanted i.c. with the 5×10^4 T9 glioma cells and were immunized s.c. 5 days post-implantation. Control nude rats were not immunized. Analysis of the level of MSC infiltrate in the tumors 15 days after tumor inoculation revealed that there were no differences in the degree of MSC infiltration between control and vaccinated nude rats, which was similar to that of non-vaccinated, immunocompetent rats (Table 1). These results indicate that, in the absence of a T cell response, there is no trafficking of MSC to the i.c. T9 gliomas and MSC levels remain unchanged. In the T9 + vaccination model, enhanced tumor progression is a result of increased levels of MSC in the tumor. Accordingly, there were no differences in glioma size between the control and vaccinated nude rats. It is interesting to note that the size of the gliomas from the nude rats is comparable to that of the tumor from immunocompetent, T9 + vaccinated rats (Figure 2). This suggests that MSC can create an immunosuppressive environment within the tumor that permits the tumor to grow at a similar rate observed in T cell deficient rats.

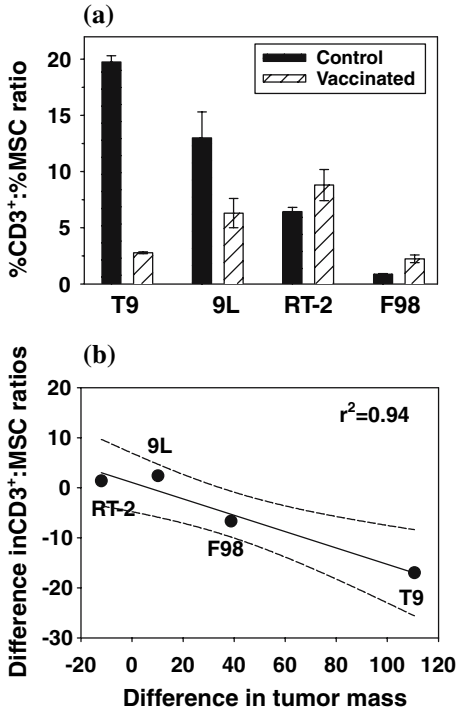

Figure 4. Comparison of $CD3^+$ cells: MSC ratio in the glioma infiltrate of control or vaccinated rats. (a) A decrease in the ratio in the infiltrate of vaccinated rats as compared to non-vaccinated controls is indicative of a more immunosuppressive environment which is permissive to tumor progression. (b) The differences in the ratios of $CD3^+$ T cells: MSC between control and vaccinated animals correlates to increased glioma growth after immunization. Correlation value: $R^2 = 0.94$, 95% confidence interval.

MSC levels in the cellular infiltrate of several rat gliomas

The results from the nude rat experiments suggest that the activation of T cells is an essential prerequisite for MSC mobilization and tumor infiltration. Therefore, we reasoned that the degree of MSC infiltration, in response to immunization and T cell activation, may correlate with tumor immunogenicity.

We briefly characterized the immunizing potential of several syngeneic glioma lines. Rats were vaccinated by two weekly injections of 1×10^7 irradiated T9, RT-2, 9L, F98 or D74 cells and then were challenged in the brain with a lethal dose of the respective tumors. The results demonstrated that the T9 glioma (4/4 survive) and RT-2 glioma (4/4 survive) are moderately immunogenic, the 9L glioma (3/4 survive) is weakly immunogenic and the F98 glioma (0/4 survive) and D74 glioma (0/4 survive) are poorly immunogenic. Additionally, Tzeng et al. reported that the F98 glioma is more immunogenic than the D74 glioma [29].

Rats were implanted i.c. with T9, 9L, F98, D74 or RT-2 glioma cells using a dose that was predetermined to generate tumors of comparable size in control rats after 15 days and were immunized with 5×10^6 irradiated cells of the respective tumor 5 days post-implantation. The level of MSC infiltration of the gliomas from vaccinated and control rats was assessed at day 15 and are shown in Table 1. The more immunogenic tumors (T9, RT-2 and 9L) of the vaccinated rats contained a substantial degree of MSC infiltration. RT-2 gliomas of control and immunized rats were highly infiltrated by MSC, possibly providing insight into the extremely aggressive nature of this tumor and an upper limit of MSC infiltration. Vaccination also induced increases in the level of MSC infiltrate in the poorly immunogenic F98 and D74 tumors, although the changes were not as pronounced.

Table 1. MSC levels in glioma infiltrate

Glioma	Mean % MSC (SEM) [a]		ΔMSC
	Vaccinated	Control	
Immunogenicity			
T9 (nude)	1.11 (0.2)	1.08 (0.1)	0.03
T9	15.8 (1.3)	1.98 (0.8)	13.8[b]
RT-2	20.2 (1.6)	19.4 (1.6)	0.80
9L	6.43 (1.1)	1.21 (0.1)	5.22[c]
F98	1.28 (0.1)	0.90 (0.1)	0.38[c]
D74	3.02 (0.2)	1.42 (0.2)	1.60[c]
T9 (DC vaccine)	12.9 (3.2)	2.28 (0.1)	10.6[b]

[a]3 rats/group, experiment performed twice with similar results.
[b]$P < 0.05$.
[c]$P < 0.005$.

A comparison of the tumor mass between control and vaccinated rats is shown in Figure 3. Significant enhancement of tumor growth after vaccination was observed in rats bearing F98 and D74 gliomas, although not as striking as observed in the T9 glioma model. There was a trend towards increased tumor size in rats bearing a 9L glioma after vaccination. Liu et al. [30,31] has recently reported a similar observation in the 9L model, i.e. s.c. vaccination of rats with a pre-existing i.c. 9L glioma results in an increase of tumor size relative to the tumors of non-vaccinated rats. There were no significant differences in sizes of the RT-2 glioma between the vaccinated and control groups. It is interesting to note, that in these experiments, rats were inoculated with 500 RT-2 cells in order to generate tumors of a comparable size. In pilot studies with RT-2 cells, rats were implanted with 5×10^4 or 5×10^3 cells and rats became moribund in 10 and 12 days, respectively. This survival information highlights the tumorigenicity of the RT-2 glioma when implanted in the CNS even though it can be classified as a moderately immunogenic tumor. The changes in MSC infiltration correlated with the enhanced tumor growth of the vaccinated rats with the exception of the 9L glioma.

Ratio of CD3$^+$ lymphocytes and MSC in the cellular infiltrate of gliomas

In a previous study, we demonstrated that MSC can suppress the response of activated, tumor-specific T cells when stimulated with tumor Ags [10] and our current findings demonstrate that the activation of T cells is required for the mobilization of MSC. Therefore, we compared the levels of CD3$^+$ T cells to that of MSC in the tumor infiltrate of control and vaccinated rats bearing T9, 9L RT-2 or F98 gliomas. The ratios of CD3$^+$ cells to MSC in the different glioma models are shown in Figure 4. In the case of the T9 and F98 gliomas, vaccination resulted in a decrease of the CD3$^+$:MSC ratio, which we believe reflects a more immunosuppressive tumor environment, and correlates with an increase of glioma growth. In contrast, immunization of 9L tumor-bearing rats resulted in an increase of MSC infiltration which coincided with a high level of CD3$^+$ T cell infiltration, resulting in little change of the CD3$^+$: MSC ratio. A similar situation was observed in the RT-2 glioma model.

Vaccination with dendritic cells pulsed with T9 cell lysates induce MSC mobilization

Immunization with DC pulsed with tumor cell lysate is an extremely effective method for activating tumor specific T cells. Therefore, we investigated if vaccination with DC pulsed with T9 cell lysate would result in the generation and tumor localization of MSC in the T9 model. Dendritic cells were obtained from bone marrow and cultured in the presence of IL-4 and GM-CSF for 9 days as described in materials and methods. After 9 days, the phenotype of the culture was CD11b$^+$, CD11b/c$^+$, HIS48$^-$, CD3$^-$, >95% MHC class II$^+$ and ~35% CD86$^+$, as determined by FACS analysis (data not shown). Additionally, the cultured DC could effectively induce the proliferation of allogeneic T cells (data not shown). At this point, DC were co-incubated with T9 cell lysates at a ratio of 3 tumor cell equivalents: 1 DC for ~20 h. Rats bearing a 5 days, i.c. T9 glioma were immunized s.c. with 2×10^6 DC that were pulsed 6×10^6 T9 cell equivalents and the levels of MSC was assessed 15 days post-tumor implant. The results are shown in Table 1 and demonstrate that vaccination with tumor lysate-pulsed DC results in a significant increase of tumor infiltrating MSC which corresponded to advanced tumor progression (Figure 5).

Discussion

Early tumor transplantation studies using the rat T9 glioma, clearly demonstrated that the T9 tumor is immunogenic and that immunization of rats with T9 cells effectively induces protective, cellular immunity from rechallenge with viable T9 cells [32,33]. We have repeated these experiments; however, when we altered the experimental model to that of more clinical relevance, in which we attempted to treat rats with an established i.c. T9 glioma using s.c. vaccination with irradiated T9 cells, the results were unexpected. In this regard, the immunizations were successful in that they induced a significant number of CD4$^+$ and CD8$^+$ tumor-specific T cells to cross the blood brain barrier and infiltrate the tumor, but the T cells appeared to be tolerant and the gliomas rapidly progressed [11]. In these therapeutic studies, we identified a population of immature myeloid cells which co-expressed granulocyte and monocyte lineage markers and significantly infiltrated the gliomas of the vaccinated rats [10]. Furthermore, we demonstrated that these myeloid cells were MSC and when purified, inhibited T cell responses to tumor Ags and enhanced tumor progression when passively transferred to glioma-bearing recipients [10]. We believe that MSC play an immunoregulatory role in the CNS by suppressing the function of reactive T cells which are potentially damaging in the brain.

When nude rats were used as host animals in the T9 + vaccination paradigm, immunization did not result in elevated levels of MSC infiltration in the glioma. This indicates that the presence of functional T cells is needed for the generation of MSC. In the T9 + vaccination model, it is possible that myeloid progenitor cells are stimulated by growth factors secreted by activated T cells (which are known to produce various colony stimulating factors), in addition to tumor-derived factors, to give rise to MSC. Bronte et al. [34] have reported that unopposed GM-CSF production by tumors results in the generation of MSC. We were not able to detect GM-CSF in the serum of moribund T9 + vaccinated rats by ELISA (unpublished results). Currently, we are in the process of selectively depleting immunocompetent Fischer rats of $CD4^+$, $CD8^+$, or NK/NKT cells in order identify which lymphocyte subset(s) is essential for MSC infiltration and unregulated glioma growth. It is interesting to note that in the nude rat experiments, the size of the tumors in the control and vaccinated nude rats is similar to that of the tumor in normal T9 + vaccinated rats. This observation may reflect the degree of immunosuppression within the glioma of the vaccinated rats. In this regard, even though the gliomas are significantly infiltrated by tumor-specific T cells [10,11], the tumor progresses at a similar rate to that of gliomas implanted in T cell-deficient animals.

Based upon the results from the nude rat experiments, we postulated that activation of T cells by the immunization with irradiated tumor cells is an essential step in MSC mobilization and, therefore, the degree of MSC infiltration may correlate to tumor immunogenicity. When we repeated our studies using the 9L, RT-2, F98 and D74 gliomas, the more immunogenic gliomas (T9, RT-2 and 9L) contained a higher level of MSC infiltration as compared to the less immunogenic F98 and D74 tumors. We also analyzed the level of $CD3^+$ T cell infiltration in response to vaccination in the gliomas. Because MSC can directly inhibit T cell activity, we believe that the degree of immunosuppression in the gliomas can be related to the ratio of $CD3^+$ T cells to MSC present in the glioma. A change in this ratio correlated well with increased tumor progression, reflecting a more suppressive environment.

Barth et al. [23] reported that the T9 and 9L cell lines originated from the same tumor over 30 years ago and may be considered to be the same tumor. Because of this, we were concerned that the results from the experiments with the 9L glioma differed from that obtained with the T9 glioma. In this regard, the 9L gliomas of vaccinated rats were less infiltrated by MSC and did not display a significant degree of enhanced tumor progression as the T9 + vaccinated rats. Because of the various laboratories and numerous passages that these glioma lines have experienced over time, it is not unlikely that they have drifted, acquiring slight changes in immunogenicity and tumorigenicity which may account for the differences we observed in our experiments. In support of this, we have demonstrated in our laboratory that rats immunized with T9 cells (obtained from Dr J. Yoshida, Nagoya University, Japan) are 100% protected from a challenge with viable T9 cells, but are partially protected from a subsequent challenge with 9L cells (~75%, a gift from Dr C. Kruse, University of Colorado Health Science Center, CO), suggesting that these two lines share common glioma Ags but have diverged over time so they are not completely the same (unpublished results). Recent reports from Liu et al. [30,31] have shown results similar to ours in that vaccination of rats bearing an i.c. 9L glioma results in a modest increase in tumor progression and reduced survival, supporting our findings.

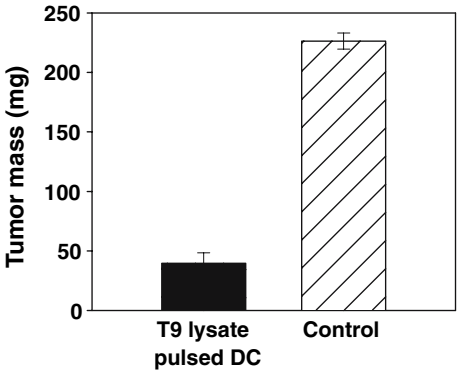

Figure 5. Vaccination with T9 lysate pulsed DC induces MSC infiltration and enhanced glioma growth. Rats bearing a 5 days T9 glioma were immunized with 2×10^6 DC that were pulsed with T9 cell lysate. MSC levels and tumor mass were analyzed at 15 days and compared to non-vaccinated controls ($n = 3$). $^*P < 0.005$.

As a final experiment, we showed that MSC infiltration of T9 gliomas can be induced by immunization with DC pulsed with T9 cell lysate and that gliomas with elevated MSC levels exhibited unregulated progression. These results are similar to that obtained when rats are immunized with irradiated T9 cells. However, the results are in contrast with those obtained by other researchers using the rat 9L glioma model and vaccination with either DC pulsed with tumor peptides [8] or tumor apoptotic bodies [35]. In these studies, a significant number of DC vaccinated rats bearing an i.c. 9L glioma were long term survivors. However, these studies differed in the source of tumor Ag used for loading DC and methods of use for DC culture and maturation. Additionally, in our hands, the 9L and T9 gliomas are not identical. Interestingly, we recently reported that T9 cells genetically altered to secrete a high level of IL-2 exhibit unregulated tumor progression and high levels of MSC, even though they were markedly infiltrated by $CD4^+$, $CD8^+$, and NK T cells representing a different scenario in which MSC co-localize with activated T cells in an intracerebral glioma [36].

Immunoregulatory MSC present in gliomas can suppress the function of tumor-infiltrating, activated T cells, and therefore play a role in brain-tumor associated immunosuppression. The inadvertent generation of MSC in clinical trials involving vaccine-based strategies may represent a significant obstacle to successful brain tumor immunotherapy. However, we believe that modulating MSC populations or their

function may improve the efficacy of current immunotherapeutic approaches for glioma patients.

Acknowledgements

The authors would like to express their gratitude for the support from the Martha Betts Foundation and the Lind Lawrence Foundation and are equally grateful for the financial assistance from the Cullather, Hord and Feichter families. This work was supported in part by The MCV Brain Tumor Foundation (M.R.G. and R.E.M.). Flow cytometry was supported in part by NIH Grant P30 CA16059.

References

1. Surawicz TS, Davis F, Freels S, Laws ER Jr., Menck HR: Brain Tumor survival: results from the National Cancer Data Base. J Neurooncol 40(2): 151–160, 1998
2. Nouri-Shirazi M, Banchereau J, Fay J, Palucka K: Dendritic cell based tumor vaccines. Immunol Lett 74(1): 5–10, 2000
3. Mach N, Dranoff G: Cytokine-secreting tumor cell vaccines. Curr Opin Immunol 12(5): 571–575, 2000
4. Hipp JD, Hipp JA, Lyday BW, Minev BR: Cancer vaccines: an update. In Vivo 14(5): 571–585, 2000
5. Eisenbach L, Bar-Haim E, El Shami K: Antitumor vaccination using peptide based vaccines. Immunol Lett 74(1): 27–34, 2000
6. Tjoa BA, Salgaller ML: Progress in active-specific immunotherapy of brain malignancies. Expert Opin Investig Drugs 9(9): 2093–2101, 2000
7. Parney IF, Hao C, Petruk KC: Glioma immunology and immunotherapy. Neurosurgery 46(4): 778–791, 2000
8. Liau LM, Black KL, Prins RM, Sykes SN, DiPatre PL, Cloughesy TF, Becker DP, Bronstein JM: Treatment of intracranial gliomas with bone marrow-derived dendritic cells pulsed with tumor antigens. J Neurosurg 90(6): 1115–1124, 1999
9. Yu JS, Wheeler CJ, Zeltzer PM, Ying H, Finger DN, Lee PK, Yong WH, Incardona F, Thompson RC, Riedinger MS, Zhang W, Prins RM, Black KL: Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. Cancer Res 61(3): 842–847, 2001
10. Prins RM, Scott GP, Merchant RE, Graf MR: Irradiated tumor cell vaccine for treatment of an established glioma. II. Expansion of myeloid suppressor cells that promote tumor progression. Cancer Immunol Immun 51(4): 190–199, 2002
11. Graf MR, Prins RM, Hawkins WT, Merchant RE: Irradiated tumor cell vaccine for treatment of an established glioma. I. Successful treatment with combined radiotherapy and cellular vaccination. Cancer Immunol Immun 51(4): 179–189, 2002
12. Young MR, Young ME, Wright MA: Myelopoiesis-associated suppressor-cell activity in mice with lewis lung carcinoma tumors: Interferon-gamma plus tumor necrosis factor-alpha synergistically reduce suppressor cell activity. Int J Cancer 46(2): 245–250, 1990
13. Apolloni E, Bronte V, Mazzoni A, Serafini P, Cabrelle A, Segal DM, Young HA, Zanovello P: Immortalized myeloid suppressor cells trigger apoptosis in antigen-activated T lymphocytes. J Immunol 165(12): 6723–6730, 2000
14. Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, Zanovello P, Segal DM: Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. J Immunol 168(2): 689–695, 2002
15. Bronte V, Apolloni E, Cabrelle A, Ronca R, Serafini P, Zamboni P, Restifo NP, Zanovello P: Identification of a CD11b(+)/Gr-1(+)/CD31(+) myeloid progenitor capable of activating or suppressing CD8(+) T cells. Blood 96(12): 3838–3346, 2000
16. Gabrilovich DI, Velders MP, Sotomayor EM, Kast WM: Mechanism of immune dysfunction in cancer mediated by immature Gr-1+ myeloid cells. J Immunol 166(9): 5398–5406, 2001
17. Bronte V, Serafini P, Apolloni E, Zanovello P: Tumor-induced immune dysfunctions caused by myeloid suppressor cells. J Immunother 24(6): 431–446, 2001
18. Bronte V, Wang M, Overwijk WW, Surman DR, Pericle F, Rosenberg SA, Restifo NP: Apoptotic death of CD8+ T lymphocytes after immunization: induction of a suppressive population of Mac-1+/Gr-1+ cells. J Immunol 161(10): 5313–5320, 1998
19. Bobe P, Benihoud K, Grandjon D, Opolon P, Pritchard LL, Huchet R: Nitric Oxide mediation of active immunosuppression associated with graft-versus-host reaction. Blood 94(3): 1028–1037, 1999
20. al Ramadi BK, Brodkin MA, Mosser DM, Eisenstein TK: Immunosuppression induced by attenuated *Salmonella*. Evidence for mediation by macrophage precursors. J Immunol 146(8): 2737–2746, 1991
21. Cauley LS, Miller EE, Yen M, Swain SL: Superantigen-induced CD4 T cell tolerance mediated by myeloid cells and IFN-gamma. J Immunol 165(11): 6056–6066, 2000
22. Benda P, Someda K, Messer J, Sweet WH: Morphological and immunochemical studies of rat glial tumors and clonal strains propagated in culture. J Neurosurg 34(3): 310–323, 1971
23. Barth RF: Rat brain tumor models in experimental neuro-oncology: the 9L, C6, T9, F98, RG2 (D74), RT-2 and CNS-1 gliomas. J Neurooncol 36(1): 91–102, 1998
24. Beckman WC Jr., Powers SK, Brown JT, Gillespie GY, Bigner DD, Camps JL, Jr: Differential retention of rhodamine 123 by avian sarcoma virus-induced glioma and normal brain tissue of the rat in vivo. Cancer 59(2): 266–270, 1987
25. Swenberg JA, Koestner A, Wechsler W: The induction of tumors of the nervous system with intravenous methylnitrosourea. Lab Invest 26(1): 74–85, 1972
26. Koestner A, Swenberg JA, Wechsler W: Transplacental production with ethylnitrosourea of neoplasms of the nervous system in Sprague-Dawley rats. Am J Pathol 63(1): 37–56, 1971
27. Graf MR, Merchant RE: Interleukin-6 transduction of a rat T9 glioma clone results in attenuated tumorigenicity and induces glioma immunity in Fischer F344 rats. J Neurooncol 45(3): 209–218, 1999
28. Grauer O, Wohlleben G, Seubert S, Weishaupt A, Kampgen E, Gold R: Analysis of maturation states of rat bone marrow-derived dendritic cells using an improved culture technique. Histochem Cell Biol 117(4): 351–362, 2002
29. Tzeng JJ, Barth RF, Orosz CG, James SM: Phenotype and functional activity of tumor-infiltrating lymphocytes isolated from immunogenic and nonimmunogenic rat brain tumors. Cancer Res 51(9): 2373–2378, 1991
30. Liu Y, Ng K, Lillehei KO: Time course analysis and modulating effects of established brain tumor on active-specific immunotherapy. Neurosurg Focus 9: 1–9, 2000
31. Liu Y, Ng K, Lillehei KO. Vaccination induced tumor progression in a 9L intracranial glioma model. Proceedings of the American Association for Cancer Research, 94th Annual Meeting, 44, 876, 2003
32. Denlinger RH, Axler DA, Koestner A, Liss L: Tumor-specific transplantation immunity to intracerebral challenge with cells from a methylnitrosourea-induced brain tumor. J Med 6(3–4): 249–259, 1975
33. Blume MR, Wilson CB, Vasquez DA: Immune response to a transplantable intracerebral glioma in rats. In: Sane K, Ishi S, LeVay D: Recent Progress in Neurologic Surgery. Amsterdam: Excerpta Medica; pp 129–134, 1974
34. Bronte V, Chappell DB, Apolloni E, Cabrelle A, Wang M, Hwu P, Restifo NP: Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8+ T cell

responses by dysregulating antigen-presenting cell maturation. J Immunol 162(10): 5728–5737, 1999
35. Witham TF, Erff ML, Okada H, Chambers WH, Pollack IF: 7-Hydroxystaurosporine-induced apoptosis in 9L glioma cells provides an effective antigen source for dendritic cells and yields a potent vaccine strategy in an intracranial glioma model. Neurosurgery 50(6): 1327–1334, 2002
36. Graf MR, Prins RM, Poulsen GA, Merchant RE: Contrasting effects of interleukin-2 secretion by rat glioma cells contingent upon anatomical location: accelerated tumorigenesis in the central nervous system and complete rejection in the periphery. J Neuroimmunol 140(1–2): 49–60, 2003

Address for offprints: Martin R. Graf, Department of Neurosurgery, Virginia Commonwealth University Medical Center, 1200 East Broad Street, P.O. Box 980631, Richmond, Virginia 23298-0631; Tel.: 804-828-5207; Fax: 804-828-0374; E-mail: mgraf@hsc.vcu.edu