# Therapeutic vaccination against malignant gliomas based on allorecognition and syngeneic tumor antigens: Proof of principle in two strains of rat

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**Summary**     In the present study we investigated whether allogeneic glioma cells can be utilized to evoke prophylactic or therapeutic immune-mediated elimination of syngeneic glioma in two rat strains. Fisher 344 and Sprague—Dawley (SD) rats were injected with two syngeneic glioma cell lines, 9L and C6, respectively, resulting in progressive tumor growth. 9L is syngeneic to the Fisher 344 and allogeneic to the SD rats, while C6 cells are syngeneic to SD rats and allogeneic to Fisher 344 rats.

Both rat strains were subcutaneously injected with their respective allogeneic tumor cells, which proved unable to grow progressively. The allogeneic cells were either rejected immediately in SD rats or within 25 days in Fisher rats, after limited tumor outgrowth. Both rat strains were subsequently challenged with their respective syngeneic glioma tumor cells and once more 10 days later with a fivefold higher dose. SD rats, even after reinjection with five times the original dosage of C6 cells, remained tumor free for at least 360 days. Similarly, Fisher rats, after initially rejecting allogeneic tumors, failed to develop syngeneic tumors.

To determine anti-tumor immunity against established glioma tumors under more demanding therapeutic conditions, rats were first injected subcutaneously with their respective syngeneic tumor and vaccinated once or repeatedly (at 5-day intervals) with a mixture of the allogeneic or xenogeneic cells, with or without a lysate from the same syngeneic tumor, which served as a therapeutic vaccine preparations. The control group received either no treatment or syngeneic

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instead of allogeneic cells. In both strains of rats, we demonstrated that the therapeutically vaccinated groups were able to significantly reduce tumor growth, while complete rejection of tumors was noted in the SD rats. Immunization with syngeneic tumor cells alone failed to evoke anti-tumor immunity.

We conclude that therapeutic immunization with a combination of allogeneic cells and syngeneic lysates induces rejection of malignant gliomas and offers a protective effect against challenge with syngeneic tumor cells. This immunization approach may prove useful as a post-surgery adjuvant therapy in future cancer treatment protocols, or even as a stand-alone therapeutic tumor vaccination.

## Introduction

In the United States alone over 18,000 primary brain tumors are estimated to occur each year. Of these 18,000, over 60% are diagnosed gliomas. Glioblastoma multiforme (GBM) is the most common and malignant of all gliomas, with 75% of patients dying within 18 months of diagnosis [1]. The prognosis for this tumor is very poor. The median survival time of untreated tumors is 3 months, with death most commonly due to cerebral edema or increased intracranial pressure. Even with the best available current therapy, which includes radiation, chemotherapy and surgery, the median survival does not extend beyond 14 months. These tumors are inevitably recurrent either locally, usually within 2 cm of the original tumor, or at distant sites. Treatment of these recurrent lesions by a second surgery and further chemotherapy may increase the symptom free interval, but the 5-year survival remains 10% [1–3].

It has been shown that the progression of certain cancers is associated with the expression of tumor-specific antigens and tumor antigen-specific immune responses [4]. Hence, theoretically, effective tumor rejection and immunity can be achieved by vaccination with tumor-associated antigen, the holy grail in tumor immunology. However, active immunotherapy for cancer has shown minimal clinical success. It has been clear that even with a fully functioning immune system, it is possible for tumors to evade recognition through the use of elusive escape strategies [5]. Although poorly understood, several mechanisms of tumor escape have been identified. For example, a change of or loss of MHC class I receptors is associated with the genesis of various tumors, while the presence of intact MHC class I molecules has been shown to participate in cancer resistance [6]. Other mechanisms include unresponsiveness to interferons [5], as well as tumor-induced immunosuppression as a result priming for and influx of inhibitory regulatory T cells [7] and associated induction of immunosuppressive molecules including IL-10, CTLA-4 and related factors.

On the other hand, there is increasing evidence that the immune system can be engaged to combat cancer. This is supported by the observations that a deregulated immune system hampers rejection of cancer, while spontaneous rejection or inhibition of malignant tumors is associated with a well-functioning immune system [8,9]. A recent study in colorectal tumor patients demonstrated that adaptive Th-1 immune gene expression and high immune cell densities of CD3, CD8 and CD45RO cells in tumor regions correlates positively with patient survival [10]. Interestingly, it has also been suggested that autoimmune diseases may contribute to a better prognosis in patients with malignant tumors [11,8]. In these patients, the majority of the IgG specificities identified share considerable homology with both human and microbial peptides [12]. This has lead to the hypothesis that molecular mimicry may initiate the observed anti-tumor autoimmunity. Studies related to this have shown long-term remission of malignant brain tumors after intracranial infection in four patients [13], and improved survival of cancer patients with microbial infection [14,15]. This brings into question whether molecular mimicry-induced ''autoimmunity'' can be employed to treat tumors. Importantly, significant homology has been shown to exist between human proteins and proteins from other species [16]. Moreover, use of artificial pathogen invasion signals, such as CpG motifs, or other innate immunity agonists, initiates and augments antigen-specific immune reactions [17], and may break tolerance to self-tumor antigens, mimicking microbial infections during immunotherapy or vaccination [18,19]. Alternatively, xenogeneic antigen from endothelial cells is able to break immune tolerance against autologous angiogeneic endothelial cells [14]. This suggests that self-tolerance to tumors may be broken by cross-reactivity against a homologous foreign antigen.

In the present study, we combine the principles of immune-based allorecognition and administration of syngeneic tumor antigen to overcome tolerance to self-tumor-associated antigens and to develop a novel approach to the treatment of tumors. It is well known that genetically identical individuals can accept tissue from one another, while tissue transplanted into heterozygous individuals will produce an immune response and eventual tissue rejection. Recognition of intact, same-species, non-self major histocompatibility molecules, on the surface of donor cells results in direct, immune-mediated elimination, is referred to as acute allograft rejection [20,21]. Indirect allorecognition results from recognition of donor histocompatibility molecules that are internalized, processed, and presented by self-MHC molecules on host antigen presenting cells. After xenotransplantation, tissues or cells are transferred across species, which causes even faster rejection by processes analogous to those seen in allografts. Hence, identical twins and genetically close family members are less likely to reject transplanted tissue since they have similar HLA loci [22]. This is based on the fact that the MHC class I genes are expressed co-dominantly, and in most cases are inherited in intact form without recombination [23]. Therefore, homozygous, syngeneic rats could theoretically accept a brain tumor from a homozygous donor.

However, more critically, they would reject a brain tumor from a heterozygous donor based on direct or indirect allo-immune rejection [20,21]. MHC class I molecules play an important role in the immune surveillance of tumors by monitoring of mitochondrial DNA integrity. One of the roles of MHC I molecules is to eliminate cells carrying mitochondrial mutations [6]. Human glioma cells carry multiple mutations in both the mitochondrial DNA and in the mitochondrial complex [24]. Hence, gliomas of the same histological type/grade are likely to carry similar mutations in their DNA and have similar abnormal surface proteins associated with both MHC class I molecules and the cell membrane. Experimental data suggests that not only MHC class I molecules are involved in immune surveillance against cancer, but also that the altered phenotype of the MHC class I molecule is linked to a variety of different tumors. Therefore, if two heterozygous individuals develop a tumor of a similar type and histological grade, then transplantation of tumor tissue from one individual to another will not only induce rejection of the transplanted tissue, but may also prime the immune system to peptides shared between these tumors and other tumors sharing similar peptides.

In this paper we show *in vivo* proof of principle experiments demonstrating that allogeneic tumors can be used to vaccinate against an established syngeneic tumor, resulting in inhibition of tumor growth or complete tumor elimination. Application of this technique in human patients may not only lead to eventual rejection of the primary tumor, but may also lead to a lasting immunologic memory, preventing the patient from developing tumor recurrence.

## Materials and methods

### Cell lines and cell culture

The cell lines used in this experiment were the rat glioma cell lines (9L, C6, RG2), and the human glioma cell lines (U87, LN229). All lines were obtained from the American Type Tissue Collection (ATTC), and grown in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Grand Island, NY) supplemented with 10% heat-killed fetal calf serum (FCS), 5% penicillin—streptomycin, and Hepes buffer in a humidified incubator at 37 °C in a 5% $CO_2$ atmosphere.

### Cell lysate antigen preparation

$1.0 \times 10^6$ cells were placed in a 5-ml tube in culture medium and centrifuged for 5 min at $2.5 \times 10^3$ rpm. The supernatant was discarded and 150 μl of sterile distilled water was added to the tube. The cell/water solution was mixed well and transferred to a 1.0-ml Eppendorf tube and centrifuged at $1.0 \times 10^4$ rpm for 10 min. The supernatant was not discarded and this preparation was used for cell lysate injections.

### *In vivo* studies

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California. All rats were maintained in a specific pathogen free (SPF) environment. For the experiment, we used Sprague—Dawley (SD) and Fisher 344 rats. All rats were males and between the ages of 4—6 weeks. Rats were obtained from Harlan (Indianapolis, IN). In the subcutaneous tumor model, C6 and 9L were collected using only DMEM to wash them from the tissue culture flasks. Syringes were then prepared containing 100,000—150,000 cells suspended in 150 μl.

Sprague—Dawley rats were divided into two major groups (Table 1). SD-A (three rats) were injected with the 9L allogeneic cell line, while SD-B (nine rats) were implanted with the C6 glioma, a syngeneic like glioma cell line for SD rats. SD-A rats, which never formed tumors, were tested for immune memory by challenging them with syngeneic C6 cells (100,000 cells). They were re-challenged with 500,000 C6 cells 10 days later, and checked for formation of a flank tumor.

Once a palpable flank tumor developed in the SD-B rats, they were further divided into two groups. The control group (SD-B1; $n = 5$ rats) received no injections. In the therapeutic treatment group (SD-B2; $n = 4$) rats were injected with a combination of allogeneic 9L cells, allogeneic 9L lysate, and syngeneic C6 lysate. On day 27, four of the five SD-B1 were sacrificed. At this time, one of the control rats, rat number (#) 9, started receiving the same treatment protocol as SD-B2 rats.

Fisher rats were also divided into two major groups (Table 2). The control group (Fisher-A; three rats) were injected with the allogeneic C6 cell line. They initially formed tumors that were subsequently rejected. They were tested for immune memory by challenging them after 40 days with 100,000 syngeneic 9L cells followed by a re-challenge 10 days later with 500,00 cells and checked for tumor growth.

In the therapeutic group (Fisher-B; $n = 8$), rats were first implanted with the syngeneic 9L cell line. Once a palpable flank tumor developed in the Fisher-B rats, they were further subdivided into three subgroups. Fisher control group ($n = 3$) rats received injections of syngeneic 9L cells, syngeneic RG2 (rat glioma) cells, or medium only (Fisher B1). One Fisher treatment group ($n = 5$) rats received a combination of allogeneic C6 cells only or allogeneic C6 cells and lysate (Fisher B2), or xenogeneic human glioblastoma cell lines U87 and LN229 cells (Fisher B3) (Table 2).

### Tumor growth analysis

All tumors were detected and confirmed through visual inspection and palpation. Once discovered, the area around the tumor was further exposed by careful shaving with an electric razor. At the time of injection, tumor size was measured in millimeters using Vernier calipers. Measurements were taken in the cranial/caudal (length), superior/inferior (height), and medial/lateral (width) direction. Tumor volume was calculated by length × width × height × 0.5. The mean tumor volume for each treatment group was calculated. For SD rats the tumor volumes of the treatment groups were compared to relevant control groups at 27 days post-injection, and for Fisher rats at 35 days, using the Student's *t*-test calculation as described before [25]. Differences were considered significant if a *p* value was <0.05.

**Table 1**  Experimental design of animal studies in Sprague—Dawley rats

| Immunization | Vaccine | Group size | Tumor challenge | Outcome |
|---|---|---|---|---|
| Prophylactic (group A) | Allogeneic 9L cells (100,000 cells) | $n = 3$ | Syngeneic C6 (100,000 cells) at 20 days after 9L ''immunization'' and C6 500,000 cells, again 10 days later | Immediate, complete allogeneic 9L and subsequent syngeneic C6 tumor rejection |
| Therapeutic (group B2) | Allogeneic 9L cell lysates (50,000 cells), syngeneic C6 cell lysates (50,000 cells), and 9L allogeneic cells (50,000 cells). Rats #5—8 and later #9 | $n = 4$—5 (rat #9) | C 6 (100,000 cells) | Complete C6 tumor rejection |
| Control group (group B1) | Saline or no injections rats #1—4 and initially #9 | $n = 5$—4 (rat #9) | C 6 (100,000 cells) | Progressive C6 tumor growth |

**Table 2**  Experimental design of animal studies in Fisher rats

| Immunization | Vaccine | | Group size | Tumor challenge | Outcome |
|---|---|---|---|---|---|
| Prophylactic (group A) | Allogeneic | Allogeneic C6 cells (100,000 cells) | $n = 3$ | Syngeneic 9L (100,000) cells at 40 days after C6 ''immunization'', and 500,000 9L cells, again 10 days later | Minimal 9L tumor outgrowth and ultimate rejection |
| Therapeutic (group B1) ''control group'' | Syngeneic or medium | 100,000 cells RG2 cells (rat #1), 100,000 cells 9L cells (rat #2) or medium only (rat #3) | $n = 3$ | Syngeneic 9L (100,000) cells | Non-reduced tumor growth |
| Therapeutic (group B2) | Allogeneic cells/lysates and/ or syngeneic lysates | Mixture of (150,000) C6 allogeneic cells (Rat #4) and C6 (100,000) allogeneic cells plus allogeneic lysate (100,000 cells) (rat #5). And syngeneic 9L cell lysate (100,000 cells) (rat #8) | $n = 3$ | Syngeneic 9L (100,000) cells | Reduced 9L tumor outgrowth, except for rat #8 |
| Therapeutic (group B3) | Xenogeneic cells | 9L syngeneic lysate (100,000 cells) plus U87 cells (50,000) plus LN229 cells (50,000) (rat #6) U87 (50,000) plus LN229 (50,000) xenogeneic cells (rat #7) | $n = 2$ | Syngeneic 9L (100,000) cells | Reduction in tumor size |

## Harvesting subcutaneous tumor tissue for immunohistochemistry

All experimental animals were euthanized with an overdose of pentobarbital. Tumors were removed and dissected under sterile conditions, cut into four pieces and stored at −80 °C. All tumor sections were cut at 7 μm and stained by immunohistochemistry as described before [26]. Briefly, tumor samples taken from the Fisher 344 rats were frozen in optimum temperature compound (OTC) and cut into 7 μm sections on a cryostat. These sections were dried, fixed with acetone, and washed well with PBS for 1—2 min. Blocking was done using the immune serum from the species the secondary antibody was obtained from. Slides were washed thoroughly again and then stained with primary antibody against either CD4, CD57 (Nora Castro Lab Ltd., Burlingame, CA), CD8, dendritic reticulum cells (DRC) (Dako Corporation, Carpenteria, CA), CD20, or CD68 (Ventana, Tucson, AZ). Slides were washed again and a secondary biotinylated antibody was added. They were rinsed again and placed in a solution of 3% hydrogen peroxidase and nine parts 1% sodium azide in PBS. Slides were then rinsed and ABC was added for 30—40 min. They were washed with PBS and developed using diaminobezidine tetrahydrochloride and counterstained. Photographs of all slides were taken by light microscopy.

## Results

### Immunization with allorejected, non-syngeneic tumors in both Fisher and Sprague—Dawley rats primes for prophylactic immunity against syngeneic tumor challenge.

Most experimental studies of glioblastoma make use of small laboratory animal models. The most frequently used immunocompetent host models employ two different strains of rat, the Sprague—Dawley and the Fisher 344 rats [27]. C6 is a syngeneic-type cell line for the SD rats, while the 9L and RG2 cell lines are syngeneic for the Fisher 344 rats [27,28].

In a prophylactic setting we examined whether the SD and Fisher 344 rats initially injected with an allogeneic cell line would be able to reject a syngeneic cell line. SD rats were seeded with the allogeneic 9L cell line (SD-A). Each of the SD rats completely rejected the 9L tumor without visible or palpable tumor growth. Twenty days later, all ''immunized'' SD rats, were injected in the contra-lateral hind flank with syngeneic C6 tumor cells, that readily formed a tumor in naïve SD rats, using 100,000 cells first, and a fivefold higher tumor cells (500,000 cells) 10 days later. The rats were monitored every 3 days for any sign of visual or palpable tumor growth. In these SD rats, no visual or palpable tumor developed. Remarkably, at 360 days, all SD rats immunized with the allogeneic tumor cells remained tumor free (data not shown).

A similar procedure was used to prophylactically immunize three Fisher rats. They were injected with the allogeneic C6 cell line (Fisher-A). Although initially well-circumscribed tumors did form, they were subsequently rejected within 40 days. These animals were subsequently seeded with 100,000 syngeneic C6 cells first, and a fivefold higher amount of C6 cells (500,000 cells) 10 days later. The rats were monitored every 3 days for any sign of visual or pal-

pable tumor growth. In these Fisher rats, a relatively small (<1 cm × <1 cm × 1 cm) growth developed at the injection site. This growth was noticeable only after palpation, and became progressively smaller and completely undetectable by 10 days. At 360 days, all immunized Fisher rats remained tumor free (data not shown).

These results demonstrate that in both strains, allorejection of non-syngeneic tumors induces effective prophylactic immunity against syngeneic tumor challenge.

### Allo-response-based therapeutic vaccination against C6 tumors in Sprague—Dawley rats

In order to assess anti-tumor immunity in a therapeutic situation, SD rats (n = 9) were each injected with the C6 cell line, which resulted in undiminished tumor growth in untreated animals. All SD rats developed visible tumors within 10 days. At this point, five rats were kept as a control group (SD-B1), while the remaining four rats were placed into treatment groups (SD-B2). On day 27, rats #1—4 were sacrificed and an attempt was made to ''rescue'' rat #9. At this time, rat #9 entered the treatment group and started to receive the same therapeutic vaccine injections as given to the SD-B2 group.

The uncontrolled tumor growth in the control group and the diminished tumor growth in the treatment group are depicted in Fig. 1. In the treatment group (SD-B2, rats #5—8), individual rats were immunized with a mixture of allogeneic and syngeneic lysates, as well as allogeneic 9L cells per subcutaneous (s.c.) injection. One rat (#5) was treated very early. After 5 days, it had a palpable flank tumor and received only one therapeutic injection, contra-lateral to the tumor, of a mixture consisting of allogeneic 9L lysates (50,000), syngeneic C6 lysates (50,000), and 9L allogeneic cells (50,000). Remarkably, within 5 days after injection, the tumor resolved. Rats #6—8 (SD-B2 rats) all developed visible tumors within 18 days post-injection. At this time, they each received a first injection of a mixture containing 50,000 allogeneic 9L lysate cells, plus 50,000 syngeneic C6 lysate cells and 50,000 9L allogeneic cells. These injections were repeated on days 23 and 28. Rat #6 received an additional treatment at day 33, 15 days after initiation of immunotherapeutic treatment. The untreated rats (SD-B1, rats #1—4) were sacrificed 27 days post-injection because of their tumor size. When compared to the tumor progression in the untreated rats (rats #1—4), rats #5—8 (SD-B2) eventually showed complete resolution of their tumors by day 50.

Rat #9 began the experiment within the non-treated group, and then was treated after sacrificing rats #1—4 (day 27). Rat #9 received five injections every 5 days with a mixture of allogeneic 9L lysates (50,000) plus C6 syngeneic lysates (50,000) and 9L allogeneic cells (50,000). This animal was sacrificed for histological analysis at day 55, when the tumor size had reduced to 11% of the size measured at the initiation of immunotherapeutic immunization.

### Allo-response-based therapeutic vaccination against 9L tumor growth in Fisher 344 rats

Fig. 2 shows tumor growth and response to the immunotherapeutic treatment of eight Fisher rats (Fisher-B) implanted
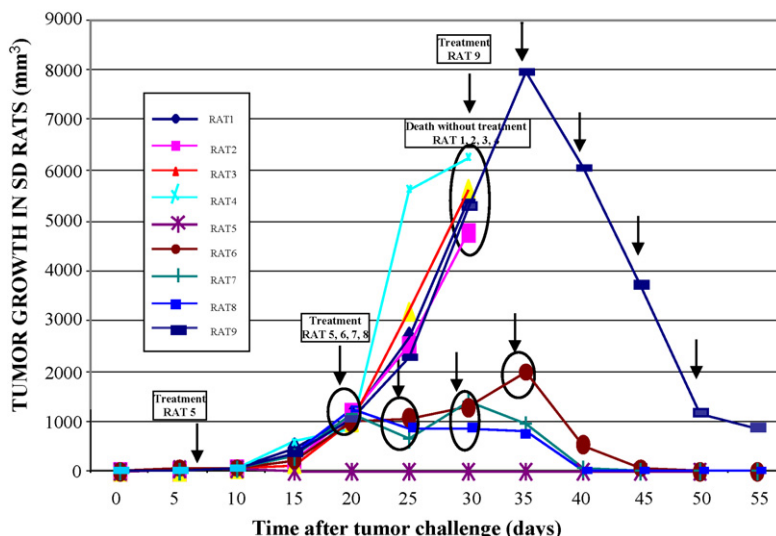
**Figure 1**   Graph charting tumor progression in nine SD rats with subcutaneously implanted syngeneic tumor (C6). Rats were placed in either control or treatment groups as previously described. Tumor progression was determined through measurements of tumor volume (mm$^3$). Rats #1—4 received no treatment after C6 tumor implantation. Rats #5—8 received one or more therapeutic vaccination(s) with allogeneic 9L cells and lysates with syngeneic C6 lysate. Rat #9 was allowed to form a relatively large tumor before it was transferred to the treatment group to become immunized similar to rats #5—8.

with 9L cells. Rats #1—3 (Fisher B1) received therapeutic contra-lateral flank injections at day 10, with either syngeneic RG2 (100,000 cells; rat #1) or 9L (100,000 cells; rat #2), or medium alone (rat #3). There was notable reduction in tumor growth over time, while a more pronounced tumor growth was noted in the RG2 treated rat (#1). By contrast, rats #4—7 (Fisher B2 and B3) were immunized ther-apeutically with, either C6 allogeneic cells only (rat #4), a mixture of C6 allogeneic cells and C6 allogeneic lysate (rat #5), U87 and LN229 xenogeneic cells only (rat #7) or mixed with 9L cell lysate (rat #6). In particular rats #5—7 showed a significant reduction in tumor outgrowth ($p < 0.05$), while rat #8, receiving 9L syngeneic lysate only, demonstrated no inhibition of tumor growth (Fig. 2).
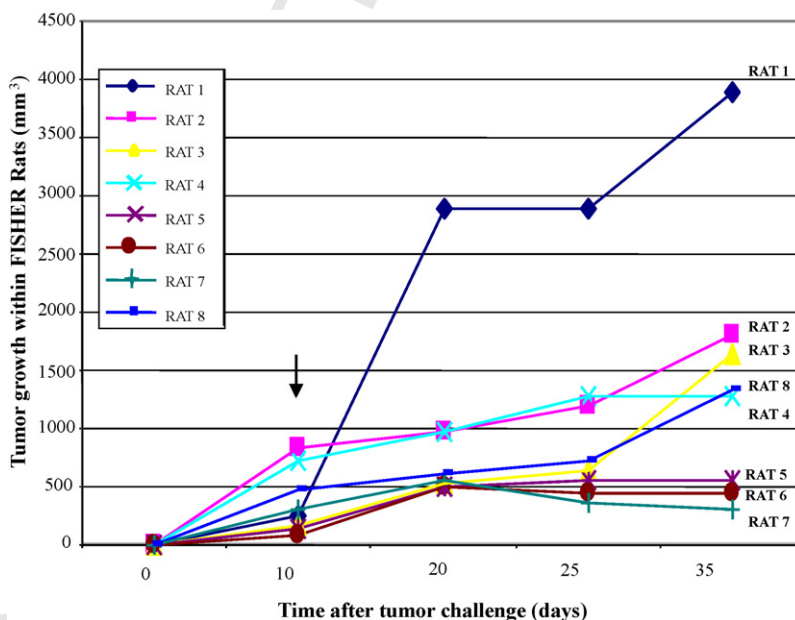


**Figure 2**   Graph charting tumor progression in Fisher 344 rats with subcutaneously implanted syngeneic tumor (9L). Control rats were injected with syngeneic RG2 cells (rat #1), syngeneic 9L cells (rat #2), or medium alone (rat #3). Rat #1 formed an extremely large tumor. Treatment group rats received allogeneic C6 cells alone (rat #4), allogeneic C6 cells and lysate (rat #5) (group B2), syngeneic 9L lysate and xenogeneic U87 and LN229 cells (rat #6), or xenogeneic U87 and LN229 cells alone (rat #7) (group B3). Rat #8 was treated with syngeneic 9L cell lysate alone. Tumor progression was determined through measurements of tumor volume (mm$^3$).
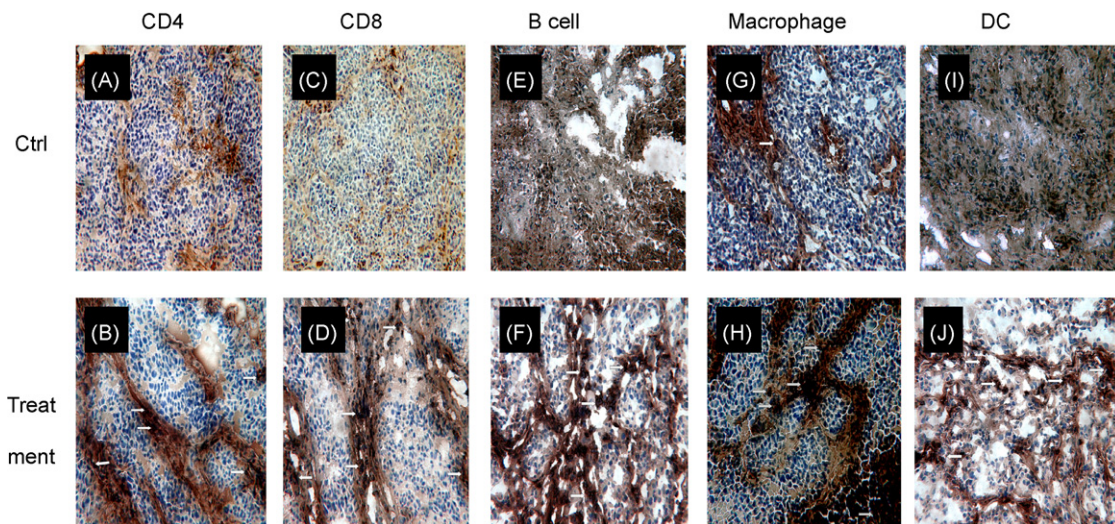
**Figure 3**    Representative tumor sections taken from control (A, C, E, G, and I) and treated (B, D, F, H, and J) Fisher 344 rats. Sections were cut at a thickness of 7 μm and, according to the previously described protocol [27], stained with an antibody directed against either the CD4 receptor (A and B), CD8 receptor (C and D), B-lymphocytes (CD 20) (E and F), macrophages (CD 68) (G and H), or a dendritic cell marker (DRC) (I and J). Small white arrows indicate the location of cells staining positively for the respective marker. The magnification of both control and treatment sample is 40×.

All Fisher 344 rats were sacrificed at day 40, when some of the rats started to develop hind limb paralysis. The tumors from each of these rats were removed and processed for immunohistological staining of immune cells. Within the tumors of the positive treatment groups we noted significantly greater numbers of CD4, CD8, B-lymphocyte (CD20), macrophages (CD68), and dendritic cells when compared to the control tumors (Fig. 3).

## Discussion

Anti-tumor immunotherapy based on an effective therapeutic vaccine, with an acceptable safety profile, is the great hope for cancer treatment. A vaccine will theoretically program the patient's immune system to attack malignant, and even metastasized, tumor antigen-expressing cells, and ideally trigger immunological memory to provide a durable anti-tumor immune response. To achieve this goal, many different vaccination strategies are currently being investigated in animal models and clinical trials. Examples include immunizations based on patient-derived dendritic cells loaded in vitro with tumor antigens or peptide fragments [29,30], virus-modified or cytokine transfected autologous or allogeneic tumor cells [31], plasmid DNA and viral or bacterial vector delivering genetically encoded tumor antigens, as well as the more classical antigen in adjuvant strategies.

In the present study, we demonstrate in two rats strains that allorejection of non-syngeneic tumors induces effective prophylactic immunity against subsequent syngeneic tumor challenges. In addition, we show that for established syngeneic tumors, therapeutic immunization with different mixtures, containing either allogeneic cell lysates plus syngeneic cell lysates, and allogeneic cells, evokes effective reduction in tumor growth in SD rats. Similarly, in Fisher rats, established tumor growth can be inhibited significantly by therapeutic immunization using either allogeneic or xenogeneic cells only, or a vaccine containing xenogeneic cells in a combination with lysates of syngeneic tumor cells. By contrast, immunization of Fisher rats with syngeneic cells or syngeneic lysate alone failed to reduce tumor outgrowth. Our results support the conclusion that it is feasible to program effective tumor antigen-specific responses as a result of anti-allogeneic or xenogeneic cell immunization. In general, cell- or cell lysate-based tumor vaccines may be more attractive when compared to single antigen or polypeptide-based vaccines, since they theoretically evoke a broader multi-targeted therapeutic response. Due to the polyclonal immune response induced, they are less likely to result in therapeutic escape than most cancer treatments in use today.

In the prophylactic setting the SD rats rejecting the 9L tumor (SD-A) and the Fisher 344 rats rejecting the C6 tumor (Fisher-A) were reinjected in the contra-lateral flank with a higher dose of 500,000 cells of syngeneic cell line (9L for Fisher and C6 for SD). Both strains remained tumor free at 360 days. These results suggest that the injection of allogeneic cell lines evokes protection against subsequent challenge with syngeneic cell lines, demonstrating that the injection of the allogeneic cells lead to an immune response and the development of immune memory. Since C6 and 9L cell lines likely share critical tumor antigens, the development of C6 tumors is inhibited. The observed time line difference between SD and the Fisher rats in terms of allogeneic tumor rejection (SD rats rejected the 9L cell line without development of a tumor, while Fisher 344 rats took about 40 days to completely reject the C6 tumor), may be explained by a less effective immune response in Fisher rats. This may possibly result from less 9L immunogenicity, or is due to reduced susceptibility of 9L cells to immune attack; 9L is a gliosarcoma cell line, while C6 is a glioma cell line. On the other hand, C6 cells may be more immunogenic for SD rats than the 9L cells for Fisher rats.

In the more demanding therapeutic setting, untreated SD rats injected with C6 gliomas developed significant tumors within 5—15 days. These tumors grew without rejection, and rats had to be sacrificed eventually due to unacceptable tumor size and limb paralysis. The treated SD rats initially developed C6 tumors at comparable rates and sizes as the control group. However, these tumors gradually decreased in size and were no longer detectable 25 days after the initiation of therapeutic vaccination with a mixture of allogeneic and syngeneic cells and syngeneic cell lysates. Strikingly, even rat #9, rescued relatively late from the untreated control group, showed significant reduction in the size of tumor after treatment began. Together, these results demonstrate that repeated subcutaneous injection of this cocktail leads to a reduction in tumor size by triggering immunological awareness, likely directed at tumor antigens shared between the syngeneic and allogeneic cells.

There is some debate in the literature about the C6 cell line and whether or not it is syngeneic to any strain of rat [27]. However, even if the cell line may not be strictly syngeneic, it developed into subcutaneous flank tumors in SD rats without rejection. Those rats not given treatment were sacrificed when tumor size became incompatible with life.

All of the Fisher 344 rats developed flank tumors at 15 days. Unlike the SD controls, the Fisher controls either received injections with two different syngeneic cell lines (9L and RG2) or with medium alone. There was no inhibition of tumor growth in these rats. This demonstrates that the injection of whole syngeneic tumor cells does not evoke an effective anti-tumor immune response, as a result of immunological tolerance to syngeneic cells. Indeed, when these rats were sacrificed, tumor sections did not stain positively for CD4, CD8, macrophages, B-lymphocytes, and dendritic cells. By contrast, the tumors in the treated Fisher 344 rats showed different growth profiles. Rat #4 received allogeneic C6 cells only and showed growth inhibition after day 35. Especially, rats #5—7 showed decreased tumor growth when compared to syngeneic or medium treated controls. Rat #5 was treated was treated with both allogeneic C6 cells and lysate. Rat #6 was treated with 9L syngeneic lysate and xenogeneic U87 and LN229 cells. Rat #7 was treated with xenogeneic LN229 and U87 cells. Rat #8, which was treated with 9L syngeneic cell lysate, had an initial delay in tumor growth. However, this effect was not lasting, as by day 25 the tumor was similar in size to controls. These data suggest that syngeneic lysate may exert a temporary protective effect, however, a lasting protective effect was noted more clearly for allogeneic cells plus lysate, and for cell injections involving the xenogeneic U87 and LN229 tumor cell lines. Interestingly, the protective effect of the allogeneic cells appeared more pronounced when a lysate was added as compared to whole cell preparation only, as suggested by comparing rat #4 versus #5.

When tumor-rejecting rats were sacrificed, their tumor sections stained generally more positive for CD4, CD8, macrophages, B-lymphocytes, and dendritic cells, in contrast to the control group, which had tumors with intact architecture and a paucity of all of the above-mentioned immune cells. These results demonstrate that immunocompetent rats, which develop syngeneic tumors without rejection, show less or no immune cell infiltration, suggesting an escape from immune recognition due to immune ignorance [32].

When comparing the therapeutic vaccinations in SD and Fisher rats it is also worthy to note that while all of the treated SD rats rejected the C6 tumor, none of the Fisher rats has complete tumor remission within 40 days. This may be explained by the fact that syngeneic lysate was not added to either the allogeneic or the xenogeneic cells in Fisher rats, as it was done in the SD rats. Hence, addition of syngeneic lysate may significantly contribute to tumor rejection and will be examined in follow-up studies.

Collectively, pooled results from these experiments confirm that experimental vaccines based on allogeneic or xenogeneic cells only or combined with syngeneic cell lysates, are safe and protective in early and advanced malignant glioblastoma. These results lead us to conclude that ''non-self'' injections of allogeneic cells and/or allogeneic lysate, as well as xenogeneic cell lines, can break self-anti-tumor tolerance. These cells likely contain antigen determinants shared with the syngeneic tumor, leading to a reduction in tumor growth. The exact immunological mechanisms underlying the observed anti-tumor immunity remains to deciphered in further studies. Although these were small pilot treatments, in a limited number of animals per therapeutic effect, the inhibition of tumor growth within the treatment groups was statistically significant when compared with control or untreated animals. Our results support the viability of this cancer vaccine strategy as an adjuvant treatment to prevent tumor relapse in cancer patients.

The impact of these data may be far reaching when translation to patients is possible to certain degree. Glioblastoma multiforme is the most common and malignant of all gliomas, and cannot be cured by surgery, radiation therapy, chemotherapy, with 75% of patients dying within 18 months of diagnosis [33]. The use of allogeneic/syngeneic/or xenogeneic cell lines and lysates may lead to a reduction in tumor size and perhaps rejection, thereby increasing survival. In the future, allogeneic cell lines and lysates may also be used as vaccine components for other cancers.

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