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CD8\(^+\) T Cell–Independent Tumor Regression Induced by Fc-OX40L and Therapeutic Vaccination in a Mouse Model of Glioma

Katherine A. Murphy,* Jami R. Erickson,* Charles S. Johnson,† Charles E. Seiler,‡ Jessica Bedi,* Peisheng Hu,§ G. Elizabeth Pluhar,‡ Alan L. Epstein,§ and John R. Ohlfest*‡

Despite the growing number of preclinical and clinical trials focused on immunotherapy for the treatment of malignant gliomas, the prognosis for this disease remains grim. Although some promising advances have been made, the immune response stimulated as a result of immunotherapeutic protocols has been inefficient at complete tumor elimination, primarily due to our lack of understanding of the necessary effector functions of the immune system. We previously demonstrated that a tumor lysate vaccine/Fc-OX40L therapy is capable of inducing enhanced survival and tumor elimination in the GL261 mouse glioma model. The following experiments were performed to determine the mechanism(s) of action of this therapy that elicits a potent antitumor immune response. The evidence subsequently outlined indicates a CD8\(^+\) T cell–independent and CD4\(^+\) T cell–, NK cell–, and B cell–dependent means of prolonged survival. CD8\(^+\) T cell–independent tumor clearance is surprising considering the current focus of many cancer immunotherapy protocols. These results provide evidence for CD8\(^+\) T cell–independent means of antitumor response and should lead to additional examination of the potential manipulation of this mechanism for future treatment strategies.


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pressive molecules, such as TGF-β. This biases the brain and the draining lymph nodes for induction of a Th2 response (20). In addition, even when a CD8\(^+\) T cell response is generated, the immunosuppressive nature of the brain can prevent effector function and limit tumor elimination (21).

The clinical success of cancer treatments such as CTLA-4 and PD-1 blockade has led to an increased interest in immune-modulatory agents (22, 23). Signaling through costimulatory molecules, including members of the TNFR superfamily, such as 4\(\beta\)BB, OX40, and glucocorticoid-induced TNFR, can lead to T cell expansion and upregulation of effector cytokine production, and can break tolerance (24). These receptors can be targeted by agonist Abs or ligand fusion proteins, in which the corresponding ligands are fused to Ig proteins for systemic delivery in vivo (24).

Recently, we described an efficacious combination therapy involving tumor lysate and adjuvant vaccines with FC-OX40L costimulation in a murine brain tumor model (25). The work described in this study aims to dissect the mechanisms at work in this potent antitumor therapy in a mouse GBM model. Our results indicate a CD4\(^+\) T cell–, B cell–, and NK cell–dependent means of tumor eradication, whereas CD8\(^+\) T cells appear to be unnecessary for enhanced tumor-free survival. The following work should aid in the understanding of mechanisms at play in an effective antitumor response and guide future therapeutic designs. Our previous research and current work suggest an alternative means of tumor eradication to the canonical CD8\(^+\) cytotoxic T cell mechanism, and may shed light on routes of immune modulation that result in effective tumor clearance in GBM.

Materials and Methods

**Animal models and cell lines**

GL261-Luc culture conditions have been described previously (26). Animals were maintained in a specific pathogen-free facility, according to the University of Minnesota Institutional Animal Care and Use Committee guidelines. Seven-week-old wild-type (WT) C57BL/6J, B6.129S2-\(C{\text{D}}{\text{a}}\text{a}^{+}\)m1Rav\(\beta\) (CD8a knockout [KO]), C57BL/6-\(P{\text{r}}{\text{f}}{\text{m}}{\text{s}}\text{e}^{+}\) (perforin KO), and B6.129S2-\(I{\text{g}}{\text{l}}{\text{m}}{\text{u}}^{+}\)c139.1 (IL-17 KO, FcγR KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Five- to 7-week-old B6.129P2-Fcer1gtm1Rav\(\beta\)N12 (FcγR KO) mice were provided from A. Waismann (University of Mainz, Mainz, Germany) and K. Rajewsky (Harvard Medical School, Boston, MA) (27, 28). Tumors were established by intracranial inoculation of 15,000 GL261-Luc glioma cells in 1 \(\mu\)l HBSS (Life Technologies) into animals anesthetized with a ketamine/xylazine mixture (54.7 mg/ml ketamine and 9.26 mg/ml xylazine). Vaccines, consisting of 65 \(\mu\)g DNA, were delivered at a rate of 0.2 \(\mu\)l HBSS between 2.5 mm lateral, 0.5 mm anterior from bregma, and 3 mm ventral to the surface of the brain and delivered at a rate of 0.2 \(\mu\)l/min over 5 min (26).

**Vacccine production and delivery**

Vaccines were generated, as previously described (25). Tumor cells were washed three times with PBS, resuspended in PBS, and flash frozen with liquid nitrogen. Cells were subjected to five cycles of freezing in liquid nitrogen and thawing in a 37°C water bath, vortexing after each round, to induce cell lysis. Trypan blue dye exclusion was used to verify complete cell death. A Pierce bichinonic acid assay kit (Thermo Scientific) was used to determine protein concentration of the lysates. Purified, endotoxin-free \(C{\text{p}}{\text{g}}\) 1826, an unmethylated oligodeoxynucleotide sequence (5\'-tctcagactgctcaggtt-3') with a full phosphorothioate backbone (Integrated DNA Technologies, Coralville, IA), was resuspended in 1\% Tris-EDTA buffer. Vaccines, consisting of 65 \(\mu\)g tumor lysate and 50 \(\mu\)g \(C{\text{p}}{\text{g}}\) 1826, brought to a final volume of 100 \(\mu\)l with saline, were delivered by intradermal injection above the shoulders.

Costimulatory fusion protein production and delivery

Fc-OX40L was developed and verified previously (29). Fc-OX40L was given at 50 \(\mu\)g/dose, brought to a final volume of 100 \(\mu\)l/dose with PBS, and delivered by i.p. injection. Animals received vaccine (intradermal) and Fc-OX40L (i.p.) on days 7, 10, and 13 postinoculation and Fc-OX40L (i.p.) days 15–19, unless otherwise stated.

**Lymphocyte depletion**

Specific lymphocyte populations were depleted by i.p. injection of depleting Abs. A total of 100 \(\mu\)g anti-NK1.1 (clone PK136; eBioscience) or anti-CD4 (clone GK1.5; eBioscience) or 200 \(\mu\)g anti-CD8 (clone 53-6.7; eBioscience) was delivered for 2 d before the first immunization, then 1 d before each additional vaccine, and 1 d prior to beginning FC-OX40L treatment. Depletion was verified by euthanizing one animal from each group on day 3 before the first immunization and analyzing splenocytes by flow cytometry.

**Flow cytometry**

Brain-infiltrating lymphocytes were harvested from animals on day 25 postinoculation. Mice were euthanized with a ketamine/xylazine mixture and perfused with PBS to flush the capillaries. The brains were removed, minced with a razor, dissociated with TrypLE (Invitrogen), and passed over a 70-\(\mu\)m cell filter. Leukocytes were collected from a two-layer Percoll gradient (70 and 30%). Cell counts were obtained using trypan blue dye exclusion and a hemocytometer. Cells were stained with the following Abs for phenotypic analysis: CD3 (clone 17A2; eBioscience), CD4 (clone GK1.5; eBioscience), CD8 (clone 5H10; Invitrogen), NK1.1 (clone PK136; eBioscience), MHC II (clone M5.114.15.2; eBioscience), CD11b (clone M170; eBioscience), CD11c (clone N418; eBioscience), FoXP3 (clone FJK-16s; eBioscience), and perforin (clone eBioOMAK-D; eBioscience). Intracellular staining of FoXP3 was achieved by utilizing the FoXP3/transcription factor intracellular staining kit, according to the manufacturer’s instructions (eBioscience). The BD Cytofix/Cytoperm plus kit (BD Biosciences) was used to gain intracellular staining of perforin. Flow cytometric analyses were performed on a BD Biosciences FACSCanto, and data were analyzed using FlowJo software (Tree Star). The percentage of stained cells was multiplied by the total number of viable cells, determined previously by trypan blue dye exclusion, to obtain the total number of stained cells, then divided by tumor burden (p/s) to obtain the number of cells relative to tumor size.

To detect tumor-reactive serum Abs, cultured GL261 cells were harvested and incubated with serum (1:100, by volume), washed thoroughly, and stained with a fluorescently labeled rat anti-mouse IgG (Jackson ImmunoResearch Laboratories) or IgM (clone II/41; eBioscience) Abs. Samples were analyzed on a BD Biosciences FACSCanto, and mean fluorescence intensities were determined using FlowJo software (Tree Star).

**Histopathology**

Tumor-bearing animals were euthanized and perfused with phosphate-buffered water and 4% paraformaldehyde. Formalin-fixed tumor-bearing brains were serially sectioned and processed into paraffin blocks using standard histology techniques, sectioned to 4-\(\mu\)m thickness, and stained with H&E. Slides were evaluated by a board-certified anatomic pathologist by light microscopy.

**Suppression assay**

T cells were isolated from a naive WT spleen using the mouse CD8a T cell isolation kit from Miltenyi Biotec and stimulated in vitro with plate-bound CD3 (1 \(\mu\)g/ml) (eBioscience; 2C11) in a round-bottom plate. CD11b\(^+\) cells isolated from spleen and brains of tumor-bearing animals with the CD11b isolation kit from Miltenyi Biotec were plated with 5 \(\times\) 10\(^5\) CD8\(^+\) T cells. Tritiated thymidine was added to cells after 48 h and allowed to incubate for an additional 24 h. Proliferation was determined by thymidine incorporation. Suppression was calculated by normalizing to CD3-stimulated T cells in the presence of CD11b\(^+\) cells isolated from naive animals, percent suppression = \((\text{T cells + CD3 + naive CD11b}^{+}) − (\text{T cells + CD3 + tumor CD11b}^{+})) / (\text{T cells + CD3 + naive CD11b}^{+})\) \times 100.

**Western blots**

Tumor tissue was sonicated in radioimmununoprecipitation assay buffer (25 mM Tris-HCl, 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 0.15 M NaCl, 1 mM EDTA) with protease and phosphatase inhibitor mix (1:100; Calbiochem). Protein concentrations were determined using the Pierce bicinchoninic acid assay kit (Thermo Scientific). For SDS-PAGE, lysates were made 2 mg/ml with Laemmli reducing sample buffer. Protein
Increased survival and tumor clearance is CD8+ T cell independent or that CD8+ T cells somehow block the response to immunotherapy. These data suggest that the immune response responsible for treating GBM.

**Results**

Lymphocyte depletion reveals the importance of CD4+ T cells and NK cells in vaccine/Fc-OX40L treatment of GBM

The role of specific lymphocyte subsets contributing to tumor eradication by vaccine/Fc-OX40L treatment was assessed by depleting CD8+, CD4+, and NK cells. Depletion was verified by flow analysis of isolated splenocytes from one animal in each depletion group. Survival of the tumor-bearing depleted animals was compared with replete tumor-bearing animals. Interestingly, CD4 depletion completely abrogated the survival benefit conferred from treatment (Fig. 1A). NK depletion also had an effect on survival, albeit to a lesser magnitude than CD4 depletion. NK-depleted animals survived significantly longer than nondepleted untreated animals, but never as long as nondepleted animals that were treated with vaccine/Fc-OX40L. Surprisingly, CD8 depletion did not negatively affect overall survival (Fig. 1A). To confirm these unexpected findings suggesting the limited role of CD8+ T cells, CD8 KO mice were challenged with GL261 and treated with vaccine/Fc-OX40L. The CD8 KO mice had prolonged survival, confirming the previous results using CD8-depleted mice (Fig. 1B). These data suggest that the immune response responsible for increased survival and tumor clearance is CD8+ T cell independent or that CD8+ T cells somehow block the response to immunotherapy. Thus, contrary to many tumor models in which immunotherapy is employed, these data suggest our vaccine therapy elicits an immune response dominated by CD4+ T cells and NK cells and not CD8+ T cells.

B cell–dependent mechanism of vaccine/Fc-OX40L treatment

To determine the extent to which B cells were necessary for the Fc-OX40L treatment, WT and μMT (B cell–deficient) animals were inoculated with GL261 tumor cells and treated with vaccine and Fc-OX40L. Treatment benefit was lost in the μMT animals (Fig. 2A), suggesting that B cells are necessary for proper immune response and eradication of tumor.

Ab response to vaccine/Fc-OX40L treatment

The B cell dependency noted for treatment efficacy may be mediated through different mechanisms of B cell action. B cells can act as APCs, although this role is less well described for B cells than for dendritic cells. B cells also differentiate into Ab-secreting cells (plasma cells) and exert effector function through Ab-mediated mechanisms. To test the extent to which Abs were playing a role in our tumor model, serum was collected from saline-, vaccine-, Fc-OX40L–, and vaccine/Fc-OX40L–treated animals at day 25 postinoculation. This time point was chosen because the treatment regimen had been completed, but the untreated animals were not yet moribund. Serum was also collected at days 35 and 45 for vaccine/Fc-OX40L–treated animals to assess the later time points when the tumors began to regress. There were no significant differences in the presence of IgM Ab binding to tumor cells among treatment groups as measured through flow cytometry (data not shown). All groups had elevated levels of tumor-reactive IgG Abs relative to serum from a normal (nontumor-bearing) animal (Fig. 2B). Tumor-reactive IgG levels at day 25 were similar among groups, but there was a trend to increasing IgG levels at days 35 and 45 in vaccine/Fc-OX40L–treated animals. The similarity in IgG levels at day 25 suggests an endogenous Ab response in tumor-bearing animals, which may indicate that Fc-OX40L is not involved in inducing Ab secretion. The trend toward increasing levels of IgG over time in the vaccine/Fc-OX40L–treated animals indicates that Fc-OX40L may aid in the maintenance of, or help promote, the Ab response over time.

Ab deposition in tumor

To determine whether Ab was being deposited in the tumors of the vaccine/Fc-OX40L–treated animals and to gain insight into the kinetics of this event, animals were inoculated with GL261 tumor, treated with vaccine/Fc-OX40L, and euthanized on days 25, 35, and 45. Saline- and vaccine-only controls were also collected on day 25. Western blot analysis revealed the presence of IgG in the tumors of animals treated with vaccine/Fc-OX40L (Fig. 2C). Bands consistent with IgG H and L chain (25 and 25 kDa, respectively) were detected in the tumors of animals treated with vaccine/Fc-OX40L. The 50-kDa band consistent with the H chain of IgG was not detected in the tumors of saline-treated animals. The presence of a 25-kDa band in these tumors is most likely due to the cross-reactivity of the Ab with the L chain of the other Igs. IgG was detected in the majority of the vaccine-only–treated tumors as well. Interestingly, whereas all of the tumors analyzed from vaccine/Fc-OX40L–treated mice at day 25 contain IgG, the number of animals with detectable IgG at the later time points decreases at day 35 (4 of 5) and day 45 (3 of 5). This may be due in part to the decreasing tumor burden at these later time points.

IgM was detected in the tumors of animals of all treatment groups, although with varying penetrance (Fig. 2D). The H chain of IgM was detectable and appears to be most penetrant at day 35 in vaccine/Fc-OX40L–treated animals. It is important to note that several gels were run to analyze these samples, so whereas the presence or absence of Ig can be noted, the relative amounts cannot be accurately compared. The presence of IgM and absence of IgG in the tumors of saline-treated mice suggest that infiltration of class-switched Ig may be impaired.

**Analysis of brain-infiltrating cells**

Flow cytometry analysis was performed on lymphocytes isolated from the brains of tumor-bearing animals. Glioma-bearing WT animals treated with saline, vaccine only, Fc-OX40L only, and vaccine/Fc-OX40L, were euthanized on day 25. One day prior to euthanasia, tumor burden was assessed by bioluminescence imaging and revealed a significant difference in the tumor size among the groups. Animals receiving Fc-OX40L as part of the treatment showed significantly less tumor burden (Fig. 3A). Lymphocytes harvested from the brains were counted and plotted as absolute numbers. The number of brain-infiltrating cells observed in the WT animals receiving vaccine/Fc-OX40L treatment was not significantly greater than saline-treated animals (Fig. 3A). The large variation in tumor size may distort the true value of infiltrating cells present in the brain, as larger tumors may contain more in-
filtrating cells simply due to volume. To compensate for this difference, the number of brain-infiltrating cells was normalized to the tumor burden determined by bioluminescence imaging (p/s). This analysis revealed that, based on tumor burden, there were more lymphocytes infiltrating the tumors of Fc-OX40L–treated WT animals (Fig. 3A).

Phenotypic staining assessed the composition of the lymphocyte population harvested from the brains of glioma-bearing animals. The T cell population was determined by analyzing the CD3+CD4+ and CD3+CD8+ populations. An increase in the infiltration of CD4+ T cells was observed for the WT animals treated with Fc-OX40L (Fig. 3B), consistent with the earlier finding that CD4+ T cells are necessary for efficacy of treatment (Fig. 1A). Interestingly, an increase in infiltration of CD8+ T cells was observed in the vaccine/Fc-OX40L–treated animals (Fig. 3C), despite the fact that these cells are not necessary for tumor clearance (Fig. 1). Although these cells are present, this analysis does not indicate whether this population is actively functioning as killer cells (data not shown). Importantly, CD4+ T cells also did not demonstrate expression of perforin.

Ligation of OX40 on the surface of Tregs can decrease the suppressive function of this population (30, 31). It is reasonable to assume that one mechanism of Fc-OX40L in tumor eradication is to suppress the regulatory function of these cells, thus allowing the effector cells to kill. Analysis of the CD3+CD4+Foxp3+ Treg population in the brains demonstrates an increase in the presence of Tregs in the tumors of animals treated with Fc-OX40L (Fig. 3D). This is contrary to what was expected; however, it is possible that the increased immune infiltration observed triggers an influx of Tregs to balance the inflammatory response. This analysis also does not indicate whether this population is actively suppressing effector cells; functional analysis of isolated cells would be required to fully understand the effect on Tregs.

The NK cell population was of particular interest as its depletion resulted in loss of treatment efficacy (Fig. 1A). The total CD3negNK1.1+ population, which excludes NKT cells, was assessed and observed to be increased in Fc-OX40L–treated animals only when normalized to tumor burden (Fig. 3E). Im-

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**FIGURE 1.** In vivo depletion of lymphocytes. Necessity of lymphocyte populations for treatment efficacy was determined through in vivo depletion of CD4+, CD8+, and NK cells. Vaccine (tumor lysate and Cpg) was administered on days 3, 7, and 10, and Fc-OX40L, was delivered days 17–21 postinoculation. Depleting Abs were administered i.p. on days 1 and 2 postinoculation and subsequently 1 d before each vaccination (days 6 and 9) and the day before the first Fc-OX40L administration (day 16). (A) Glioma-bearing animals were treated, and survival of animals depleted of lymphocyte populations was compared with nondepleted saline and vaccine/Fc-OX40L–treated animals. (B) To verify the depletion results, the survival of glioma-bearing animals was assessed in WT animals compared with CD8 KO animals. Data represent one experiment; results have been reproduced one additional time, and statistical significance was determined by log-rank test.

<table>
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<th>Group</th>
<th>Median Survival (days)</th>
<th>P value (vs Vaccine/Fc-OX40L No Depletion)</th>
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<td>&lt;0.0001</td>
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<tr>
<td>Vaccine/Fc-OX40L No Depletion</td>
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<table>
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<th>P value (vs WT Vaccine/Fc-OX40L)</th>
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</tr>
<tr>
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pressively, the CD3∑NK1.1∑perforin∑ population was increased in the Fc-OX40L–treated groups (Fig. 3F), demonstrating that NK cells are present and presumably functional. An intriguing observation was the presence of a distinct CD3∑NK1.1∑ population, NKT cells, in the brains of Fc-OX40L–treated animals (Fig. 3G), and, surprisingly, a significant number of these cells expressed perforin (Fig. 3H).

The most noticeable difference among the treatment groups was a granular cell population in the vaccine/Fc-OX40L treatment groups that was absent in the saline and the vaccine treatment groups (Fig. 3I). Phenotypic analysis revealed that the majority of cells in this population are CD11b∑MHC IIneg, indicative of neutrophils, which like NK cells are able to kill Ab-coated cells through Ab-dependent cell-mediated cytotoxicity (ADCC) (32, 33).

**FIGURE 2.**  B cell–dependent mechanism of treatment efficacy and Ab response. (A) To assess the role of B cells in treatment efficacy, the survival of WT animals was compared with μMT animals receiving treatment. Data represent one experiment; results were reproduced two additional times, and statistical significance was determined by log-rank test. (B) The presence of tumor-reactive Abs in the serum of animals was determined through flow cytometry analysis. Glioma-bearing animals were euthanized at noted time points, and sera collected. Tumor-reactive IgG Abs were plotted based on mean fluorescence intensities. The presence of Igs was detected in tumors harvested from brains of glioma-bearing animals at noted time points. n = 5–11/treatment group. Statistical significance is indicated as *p < 0.05, determined by Mann–Whitney U test. Tumors were run on SDS-PAGE and probed for (C) IgG and (D) IgM to detect the presence of these Igs. Tumors from μMT animals and normal (nontumor-bearing) brains from WT animals were used as negative controls.

Involvement of the FcR and Ab

The indication that Ab production may play a significant role in tumor elimination combined with the loss of treatment efficacy with NK cell depletion (Fig. 1A) led to the investigation of the role that ADCC may play in treatment-induced tumor regression. ADCC requires the Fc portion of Igs bound to target Ag to bind to FcR on the surface of effector cells, most commonly NK cells. This binding induces lysis of the target cell by the effector cell, generally through the production and release of perforin and granzymes. To test whether this mechanism was the link between the NK cell and the Ab results, treatment efficacy was tested in mice deficient for the γ-chain of the FcR (FcRγ KO). These mice lack the FcRs capable of binding IgG and IgE, and therefore ADCC action mediated through these Igs would not be possible.
Whereas there was a significant difference in the overall survival of saline-treated WT and FcRγ KO animals (p = 0.0146), suggestive of an endogenous response involving the FcR, the overall survival of WT and FcRγ KO animals that received vaccine/Fc-OX40L treatment did not differ significantly (Fig. 4A). However, there is clearly a trend toward an intermediate phenotype that is best illustrated in the tumor burden of these animals. Bioluminescence imaging was performed to track changes in tumor burden. These studies showed that the rate of tumor growth was rapid in saline-treated mice, but was slowed by vaccine/Fc-OX40L treatment especially in the WT mice compared with the FcRγ KO mice (Fig. 4B).

To verify whether indeed Abs were necessary for the efficacy of our treatment, we tested our therapy in an animal model deficient for plasma cells (IgMi mice) (27, 28). We observed an intermediate phenotype in response to treatment in these mice. The IgMi vaccine/Fc-OX40L–treated animals survived significantly longer than the saline-treated animals, but did not reach the full survival benefit of the WT animals (Fig. 4C). Once again, the intermediate phenotype in response to treatment is reflected in the tumor burden of these animals, as measured by bioluminescence imaging (Fig. 4D).

Overall, survival after vaccine/Fc-OX40L treatment of the plasma cell-deficient mice paralleled that of FcRγ KO, suggesting that the Ab dependence is likely through a FcR-based mechanism. Whereas Ab-mediated mechanisms are not required for complete tumor clearance, Ab does play a role in controlling tumor growth early in the course of treatment based on the greater tumor burden seen in the IgMi and FcRγ KO mice.

A mechanism of NK killing through ADCC is through the release of perforin and granzymes, and we observed an increase in the perforin-expressing NK cells after treatment; however, when the efficacy of the vaccine/Fc-OX40L treatment was tested in perforin-deficient (perforin KO) animals, we found that treatment was not perforin dependent (Fig. 5).

**Tumor infiltration of granular population with Fc-OX40L treatment**

The granular population observed by flow cytometry (Figs. 3I, 6A) was further analyzed to determine its role in the therapeutic response. Histologic analysis revealed that tumors from multiple vaccine/Fc-OX40L–treated animals were infiltrated by a moderate to large number of neutrophils, which in some areas of the tumor formed dense aggregates and replaced neoplastic cells. Neutrophils were characterized by a multilobed nucleus and a homogeneous eosinophilic cytoplasm. Tumors in saline-treated mice were infiltrated by a moderate number of mononuclear cells, but neutrophils were not a prominent component of the inflammatory cell infiltrate as determined by a pathologist (Fig. 6B). It is important to note that this population is also consistent with the myeloid-derived suppressor cell population, which is often in-
duced by the tumor itself and generally only distinguishable from neutrophils by functional assays. Thus, a suppression assay was performed, revealing that the CD11b+ cells isolated from the brains of vaccine/Fc-OX40L–treated animals were not functionally suppressive (Fig. 6C). Furthermore, when nontumor-bearing animals were treated with Fc-OX40L, there was a dramatic increase in the presence of CD11b+ cells in the blood, suggesting that the treatment, rather than the tumor, is responsible for neutrophil recruitment (Fig. 6D).

**Discussion**

Despite the increasing interest in utilizing immunotherapeutic approaches in cancer treatment, the prognosis for patients diagnosed with glioma remains poor. Advances in the field of cancer
immunotherapy have been incremental, and an inherent hurdle to progress is the lack of a basic understanding of the mechanisms needed for an effective immune-based antitumor response. One route of gaining insight into these mechanisms is to examine the immune players at work during an effective antitumor response. Because prior studies by our laboratory have identified a potent antitumor treatment in a murine model of glioma (25), we were in the position to define the mechanisms responsible for tumor clearance.

In the present report, our data demonstrate that this response is dependent on CD4+ T cell and NK cell responses, as efficacy is lost upon depletion of these cells. In contrast to many other studies, our data indicate that the CD4+ T cell response in this model was critical for tumor regression. In this immunotherapeutic model, however, further work remains to be done to identify the exact mechanism of CD4+ T cell action. CD4+ T cells could potentially be orchestrating the activation and infiltration of other immune cells that carry out the cytotoxic functions. In contrast, CD4+ T cells are capable of cytolytic function through their direct and indirect killing in viral and tumor models (6, 12, 34). It is possible that our model may be reproducing the results observed in these previous publications. Whereas the exact mechanisms remain to be elucidated, the perforin-independent tumor regression would suggest that if CD4+ T cells are mediating a cytotoxic response, pathways such as TRAIL and FasL should be examined.

Many cancer vaccines have been designed to induce a robust CD8+ T cell response. Surprisingly, we did not observe a dependency of CD8+ T cells, as CD8 depletion did not alter survival outcome. An increase in the median survival of animals depleted of CD8+ T cells was observed (Fig. 1A), initially suggesting that CD8+ T cells may have a deleterious effect on survival. However, the overall survival did not achieve statistical significance and further analysis of survival in CD8 KO animals did not reveal any difference in median survival. Although tumor-infiltrating CD8+ T cells were observed in vaccine/Fc-OX40L–treated animals (Fig. 3C), the results of the depletion study suggest these cells may be functionally inactive. It is also possible that, in an attempt to prevent tissue damage from an inflammatory response, the CD8+ T cells may be suppressive. Previous laboratories have shown that CD8+ T cells express the suppressive cytokine IL-10 in response to viral infections in the lung and brain, where prolonged activation may cause deleterious bystander effects (35, 36). These remain attractive theories that need to be tested, and the optimized strategy of vaccine/Fc-OX40L in the GL261 glioma model presents as a robust model to pursue these questions in the future.

Additionally, B cells are required for the efficacy of treatment. B cells may be necessary to act as APCs for CD4+ T cell activity or they may be differentiating into plasma cells and secreting tumor-reactive Abs or Abs required for ADCC. The observation that tumor-bearing animals generate tumor-reactive Abs and the deposition of Ab in the tumors of vaccine/Fc-OX40L–treated animals points in this direction. The loss of control of tumor growth in FcRy KO and IgMi mice suggests that ADCC mechanisms may be a contributing factor in the therapeutic response; however, due to the incomplete loss of efficacy, there are obviously other mechanisms contributing to tumor clearance.

The infiltration of tumor by immune cells is key in understanding the mechanism of tumor clearance. The most notable observation was the infiltration of NK cells, NKT cells, and the large granular population. Recruitment and activation of NK cells, NKT cells, and neutrophils may be mediated by cytokine secretion by CD4+ T cells activated through OX40 ligation. Another explanation could involve direct activation by OX40L. Although the majority of research conducted on OX40:OX40L interactions has focused on activated T cells, there have been some reports indicating that NK cells (37), NKT cells (38), and neutrophils (39) may express the OX40 receptor. It is possible that Fc-OX40L administration is acting directly on the innate immune cells to induce a productive antitumor response, although this remains controversial.

The observed infiltration of innate immune cells is particularly interesting in light of our Ab data (Figs. 2, 4), due to the known link between the NK cells, neutrophils, and ADCC. It is possible that these cells are actively killing Ab-coated tumor cells and inhibiting growth in a FcR-dependent mechanism. The presence of a large granulocyte population remains an interesting potential mechanism of tumor clearance. These cells were found to infiltrate into the tumors of animals treated with Fc-OX40L, and these mice also demonstrated the greatest response to treatment.

As shown by our data, multiple mechanisms of tumor clearance may be occurring in this tumor model. Too often in the design of immunotherapeutic studies, particular immune subsets are targeted while ignoring the remainder of the immune system. This is particularly noted for CD8+ T cell–biased therapies. Many vaccines have focused on CD8+ T cell–restricted Ags or adoptive transfer exclusively (2). Even in the case of CD8+ T cell–mediated tumor clearance, recruitment of additional immune cells is necessary, particularly CD4+ Th1 cells that are needed for mainte-
The observation of an increase in the influx of innate cells to the tumor site upon treatment and the presence of a tumor-reactive Ab response may be linked through a FcR-dependent mechanism. A deeper understanding of immune-mediated mechanism of tumor clearance will certainly drive the design of immunotherapy regimens in the clinic with the hope of fully harnessing the power of the immune system to mediate lasting tumor clearance with memory. Considering that the CNS may provide unique challenges for tumor immunology, it is entirely possible that different cell populations may be involved in effective tumor clearance than that found in solid tumors of peripheral tissues.

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**Figure 6.** Observation of a distinct granular population. (A) Flow cytometry scatter plots of brain-infiltrating cells revealed a distinct granular population. Representative plot, data from three independent experiments reproduce this result. (B) Histologic analysis demonstrates a dense aggregate of neutrophils (arrows) within the tumor of a vaccine/Fc-OX40L–treated animal that is absent in a saline-treated animal. H&E stain. (C) The suppressive capacity of CD11b+ cells was determined by incubating these cells with CD8+ T cells and plate-bound CD3 at a ratio of 1:1. T cell activation was measured by [3H]-thymidine uptake and graphed as percent suppression, n = 3 for vaccine/Fc-OX40L and saline brain-infiltrating lymphocytes, n = 1 for saline spleen; all samples were performed in triplicate. This experiment was performed once. (D) Analysis of blood from tumor- and nontumor-bearing animals at day 14. Mean values are shown as ±SEM; n = 3–5/group. Statistical significance, determined by unpaired Student t test, is indicated as *p < 0.05.
Disclosures
The authors have no financial conflicts of interest.

References