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Vaccine Injection Site Matters: Qualitative and Quantitative Defects in CD8 T Cells Primed as a Function of Proximity to the Tumor in a Murine Glioma Model

John R. Ohlfest,* Brian M. Andersen,* Adam J. Litterman,* Junzhe Xia,* Christopher A. Pennell, † Lauryn E. Swier,* Andres M. Salazar,‡ and Michael R. Olin*

Malignant gliomas are lethal brain tumors for which novel therapies are urgently needed. In animal models, vaccination with tumor-associated Ags efficiently primes T cells to clear gliomas. In clinical trials, cancer vaccines have been less effective at priming T cells and extending survival. Generalized immune suppression in the tumor draining lymph nodes has been documented in multiple cancers. However, a systematic analysis of how vaccination at various distances from the tumor (closest to farthest) has not been reported. We investigated how the injection site chosen for vaccination dictates CD8 T cell priming and survival in an OVA-transfected murine glioma model. Glioma-bearing mice were vaccinated with Poly:ICLC plus OVA protein in the neck, hind leg, or foreleg for drainage into the cervical, inguinal, or axillary lymph nodes, respectively. OVA-specific CD8 T cell number, TCR affinity, effector function, and infiltration into the brain decreased as the vaccination site approached the tumor. These effects were dependent on the presence of the tumor, because injection site did not appreciably affect CD8 T cell priming in tumor-free mice. Our data suggest the site of vaccination can greatly impact the effectiveness of cancer vaccines. Considering that previous and ongoing clinical trials have used a variety of injection sites, vaccination site is potentially a critical aspect of study design that is being overlooked.

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A ctive tumor immunotherapy shows great promise in animal models but has yet to achieve widespread success in the clinic. Vaccines have been extensively tested in clinical trials for the treatment of glioma. Glioma patients have been vaccinated in multiple sites including the scalp drilling into the axilla (1), the anterior upper thigh (2), the upper arm (3), and the cervical regions (4, 5). Data are insufficient to correlate response rates with vaccination site. Few basic studies have examined priming after vaccination as it relates to anatomic location.

The sentinel lymph nodes (draining lymph node [DLN] nearest to the tumor) are in direct lymphatic drainage from the primary tumor (6, 7) and are the DLNs most prone to immune suppression (8–10). In breast cancer and melanoma patients, T cells isolated from the sentinel lymph nodes have suppressed activation and proliferation in response to various mitogens compared with T cells isolated from the more distal lymph nodes (11–14). Multiple mechanisms contribute to this local suppression. Tumor-elaborated soluble factors, such as TGF-β and PGE2 (15–18), can act at the tumor site or DLN to dampen T cell reactivity. In experimental systems, additional documented mechanisms of local immune suppression at the DLN include regulatory T cell–mediated killing of tumor Ag-presenting dendritic cells (DCs) (19) and TCR affinity, effector function, and infiltration into the brain decreased as the vaccination site approached the tumor. These effects were dependent on the presence of the tumor, because injection site did not appreciably affect CD8 T cell priming in tumor-free mice.

With respect to brain tumors, the immunologically specialized nature of the brain and its draining cervical lymph nodes must also be considered. Initial experiments revealed that vaccination with Ag into the brain can trigger higher Ab titers compared with vaccination in the periphery (21). In contrast, Th1-mediated, delayed-type hypersensitivity responses are absent or blunted when the same Ag is delivered to the brain (reviewed in Ref. 22). These findings support a model whereby the cervical lymph nodes have an intrinsic Th2 bias in steady-state conditions. Additional experiments showed that CD8 T cells undergo initial expansion after intracerebral tumor cell challenge, but fail to differentiate into CTLs (23). However, it was unclear whether this was due to tumor-induced immune suppression, a lack of costimulation, or an intrinsic bias against CTL development in the cervical lymph nodes. Normal mouse cerebrospinal fluid can suppress CD8 T cell activation in ex vivo assays, which is restored by a TGF-β blocking Ab (24), implicating brain-derived TGF-β as one soluble mediator of CTL suppression in the cervical lymph nodes.

Despite support for Th2 immune deviation in the brain DLNs, there is evidence that CD8 T cell responses play a tumoricidal role in human gliomas. Infiltration of CD8 T cells is a positive prognostic factor in glioma patients (25). Furthermore, immunological synapses between CD8 T cells and glioma cells have been documented in humans (26). Interestingly, glioblastoma patients receiving autologous tumor lysate-pulsed DC vaccines had superior survival when their gene expression was of mesenchymal rather than the proneural molecular signature; the mesenchymal signature is inflammatory and was correlated with significantly more infiltrating CD8 T cells at the tumor site compared with proneural tumors (27). Regardless of these spontaneous or vaccine-induced
T cell responses, global immune suppression has been widely accepted to occur in glioma patients. Many studies done to establish this conclusion were conducted with leukocytes harvested after treatment with glucocorticoids or chemotherapy, clouding the contribution of the tumor versus the treatment on dampened immunity. More recent data suggest that treatment with glucocorticoids and alkylating chemotherapy plays a significant role in inducing global immune suppression, because both drugs are associated with rapid posttreatment lymphopenia, and elevation in regulatory T cell or myeloid-derived suppressor cell frequency (28, 29). The severity of lymphopenia following the standard of care (steroids, chemotherapy, and radiation) negatively correlates with overall survival in glioblastoma patients (30).

There is stronger experimental evidence for profound local immune suppression at the tumor site in gliomas. Studies in spontaneous murine models suggest that gliomas accrue immune-suppressive cell populations even at early, asymptomatic stages (19). As gliomas develop, recruitment of myeloid-derived suppressor cells is mediated, in part, through a cyclooxygenase-2 and PGE2 axis (31), decreasing CTL recruitment to the tumor bed. Local immune suppression is further enforced by hypoxia, leading to reduced effector function of T cells via increased production of immunosuppressive factors (32). Moreover, glioma cells express inhibitory ligands that can trigger T cell tolerance including HLA-E and B7-H1 (33, 34). However, little is known about how these mechanisms of suppression at the tumor site impact priming responses in the cervical lymph nodes. One purpose of this study was to shed insight on this poorly understood area.

Based on evidence that much of the tumor-derived immune suppression in other malignancies is anatomically graded rather than global (8–10, 35, 36), we reasoned that vaccination distal to gliomas could be exploited to improve active immunotherapy. We focused our investigation on the relation between vaccination sites and the ensuing CD8 T cell response. Vaccination in close proximity to the tumor DLN (cervical or axillary regions) resulted in no survival benefit. In contrast, vaccinations in DLN further from the brain (in the inguinal region) increased survival of glioma-bearing mice. Mice vaccinated in the cervical region had CD8 T cells with lower effector function of T cells via increased production of immuno-suppressive factors (32). Moreover, glioma cells express inhibitory ligands that can trigger T cell tolerance including HLA-E and B7-H1 (33, 34). However, little is known about how these mechanisms of suppression at the tumor site impact priming responses in the cervical lymph nodes. One purpose of this study was to shed insight on this poorly understood area.

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bated for an additional 20 min at 4˚C; cells were then washed twice and analyzed by flow cytometry.

Brain-infiltrating lymphocyte isolation

Brain-infiltrating lymphocytes (BILs) were prepared by perfusing four mice per treatment group with PBS. Brains were minced into a crude suspension in cold RPMI 1640 complete media with 10% FBS. Suspensions were then passed through a 40-μm filter, washed once in cold PBS, and spun on the following Percoll gradient: 3 ml 70% (v/v, in PBS) bottom, 3 ml 37% middle, and 4 ml 30% with resuspended pellet. BILs were centrifuged at 800 relative centrifugal force at 4˚C for 20 min and collected from the cell interface and washed in cold RPMI 1640 complete media with 10% FBS.

CTL analyses

These assays were conducted as previously described (40). In brief, DLNs were harvested, dissociated, and lymphocytes were incubated with CFSE-labeled GL261 or GL261-OVA cells for 4 h at E:T ratios of 0:1 and 25:1, and analyzed for cytotoxicity according to the manufacturer’s protocol (Immunocytometry). After incubation, the percentage of CFSE-labeled target cells that stained with 7-AAD was determined by flow cytometry. Data were reported as specific lysis by correcting for background 7-AAD staining using the following formula: % CFSE+ 7-AAD+ at 25:1 E:T ratio – % CFSE+ 7-AAD+ at 0:1 E:T ratio.

IFN-γ detection

Lymphocytes isolated from DLNs were plated at a concentration of 5 × 10^5 cells/well and pulsed with 2 μg SIINFEKL peptide for 12 h. After incubation, 50 μl culture supernatant was analyzed for IFN-γ using a flow cytometric bead array according to the manufacturer’s protocol (BD Biosciences). Cells were then harvested, surface-stained with CD8 and SIINFEKL/Kb dextramer, then intracellularly stained for IFN-γ according to the manufacturer’s protocol (BD Biosciences). To determine the number of secreted molecules of IFN-γ per Ag-specific CD8 T cell, IFN-γ concentration was converted to molarity and divided by the number of plated cells per well. In addition, lymphocytes were harvested from the lymph nodes, stained for CD8, and intracellularly stained for IFN-γ. Flow cytometry was used to determine the fraction of viable cells that were Ag-specific CD8 T cells, and a hemocytometer was used to determine the number of viable cells plated.

Statistical analysis

Statistical comparisons were made by ANOVA, followed by post hoc comparisons using a two-tailed t test. Differences in animal survival were evaluated by log-rank test. All tests were performed with Prism 4 software (GraphPad Software). The p values <0.05 were considered significant. Column statistics were calculated to validate significance within the values of each treatment group.

Results

Vaccination in the hind leg enhances survival benefit

We previously demonstrated that vaccinating glioma-bearing mice with a poly-Ag tumor lysate/CpG vaccine enhanced survival (40). However, these previous studies relied on vaccination in two sites in the same animal: the hind leg and the neck, which is in contrast with most clinical trials that use single vaccination sites. To investigate potential differences in efficacy of vaccination among three different sites, we vaccinated glioma-bearing mice in the hind leg, foreleg, or hind leg and neck (Fig. 2B). There were several notable differences in CD8 T cell priming among three different sites. In the blood, dextramer-staining cells compared with mice vaccinated in the back of the neck, foreleg, or saline control, which had no survival benefit (Fig. 1A).

Although this experiment was suggestive of differential T cell priming, the use of a poly-Ag lysate vaccine did not permit tracking of Ag-specific T cells. To test an Ag-specific response, we developed a model whereby mice bearing intracranial GL261-OVA were vaccinated with OVA plus Poly-ICLC in three different sites. In this experiment, there was an incremental decrease in survival as the vaccination site approached the tumor. Mice vaccinated in the back of the neck showed no survival benefit compared with control animals, whereas mice vaccinated in the hind leg demonstrated the greatest survival benefit (Fig. 1B).

CD8 T cell priming is suppressed through an anatomic gradient

We measured priming of an endogenous CD8 T cell response against the OVA Ag by flow cytometry using a SIINFEKL/H2-Kb dextramer. Mice were inoculated with GL261-OVA and vaccinated in the back of the neck, foreleg, or the hind leg for drainage into the cervical, axillary, or inguinal lymph nodes, respectively. In all tissues analyzed, there was a stepwise decline in the frequency of dextramer-binding CD8 T cells as the vaccination site approached the tumor in the following order: hind leg > foreleg > neck (Fig. 2B). There were several notable differences in CD8 T frequency in each tissue. In the blood, dextramer-binding CD8 T cells averaged from ~11 to 28% of the CD8 T cell compartment depending on the site of priming. Mice primed in the rear leg had roughly twice as many dextramer-staining cells compared with other sites. In the lymph nodes draining the respective vaccination site, CD8 T cell frequency was much lower than blood, averaging ~1.5–2.8% depending on the site of priming. The BILs followed a similar trend compared with the vaccine DLNs, but at much higher frequency, ranging from 18 to 60% of the CD8 T cells during dextramer binding. There was a 3-fold increase in the dextramer-binding CD8 T cell frequency in mice vaccinated in the hind leg compared with back of the neck. There was also a change in the absolute number of BILs that paralleled the trend of CD8 T cell frequency, but this failed to reach statistical significance because of high animal-to-animal variability (Fig. 2B).

Presence of the tumor locally alters the affinity of the TCR

Subsequent experiments were conducted to determine whether the lower T cell priming was due to an intrinsic bias of the cervical
lymph nodes themselves or from the presence of the tumor. These experiments also addressed how the presence of the cognate Ag (OVA) being expressed in the tumor impacted priming. To answer these questions, we inoculated mice with the GL261-OVA or the parental GL261 cells; saline injection was performed in the “no tumor” group of mice to control for brain injury. Mice were vaccinated with OVA plus Poly:ICLC in the back of the neck for drainage into the cervical lymph nodes. Whole blood was collected and analyzed as described above. As shown in Fig. 3A, the presence of the tumor reduced the dextramer-binding CD8 T cell frequency by half regardless of whether the tumor expressed OVA. GL261 and GL261-OVA groups were not significantly different. Thus, the presence of the tumor in the brain is responsible for the suppression in priming in the cervical lymph nodes. Furthermore, the change in priming is not dependent on expression of the Ag used for vaccination in the tumor.

We then focused on qualitative differences in CD8 T cells primed in the rear leg or neck, exclusively in tumor-bearing mice, because this is most relevant to a clinical scenario. The MFI of dextramer-binding CD8 T cell frequency by half regardless of whether the tumor expressed OVA. GL261 and GL261-OVA groups were not significantly different. Thus, the presence of the tumor in the brain is responsible for the suppression in priming in the cervical lymph nodes. Furthermore, the change in priming is not dependent on expression of the Ag used for vaccination in the tumor.

FIGURE 2. T cell priming is suppressed as vaccination site approaches the tumor. Glioma-bearing mice were vaccinated for 4 consecutive days and boosted on day 7. On day 8, mice were sacrificed and analyzed using flow cytometry. (A) Gating scheme used. (B) Whole blood, lymph nodes, and BILs were analyzed for an endogenous CD8 T cell response. Error bars are representative of SD (n = 8 mice/group for blood and lymph nodes, n = 4 for BILs; *p < 0.05, **p < 0.01, t test). Data are representative of two independent experiments.

Effector function of CD8 T cells is changed as a consequence of vaccination site

We investigated how vaccination site alters effector cytokine elaboration and cytotoxic function of CD8 T cells. Mice were primed at multiple sites as before, and the lymph nodes draining the vaccination sites were harvested and used as a source of effector cells. Lymphocytes were stimulated for 12 h in vitro with the OVA-
derived SIINFEKL peptide or unpulsed as a control. There was a >10-fold increase in soluble IFN-γ measured in responder cells from the inguinal lymph node compared with axillary (Fig. 4A). Cells from the cervical lymph node completely failed to elaborate IFN-γ in response to stimulation with SIINFEKL.

To better characterize the responding cells, we used flow cytometry to determine the frequency of dextramer-binding cells in the CD8 gate secreting IFN-γ, and the amount of IFN-γ produced per cell, after peptide stimulation in vitro. The number of soluble IFN-γ molecules elaborated per dextramer-binding CD8 T cell was calculated (Fig. 4B), revealing large differences in functional capacity on a per cell basis as affected by vaccination site. Consistent with previous experiments, mice primed in the neck in the absence of a tumor had a significant restoration of effector function as measured by IFN-γ response to SIINFEKL stimulation.

Intracellular flow cytometry confirmed that dextramer-binding CD8 T cells were the source of IFN-γ (Fig. 4C). To assess the in vivo effector function of tumoricidal T cells, unstimulated lymphocytes were isolated from the various lymph nodes and analyzed intracellularly for IFN-γ production. Mice primed in the hind leg had a significant increase of IFN-γ compared with the other treatment groups (Fig. 4D). This response correlated with previous experiments demonstrating that priming near the tumor environment had suppressed effector function.

We next investigated CD8 T cell tumoricidal function in tumor-bearing mice primed with OVA and Poly.I:CLC vaccination. Lymphocytes isolated from lymph nodes draining the vaccination site were used as a source of CTLs. CTLs from mice vaccinated in the hind leg had significantly greater tumoricidal function compared with mice vaccinated in the foreleg (Fig. 4E). Cells isolated from the cervical lymph nodes had negligible killing activity, but tumoricidal function was restored in tumor-free mice. Killing was mostly dependent on the expression of OVA because parental GL261 targets had diminished specific lysis. However, cells primed by vaccination in the hind leg or foreleg had tumoricidal function above background against parental GL261, implying...
spontaneous epitope spreading to endogenous GL261 Ags in these lymph nodes. Also consistent with this explanation is the finding that CTLs from tumor-free mice that were vaccinated with OVA had no tumoricidal function against parental GL261 targets (Fig. 4E). In addition, no significant killing activity was measured within saline-vaccinated mice at any site, ruling out differences in baseline killing function in cervical, axillary, and inguinal lymph nodes (data not shown).

Discussion

Tumor-induced immune suppression consists of several functionally distinct mechanisms, most of which have been reported to corrupt not only the tumor microenvironment but also local secondary lymphoid tissues in direct drainage of the tumor site (41, 42). In this study, we examined for the first time, to our knowledge, the relation between vaccination site and treatment efficacy in a glioma model. From an efficacy standpoint measured by survival, the results were generalizable to different vaccine adjuvants (CpG or Poly:ICLC) and different Ag sources (lysate or single protein). Our data demonstrate a profound suppression of CD8 T cell priming within the cervical lymph nodes. This suppression is tumor dependent, but is not dependent on expression of the vaccine Ag in the tumor. Such suppression is likely to occur from secreted tumor-derived factors that reach secondary lymphoid organs rather than direct contact with tumor cells because GL261 does not metastasize to extracranial sites. A clear limitation to our study is the use of mice with recently established tumors to explore these questions. At best, this is a model of minimal residual disease in a clinical setting. How this animal model relates to humans who have coevolution of the immune system and tumor for months is an open question. Nonetheless, the results are informative and raise intriguing questions that can be explored in human subjects.

Tumor vaccines break tolerance by reversing anergy and/or cross-priming naive CD8 T cells. Crucial to both processes is presentation of Ag on MHC class I and induction of costimulatory signals by DCs (43). We sought to characterize the suppressive mechanisms present in our tumor model from site-based differences in response to vaccination. We found no difference in expression of CD80, CD86, or OX40L in DCs in the cervical lymph nodes of glioma-bearing and tumor-free mice (data not shown). Differential expression of other costimulatory molecules cannot be ruled out, although a myriad of other suppressive mechanisms exists. Hyperactivation of immune checkpoint molecules such as CTLA-4 or PD-1 may occur from the local presence of tumor-derived factors. Such a mechanism has been associated with anergic or tolerant CD8 T cells whose tumoricidal function can be restored by Ab-mediated blockade (44). In addition, studies report increases in DC expression of PD-L1 in tumor DLNs (45). Also, local downregulation of MHC II expression has been shown to limit CD4 help to tumor-specific CD8 T cells (46), but we did not detect differences in expression of MHC class II by DCs in our studies (data not shown). Regulatory T cells are often increased in tumor DLNs (47, 48). We have not tested regulatory T cells levels in our model. Clearly, many additional studies are needed to more fully characterize the mechanisms of immune suppression in the cervical lymph nodes of glioma-bearing mice.

TCR diversity exists in the CD8 T cell precursor clones capable of responding to vaccination. Differential expansion of clones...
occurs in a hierarchy according to TCR affinity, enabling high-affinity clones to preferentially expand and dominate the response (39, 49, 50). We found relatively weaker TCR affinity of CD8 T cells specific for the OVA Ag when vaccination drained to the cervical lymph nodes of mice with gliomas. It is unlikely that thymic emigrant populations could contribute to this effect, as 1 wk elapsed from time of tumor implantation to vaccination. Rather, this difference may be because of other factors including relatively greater expansion of cells with weaker binding to cognate Ag, destabilized TCR coreceptor complex (51–53), or perhaps more likely is that cells with stronger TCR signaling are selectively deleted. Additional work is needed to address these possibilities. This study is the first, to our knowledge, to demonstrate a meaningful difference in TCR affinity as it relates to vaccination site in tumor-bearing hosts. We postulate that differences in TCR affinity may underlie the inferior effector functions of CD8 T cells from mice vaccinated near the tumor DLNs, because only high levels of Ag triggered TCR signaling ex vivo in these mice (Fig. 3C). Accordingly, because of impaired TCR signaling, it is unlikely that levels of peptide–MHC I are sufficiently high in situ to trigger CD8 T cell tumoricidal function in animals vaccinated near the tumor. Lower TCR affinity and poor effector function would account for the inferior survival of animals vaccinated nearer to the tumor DLNs.

This study has potential implications for the design of translational research and clinical trials, where injection site is variable and often arbitrarily chosen. These data provide guidelines for a strategic choice of vaccination site in humans and raise several questions. For example, in metastatic melanoma, would better immune responses be achieved if vaccination sites were consistently altered to be farthest away from active tumors? What is the immune competence of such distal lymph nodes related to those that have been studied to date, which are only located around the tumor and resected as part of standard of care? Our results call for testing if anatomically graded suppression occurs in humans, whose anatomic scale may follow different rules than that of mice.

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Disclosures

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References


