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Examining T Cells at Vaccine Sites of Tumor-Bearing Hosts Provides Insights to Dysfunctional T Cell Immunity

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Abstract

When tumor vaccines are administered as cancer immunotherapy, cellular interactions at the vaccine site are crucial to the generation anti-tumor immunity. Examining interactions at the vaccine site could provide important insights to the success or failure of vaccination. Our laboratory previously showed that while administration of a cell-based vaccine to tumor-free mice leads to productive anti-neuroblastoma immunity, vaccination of tumor-bearing mice does not. The goal of this study was to examine immune effectors at the vaccine site to identify mechanisms responsible for the generation of ineffective anti-tumor immunity in tumor-bearing mice. The results of this study show that vaccine sites of tumor-bearing mice contained significantly fewer T cells than vaccine sites of tumor-free mice. Similar migration and proliferation of T cells was observed in the vaccine sites of tumor-bearing and tumor-free mice, but T cells in the sites of tumor-bearing mice were more apoptotic. T cells at the vaccine sites of both tumor-free and tumor-bearing mice had an effector-memory phenotype and expressed activation markers. Despite the activated phenotype, T cells from tumor-bearing mice elicited defective anti-tumor immune responses. Although T cells from vaccine sites of tumor-bearing mice were capable of producing inflammatory cytokines, the T cells from tumor-bearing mice produced lower levels of cytokines compared to T cells from the tumor-free mice. Remarkably, this defect appears to be systemic, affecting distal T cells in tumor-bearing mice. This study demonstrates that the defective vaccine-induced immune response to neuroblastoma in tumor-bearing hosts originates as a result of tumor burden, resulting in poor anti-tumor immunity.

INTRODUCTION

Neuroblastoma is the most common pediatric extracranial solid tumor ¹, accounting for 12% of all pediatric cancer deaths ². Patients over one year of age and those diagnosed with stage III or stage IV disease are considered high-risk ^{3,4}. Current treatment regimens for high-risk neuroblastoma patients include surgery, chemotherapy, radiation therapy, and autologous hematopoietic stem cell transplantation ⁵. Despite aggressive therapy children with high-risk

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disease (approximately half of the new neuroblastoma cases each year) have a long-term survival rate of less than 40% ⁴.

Novel therapeutic approaches are needed to improve the outcomes for high-risk neuroblastoma patients. Immune-based approaches to cancer therapy are promising because of the directed specificity to tumor antigens ^{6–8}. Current approaches that target the immune response to neuroblastoma include administration of cytokines, antibodies, vaccines, and adoptive T cell transfer. Unfortunately, these immune therapies have not been very successful in treating high-risk patients due to targeting unknown tumor antigens, the inability to identify tumor-reactive T cells, and the immunosuppressive milieu surrounding the tumors. Unraveling the mechanisms of T cell activation at the vaccine site and the suppressive influence of tumor will enable development of more effective anti-tumor vaccine strategies.

For our studies, an aggressive mouse model of neuroblastoma has been employed in which the tumor cells have been genetically modified to express the immune co-stimulatory molecules CD54, CD80, CD86, and CD137L to create a whole cell-based tumor vaccine ⁹. This modified vaccine cell line is referred to as AGN2a-4P. A strong T cell-mediated immune response to the AGN2a-4P vaccine results in protection from live neuroblastoma tumor challenge ⁹, and this vaccine is able to treat established tumors immediately after hematopoietic stem cell transplantation ¹⁰, but administration of the AGN2a-4P vaccine to tumor-bearing mice does not eliminate established tumors in non-transplanted mice ¹¹. These data indicate that while the AGN2a-4P vaccine is able to induce a ‘protective’ anti-tumor immune response, it is unable to elicit an effective immune response against established tumors.

Most investigations into tumor-specific T cell defects have focused on tumor-infiltrating T cells or T cells in peripheral lymphoid tissues. To better understand the mechanisms responsible for defective tumor vaccine-induced immune responses, examining T cell responses in draining lymphoid tissues or the sites of vaccination could prove to be more informative. Our laboratory adopted a method developed by Corthay et al. ¹², where we used growth factor reduced (GFR) matrigel to capture immune cells that infiltrate vaccine sites ¹³. The resulting matrigel plugs can be isolated to investigate cells that have migrated into the vaccine site. Using this method, we found that a variety of immune cells, including T cells (CD4⁺ and CD8⁺), B cells, monocytes/macrophages, dendritic cells and granulocytes, migrate into the vaccine sites of tumor-free mice ¹³. Activation of tumor-specific T cells at the vaccination site is a rapid event that occurs early, and effector T cells at the vaccination site play a dominant role in generating an effective anti-tumor immune response ¹². However, the previous studies did not investigate the anti-tumor immune response at vaccine sites of animals with ‘established’ neuroblastoma.

In the current study, we sought to characterize and compare immune responses at the vaccine sites of tumor-bearing mice versus tumor-free mice. Investigating the vaccine site provided insights into why tumor-bearing mice fail to generate an effective anti-tumor immune response ¹⁰. Decreased numbers of T cells were seen in the vaccine sites of tumor-bearing mice, while the numbers of other immune cells were unaffected. It appeared that the decreased T cell numbers in tumor-bearing mice vaccine sites were a result of increased apoptosis. Although T cells present at the vaccine site of both tumor-bearing and tumor-free mice displayed an effector-memory phenotype, T cells from the vaccine sites of tumor-bearing mice produced decreased levels of cytokines in response to tumor antigens or polyclonal stimuli *ex vivo*. Interestingly, the functional T cell defect observed in tumor-bearing mice was not specific to tumor-reactive T cells. These studies indicate that defective

tumor immunity occurs systemically and early during generation of the immune response, and not exclusively during the effector phase.

MATERIALS AND METHODS

Mice

Male A/J mice, 6 to 8 weeks old, were purchased from the Jackson Laboratory. All mice were housed in the Medical College of Wisconsin (MCW) Biomedical Resource Center (Milwaukee, WI), which has been accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. All animal work was approved by the MCW Institutional Animal Care and Use Committee.

Cell lines

The strain A-derived mouse neuroblastoma cell line, AGN2a, was used as the tumor model. Cell-based AGN2a vaccines consisted of cells previously modified to express the immune co-stimulatory molecules CD54, CD80, CD87, and CD137L⁹. This modified cell line is referred to as AGN2a-4P. Prior to use, expression of the co-stimulatory molecules was checked by flow cytometric analysis. Cells were thawed and cultured for 2 weeks prior to use.

Tumor vaccination and matrigel processing

Mice inoculated with live tumor cells (i.e., tumor-bearing) were injected subcutaneously (s.c.) in the hind flank with AGN2a tumor cells (10^5) in 0.2 ml phosphate buffered saline (PBS). For vaccination, mice received two s.c. injections of irradiated (3000 rads) AGN2a-4P cells (2×10^6). The first AGN2a-4P vaccine in 0.2 ml PBS was given 6 days after tumor inoculation. The second vaccine was given 7 days after the first vaccine (13 days after tumor inoculation), and the vaccine consisted of irradiated AGN2a-4P cells (10^6) suspended in 0.3 ml ice-cold GFR matrigel (BD Bioscience, San Diego, CA). The AGN2a-4P/matrigel suspension was injected s.c. on the contralateral flank of anesthetized mice. Mice remained under the anesthesia for 20 minutes after matrigel injection to allow the matrix/cell mixture to solidify. After the indicated number of days, the matrigel plugs were surgically removed from euthanized mice, and the plugs were cut into small pieces and treated with 1 mg/ml collagenase (Roche, Indianapolis, IN or Sigma, St. Louis, MO) for one hour at 37°C and 5% CO₂. The matrices were then processed into single cell suspensions by passing the collagenase-treated fragments through thin wire mesh. Cells were washed two times with PBS/bovine serum albumin (BSA) and resuspended in PBS/BSA for analysis.

Antibodies

Monoclonal antibodies (mAbs) to the following antigens were obtained from BD Biosciences Pharmingen (San Diego, CA): caspase3, CD54 (3E2), CD137L (TKS-1), CD80 (16-10A1), CD86 (37.51), CD49b (DX5), PD-1 (J43), Ki-67 (B56), CD44 (1M7), and I-A^k (11-5.2). mAbs to the following antigens were obtained from eBioscience (San Diego, CA): CD3 (145-2C11), CD4 (RM4-5), CD8a (53-6.7), CD45 (30-F11), CD62L (MEL-14), CD25 (PC61), Foxp3 (FJK-16s), CD69 (H1.2F3), Gr-1 (RB6-8C5), CD11c (N418), CD11b (M1/70), F4/80 (BM8), and NKp46. Control antibodies included purified mouse IgG2b, rat IgG2a, and rat IgG2b.

Flow cytometric analysis

Staining of cell surface markers was conducted as previously described¹⁴. Ki-67 and Foxp3 intracellular staining was performed according to the manufacturers' protocols (BD Pharmingen and eBioscience). 7-aminoactinomycin D (7AAD) was added to each sample as

a viability marker to exclude dead cells from analysis. All stained cells were run on a BD LSR II flow cytometer (BD Biosciences). The resulting data was analyzed using Flow-Jo software (Tree Star Inc., Ashland, OR).

T cell purification and ELISPOT assays

Pooled cells, either from spleens or matrigel vaccine sites, were labeled with anti-CD8-conjugated magnetic microbeads (Miltenyi Biotec, Auburn, CA). CD8 T cells were positively selected by immunomagnetic sorting using a Miltenyi automated immunomagnetic sorter (autoMACS). CD8 T cell purity was determined by flow cytometric analysis. To assess the number of tumor-specific IFN- γ producing T cells, an enzyme-linked immunosorbent spot (ELISPOT) assay was conducted using a kit from BD Bioscience Pharmingen. For the final step of the ELISPOT assay, extravidin alkaline phosphatase (Sigma-Aldrich Corp, St Louis, MO) was added, and the spots developed by adding BCIP/NBT substrate (Sigma-Aldrich Corp). The number of spots was quantified using a Cellular Technology Limited (CTL) ImmunoSpot Analyzer (CTL Analyzers, LLC, Cleveland, OH).

Cytokine production assays

Spleens were isolated, processed into single cell suspensions and pooled. CD8 T cells were enriched by autoMACS immunomagnetic sorting. The CD8 cell purity after AutoMACS sorting was >85%. The AutoMACS sorted cells were further purified by flow cytometric sorting using a FACSaria cell sorter (BD Biosciences) to obtain CD8 T cells purities of >99%. For IL-2, GM-CSF, TGF- β and IFN- γ secretion analysis, 10^4 purified CD8⁺ T cells were plated in 96-well round-bottoms plates in DMEM plus 5% FBS. The cells were stimulated with either AGN2a cells, α CD3/ α CD28-conjugated beads (bead:cell ratio of 1:1, Life Technologies, Carlsbad, CA), or received no stimulation (negative control). After 24 hours, culture supernatants were collected and tested for presence of cytokines using Bio-Plex Analysis (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. All samples were assayed in duplicate and quantified using a Bio-Plex protein array reader. Data from the Bio-Plex analysis were automatically processed and analyzed by Bio-Plex Manager Software 4.1 using standard curves generated from recombinant cytokine standards.

Statistics

All experiments were conducted in duplicate or triplicate. Each experimental group contained at least 3 mice. GraphPad Prism (La Jolla, CA) software was used to perform statistical analyses. The Student's t-test and/or ANOVA were used to compare results from the ELISPOT, cytokine, proliferation, and activation assays, as well as flow cytometric analyses. P-values less than 0.05 were considered as significant.

RESULTS

Matrigel plugs (vaccine sites) harvested from tumor-bearing mice have decreased numbers of T cells as compared to vaccine sites from tumor-free mice

Our laboratory previously demonstrated that cell-based vaccines embedded in GFR matrigel can allow for the isolation and characterization of immune cells that have migrated into the vaccine site¹². However, the influence of active disease (i.e., neuroblastoma present at the time of vaccination) on the immune response at the vaccine site was unknown. To address this issue, matrigel plugs were isolated from tumor-free and tumor-bearing mice and analyzed to investigate the influence of neuroblastoma on cellular components. Mice were inoculated subcutaneously in the hind flank with either 10^5 live wild-type AGN2a cells to generate tumor-bearing hosts (day 0; Figure 1A). Tumor-free hosts were inoculated with 0.2

ml of PBS subcutaneously. Both groups of mice received two weekly subcutaneous vaccinations with 2×10^6 irradiated AGN2a-4P cells; the first vaccine consisted of cells suspended in PBS, and the second vaccine consisted of cells in matrigel. Spleens, draining lymph nodes, and matrigel plugs (i.e., vaccine sites) were harvested five days after the second vaccination (day 18) for analysis. The tissues and matrigel plugs were processed into single cell suspensions and analyzed to determine cellular composition (Figure 1B). The total number of cells infiltrating matrigel plugs from tumor-free was significantly higher than the cell numbers in matrigel plugs from tumor-bearing mice ($P < 0.05$). Flow cytometric analysis of cells infiltrating the matrigel plugs was performed to determine numbers of T cells, B cells, monocytes/macrophages, DCs, NK cells, granulocytes, and regulatory T cells. The only significant difference between the matrigel plugs of tumor-free and tumor-bearing mice was the number of T cells. When CD4 and CD8 T cell subsets were examined individually, significant decreases in both subsets were observed in the matrigel of tumor-bearing mice (Figure 1C) ($P < 0.001$). No differences in cell numbers or cellular composition were observed in the spleens or draining lymph nodes of vaccinated tumor-free and vaccinated tumor-bearing mice (data not shown). Based on these results, additional T cell analyses were performed.

T cells harvested from the vaccine sites of tumor-bearing mice show increased apoptosis

The decrease in T cell numbers at the vaccine sites of tumor-bearing mice as compared to tumor-free mice could be the result of several factors that include: (1) Reduced migration into the vaccine site, (2) Reduced proliferation or, (3) An increase in apoptosis. To investigate these factors, the kinetics of T cells infiltrating the matrigel plugs, percentages of proliferative T cells, and percentages of apoptotic T cells were determined. Following the experimental design in Figure 2A, CD4 and CD8 T cell numbers in harvested matrigel plugs were determined on days 14, 16, 18 and 20 after tumor inoculation in order to determine the kinetics of T cells present at the vaccine sites over time. On days 14 and 16 after tumor inoculation (days 1 and 3 after the second vaccine), there were similar numbers of T cells in the matrigel of tumor-free and tumor-bearing mice (Figure 2B). However, by day 18, a decrease in T cell numbers was evident in the matrigel plugs of tumor-bearing mice (Figure 2B). T cell numbers in the spleens of tumor-free and tumor-bearing mice were also examined at the same time points, and no differences were evident (data not shown). When total cell numbers in the matrigel plugs were analyzed on days 14 and 16 after tumor inoculation, similar numbers were observed in tumor-free and tumor-bearing mice (Figure 2C). By day 18, total cell numbers were significantly decreased in the matrigel of tumor-bearing mice relative to those of tumor-free mice (Figure 2C).

CD4 and CD8 T cells were analyzed for Ki-67⁺ expression at days 16 and 18 (see timeline in Figure 2A) to determine if altered proliferation is responsible for the decrease in T cell numbers. The percentages of Ki-67⁺ T cells did not differ between the matrigel plugs of tumor-bearing and tumor-free mice at either time point (Figure 3A). These results suggest that T cells from the matrigel of tumor-bearing mice are equally proliferative, and thus a defect in proliferation is not responsible for the decreased T cell numbers in the matrigel of tumor-bearing mice.

The apoptotic state of T cells in tumor-bearing and tumor-free mice was next examined. Spleens and matrigel plugs were isolated days 14, 16 and 18 after tumor inoculation (same timeline as in Figure 2A). The percentages of apoptotic CD4 and CD8 T cells were determined by staining for caspase-3 expression, and representative staining of CD8 T cells at day 5 after second vaccination (day 18 post tumor inoculation) is shown in Figure 3B. While no change in percentages of caspase-3⁺ T cells was evident at day 16 (Figure 3C), at day 18 there was a significant increase of caspase-3⁺ CD8 T cells in the matrigel of tumor-bearing mice (Figure 3D) ($P < 0.001$). Although a marked increase in percentages of

caspase-3⁺ CD4 T cells was observed in the matrigel of tumor-bearing mice on day 18, the increase was not statistically significant. The increase in percentages of caspase-3⁺ CD8 T cells at the vaccine sites of tumor-bearing mice suggests that increased apoptosis is at least partly responsible for the decreased numbers of T cells present at these sites.

T cells from the matrigel plugs of both tumor-free and tumor-bearing mice display an effector-memory phenotype

T cells from tumor-bearing mice are defective at eliciting an anti-tumor immune response to AGN2a cells both *in vitro* and *in vivo*¹⁰. Cells were analyzed for expression of CD44 and CD62L by flow cytometry to address whether T cells in tumor-bearing mice have a diminished ability to become activated and differentiate into cells that exhibit an effector-memory phenotype. Representative flow cytometric contour plots from splenocytes demonstrated the typical T cell populations in which the majority of CD8 T cells exhibited a CD62L^{hi}CD44^{lo} naïve phenotype (Figure 4A – top contour plots). In contrast, the majority of T cells at vaccine sites of both tumor-free and tumor-bearing mice exhibited a CD62L^{lo}CD44^{hi} effector-memory phenotype (Figure 4A – bottom contour plots). The results from both spleens and matrigel plugs are shown in Figure 4B, and no differences were observed between tumor-free and tumor-bearing mice. Similar results were also observed for CD4 T cells (data not shown). In addition, CD8 T cells from the matrigel of both tumor-free and tumor-bearing mice expressed the activation markers CD69, CD49d, CD25 and PD-1 (Figure 4C). Therefore, the CD8 and CD4 T cells harvested from spleens and vaccine sites are activated, and the T cells harvested from vaccine sites have an effector-memory (T_{EM}) phenotype regardless of the presence of tumor.

CD8 T cells from matrigel plugs in tumor-bearing mice produce decreased levels of inflammatory cytokines as compared to CD8 T cells from tumor-free mice

CD4 and CD8 T cells from vaccine sites were next examined for cytokine production to determine if functional differences exist between cells from tumor-bearing and tumor-free mice. Isolated T cells were co-cultured overnight with either AGN2a tumor cells (CD8 cells only) or αCD3/αCD28 beads (both CD8 and CD4 cells) (Figure 5). Supernatants were collected and analyzed for IL-2 (Figure 5A), GM-CSF (Figure 5B), TNF-α (Figure 5C), and IFN-γ (Figure 5D). CD4 and CD8 T cells from the matrigel of tumor-free mice produced elevated levels of all four cytokines in response to polyclonal stimulation (αCD3/αCD28 beads) when compared to T cells from tumor-bearing mice. CD8 T cells from the matrigel of tumor-free mice also had elevated cytokine levels compared to tumor-bearing CD8 T cells in response to tumor cells. T cells from the spleen produced only minimal levels of these cytokines in response to either tumor cells or αCD3/αCD28 beads (data not shown). These results show that while T cells from the vaccine sites of tumor-bearing mice were capable of producing inflammatory cytokines, they produced significantly lower levels of cytokines as compared to T cells from the vaccine sites of tumor-free mice.

Frequencies of IFN-γ-producing CD8 T cells are decreased in the matrigel plugs of tumor-bearing mice

Results from the previous experiments showed that CD8 T cells from vaccine sites of tumor-bearing mice, as a population, produce less IFN-γ than T cells from vaccine sites of tumor-free mice. To assess whether the decreased IFN-γ production is due to decreased frequencies of IFN-γ-producing T cells, CD8 T cells were tested in ELISPOT assays. Using the experimental approach outlined in Figure 1A, CD8 T cells were isolated and purified from both spleens and matrigel plugs (vaccine sites) of tumor-free and tumor-bearing mice and tested for responses to tumor antigens and non-specific polyclonal stimulation. Relatively few spleen-derived CD8 T cells produced IFN-γ in response to tumor antigens (AGN2a cells), regardless of whether mice were tumor-free or tumor-bearing at the time of

vaccination (Figure 6A). Higher frequencies of splenic CD8 T cells from both vaccinated tumor-free and tumor-bearing mice produced IFN- γ in response to non-specific polyclonal stimulation (α CD3/ α CD28 beads) (Figures 6B). Even higher frequencies of CD8 T cells from the vaccine sites of tumor-free mice produced IFN- γ in response to AGN2a-4P cells (Figure 6A – white bars) or α CD3/ α CD28 beads (Figure 6B – white bars). CD8 T cells from the vaccine sites of tumor-bearing mice had significantly decreased frequencies of IFN- γ -producing cells in response to both tumor antigens and α CD3/ α CD28 beads as compared to CD8 T cells from the vaccine sites of tumor-free mice. Notably, there is no difference in frequencies of IFN- γ producing cells between splenic CD8 T cells and CD8 T cells from the matrigel plugs of vaccinated tumor-bearing mice in response to α CD3/ α CD28 beads. These data indicate that CD8 T cells from the vaccine sites of tumor-bearing mice are capable of producing IFN- γ , but there are fewer IFN- γ producing cells in comparison to CD8 T cells from the vaccine sites of tumor-free mice.

Presence of tumor globally affects the activation of antigen-reactive CD8 T cells

To further confirm that the functional defect in T cells at vaccine sites of tumor-bearing mice is not restricted to tumor-specific T cells, but is also a property of non-tumor-specific T cells, mice were vaccinated on contralateral sides with matrigel-embedded AGN2a-4P vaccine cells and allogeneic (FVB) splenocytes (Figure 7A). Following the experimental approach in Figure 1A, the matrigel plugs were harvested on day 18 after tumor inoculation (5 days after the second vaccine) for T cell analysis. The absolute numbers of CD4 and CD8 T cells present in the matrigel were significantly reduced in both vaccine sites (tumor antigen matrigel plug and alloantigen matrigel plug) of tumor-bearing mice as compared to tumor-free mice (Figure 7B). Isolated CD8 T cells were re-stimulated with T cell-depleted FVB splenocytes and analyzed in IFN- γ ELISPOT assays to determine the functional status of CD8 T cells harvested from the alloantigen vaccine sites. Figure 7C shows a significant reduction in the number of alloantigen-stimulated CD8 T cells producing IFN- γ in tumor-bearing mice as compared to tumor-free mice. These data demonstrate that the presence of tumor confers global, as opposed to tumor-specific, T cell anergy.

DISCUSSION

An experimental cell-based neuroblastoma vaccine induces protective anti-tumor immunity in healthy mice⁹. Unfortunately, this tumor vaccine is unable to eliminate established tumors in the absence of hematopoietic stem cell transplantation¹⁰. This observation is not unique to our laboratory, as other investigators have shown that vaccine-based immunotherapy can induce anti-tumor immunity that does not fully eliminate established tumors^{15–17}. We chose to examine the initiation of the immune response within vaccine sites in an effort to understand why anti-tumor immune responses are ineffective in the presence of established tumors. This approach did provide new insights into why anti-tumor T cell reactivity is defective in mice with established neuroblastoma.

We compared the vaccine sites of tumor-free and tumor-bearing mice to determine why vaccination of tumor-bearing hosts is ineffective at eliminating established tumors. Neuroblastoma tumor vaccine cells (AGN2a-4P) were embedded in a solidified matrix *in vivo*, as previously described¹², to localize vaccine sites and allow for the isolation and characterization of cells that have migrated to those sites. Significant decreases in T cell numbers were observed within the vaccine sites of tumor-bearing mice, but numbers of other immune cells were unaffected when compared to vaccine sites of tumor-free mice. Increased T cell apoptosis in vaccine sites of tumor-bearing mice appears to be at least partly responsible for the decreased cell numbers, while altered migration or cell proliferation do not appear to be involved. Mechanisms responsible for the increased apoptosis at the vaccine sites of tumor-bearing mice are not known, though ongoing studies in our laboratory

are examining possible mechanisms. These results are similar to previous studies in which there was a high rate of spontaneous tumor-specific T cell apoptosis both in the tumor microenvironment and periphery of tumor-bearing hosts^{18–20}. Our laboratory demonstrated that the AGN2a neuroblastoma is immune suppressive via production of macrophage migration inhibitory factor (MIF)^{21–23}, though these studies demonstrated that MIF was not directly responsible for the T cell inhibition. We also speculate that other soluble factors produced by this tumor may be responsible for the increased apoptosis. Unpublished *in vitro* and *in vivo* studies by our laboratory have ruled out some of the more common immunosuppressive factors including TGF- β , FasL, IL-10, and indolamine 2,3-dioxygenase (IDO) (data not shown). In addition, unpublished data has demonstrated that the immunosuppressive factor is a membrane-associated protein that is heat and trypsin-sensitive. The inability to isolate this molecule in a functional form using a variety of methods has made identification of this factor challenging. Although the suppressive factor has yet to be identified, we hypothesize that the suppressive factor is found in tumor-derived microvesicles, and further studies are being conducted to identify this factor. Identification of this factor should allow us to more easily identify the mechanism by which the tumor suppresses T cell function.

Not surprisingly, the majority of T cells at the vaccine sites of tumor-free mice exhibited an effector-memory phenotype (CD44^{hi}CD62L^{lo}). The accumulation of T cells at the vaccine site is due to tumor antigen-specific cells that migrate to and divide within the site of antigen deposition. CD8 T cells appear to be highly enriched for tumor-reactivity due to the response evident against AGN2a-4P stimulation *ex vivo*. Therefore, a benefit of utilizing the matrigel plug as the vaccine site allows for isolation of tumor-specific T cells in a model where it has been extremely difficult to isolate tumor-specific T cells due to the lack of known tumor antigens. It is noteworthy that previous studies suggested that effector-memory T cell subsets are preferentially targeted for apoptosis in tumor-bearing hosts^{19,20}. Therefore, in tumor-bearing mice the activated T cells in the vaccine site may be prone to apoptosis.

T cells at the vaccine sites of tumor-bearing mice did not mediate the same effector response as T cells from the vaccine sites of tumor-free mice despite having a similar effector phenotype. However, the T cells were not completely dysfunctional as they still produced cytokines at levels similar to those of naïve T cells activated by polyclonal stimulation (i.e. α CD3/ α CD28 beads; data not shown). This is contrary to other reports showing that IFN- γ and other cytokines (for example, IL-2) are only produced by tumor-specific T cells in tumor-bearing animals if proximal TCR signaling is bypassed²⁴. As indicated by our results, T cells at the vaccine sites of tumor-bearing mice do have a functional TCR since both AGN2a tumor cells and α CD3/ α CD28 beads were able to induce cytokine production, although not to the level of T cells from vaccine sites of tumor-free mice. Not only were cytokine production levels decreased by T cells at the vaccine sites of tumor-bearing mice, but the numbers of IFN- γ -producing T cells were also decreased. Despite these results, it is important to note that T cells from the vaccine sites of tumor-bearing mice still produced higher levels of cytokines than T cells from the spleens of non-vaccinated naïve mice, vaccinated tumor-bearing mice, or vaccinated tumor-free mice. Therefore, while T cells from vaccinated tumor-bearing mice appear to be dysfunctional in regards to cytokine production levels, these T cells are still capable of some cytokine production. Although they appear to be functional in regards to their ability to make cytokines, the tumor-bearing T cells are not able to elicit an anti-tumor immune response of sufficient strength to eliminate established tumors.

Interestingly, the decreased cytokine production by tumor-bearing T cells in the vaccine site was not restricted to tumor-reactive T cells, since T cells from allogeneic (FVB) vaccine

sites of tumor-bearing mice also had decreased numbers of T cells that produced IFN- γ . Therefore, the presence of established tumor had a global effect on T cells, rendering all CD8 T cells at the vaccine site dysfunctional. Mechanisms responsible for this T cell hypo-responsiveness are unknown, but they could include clonal exhaustion due to chronic antigen exposure²⁴, activation of suppressive cells (i.e., T_{reg})^{14,24–26}, and/or antigen exposure in the absence of co-stimulation^{27–34}.

A major limitation of this study is the inability to use tetramers or other tools to assess the function of tumor-specific T cells directly. Further studies in our laboratory will include the development of H-2^k tetramers for identified neuroblastoma antigens or dominant epitopes. These studies will allow us to investigate the function of tumor-specific T cells directly. Alternatively, the 9464D neuroblastoma from C57BL/6 mice could be utilized, though these studies are in their infancy.

In summary, T cell function is defective at the tumor vaccine sites of neuroblastoma-bearing animals, and it appears that the defective T cell function is the result of an unidentified tumor-derived suppressive factor that acts systemically. Numbers of both CD4 and CD8 T cell subsets were decreased, which correlated with increased apoptosis. The increased apoptosis of T cells in the vaccine site offers a therapeutic target for advancing neuroblastoma immunotherapy. Although T cells at the vaccine sites of tumor-bearing mice have an effector-memory phenotype (CD44^{hi}CD62L^{lo}), these T cells fail to elicit effective anti-tumor immune responses *in vivo*, and they have a general defect in cytokine production to tumor antigens. Somewhat surprisingly, the presence of established neuroblastoma also inhibited vaccine responses to third party antigens, indicating a tumor-derived global effect on antigen reactivity. The reason for this potent T cell inhibition, and approaches to overcome this inhibitory effect, are ongoing.

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Figure 1. Phenotype of immune cells infiltrating the vaccine sites of tumor-bearing and tumor-free mice

(A) Experimental timeline: Tumors were established in the right hind flank of A/J mice by subcutaneous injection of 10⁵ AGN2a wild-type cells on day 0. On day 6, mice received 2x10⁶ AGN2a-4P vaccine cells suspended in PBS. On day 13, mice received 2x10⁶ AGN2a-4P vaccine cells embedded in matrigel. On day 18, spleen, lymph nodes, and matrigel plugs were processed for analysis. (B) Absolute numbers of infiltrating T cells (7AAD⁻CD45⁺CD3⁺CD4/8⁺) B cells (7AAD⁻CD45⁺CD3⁺B220⁺), monocytes/macrophages (7AAD⁻CD45⁺CD3⁺F4/80⁺), DCs (7AAD⁻CD45⁺CD11c⁺I-A_k⁺), NK cells (7AAD⁻CD45⁺CD3⁺CD335⁺), and Tregs (7AAD⁻CD45⁺CD3⁺CD4⁺CD8⁻Foxp3⁺). (C) Absolute numbers (left) and percentages (right) of CD4 T cells (7AAD⁻CD45⁺CD3⁺CD4⁺CD8⁻) and CD8T cells (7AAD⁻CD45⁺CD3⁺CD4⁺CD8⁺) captured in matrigel plugs and harvested 5 days after the second immunization. (B & C) White bars represent vaccinated tumor-free treatment groups (TF VACC). Gray bars represent vaccinated tumor-bearing treatment groups (TB VACC). Values plotted are the average number obtained from three separate experiments. Each experiment includes at least 3 mice from each treatment group. *p-value < 0.01

Figure 2. A decrease in the number of T cells at tumor-bearing vaccine sites is detected five days after the second vaccination

(A) Experimental timeline: Tumors were established in A/J mice by subcutaneous injection of 10⁵ AGN2a cells on day 0. On day 6, mice were injected with 2×10⁶ irradiated AGN2a-4P vaccine cells in PBS. On day 13, mice received 2×10⁶ irradiated AGN2a-4P vaccine cells embedded in matrigel. Spleens, lymph nodes, and matrigel plugs were processed for analysis on days 14, 16, 18, and 20. (B) Absolute numbers of CD4 T cells (7AAD⁻CD45⁺CD3⁺CD4⁺CD8⁻) and CD8 T cells (7AAD⁻CD45⁺CD3⁺CD4⁻CD8⁺) from matrigel plugs harvested on days 14, 16, and 18. (C) Total numbers of cells harvested from vaccine sites on days 14, 16, 18, or 20. (B and C) White bars represent TF VACC groups and gray bars represent TB VACC groups. Data is representative of three separate experiments. *p-value < 0.01

Figure 3. Decreased numbers of T cells in vaccine sites of tumor-bearing hosts appear to be due to increased apoptosis

A/J mice were inoculated subcutaneously with 10^5 viable AGN2a cells on day 0. Identical to the timeline in Figure 2, mice received two vaccines containing 2×10^6 irradiated AGN2a-4P cells, the first in PBS and the second embedded in matrigel. Percentages of CD4 and CD8 T cells that were caspase 3^+ and 7AAD $^-$ were determined by flow cytometry. (A) Combined data showing the percentages of Ki-67 $^+$ CD4 and CD8 T cells from matrigel plugs as determined by flow cytometry. White bars represent tumor-free treatment groups. Gray bars represent tumor-bearing treatment groups. The results are the combined data from three separate experiments (at least 3 mice per group). (B) Representative flow cytometric plots of CD8 T cells that were caspase 3^+ from the TF VACC group, TB VACC group, and a serum-starved control group. The top panels show control FMO staining that was used for gating. The bottom panels show that CD8 T cells that were caspase 3^+ . (C and D) Combined data showing the percentages of caspase 3^+ CD4 and CD8 T cells harvested from the spleens and vaccine sites of tumor-free and tumor-bearing mice on day 16 and day 18. *p-value < 0.01

Figure 4. The majority of T cells at vaccine sites of both tumor-free and tumor-bearing mice express activation markers and have a T_{EM} phenotype

The experimental design in Figure 1 was used for these experiments. (A) Representative flow cytometric plots showing CD44 and CD62L expression on CD8 T cells from the spleens (top row) and vaccine sites (bottom row) in tumor-free and tumor-bearing mice. (B) The percentages of CD44^{lo}/CD62L^{hi}, CD44^{hi}/CD62L^{hi}, and CD44^{hi}/CD62L^{lo} CD8 T cells in the spleens (left) and vaccine sites (right) from TF VACC (white bars) and TB VACC (gray bars) are depicted. (C) The percentages of PD1⁺, CD25⁺, CD69⁺ and CD49b⁺ CD8 T cells from the vaccine sites of TF VACC (white bars) and TB VACC (gray bars) mice are shown. The T cell populations analyzed were pre-gated on 7AAD⁻CD45⁺CD3⁺ cells. The data is representative of three separate experiments. *p-value < 0.01

Figure 5. Decreased cytokine production by T cells from the vaccine sites of tumor-bearing mice
 The experimental timeline from Figure 1A was used for these experiments. On day 18, CD4 and CD8 T cells were isolated from spleens for Bio-Plex cytokine analysis. T cells were co-cultured overnight either with α CD3/ α CD28 beads or AGN2a-4P cells (CD8 T cells only). After 24 hours, culture supernatants were collected to determine concentrations of (A) IL-2, (B) GM-CSF, (C) TNF- α , and (D) IFN- γ . Data from one of two independent experiments is shown, where the T cells are pooled from at least 5 mice. White bars represent TF VACC and gray bars represent TB VACC treatment groups. *p-value < 0.01

Figure 6. CD8 T cells from the vaccine sites of tumor-bearing mice produce decreased amounts of IFN- γ

The experimental timeline from Figure 1 was used for these experiments. On day 18 (5 days after the second vaccine), CD8 T cells from spleens and vaccine sites were isolated and tested for IFN- γ production in ELISPOT assays. For the assay, either (A) live AGN2a-4P cells or (B) α CD3/ α CD28 beads were used to stimulate the CD8 T cells overnight in culture. Results are shown from one of 3 independent experiments, where the CD8 T cells were pooled from at least 5 mice. White bars represent TF VACC treatment groups, and gray bars represent TB VACC treatment groups. *p-value < 0.01

Figure 7. Established tumor globally inhibits the activation of antigen-reactive CD8 T cells
 (A) The experimental timeline from Figure 1 was used for these experiments. AGN2a tumor was inoculated in both the right and left hind flanks to produce tumor-bearing mice. In all mice, the AGN2a-4P vaccine was injected subcutaneously in the left shoulder, and a “vaccine” consisting of irradiated allogeneic FVB splenocytes was administered subcutaneously in the right shoulder. On day 18, CD8 T cells from the spleen and each of the matrigel plugs were isolated for analysis. (B) Absolute numbers of CD4 and CD8 T cells isolated from the AGN2a-4P and FVB (allogeneic) vaccine matrigel plugs of the tumor-free vaccinated mice (open squares) and tumor-bearing vaccinated mice (enclosed circles) were determined. (C) CD8 T cells isolated from the allogeneic vaccine matrigel plugs were tested in IFN- γ ELISPOT assays after overnight stimulation with FVB (allogeneic) splenocytes.