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Research Article

Tumor-Derived Extracellular Vesicles Suppress the Antigen-specific Immune Response via Toll-like Receptor 4

Michael R Olin[1*], Brian M Andersen[2], Zhengming Xiong[1], Rob L Shaver[1], Lynne Bemis[3], Mark Sanders[4]

[1]Department of Pediatrics, University of Minnesota, Minneapolis, USA

[2]Department of Neurology, New York-Presbyterian/Weill Cornell Medical Center, New York, USA

[3]Department of Biomedical sciences, University of Minnesota, Duluth, USA

[4]University Imaging Center, University of Minnesota, Minneapolis, USA

[*]**Corresponding author:** Michael R Olin, 3-136 CCRB, 2231 6th St SE, Minneapolis, MN 55405, USA. Tel: +16126263123; Fax: +16126243514; E-mail: olin0012@umn.edu

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Abstract

Background: Progression to a productive immune response involves passing through immunological checkpoints that act as barriers to effective immunotherapy. Thus, as tumors exploit multiple immune suppressive factors to inactivate the immune system, treatments against cancer have often failed to fully overcome the complex interplay between the immune system and the immunosuppressive tumor microenvironment. Recently, interest in the immunosuppressive effects of tumor-secreted exosomes has increased. Exosomes, A Subset of Extracellular Vesicles (EVs), play a role in intercellular communication that may be immunologically detrimental to develop an anti-tumor response.

Aim of the Study: We sought to investigate the suppressive role of Tumor-Derived Extracellular Vesicles (tEVs) on the ability to mount an antigen-specific response *in vivo*.

Study Design: To accomplish our objective, wildtype, Toll-Like Receptor 4 (TLR4) and Caspase-1 (Cas-1) Knockout (KO) mice were inoculated with tEVs isolated from gliomas.

Results: We show here that tEVs inhibit *in vivo* the antigen-specific immune response through TLR4. These results appear to be tumor-specific as non-tumor derived EVs failed to induce an anti-tumor response or TBM. Moreover, tEVs induce a population of suppressive Tingible-Body Macrophages (TBM) within the germinal centers of lymph nodes via the TLR4/Cas-1 axis. In addition, we determined that removal of Cas-1 pathway allows for tumor control indicating that inflammasome inhibit an anti-tumor response.

Conclusion: Tumor-derived EVs inhibit the ability to mount an anti-tumor response following immunotherapy, which may represent an important immune escape mechanism.

Keywords: Extracellular Vesicles; Immunotherapy; Inflammasomes; Toll-Like Receptors; Tumor-Derived Exosomes

Introduction

Increasing evidence indicates that the release and uptake of Extracellular Vesicles (EVs) is a crucial mode of cell-to-cell communication within tumors and the microenvironment. EVs are divided into three main subsets, i.e., apoptotic bodies, exosomes, and microvesicles, ranging from 30 to 2,000 nm in diameter. Unlike EVs derived from non-tumor cells, Tumor-Derived EV (tEVs) are enriched for tumor antigens such as melan-A and carcinoembryonic antigen [1,2], which are targets for immunotherapy [3]. However, despite preclinical and clinical data suggesting that tEVs are a rich source of tumor antigens, there is increasing evidence that they suppress antigen-specific and non-specific anti-tumor responses [4-9].

Tumor-derived EVs exert a broad array of detrimental effects on the immune system [10-12], particularly in gliomas [4]. EVs released by tumor cells contribute to angiogenesis [13], drug resistance [14], cell migration [15], and tumor progression by suppressing the immune system [16]. Studies have reported that multiple tumors block the differentiation of murine myeloid precursor cells into dendritic cells [17], carries TGF-β1, and skews IL-2 responsiveness in favor of regulatory T cells and away from cytotoxic cells [18]. Hsp72 on tEVs promotes The Immunosuppressive Activity of Myloid Derived Suppressor cells (MDSC) via autocrine activation of the IL-6/STAT3 pathway [19]. Prostate cancer-derived tEVs downregulate NKG2D expression on Natural Killer (NK) and $CD8^+$ T cells, leading to impairment of their cytotoxic function [20,21] demonstrated that different concentrations of glioma-derived EVs have distinct effects on immune activation *in vitro* [4], as high concentrations of tEVs inhibited IFN production and proliferation [4].

Toll-like receptors have been reported to be immunosuppressive. TLR4 signaling induces the differentiation of T regulatory cells [22,23] and only TLR that activates both Myeloid Differentiation primary response 88 (MyD88), which is initiates downstream signaling that results in NF-κB activation and induction of proinflammatory mediators such as TNF-α and IL-6 [23]. However, TLR4 is also associated with inflammasomes, which are multimolecular complexes containing a caspase recruitment domain (ASC) and Caspase-1 (Cas-1). These complexes are responsible for the cleavage of pro-IL-1β and pro-IL-18 proteins into their active forms [24]. Although the function of inflammasomes in tumor growth and metastasis remains controversial, studies have indicated that increased concentrations of IL-1β protein are associated with poor prognosis for cancer patients [25,26]. Published colon cancer studies demonstrate that inflammasome components provide protection against tumorigenesis in colitis-associated colon cancer. However, mice deficient in inflammasomes, such as NLRP3, NLRP12, NLRC4 and Cas-1, have increased tumorigenesis in colon cancer animal model [27,28]. In addition, inflammasomes and IL-1β have been shown to enhance tumor growth in other cancers, including melanoma and mesothelioma [29]. Here, we show *in vivo* that tEVs specifically inhibit the antigen-specific immune response through TLR4. We also demonstrate that tEV-induced a suppressive macrophage population, The Tingible Body Macrophages (TBM), which was upregulated in wildtype mice but not in TLR4 or Cas-1 knockout mice.

Materials and Methods

Cell Lines and Culture

SIINFEKL and $luciferin^+$/GFP^+ expressing GL261 cells [30] and primary human glioblastoma cells obtained from patients were cultured in DMEM/F12 (1:1) supplemented with L-glutamine, sodium bicarbonate, penicillin/streptomycin (100 U/ml), B27 and N2 supplements, and 0.1-mg/ml normocin. Cultures were maintained in 5% O_2 and supplemented semi-weekly with EGF and FGF, each at 20 ng/ml (R&D Systems, Minneapolis, MN) [31]. DC2.4, THP, and THP-ASC cell lines as well as primary murine lymphocytes were cultured in RPMI 1640 containing exosome-free fetal bovine serum. Murine transgenic OT-I cells were used to measure the OVA (SIINFEKL)-specific immune response. All cell lines were in house.

Preparation of Extracellular Vesicles

Extracellular vesicles were purified from GL261 or DC2.4 cell culture media using centrifugation at 5000 × *g* for 15 min. The supernatant was filtered using low-protein binding 0.22 μm pore filters. The filtered supernatants were separated by ultracentrifugation at 110,000 × *g* for 2 hr at 4°C using a Beckman Coulter Optima-XL ultracentrifuge and a 70Ti low-angle small volume fixed-angle rotor. The pelleted EVs were then washed with PBS and centrifuged again at 110,000 × *g* for 2 hr at 4°C. The resulting EVs were suspended in 0.5 mL PBS and stored at -80°C for later use. Purified EVs were then characterized by NanoSight, transmission electron microscopy (University of Minnesota Imaging Center), and flow cytometry as described by the manufacturer (System Bioscience, Palo Alto, CA), and submitted to System Bioscience for proteomic analysis.

Animal Models and Immunization

C57BL/6J and caspase-1 B6N.129S2-$casp^{1tm1Flv/j}$ mice were purchased from Jackson Laboratories. B6.$129^{Tlr2tm1Kir}$/J (TLR2 knockout) and C57BL/10ScNJ$^{-Tlr4lps-del}$ (TLR4 knockout) mice were a kind gift from Dr. Sabita Roy, University of Minnesota. OT-I mice were expanded in our laboratory. All mice were maintained in specific pathogen-free facilities at the University of Minnesota. Each animal study was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Minnesota.

Mice underwent intradermal vaccination with tEV and OVA in the posterior neck for four consecutive days and once more on day 7 [32]. In selected experiments, mice received purified tEVs three days prior to vaccination. Treatment groups included: i) saline; ii) 100 µg ovalbumin (OVA (Fisher Bioreagents) plus 10 µg Poly: ICLC (Oncovir); or iii) 100 µg OVA plus 10 µg Poly: ICLC (Oncovir) and 50 µg EVs in a total volume of 100µl.

Intracranial inoculation

Mice were inoculated with $1.5x10^4$ $GL261Luc^+GFP^+$ cells in the right striatum at the following coordinates: 2.5 mm lateral and 0.5 mm anterior of bregma, and 3 mm deep from the cortical surface of the brain [30,33]. In selected experiments, C57BL/6J mice were implanted with ALZET pumps for a seven-day infusion of tEVs. The pump was placed at the same location as glioma cells for delivery.

TLR activity: HEK-Blue reporter cells stably express membrane-bound human TLR2 or human TLR4 and secrete alkaline phosphatase upon TLR stimulation (InvivoGen) as previously described [34]. HEK-Blue cells were pulsed with exosomes at different concentrations and incubated for 48 hrs. HEK-Blue cell supernatant was assayed for secreted embryonic alkaline phosphatase activity using QuantiBlue colorimetric enzyme assay (InvivoGen). Enzymatic activity was recorded as an increase in OD640 after being normalized to media-only treatment.

Flow Cytometry: A Becton Dickinson Canto three-laser flow cytometer was used for data acquisition. For whole blood staining, 50 µL whole blood was obtained via retro-orbital blood collection and placed in 100 µL of a 1:10 dilution of heparin in PBS. Five µL of the MHC Dextramer H-2 Kb/SIINFEKL (Immudex) was added to the blood. Following 10 min incubation at room temperature, 1 µL (0.5 mg) of anti-mouse CD8α-Pacific Blue (clone 53-6.7; eBioscience) was added to the cells that were incubated for an additional 20 min at room temperature. Red cells were lysed by adding 1 mL of a 1:10 lysis buffer dilution (BD Pharmigen), followed by 10 min incubation at room temperature, and centrifugation twice. Cell pellets were suspended in 100 µL PBS and analyzed by flow cytometry.

Cytokine Production: Mice were vaccinated as described above, lymphocytes were isolated from draining lymph nodes, and plated in RPMI 1640 media at a concentration of 4×10^5 cells per well in 96-well plates. Lymphocytes were pulsed with 10 µg of the Dextramer H-2 Kb/SIINFEKL peptide, which contains the OVA-derived core epitope (EVSQLEQLE**SIINFEKL**TEEWTSSNVM), for re-stimulation and incubated for 12 hr. Supernatants were analyzed for cytokine production using bead arrays (BD Bioscience).

Anti-tumor Response: These assays were conducted as previously described [33]. Draining lymph nodes were harvested and dissociated, and the resulting lymphocytes were incubated with CFSE-labeled GL261 cells for 4 hr at E: T ratios of 0:1, 25:1 and 50:1. Following incubation, cytotoxicity was analyzed according to the manufacturer's protocol (Immunochemistry). Cell death was measured as the percentage of CFSE-labeled 7-AAD positive populations.

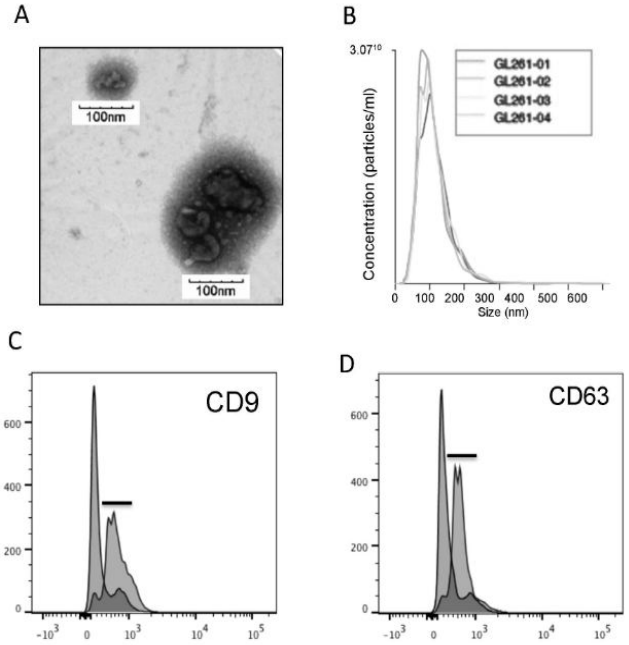
Statistical Analysis

Statistical comparisons were made by ANOVA, followed by post-hoc comparisons using a two-tailed t-test. All tests were performed with the Prism 5 software (Graph Pad Software, Inc). P values <0.05 were considered significant.

Results

Characterization of Tumor-Derived Extracellular Vesicles

As tumors have been reported to secrete EVs [35], we isolated EVs derived from murine tumor cells (GL261) by ultracentrifugation [36], and characterized them by Transmission Electron Microscopy (TEM) (Supplementary Figure 1A) and Nano Sight (Supplementary Figure 1B), and by flow cytometry for the common exosome markers, CD9 and CD63 [37] (Supplementary Figure 1C-D).



Supplementary Figure 1: Characterization of tumor-derived extracellular vesicles. Purified EVs were analyzed by **(A)** transmission electron microscopy and **(B)** NanoSight; Common EV markers **(C)** CD9 and **(D)** CD63 were assessed by flow cytometry.

The data showed that these preparations consisted of a mixture of exosomes (<100nm) and microvesicles (>100nm) [35, 38],

which we will refer to as Tumor-Derived Extracellular Vesicles (tEVs) throughout the remainder of the manuscript.

Pre-Vaccinating with Tumor-Derived Extracellular Vesicles Suppresses the Antigen- Specific Immune Response

We hypothesized that tEVs may inhibit the ability to initiate an antigen immune response. Non-tumor bearing wildtype mice were vaccinated with OVA +/- tEVs as described in the Methods. Blood was isolated and analyzed for CD8-specific proliferation using the Dextramer H-2 Kb/SIINFEKL peptide. To our surprise, the antigen-specific proliferation was enhanced with the addition of tEVs to OVA (p=0.003; Figure 1A), but cytokine production was not changed (Figure 1B-D). Thus, the addition of tEVs did not significantly alter the immune response when administered with the antigen. However, as tEVs are theoretically secreted by tumors before any treatment by immunotherapy, we pre-treated mice with GL261-derived tEVs prior to vaccination then again with the antigen OVA. In these experiments, mice pretreated with tEVs significantly suppressed proliferation of OVA-specific CD8+ cells (p=0.0004; Figure 1A; black bar). Same decrease of OVA-specific CD8+ cells isolated from draining lymph nodes was observed (data not shown). In addition, pretreating mice with tEVs decreased the production of TNFα (p=0.001), IL-2 (p=0.0001) and IFN-γ (p=0.006) in response to OVA (Figure 1B-D; black bar). In contrast, we did not observe any immune suppression following inoculation with EVs prepared from non-tumor cells (DC2.4 cells) as assessed by antigen-specific expansion (Figure 1E) or IFN-γ response (Figure 1F), demonstrating that the immune suppression is specific to tEVs. Finally, to investigate the effects of tEVs on the development of an anti-tumor immune response, lymphocytes isolated from draining lymph nodes were restimulated with OVA-expressing GL261 cells (Figure 2). We concluded that pretreating mice with tEVs failed to mount an anti-tumor response to OVA-expressing glioma cells (p=0.001).

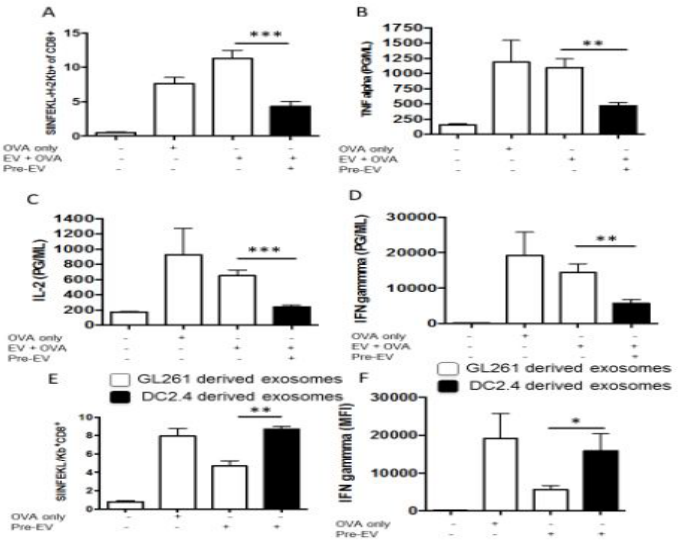


Figure 1: Pre-vaccinating with tumor-derived extracellular vesicles suppresses the antigen-specific immune response. Non-tumor bearing mice

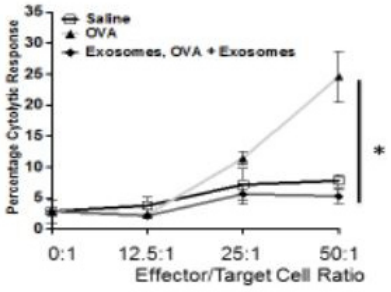


Figure 2: Tumor-derived extracellular vesicles suppress the cytolytic response. Lymphocytes were isolated from draining lymph nodes on day 8, stimulated with the SIINFEKL peptide, and incubated with OVA-transfected GL261 cells. The anti-tumor response was then analyzed by flow cytometry for a cytolytic response. Error bars are representative of standard deviation (n=5/group *P < 0.05 by t-test. Data represent the mean of three independent experiments).

Tumor-Derived Extracellular Vesicles Induce Tingible Body Macrophages

To explore the immunomodulatory effects of tEVs, draining (cervical) lymph nodes were harvested and H&E-stained. In contrast to mice treated with non-tumor-derived EVs (Figure 3A-B), mice inoculated with tEVs + OVA had well-defined germinal centers surrounded by thick marginal zones (Figure 3C-D). Interestingly, TUNEL staining of the germinal centers revealed the presence of apoptotic cells (Figure 3E-F). Given the observed immune suppression following the addition of tEVs, we hypothesized that the TUNEL positive cells could represent a small population of macrophages called tingible body macrophages (TBM). TBM are apoptotic macrophages residing in germinal centers that induce immune suppression through interactions with B cells and T helper cells [39]. To test our hypothesis, we stained the draining lymph nodes with antibodies to the macrophage markers, IBA-1 (Figure 3G) and CD3 (Figure 3H).

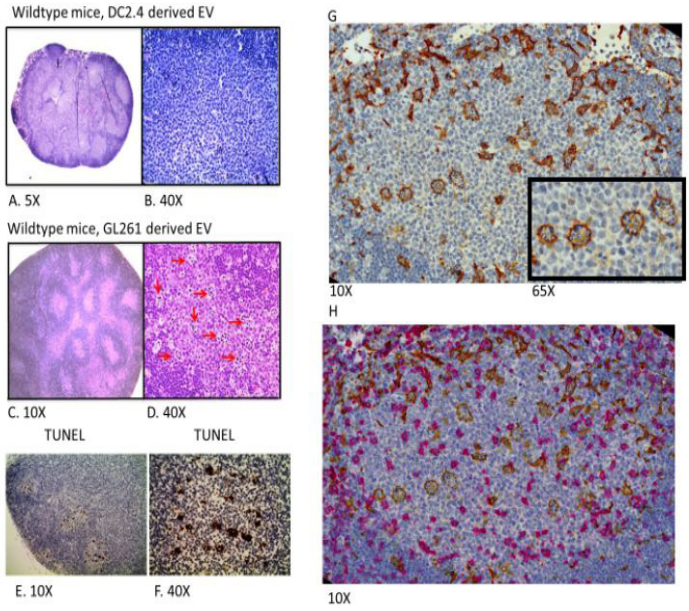
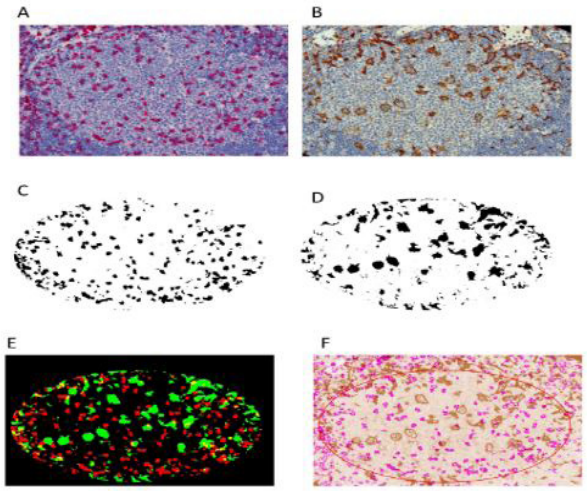


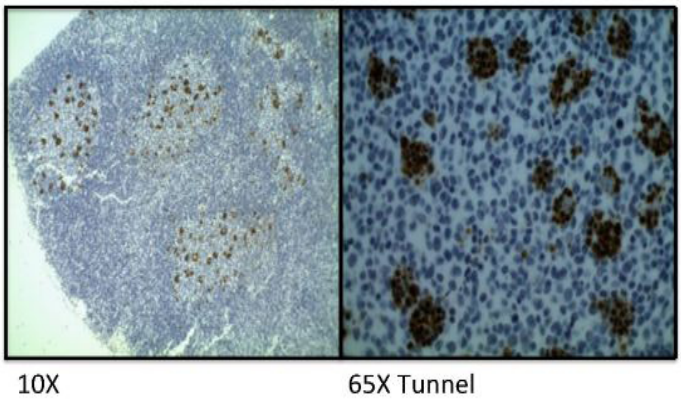
Figure 3: Tumor-derived extracellular vesicles induce tingible body macrophages. Mice were vaccinated with tumor-derived or non-tumor-derived EVs for three consecutive days, then vaccinated with OVA + tumor-derived or non-tumor derived EVs for 4 subsequent days and boosted on day 8 **(A-D)**. On day 9, lymph nodes were harvested and H&E stained **(E-F)** or stained for TUNEL, IBA-1 **(G)**, and IBA-1 + CD3 **(H)**.

These experiments revealed that 13.6% of TBM interacted with $CD3^+$ cells within a germinal center, potentially suppressing the immune response (Supplementary Figure 2A-F).



Supplementary Figure 2: Tingible body macrophages (TBM) are associated with CD3 T cells. To quantitate the percentage of TBM associated with CD3+ cells within the germinal centers, a representative region from CD3- **(A)** IBA-1- stained sections **(B)** were analyzed using Fiji/ImageJ. The images were aligned manually; **(C-D)** The CD3 and the IBA-1 images were color-deconvoluted using the Color Deconvolution plugin and the "H&E and DAB" setting. The CD3 brown image and IBA-1 magenta image was then thresholded manually, and both were post-processed by the morphological operator "Fill Holes" included in ImageJ; **(E)** area in yellow over area in green successively, the Manders coefficient was calculated using JACoP to find the percent of CD3+ regions overlapping the IBA-1+ regions; **(F)** The signals for IBA-1 and CD3 overlapped.

In our studies, mice were vaccinated with tEVs near the draining lymph nodes, resulting in the upregulation of the TUNEL-positive TBM. We previously reported that the sites of vaccination affect the strength of the $CD8^+$ T cell response in the draining lymph nodes [30]. To ensure that our results were not an artifact of vaccination near the draining lymph nodes, ALZET pumps were mounted in non-tumor bearing mice to deliver tEVs at the same location/coordinates as the inoculation of tumor cells. The results showed that tEVs filtered from the entral nervous system to the draining lymph nodes and induced TBM (Supplementary Figure 3),



Supplementary Figure 3: Tumor-derived extracellular vesicles infiltration from the CNS induce apoptotic T cells. An ALZET pump was mounted for constant tEVs delivery into the same location in the central nervous system as the tumor cells. Four days post-inoculation, cervical lymph nodes were harvested and TUNEL-stained. Shown are 2 magnifications of the same image and are representative of 3 independent experiments (n=3/treatment group).

supporting our previous findings that tEVs suppress the ability to mount an immune response.

Tumor-Derived Extracellular Vesicle-Induced Immune Suppression is TLR4-Mediated

Direct evidence indicates that anticancer drugs cause the release of exosomes from human hepatocellular carcinoma cells, and influence the release of Heat Shock Proteins (HSPs), modulating cells through Toll-like receptors [40]. Proteomics analysis of our tEV preparations demonstrated an increased expression of HSPs in tEVs compared to non-tumor derived EVs (Table 1).

Protein	GL261	DC2.4
HSP 70	27	0
HSP 71	98	29
HSP 90α	132	9
HSP 90β	169	14
HSP 105	10	0

Table 1: Heat shock protein detections. Tumor-derived and non-tumor derived extracellular vesicles were analyzed by proteomics. Heat shock protein are listed as numbers of protein detection.

Given that HSPs interact with Toll-Like Receptors (TLRs), we investigated the role of TLRs in the immunosuppression induced by tEVs. In these studies, different concentrations of tEVs were used to pulse TLR2- and TLR4-HEK293 cells transfected with an NFκB reporter to measure TLR signaling. We observed a dose-dependent increase in NFκB activity in both TLR2 (0-5 μg; p=0.04, 5-10 μg; p=0.002, 10-20 μg; p=0.001, 20-40 μg; p=0.04); (Figure 4A) and TLR4 (0-5 μg; p=0.0006, 5-10 μg; p=0.003, 10-20 μg; p=0.002, 20-40 μg; p=0.0004); (Figure 4B) cell lines pulsed with tEVs. In contrast, non-tumor derived EVs failed to activate NFκB (Figures. 4C-D). To further investigate the role of TLRs, TLR2 and TLR4 knockout mice were vaccinated with tEVs as described above. Tumor-derived EVs suppressed the induction of antigen-specific proliferation, measured as the percentage of SIINFEKL-specific CD8+ T cells, isolated from the blood of wildtype and TLR2 knockout mice, compared to wildtype mice receiving OVA only (OVA-WT; p=0.001 and OVA-TLR2; p=0.003); (Figure 4E). In addition, draining lymph nodes from TLR2 knockout mice had well-defined germinal centers containing TUNEL-positive T cells (Figure 4F). In contrast, tEVs failed to inhibit the induction of an immune response in TLR4 knockout mice (Figure 4E) and did not induce TUNEL$^+$ cells (Figure 4G).

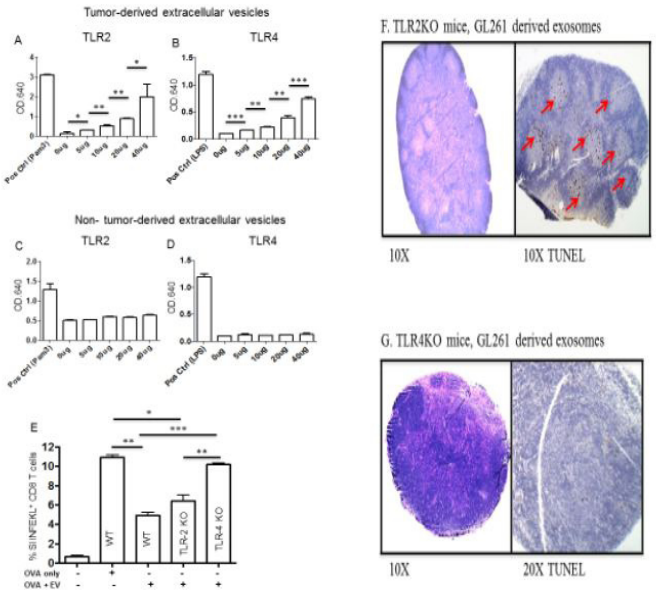


Figure 4: Tumor-derived extracellular vesicles signal through Toll-Like Receptor 4. **(A)** TLR2- or **(B)** TLR4-expressing HEK-Blue cells, which co-express an NF-κB-inducible Secreted Embryonic Alkaline Phosphatase (SEAP) reporter gene were pulsed with tumor-derived or **(C-D)** non-tumor derived EVs, and NFκB activity was assessed as the amount of secreted alkaline phosphatase; **(E)** Non-tumor bearing TLR2 and TLR4 knockout mice were vaccinated with tEVs for three consecutive days then, vaccinated with OVA + tEVs for 4 subsequent days, and boosted on day 9. On day 10, whole blood was harvested from these mice and analyzed by flow cytometry for an endogenous SIINFEKL$^+$CD8$^+$ T cell response; Lymph nodes from **(F)** TLR2 and (G) TLR4 knockout mice treated as above were harvested and TUNEL-stained. Data are representative of three independent experiments, n=4/treatment group. *$P < 0.05$, **$P<0.005$, ***$P < 0.0005$; by t-test.

These results demonstrate that tEVs induce immune suppression through the TLR4 pathway.

Tumor-Derived Extracellular Vesicles Signal Through the Inflammasome Pathway

Our studies demonstrated that TUNEL-Positive Cells (TBM) are upregulated through TLR4. Thus, we further examined the activation of pathways parallel and downstream of TLR4, i.e., inflammasomes. In these experiments, Caspase-1 (Cas-1) Knockout (KO) mice were vaccinated with OVA + GL261-derived EVs. The results showed a lack of TUNEL$^+$ cells in the germinal centers of lymph nodes (Figure 5B), as was seen in TLR4 KO mice. Because tEVs did not induce TBM in TLR4 and Cas-1 KO mice, we hypothesized that tumor growth would be reduced in these mice. To test our hypothesis, wildtype, TLR4 and Cas-1 KO mice were inoculated with GFP$^+$ GL261 cells, and glioma-bearing mice were monitored for tumor growth and survival. Wildtype and TLR4 KO mice failed to reduce tumor growth (Figure 5D); however, after 14 days, tumor growth was reduced in Cas-1 KO mice (Figure 5C). Further analysis demonstrated that Cas-1 KO mice were able to totally eradicate the tumors from the CNS (Figure 5E). These results indicate that a TLR4-independent inflammasome pathway is involved in tumor growth. Interestingly, although tumors completely regressed, Cas-1 KO still died, albeit at a slower rate than WT or TLR4 KO (Figure 5F).

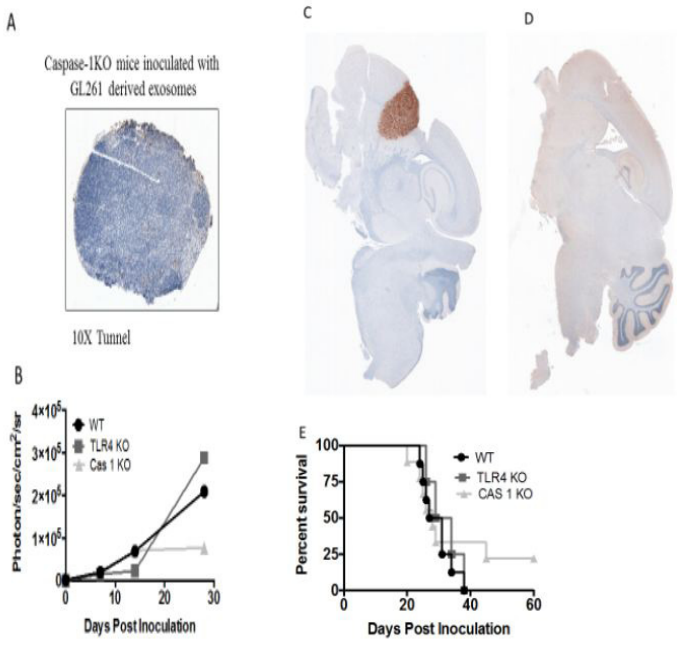


Figure 5: Tumor-derived extracellular vesicles signal through the inflammasome pathway. **(A)** The THP monocyte cell line and inflammasome KO THP-ASC were pulsed with tEVs. Mice were vaccinated with tEVs for three consecutive days, then vaccinated with OVA + tEVs and boosted on day 8; **(B)** On day 9, lymph nodes were harvested and TUNEL-stained; **(C)** Wildtype (WT), TLR4 and Caspase-1 (Cas-1) KO mice were inoculated with Luc$^+$GFP$^+$ GL261 cells and followed for tumor growth; on day 21, wildtype **(D)** and Cas-1 **(E)** knockout mice were euthanized and stained with GFP for tumor formation; (F) Another set of mice was followed for survival. Data are representative of four independent experiments; n=3/ treatment group.

Discussion

Tumor-derived EVs are promising candidates for targeting glioma progression and facilitating effective immune strategies [41,42]. Previous work revealed a direct correlation between tumor grade and tEV release, indicating that more aggressive tumors release more tEVs [2,43,44]. The highly malignant nature of gliomas and the high concentrations of circulating tEVs found in patients with other highly aggressive tumor types [43,45] suggest an important role of tEVs in promoting tumor progression and evading immune surveillance. Our data add to this narrative by demonstrating *in vivo* that glioma-derived EVs, and not non-tumor-derived EVs, suppress the antigen-specific immune response via activation of TLR4 and induction of Tingible Body Macrophages (TBM). Proteomic analysis of our EV preparations showed high levels of HSP71 and HSP90 in GL261-derived EVs compared to DC2.4-derived EVs (Table 1), suggesting that the high level of HSPs within EVs may stimulate TLRs. Indeed, *in vitro* studies with TLR2 and TLR4 cells transfected with an NFκB reporter showed that tEVs can activate both TLR2 and TLR4. However, when inoculating TLR2 and TLR4 KO mice with tEVs, the antigen-specific response, as measured by the percentages of SIINFEKL-specific CD8$^+$ T cells, was suppressed in wildtype and TLR2 KO mice, but not in TLR4 KO mice, suggesting that only TLR4 is involved in the immunosuppressive mechanism triggered by tEVs.

We also showed that mice inoculated with tEVs + OVA specifically induced the suppressive TBM in the draining lymph nodes, as shown by the presence of the macrophage markers IBA-1 and CD3 in these TUNEL-positive cells. These data suggest that TBM may play a role in downregulating germinal center reaction, where they are predominantly located. This is supported by other studies reporting that TBM release prostaglandins, thereby reducing B-cell-mediated IL-2 production [39]. Interestingly, no TBM were detected in the TLR4-KO mice. Thus, we propose that TLR4 is activating TBM, which then suppress the immune response within the germinal centers through the secretion of prostaglandins, as was reported [39]. These findings may explain the current failure of immunotherapy drugs in central nervous system tumors. Further studies will be needed to confirm that glioma-derived EVs effectively produce prostaglandins. If confirmed, that may lead to the use of prostaglandin inhibitors such as Indomethacin with immunotherapy treatments to allow the immune system to better respond to tumor antigens. Accumulating data indicate that tumor development not only depends on genetic alterations, but also on the inflammatory microenvironment [46-49].

Innate pathways regulating the inflammatory response in the tumor microenvironment are not fully understood [24]. Among those, inflammasomes have increasingly gained interest. Inflammasomes are multi-molecular complexes containing a caspase recruitment domain (ASC) and Cas-1, and are responsible for the cleavage of pro-IL-1β and pro-IL-18 proteins into their active forms [24]. In our study, Cas-1 KO mice treated with glioma-derived EVs lacked TBM in the germinal centers, as observed in TLR4 KO mice, thus implicating a TLR4/Cas-1 axis in the activation of TBM. However, only the removal of Cas-1, and not that of TLR4, resulted in total regression of gliomas without the use of a tumor vaccine, suggesting that TLR4-independent inflammasomes may be involved in controlling tumor growth (Figures 5C, D). Interestingly, survival was only increased temporarily despite total glioma regression, which may have been related to cachexia.

These investigations demonstrate a new role for tumor-derived EVs in altering the immune response via TLR4 and inflammasomes. In addition, they also suggest that EVs secreted by tumors may be a factor in the inability of tumor-derived vaccines to elicit an adequate immune response.

Acknowledgments

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