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TITLE:

In Vivo Immunogenicity Screening of Tumor-Derived Extracellular Vesicles by Flow Cytometry of Splenic T Cells

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SUMMARY:

This manuscript describes how to assess *in vivo* immunogenicity of tumor cell-derived extracellular vesicles (EVs) using flow cytometry. EVs derived from tumors undergoing treatment-induced immunogenic cell death seem particularly relevant in tumor immunosurveillance. This protocol exemplifies the assessment of oxaliplatin-induced immunostimulatory tumor EVs but can be adapted to various settings.

ABSTRACT:

Immunogenic cell death of tumors, caused by chemotherapy or irradiation, can trigger tumor-specific T cell responses by releasing danger-associated molecular patterns and inducing the production of type I interferon. Immunotherapies, including checkpoint inhibition, primarily rely on preexisting tumor-specific T cells to unfold a therapeutic effect. Thus, synergistic therapeutic approaches that exploit immunogenic cell death as an intrinsic anti-cancer vaccine may improve their responsiveness. However, the spectrum of immunogenic factors released by cells under therapy-induced stress remains incompletely characterized, especially regarding extracellular vesicles (EVs). EVs, nano-scale membranous particles emitted from virtually all cells, are considered to facilitate intercellular communication and, in cancer, have been shown to mediate cross-priming against tumor antigens. To assess the immunogenic effect of EVs derived from tumors under various conditions, adaptable, scalable, and valid methods are sought-for. Therefore, herein a relatively easy and robust approach is presented to assess EVs' *in vivo* immunogenicity. The protocol is based on flow cytometry analysis of splenic T cells after *in vivo*

immunization of mice with EVs, isolated by precipitation-based assays from tumor cell cultures under therapy or steady-state conditions. For example, this work shows that oxaliplatin exposure of B16-OVA murine melanoma cells resulted in the release of immunogenic EVs that can mediate the activation of tumor-reactive cytotoxic T cells. Hence, screening of EVs *via in vivo* immunization and flow cytometry identifies conditions under which immunogenic EVs can emerge. Identifying conditions of immunogenic EV release provides an essential prerequisite to testing EVs' therapeutic efficacy against cancer and exploring the underlying molecular mechanisms to ultimately unveil new insights into EVs' role in cancer immunology.

INTRODUCTION:

The immune system plays a pivotal role in the fight against cancer, both when incited by immune checkpoint inhibition and for the efficacy of conventional cancer therapies. Tumor cells succumbing to genotoxic therapies such as the chemotherapeutic agents oxaliplatin and doxorubicin, or ionizing radiation treatment can release antigens and danger-associated molecular patterns (DAMPs) that potentially initiate an adaptive anti-tumor immune response[1]. The most prominent DAMPs, in the context of immunogenic cell death, include find-me signals such as chemotactic ATP, eat-me signals such as the exposure of calreticulin, that promotes tumor cell uptake by antigen-presenting cells, and the release of HMGB1, that activates pattern recognition receptors, thereby enhancing the cross-presentation of tumor antigens[2]. Furthermore, type I interferons (IFN-I), induced *via* tumor-derived immunogenic nucleic acids or other stimuli, are sensed by dendritic cells, enabling them to effectively prime tumor-specific cytotoxic T cells[3,4]. Clinically, activated and proliferating $CD8^+$ T cells infiltrating the tumor provide an independent prognostic factor for prolonged survival in many cancer patients. Released from such activated T cells, IFN-γ mediates direct antiproliferative effects on cancer cells and drives Th1 polarization and cytotoxic T cell differentiation, thereby contributing to effective immunosurveillance against cancer[5,6]. Oxaliplatin is a bona fide immunogenic cell death inducer, mediating such adaptive immune response against cancer[7]. However, the plethora of initial immunogenic signals released by tumor cells under therapy-induced stress remains to be fully unveiled. Despite significant advances in cancer immunotherapy, expanding its benefits to a larger portion of patients remains a challenge. A more detailed understanding of immunogenic signals that initiate T cell activation may guide the development of novel therapies.

A heterogeneous group of membrane-enclosed structures, known as extracellular vesicles (EVs), seem to serve as intercellular communication devices. Emitted by virtually all cell types, EVs carry functional proteins, RNA, DNA, and other molecules to a recipient cell or may alter a cell's functional state just by binding to receptors on the cell surface. Their biologically active cargo varies significantly by the type and functional state of the generating cell[8]. In cancer immunology, EVs released from tumor cells have been predominantly regarded as adversarial to immunotherapy because they eventually promote invasive growth, perform metastatic niches[9], and suppress the immune response[10]. In contrast, some studies have shown that EVs can transfer tumor antigens to dendritic cells for effective cross-presentation[11,12]. EVs may provide immunostimulatory nucleic acids if they emerge under therapy-induced stress, facilitating an anti-tumor immune response[13,14]. Sensing such RNA and DNA, innate immune ligands in the tumor microenvironment have recently been shown to modulate responsiveness to checkpoint

blockade significantly[15–17]. Hence, the immunogenic role of EVs released by tumor cells under different therapy-induced stress needs to be further elucidated. Since EVs constitute a young yet growing field of research, standardization of methods is still ongoing. Therefore, sharing knowledge is essential to improve the research reproducibility on interactions between EVs and cancer immunology. With this in mind, this manuscript describes a simple protocol to assess the immunogenic effect of tumor-derived EVs *in vivo*.

This assessment is performed by generating tumor-derived EVs, immunizing recipient mice with those EVs, and analyzing splenic T cells *via* flow cytometry. EV generation is ideally performed by seeding murine tumor cells in an EV-free cell culture medium for a high degree of purity. Cells are treated with a specific cell stress stimulus, such as chemotherapy, to compare the effect of therapy-induced EVs against baseline immunogenicity of respective tumor-derived EVs. The isolation of EVs may well be performed by various techniques that should be selected according to *in vivo* applicability and local availability. The following protocol describes a precipitation-based assay with a commercial kit for EV purification. Mice are immunized twice with those EVs. Fourteen days after the first injection, T cells are extracted from the spleen and analyzed for IFN-γ production *via* flow cytometry to evaluate a systemic immune response. With this, the potential of tumor-derived EVs, emerging under different therapeutic regimens, to induce anti-tumor T cell responses is assessed relatively easily, quickly, and with high validity[13]. Therefore, this method is suitable for an immunological screening of EVs derived from cancer cells under various conditions.

PROTOCOL:

At the onset of experiments, mice were at least 6 weeks of age and were maintained under specific pathogen-free conditions. The present protocol complies with the Institutional ethical standards and prevailing local regulations. Animal studies were approved by the local regulatory agency (Regierung von Oberbayern, Munich, Germany). Possible sex-related biases were not investigated in these studies.

1. Generation and isolation of EVs derived from tumor cells after chemotherapy exposure

1.1. Culture murine B16 melanoma cells expressing ovalbumin (B16-OVA) in DMEM (containing 4 mM L-glutamine and 4.5 g/L of D-glucose) supplemented with FCS (10% v/v), penicillin (100 Units/mL), and streptomycin (100 µg/mL) at 37 °C, until cells grow steadily and are approximately 90% confluent.

NOTE: Perform this section of the protocol entirely under sterile conditions using a cell culture hood. For analysis of other cancer entities, cell lines expressing a potent antigen are preferable to assess antigen-specific T cell responses, as they allow for specific *ex vivo* antigen restimulation.

1.2. To prepare the cell culture media required for EV generation, deplete bovine EVs within FCS by ultracentrifugation at 100,000 x *g* for 24 h at 4 °C, and then discard the pellet. Alternatively, choose a commercial preparation low in animal EVs beforehand.

1.3. Harvest B16-OVA cells and wash them twice in PBS, and then seed at a concentration of 400,000 cells/mL in EV-depleted media.

NOTE: Adapt the cell concentration to the growth dynamic of the cell line under investigation so that cells do not overgrow.

1.4. Treat B16-OVA cells by adding 30 μg of oxaliplatin per mL and incubate for 24 h at 37 °C. Leave control conditions untreated. At the first use of a genotoxic substance, titrate the desired cytotoxic efficacy using cell-viability assays such as trypan blue exclusion[18].

NOTE: This assay may also evaluate EVs generated in cell cultures treated with other immune-modulating substances or ionizing irradiation besides chemotherapeutics.

CAUTION: Oxaliplatin causes skin and severe eye irritation and may cause an allergic skin reaction and respiratory irritation. Oxaliplatin is suspected of causing cancer. As a precaution, use personal protective equipment, including adequate gloves, goggles, masks, and clothing, cleaned before reuse. Avoid inhalation and wash hands thoroughly after handling. Avoid release into the environment and dispose oxaliplatin according to prevailing regulations. Obtain detailed information from the safety data sheet.

1.5. Collect cell culture supernatant. Centrifuge first at 400 x *g* for 5 min at 4 °C, and then at 2,000 x *g* for 30 min at 4 °C, each time discarding the pellet. Finally, filter through a 220 nm PVDF membrane. Use a fresh tube for each step to remove any cell debris.

NOTE: At this stage, the EV-containing supernatant may be stored at 4 °C for a day before resuming the protocol. However, it is strongly recommended to adhere to the described schedule with immediate EV purification.

1.6. Mix 1 mL of supernatant with 0.5 mL of a specific commercially available exosome isolation reagent (see **Table of Materials**) in a V-shaped 1.5 mL tube. Thoroughly pipette up and down or vortex to create a homogenous solution. Incubate overnight at 4 °C.

1.7. Centrifuge at 10,000 x *g* for 60 min at 4 °C. Carefully discard the supernatant. Remove the remaining drops by tapping the 1.5 mL tube upside down on a paper towel and by aspiration through a pipette with a fine tip without touching the EV-pellet at the bottom.

1.7.1. Thoroughly remove all fluids to prevent uncontrolled dilution of the EV-pellet. Also, execute these tasks quickly to prevent the pellet from drying out.

1.8. Resuspend the EVs in cold PBS by pipetting up and down without scratching the pellet from the tube's wall with the tip. Now, transfer the suspension step by step from the first to the last tube to pool the EVs.

NOTE: Use a volume of PBS that equals 5 μL multiplied by the number of tubes. 5 μL of the final

suspension contains the isolated EVs released from 400,000 cells under chemotherapy or at a steady state.

1.9. Preferably, use EVs directly. If this is not possible, store EV suspensions at -80 °C in siliconized vessels for up to 28 days until application.

NOTE: The EVs described here and in several other publications do not lose their respective biological function when stored at -80 °C for that time period[19].

1.10. Quantify and characterize EV isolates according to the MISEV2018 guidelines[20].

NOTE: Possible methods for quantification include nanoparticle tracking analysis (NTA)[21] and the detection of EV's membrane-bound proteins[22]. Possible approaches to further characterize EVs include electron microscopy[23] and western blot[20].

2. Immunization of mice with EVs

2.1. Plan the *in vivo* experiment with C57BL/6 mice (or other syngeneic mice corresponding to the tumor cell line), including treatment groups receiving EVs derived from treated cells, untreated cells, and PBS (vehicle), respectively.

NOTE: Preferably, use mice at the age of 6–8 weeks to prevent physiological senescence from diminishing the immune response[24].

2.2. Mix 5 µL of EV-suspension with 55 µL cold PBS for each mouse within the respective treatment group to immunize it with EVs isolated from 4.0 x 10^5 B16-OVA cells.

NOTE: This amount of EVs corresponds to approximately 2 x 10^9 particles measured by nanoparticle tracking analysis (data not shown). OVA protein mixed with an adjuvant (e.g., LPS) can be applied as a potent vaccine positive control.

2.2.1. Fill syringes (needle size 26–30 G) with 60 µL of the diluted EVs or PBS, respectively and put immediately on ice.

NOTE: In the protocol, the amount of injected EVs is normalized to the number of EV-releasing tumor cells to experimentally consider both qualitative and quantitative effects of oxaliplatin on tumor cell EV biogenesis. For some readers, normalization to a specific concentration of produced EVs may better fit their experimental setup depending on their scientific question.

2.3. Inoculate EVs or PBS subcutaneously into the mice's hindfoot as described elsewhere[25] and repeat the immunization after 7 days. Fourteen days after the first treatment, sacrifice mice, e.g., by cervical dislocation to analyze the immune response.

NOTE: Alternative subcutaneous injection routes may be used, according to the local standards.

3. Flow cytometry analysis of splenic T cells

3.1. Prepare and cool complete RPMI (cRPMI), supplementing RPMI-1640 with FCS (10% v/v), penicillin (100 Units/mL), streptomycin (100 μg/mL), L-glutamine (2 mM), and β-mercaptoethanol (50 μM).

3.2. Resect the spleen from the opened abdominal cavity. Mash the spleen with a moistened 100 μm cell strainer and the plastic plunger of a syringe and flush the splenic cells into a 50 mL tube with 5–10 mL of cRPMI. Centrifuge at 400 x *g* for 5 min at 4 °C and discard the supernatant.

NOTE: Keep cells on ice whenever possible. To analyze the local rather than the splenic immune response, resect the draining popliteal and inguinal lymph nodes, following the same protocol. In this case, skip the next step for the lysis of erythrocytes.

3.3. To remove erythrocytes from the cell suspension, resuspend the pellet with 2 mL of red blood cell lysis buffer (see **Table of Materials**) and incubate for 5 min at room temperature. Then, stop the reaction by adding cRPMI. Centrifuge at 400 x *g* for 5 min at 4 °C and discard the supernatant.

3.4. For seeding, resuspend the cell pellet in cRPMI and count the cells to place triplicates of 200,000 cells with 200 μL cRPMI into each well, using a 96-well plate with a U-shaped bottom. Incubate for 48 h at 37 °C.

NOTE: To address the antigen-specificity of activated T cells, add 1 μg/mL soluble ovalbumin (or another tumor antigen corresponding to cell line under investigation) or leave without additional stimulus, respectively. Adding the immune-dominant peptide epitope SIINFEKL, instead of full-length ovalbumin, allows for a shorter incubation period. Besides flow cytometry, the cell culture supernatant and the mice's serum after 48 h of incubation can be analyzed for various cytokines.

3.5. After 48 h, to enhance intracellular IFN-γ staining by, inter alia, blocking the Golgi-mediated secretion of proteins, add Brefeldin A (5 ng/mL), PMA (20 ng/mL), and Ionomycin (1 μg/mL) to the cell culture. Incubate for 4 h at 37 °C.

3.6. Before staining surface biomarkers, transfer splenocytes to a 96-well plate with a V-shaped bottom and wash twice with PBS. Then, add fluorescent antibodies, compatible with the locally available flow cytometer, directed against surface biomarkers, CD3, CD8, and CD4 (see **Table of Materials**), diluted 1:400, plus a fixable viability dye, diluted 1:1,000 in PBS. Resuspend pelleted splenocytes in the staining solution and incubate for 30 min at 4 °C, protected from light.

3.7. For fixation and permeabilization of splenocytes, wash twice in FACS-buffer (PBS plus 3% v/v FCS), and then resuspend in 100 μL of fixation/permeabilization buffer (see **Table of Materials**) per well. Incubate for 30 min at 4 °C, protected from light.

3.8. For staining of intracellular IFN-γ, wash splenocytes in fixation/permeabilization buffer and resuspend with fluorescent antibodies against IFN-γ, diluted 1:200 in the buffer. Incubate for at least 1 h (up to a maximum of 12 h) at 4 °C, protected from light.

3.9. Before measuring the samples by flow cytometry, wash splenocytes twice in fixation/permeabilization buffer and resuspend in FACS-buffer. Analyze the activation of cytotoxic T cells according to the gating strategy displayed in **Figure 2**.

3.9.1. First, to detect single cells, blot FSC-H against FSC-A. Then, to detect lymphoid cells, blot SSC against FSC-A. Subsequently, select living $CD3^+$, $CD4^-$, $CD8^+$ cells and determine their IFN-γ-producing subset to quantify the activation of cytotoxic T cells in the spleen.

NOTE: Include a Fluorescence-minus-one (FMO) stain with all fluorochromes except the fluorochrome targeted against IFN-γ as negative technical control.

REPRESENTATIVE RESULTS:

This protocol is intended to facilitate the straightforward and easily reproducible assessment of the immunogenicity of tumor-derived EVs. Hereby, mice are inoculated with EVs derived from *in vitro* cultures of tumor cells expressing the model antigen chicken ovalbumin (OVA). The subsequent immune response is analyzed in splenic T cells *via* flow cytometry.

Figure 1 gives an overview of the practical steps of the entire protocol. Since the work focuses on immunogenic cell death, cross-presentation, and EV-induced anti-tumor immunity, this protocol is restricted to the function of CD8+ cytotoxic T cells. As displayed in **Figure 2**, cells were gated as single cells, lymphocyte subset (by size and granularity), viable cells (excluding a life/dead marker), and $CD3^+$ $CD4^-$ $CD8^+$ cytotoxic T cells. Intracellular accumulation of IFN-γ was assessed as a surrogate marker for activation. Possible additional markers regarding T cell differentiation and exhaustion are discussed below.

Using the method described here, mice were immunized with EVs derived from OVA-expressing tumor cells cultured either under steady-state (untreated) or genotoxic stress conditions (oxaliplatin-treated). Only mice injected with EVs derived from tumor cells under genotoxic stress conditions induced potent activation of splenic cytotoxic T cells in recipient animals (**Figure 3A**). Injection of EVs derived from tumors under steady-state conditions resulted in some T cell activation, but that was not significantly different from T cell activation in mice injected with the PBS vehicle. These data show that under genotoxic stress, tumor cells can release potently immunogenic EVs. The production of IFN-γ was particularly increased when splenocytes of tumor EV-treated animals were *ex vivo* restimulated with the model tumor antigen OVA before analysis (**Figure 3B**). These data suggest that tumor-derived EVs can induce tumor antigen-specific immune responses. Interestingly, IFN-γ-production – even though to a much lesser extent – is also detected in the absence of antigen-specific restimulation. Possibly, other melanoma-associated antigens, such as the differentiation antigen TRP2[26], may be targeted by some part of the EV-induced T cell response.

FIGURE LEGENDS:

Figure 1: Pictographic overview of the protocol. (**A**) Isolation procedure of EVs generated in tumor cell cultures resembling chemotherapy. (**B**) Schedule for the immunization of mice with EVs. (**C**) Staining protocol for flow cytometry analysis of cytotoxic T cells.

Figure 2: Flow cytometry gating strategy to analyze cytotoxic T cell activation in the spleen. The numbers represent the percentage of its respective parent population. FSC-A: forward scatter area; FSC-H: forward scatter height; SSC: sideward scatter; live/dead: cell death marker.

Figure 3: EVs derived from tumor cells under genotoxic stress can induce antigen-specific T cell responses in recipient animals. (**A**) Mice were immunized with EVs derived from tumor cells cultured either under steady-state (untreated) or genotoxic stress conditions (oxaliplatin-treated). Vehicle (PBS) injections were used as a negative control. IFN-γ production by cytotoxic T cells in the spleen upon EV immunization was determined. With this, splenic cell suspensions were restimulated with ovalbumin *ex vivo* before analysis. (**B**) Mice were treated with EVs derived from tumor cells under genotoxic stress conditions as described above. Splenic T cell activation was determined after *ex vivo* restimulation either in the presence or absence of ovalbumin. Bars depict the mean per group and whiskers its standard error. The one-way analysis of variance (ANOVA) test with Bonferroni posttest was used for multiple statistical comparisons of a dataset. The significance level was set at $P < 0.05$, $P < 0.01$, and $P < 0.001$ and is indicated here with asterisks (*, **, and ***).

DISCUSSION:

This protocol provides an immunological *in vivo* assessment of EVs derived from melanoma cells under chemotherapy-induced stress while adapting to EVs emitted from various cancers under various treatments. Immunizing mice with EVs derived from oxaliplatin-treated B16-OVA cells, for instance, expands IFN-γ-producing $CD8^+$ T cells in the spleen, which are further stimulated by *ex vivo* incubation with ovalbumin, indicating a tumor-specific immune response. Thus, detection of immunogenic EVs by screening through this protocol facilitates a more comprehensive understanding of conventional cancer therapies and enables a focused investigation into the role of EVs in cancer immunology.

Of note, experiments with EVs require some special considerations. In this protocol, EVs are semi-quantitatively normalized to the number of tumor cells released within 24 h. This approach reflects the aim to identify enhanced immunogenicity, regardless of whether it emerges from alterations in quality or quantity of released EVs. Therefore, reproducible *in vivo* results rely on the constant isolation efficacy of EVs. To this end, ensuring that EV pellets are resuspended entirely and promptly to avoid desiccation is a critical step.

Additionally, EVs must be quantified and characterized, e.g., by nanoparticle tracking analysis and western blot of canonical transmembrane, luminal, and at least one negative EV-marker[20]. Quantifying and characterizing EVs controls for inconstant isolation and addresses quantitative differences in EVs or EV subsets. This type of EV characterization constitutes a part of the

minimum information that needs to be reported in studies about extracellular vesicles, according to the International Society of Extracellular Vesicles (ISEV) guidelines. However, various characterization methods are equally legitimate and should be selected concerning local availability and the individual research question. Notably, defining dosage of a substance of interest or ionizing irradiation constitutes another critical step of the protocol, which may require experimental validation to achieve an adequate level of cell death.

In general, potential contamination of isolated EVs with the treatment substance, soluble proteins, and lipoproteins must be considered. One strategy in this regard consists in reproducing the experiment with complementary EV-isolation techniques that the same type of contamination[20] may not compromise. Immunoaffinity, for instance, isolates EVs with a lower yield but higher specificity than purely precipitation-based methods and may provide an appropriate control in this regard[27]. An alternative approach is to compare wild-type cell-derived EVs with isolations from genetically engineered cells with a specific deletion of genes involved in EV biogenesis or deploy substances that reduce EVs emission[20].

Results obtained from this protocol should be complemented by a more comprehensive characterization of the EV-mediated immune response. Especially the classification of $CD8^+$ T cells into effector and effector memory cells, through CD44[28], as well as antigen-naïve and central memory cells, through CD62L[29], may convey deeper insight. Furthermore, the analysis of T helper cells, regulatory T cells, and NK cells may be of interest. For testing the anti-tumor efficacy of EVs, mice may be challenged with the corresponding cancer cells after receiving EV immunization or treated with EVs against a preestablished cancer, thereby adapting the guidelines for detection of immunogenic cell death[2,30] to this cell-free tumor derivate. However, conclusions from all these experimental setups are limited by the fact that cancer cells in a Petri dish potentially generate functionally different EVs than cancer cells embedded in a dynamic tumor microenvironment[31] that often suppresses anti-cancer immunity. Thus, assessing EVs derived from tumor/fibroblast cocultures or *ex vivo* tumor tissue may better reflect the actual situation. In a next translational step toward clinical reality, EVs from patient material may be analyzed for immunogenicity before and during therapy to assess their usability as biomarkers.

As cancer-derived EVs were recently found to – under certain circumstances – modulate the immune system, the journey of exploring their clinical potential has only just begun[32]. For an in-depth analysis of the immune mechanisms co-opted by immunogenic EVs, useful tools comprise fluorescence microscopy visualizing the EV uptake by specific cells, the deployment of mice with genetic deficiencies for specific immune pathways, and screening methods for molecular alterations in the EV content. Ultimately, identifying immunogenic tumor-derived EVs, with screening methods described herein, will enable a better understanding of the underlying mechanisms behind EV-mediated immunity and therefore constitutes a crucial step toward harnessing their potential against cancer.

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AUTHOR CONTRIBUTIONS:

F.S., H.P., and S.H. designed the research, analyzed, and interpreted the results. F.S. and S.H. wrote the manuscript. H.P. and S.H. guided the study.

DISCLOSURES:

The authors declare that there is no conflict of interest.

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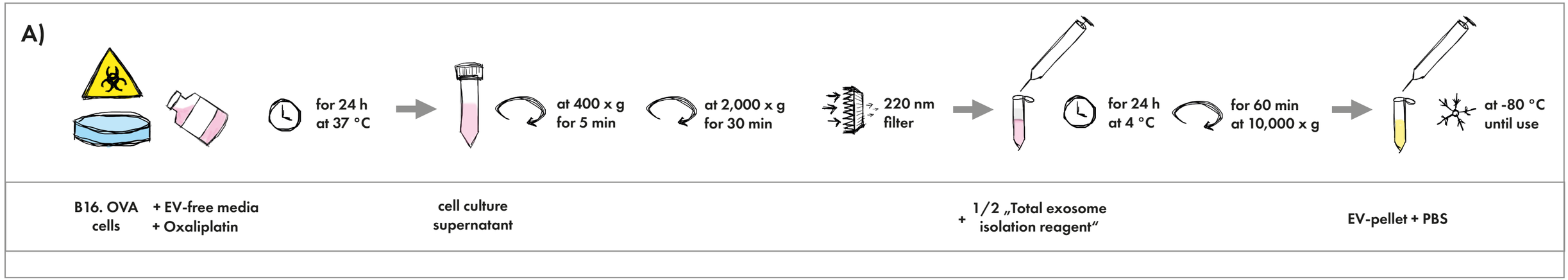
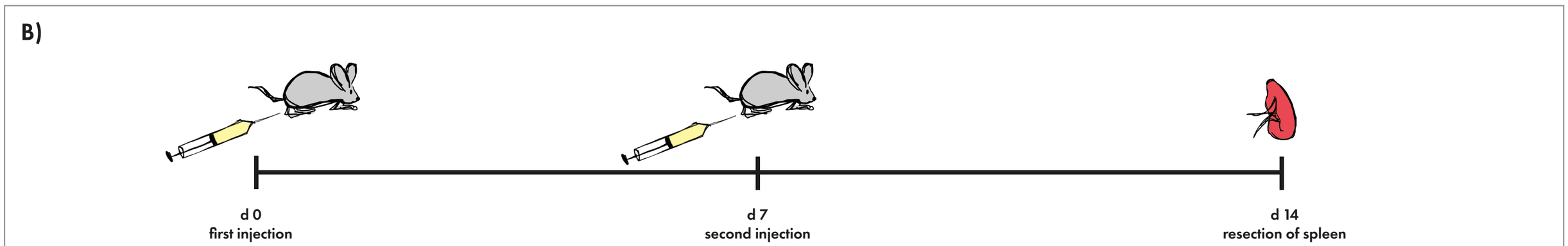
Figure 1





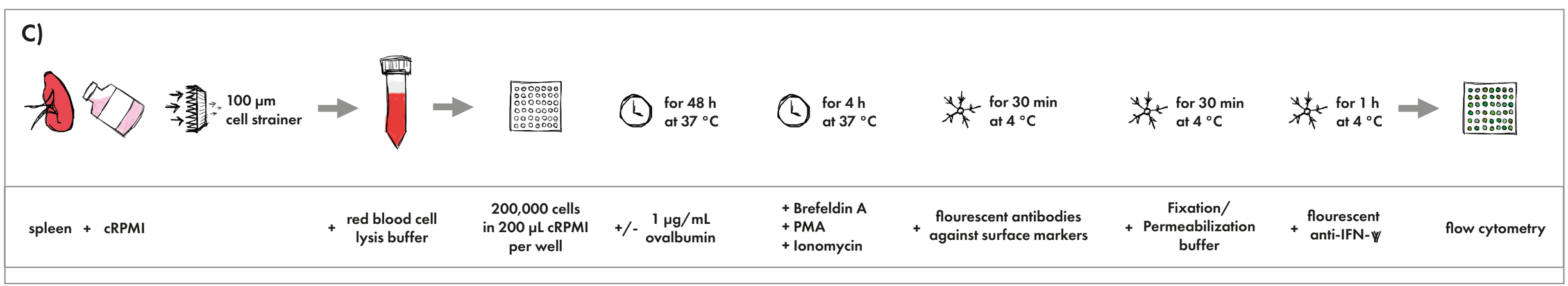


Figure 2

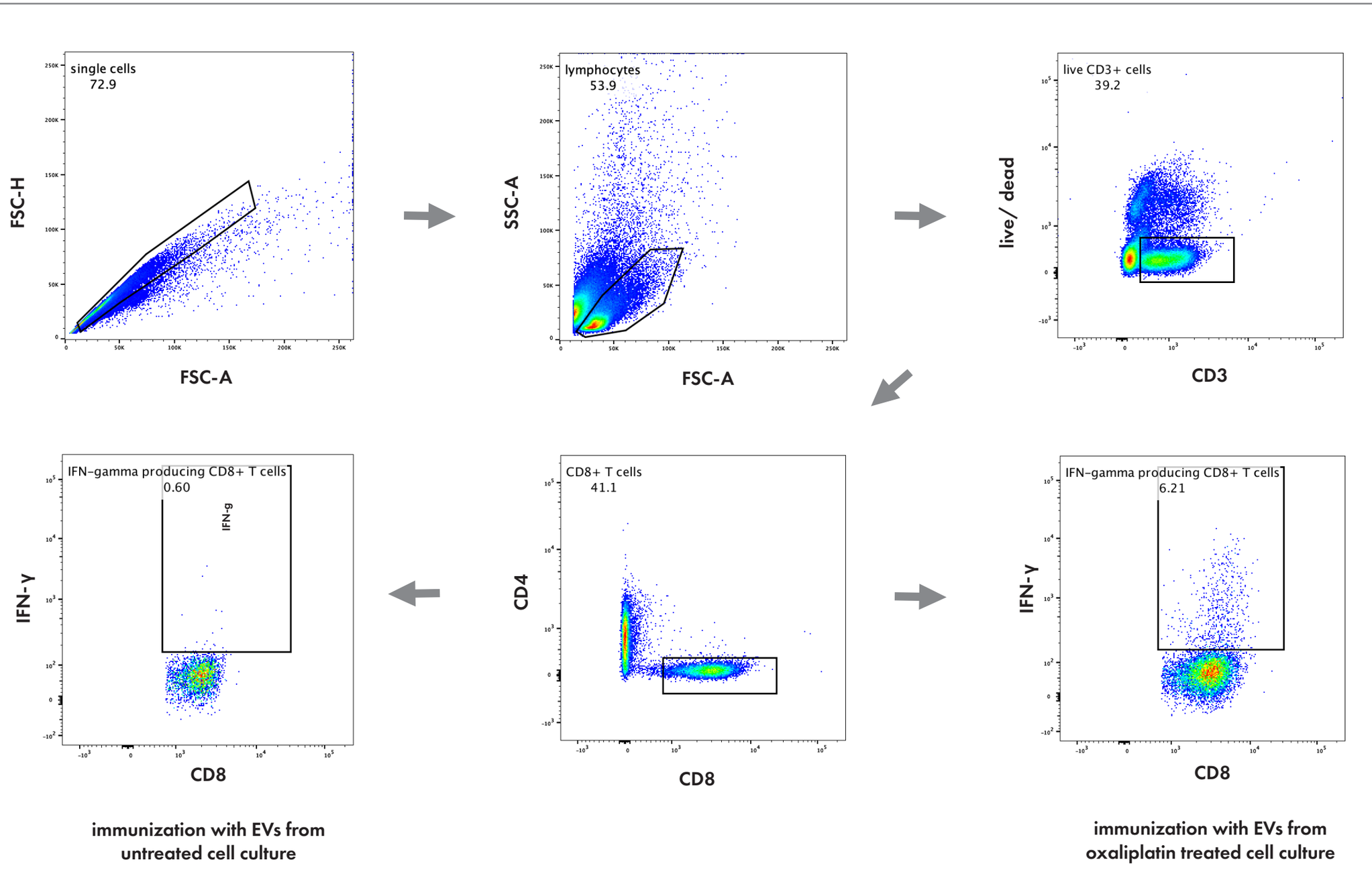
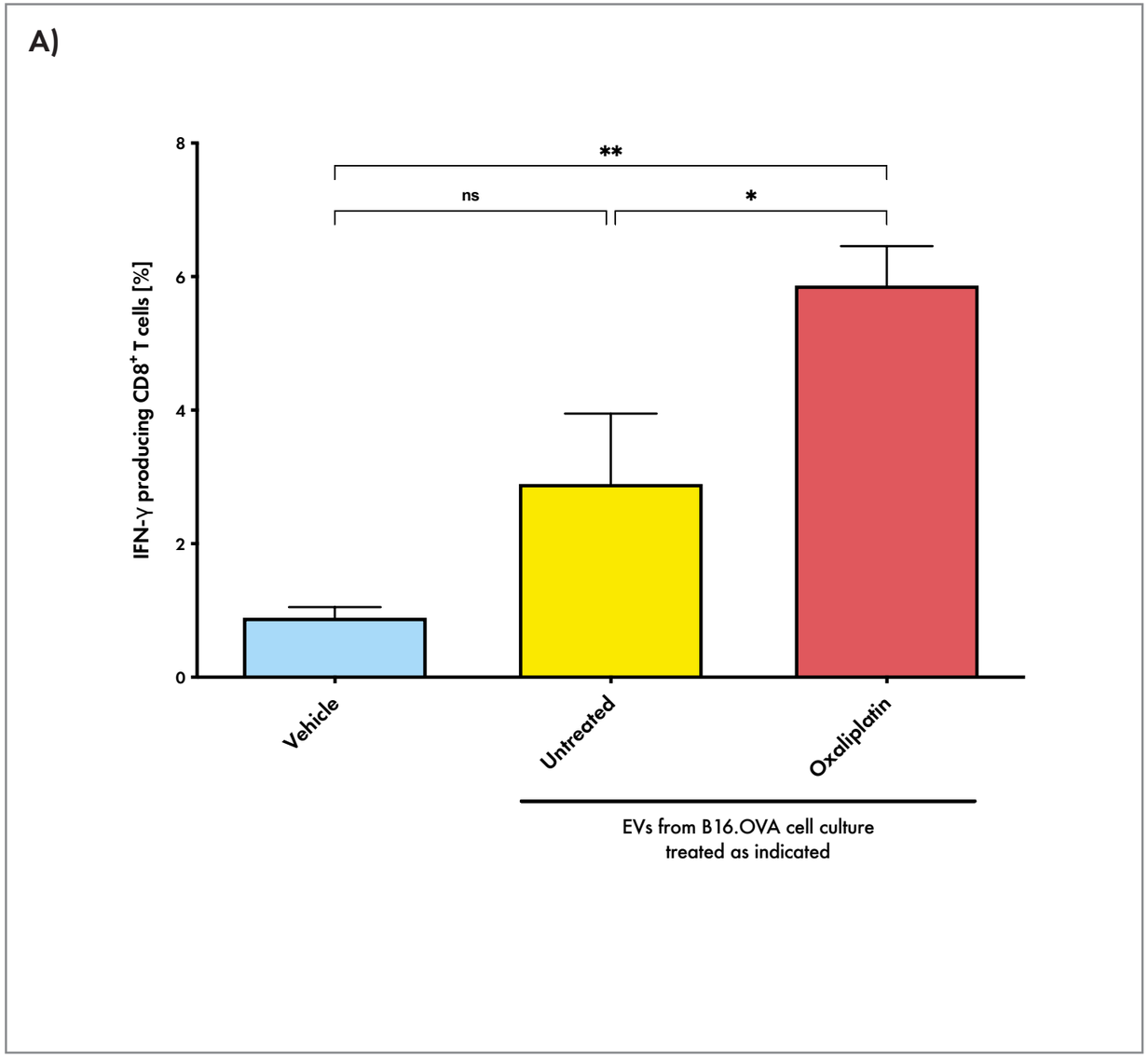
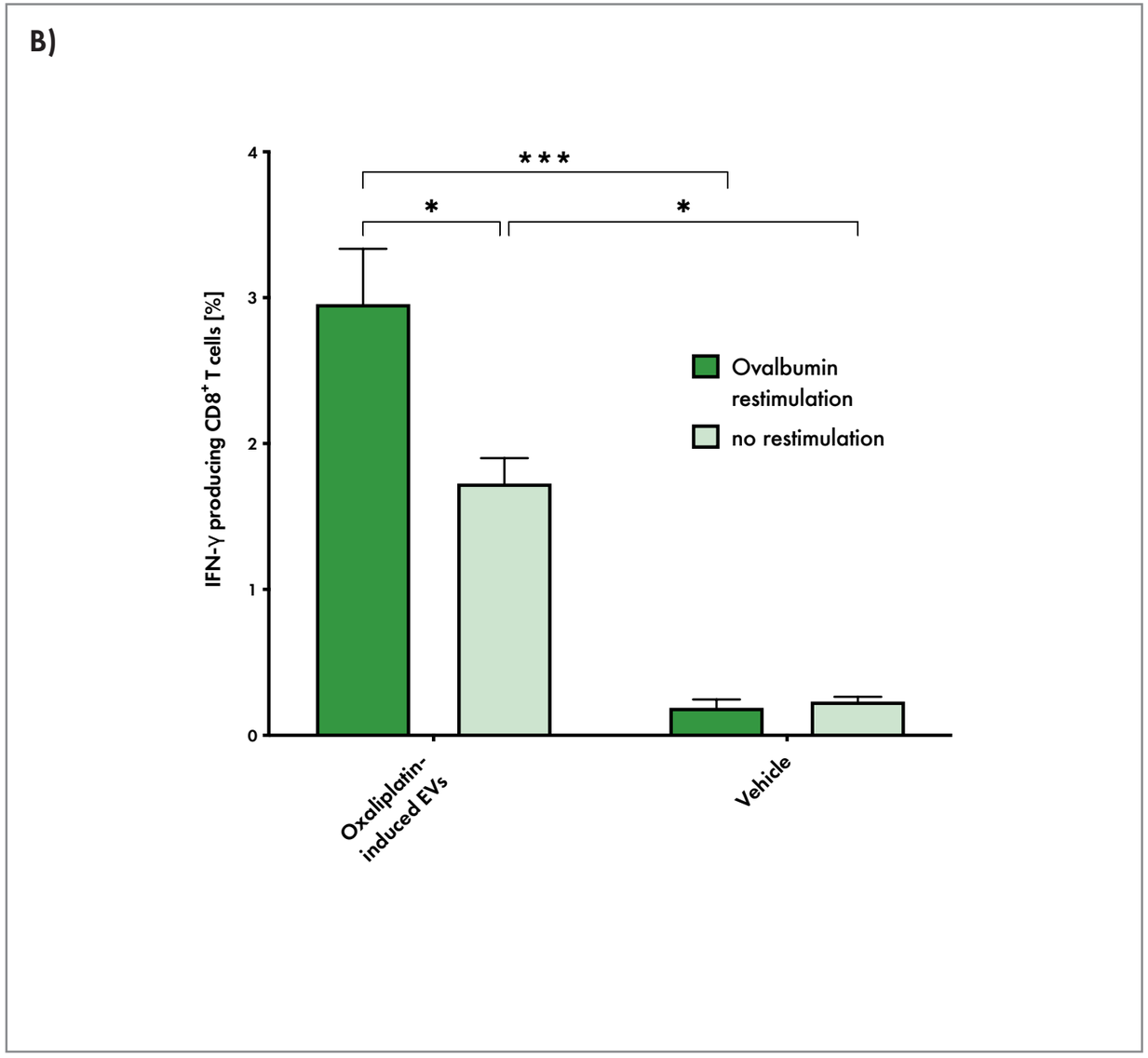


Figure 3





Point-by-point response to the editor's and reviewers' comments
Revised manuscript 62811, Stritzke et al.

We thank the editor and reviewers for their helpful comments on our manuscript. All comments are addressed in detail below. Changes in the revised manuscript can be tracked.

Editor:

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We took the time to thoroughly improve spelling and grammar.

2. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

The manuscript has now been adapted to avoid the use of personal pronouns.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Eppendorf, falcon, etc

The manuscript has been adapted to exclude commercial language.

4. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

An ethics statement has been added before the steps of the protocol that affect living animals. During the whole protocol we strictly comply with the guidelines of our institution and with local regulation by the “Regierung von Oberbayern”.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

The protocol has been adapted to use the imperative tense consistently. Furthermore, the recommendation to use a cell culture hood has been included before the steps describing EV isolation.

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

With the recent amendments, the steps of resuspending the EV pellet and analyzing flow cytometry data are now described in a more comprehensible way. For the analysis of cell viability and the hoc immunization, we have referenced publications describing these methods in detail to provide clarity.

7. Line 163: Is there any age/sex bias? Please specify.

The experiments were only performed with female mice at the age of 6 – 8 weeks. Therefore, we cannot account for any sex bias. However, immune-senescence in old mice reportedly impairs the cytotoxic T cell activation upon encounter of a novel antigen[1]. When using older mice, we would expect a similar effect in our setting. This has been clarified in the revised manuscript.

8. Line 175-177: Please specify how the animals were euthanized.

The specification that mice were euthanized by cervical dislocation has been added.

9. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

To protocol is no longer than 3 pages. We deem all steps to be essential, and should therefore be included in the video.

10. Please provide detail discussion regarding the Figures in the Representative Results

We now included a more detailed discussion regarding T cell activation and its antigen specificity in the updated Representative Results section.

11. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.

We thank the editor for this constructive critique. We have shifted the description of flow cytometry gating into the protocol section. In addition, we have edited the figure titles.

12. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol

Critical steps explicitly addressed in the Discussion include the reproducible quality of EV isolation and defining the dosage for an adequate level of cell death. Minimum information requirements for the publication of EV data are also discussed in appropriate detail.

b) Any modifications and troubleshooting of the technique

Possible modifications of flow cytometry to characterize the immune response comprehensively have now been added, including reviewer suggestions such as additional markers for flow cytometry analysis.

c) Any limitations of the technique

We discuss the need for additional EV characterization and application of complementary isolation methods following the International Society of Extracellular Vesicles (ISEV) guidelines, which we refer to in the manuscript.

d) The significance with respect to existing methods

The significance of the described protocol lies in its role as a relatively easy screening method. We emphasize this explicitly in the last paragraph.

e) Any future applications of the technique

Testing EV immunogenicity in patient material before and during therapy as a clinical biomarker is a possible future application that we have included in the Discussion.

13. Please do not use the &-sign or the word "and" when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

We have adapted the citation style to the journal's policy.

14. Figure 2: Please replace 'commas' with 'decimal points' in the percentages denoted in the panels of the figure.

Commas were replaced with decimal points.

Reviewer #1:

Stritzke and the co-authors present a very interesting study of "In vivo immunogenicity screening of tumor-derived extracellular vesicles after purification from cell culture by flow cytometry of splenic T cells". As an important component of liquid biopsy, EVs not only have a great potential to be an effective tool for tumor diagnosis, but also become cross-priming

against tumor antigens. In this study, they focus on a relatively innovative method to assess the in vivo immunogenicity of EVs. However, some major points need to further improve in this work.

1. It is suggested to supply more evidence for the quality of tumor-derived EVs, including the electron microscope image of EVs, size determination (nanoparticle tracking analysis), and western blot of EVs typical markers (CD9, CD63, CD81).

We thank the reviewer for this critical comment. EV characteristics like the ones described above are part of the minimal information for studies of extracellular vesicles (MISEV2018) required by the ISEV[2]. Raising awareness for this requirement, we explicitly refer to their guidelines in our manuscript. However, comparing the quality of a specific isolation method or comparing different EV isolations methods from the several legitimate methods listed in the MISEV18 (as suggested by another reviewer) is beyond the scope of this manuscript. Instead, we leave it to the reader to choose a method that best suits their local infrastructure and individual research question. To focus of this manuscript is a method for screening EVs (or possible alternative small particles) for their immunogenicity. To keep this methods paper straight-forward, we would refrain from in-depth description of detailed quality control steps but refer to the ISEV guidelines.

2. The protocol only studied the B16 melanoma cells, thus it is recommended to validate this method on another melanoma cell line to avoid deviation.

This is a very legitimate point. Indeed, this method has been validated with other cell death inducers, another murine melanoma (B16.F10) and pancreatic carcinoma cell lines, and another method for EV isolation (size-exclusion chromatography). However, these data are part of another manuscript in preparation and can therefore not be included in this manuscript.

3. In this study, only flow cytometry is used to test the immune response. It is recommended to use multiple methods to increase the evaluation from more perspectives.

We agree with the reviewer. In the revised manuscript, we encourage readers to use additional assays characterizing cytokine release ex vivo, using genetically engineered mouse models, and more.

4. One of the important advantages of liquid biopsy is that markers can be tested at multiple time points to show changes in the whole treatment process. Is it possible to apply EVs as biomarkers to assess the immune response in clinical practice?

Patient-derived EVs have been described as biomarkers under several circumstances (as reviewed here[3]) including as potential predictors of response to immunotherapy targeting PD-1/PD-L1 in NSCLC[4]. The immunological assessment of circulating patient-derived EVs is a highly clinically relevant endeavor, though it would require some major adaptations in the described protocol.

5. The contribution of each author in the manuscript is missing, better to update with a clear explanation.

We have now included a paragraph briefly describing the authors' contributions.

Reviewer #2:

Manuscript Summary:
This article provides description of the method to obtain vesicles from cells exposed to immunotherapy , followed by immunization. I have had some issues with how the rigor was maintained throughout by the use of nanotracking, westerns etc. These data should at least be cited from other articles by the authors. The protocol uses cells that were exposed to EVs generated from cells treated with oxaliplatin but it is not clear how oxaliplatin by itself would have these effects since it was not included in the assay. Did the EVs contribute to the effect or would the delivery of oxaliplatin have similar results?

We thank the reviewer for her/his insightful thoughts on our manuscript. However, oxaliplatin is a cytotoxic agent causing cell death via cross-linking of DNA. Oxaliplatin is not an "immunotherapy" per se, and there is no evidence that oxaliplatin alone induces an ovalbumin-specific immune response in mice. Oxaliplatin is a bona fide immunogenic cell death inducer mediating an adaptive immune response by triggering calreticulin exposure and HMGB1 release of cancer cells[5]. Essentially, this protocol provides a screening method to detect modes of immunogenic cell death in cancer that potentially involve the release of immunogenic EVs.

Regarding quantification and characterization of EVs (with nanoparticle tracking analysis, western blot, or others), we refer to MISEV2018 which resembles the current expert consensus in the field.

The gating strategy for flow cytrometry is not the best. more markers should be used.

We agree with the reviewer that more data about the induced immune response is desirable. In this protocol, however, we consciously chose to provide just the core method for detecting immunogenic EVs to preserve practicability for the reader. Multiple suggestions on how this method can be expanded, depending on the particular research question, have been added to the revised Discussion section. These suggestions include markers for T cell subpopulations and T cell function, among others.

Other concerns are listed below.

Major Concerns:
Line 121 - seeding cells at such high confluency might not be desired. Will they not overgrow after 24 hours? Explain please.

As the reviewer mentioned correctly, overgrowing of cells is not desired here. We, therefore, added a note that readers should adapt the cell concentration to their respective cell line. For B16.OVA, 400,000 cells per mL, works well in our hands.

Line 123 - explain better why oxaliplatin was used and provide references

As mentioned above, oxaliplatin was used as a bona fide immunogenic cell death inducer in cancer with high clinical relevance. The reference to the original work of Tesniere et al.[5] has been added to the manuscript.

Line 133 - was the first spin also performed at 4C and were the pellets discarded, like for the 2, 000 spin?

The reviewer is correct in this assumption. The paragraph has been edited to provide more details to the instructions.

Line 134 - The filter should be better explained - mention the make if possible. Is it 220 nm filter or 0.2 um filter?

In compliance with the policies of JoVE, we do not mention product names in our manuscript. However, this information is provided in the table of materials.

Line 136 - I am concerned with proteolysis at this stage of the preparation and I do not recommend longer storage. Are any inhibitors of proteolysis added, and are the preparations sterile and prevent bacterial growth? Cell viability assays might not provide the best therapeutic efficacy information on these EVs as their ultimate function is in promoting immunogenic responses in animals. Apart from proteolysis, I am interested in whether the structure of vesicles is affected after long term storage.

Importantly, no change in functionality after pausing the protocol for one day has been observed with the specific EVs described here. However, in the Protocol section, we strongly recommend adhering to the described timetable.

The cell viability assay refers to evaluating the treatment substance, not the EVs. Therefore, we have shifted it to step 1.4.

Line 140 - what reagent is exactly used and why is the incubation so lengthy?

These steps of the protocol resemble the instructions of the manufacturer. The manufacturer does not provide the composition of this reagent.

Line 157-158. - I am interested in the storage conditions at -80C for 28 days. Was this established by the authors? The paper is citing a review. Always cite research papers, not reviews, or indicate it very clearly in the text if you are citing a review rather than an original research paper.

Undoubtedly, direct use of EVs after their isolation is the safest option, though not always feasible. However, we have added the recommendation to apply the EVs in vivo directly. Importantly, no change in functionality after storage at -80 °C for four weeks has been observed with the specific EVs described here. Moreover, in their work, Jeyaram and Jay review published data about the impact of storage conditions on the number, content, and functionality of various EVs and, ultimately, recommends storage at -80 °C[6]. This review

thus provides more information than any single original research paper. We changed the wording to make this clear.

Line 168-170 - the number of vesicles should be first established empirically and these data should be shown. I would not pick a dose normalized to a microliter of vesicles but number of vesicles or microgram of vesicles. This approach lacks in rigor.

We partly disagree with the reviewer's opinion in this context. Normalizing the amount of EVs for immunization only allows addressing qualitative alterations in EV biogenesis. Immunization with the total amount produced by a constant number of cells, though, addresses qualitative *and* quantitative alterations contributing to the immunogenicity of EVs. Hence, the choice between the two options depends on the research question. As the latter reflects the biological situation more comprehensively, we suggest it to the reader.

Line 175-176 - why was this type of immunization chosen over other delivery types? Are mice anesthetized ? Likewise, in later sections (section 3), the sacrifice of mice is not described, and it would be good to have entire protocol spelled out.

Injection into the hindfoot was chosen due to local regulation for animal welfare. For organ harvest, mice were sacrificed by cervical dislocation. No anesthesia was used in either case. This has been integrated into the protocol.

Line 188 - how many milliliters of RMPI is used? Is the spleen just mashed with strainer and no bead-based lysis is used?

5 – 10 mL cRPMI was used. This has been added to the protocol. Spleens were mashed with a strainer as described in the protocol.

Line 191 - It is unclear why red blood cells lysis would be skipped. Please provide citation.

Due to its anatomy, spleens are rich in erythrocytes, unlike lymph nodes. The low number of erythrocytes in lymph nodes does not interfere with downstream analysis.

Line 200-204 - why is incubation 48 hours and not shorter? Also, you will likely analyze the production and not necessary secretion of cytokines if you do intracellular staining. Please fix this detail and be specific on the planned measurements.

We did ex vivo restimulation with the full-length ovalbumin protein. Incubation for 48 h was performed to give sufficient time for ovalbumin uptake, processing, and presentation by antigen-presenting cells and subsequent stimulation of the T cell receptor. Using ovalbumin's immunogenic peptide epitope SIINFEKL instead would allow for a shorter incubation period.

The reviewer is correct about the capability of intracellular staining. Therefore, in the manuscript, we consistently refer to IFN-γ production and not to its release. However, IFN-γ release upon T cell receptor stimulation relies on the inducible expression of the IFN-γ gene

via NFAT[7], which is why we consider the chosen method to be a biologically meaningful readout.

Line 206 - at which stage is the PMA + Ionomycin added after initial incubation? Last 4 hours of culture or in addition to before mentioned 48 hrs?

We thank the reviewer for pointing out the need to be more explicit here. We now specify in the manuscript that PMA, Ionomycin, and Brefeldin are added after 48 h.

Line 211 - do you mean primary antibodies conjugated to a fluorophore? Are any other markers used or just CD3/CD8/CD4? What about TCR-β, CD44, CD62L, viability markers? I do not think you have sufficient markers here. Was brefeldin added at this stage since later the intracellular IFNgamma is mentioned?

The reviewer is correct that we have used primary antibodies conjugated to a fluorochrome. “Fixable viability dye” is a stain for cell viability and was already included in the initial manuscript.

Indeed, our Protocol section describes the core of our proposed method for immunogenicity screening of EVs. In the Discussion section, however, we encourage readers to extend the scope of this method according to their needs. Our suggestions now include additional antibodies like the ones mentioned by the reviewer.

Brefeldin was added prior to surface and intracellular antibody staining.

Line 222 - It should be clear how long the staining is for - there is a large difference between 1 and 12 hours. How is this determined?

The window of 1 – 12 h allows to proceed with the protocol the following day and still yields stable results.

Line 226 - unclear why cytotoxic T cells are only mentioned and not helper T cells as well.

Cytotoxic T cell activation is a highly clinically relevant factor with an established impact on the prognosis of many cancer diseases[8]. Therefore, we have chosen cytotoxic T cells as our primary read-out.

Lines 229-232. Please do not describe "as depicted in Figure 1" but rather make statement and follow by figure citation e.g., "The immunogenicity of oxaliplatin-induced tumor cell EVs is assessed by …..(Figure 1)"

We thank the reviewer for this comment. We have taken the opportunity to rephrase the respective paragraph.

Line 256: issues with the analysis - was there any multiple testing correction used?

We thank the reviewer for this remark. Indeed, for multiple statistical comparisons of a dataset, the one-way analysis of variance (ANOVA) test with Bonferroni posttest was used. We now state this in the figure legend.

Line 282-289 - I am glad the authors acknowledged that this EV preparation technique is not optimal. However, do mention gold standard in EV preparation, such as ultracentrifugation . In general, the technique that is ere described should be accompanied by validation by nanosight, microscopy, mass spectrometry, western blotting etc to ensure no contaminants are present. The precipitation-based methods can precipitate some other lapidated structures as well, contributing to immunogenicity of the preparation. The discussed immunoaffinity might not be the best for animals if antibody is bound to the vesicles.

Regarding validation of the described method in alternative settings, EV characterization, and quantification, please see our response to reviewer #1 comment #1.

Regarding a "gold standard" in EV separation the MISEV2018 guidelines state very clearly: "There is no single optimal separation method, so choose based on the downstream applications and scientific question."[2]

Minor Concerns:
Minor stylistic edits:
One comment: in some cases, protocol reads like a series of commands "e.g. Wash", "analyze" and in other parts of the article, the protocol reads like statements" the cells are washed". Please rewrite the protocol in the consistent style. There are also some stylistic issues.

We appreciate the effort by the reviewer to point out specific phrases that should be improved from the reviewer's perspective. Building on that we have taken the chance to revisit the whole manuscript from a stylistic perspective.

Examples of some issues throughout the text.
Line 30 - "their" - whose? Do not include undefined pronouns due to reduced clarity

"Their" has been replaced.

Line 41 - "exemplary" - change to "For example"

Changed to "as an example".

Line 97 - "precipitation based assay" - should not it be "method" rather than "assay"?

We think "assay" is an accurate description in this case.

Line 113 - not sure why the author used the word "entities"

Different types of cancer are commonly categorized into entities.

Line 140. It should be "0.5" and not "0,5" mL - correct similar mistakes throughout the text

We have replaced all the decimal commas throughout the manuscript.

Line 140 - what is "eppi" ? Avoid jargon please.

We have replaced all product names.

Line 172-173: awkward sentence. Rephrase.

The sentence has been rephrased to provide clarity.

Line 233-234 - vague. Explain and reword.

This part has been explained with more detail. Further, we have moved it to the discussion section.

Line 238 - "A scheme of the proposed protocol"

In this case, we think “overview” is an accurate description.

Line 238-239 : "A) Generation of EVs derived from tumors under chemotherapy and EV isolation procedure. " How about: "A) Isolation of EVs derived from tumors under conditions mimicking chemotherapy."

We have rephrased this.

Line 301-307. Please revise and avoid such statements as "EVs are everywhere". "time to harness their potential"

The concluding statement has been rephrased in a more objective manner.

This section should be written in a more scientific language. Moreover, the use of undefined pronouns is very distracting to the reader. Consider professional editing of the text.
Figure 1. While the artwork is certainly appreciated, do consider using biorender or similar software to improve. What is " a resection"?

A resection is an operation to cut out part of an organ or a piece of tissue from the body.

Reviewer #3:

Manuscript Summary:
The authors demonstrate detection of immune responses to EVs by measuring the production of IFN-γ of CD8+ cells following in vitro stimulation of spleen cells obtained from mice previously immunized with the EVs. The various steps are clearly described.

We thank the reviewer for this positive feedback.

Major Concerns:
1) Does the data shown in Figure 3 show responses with ovalbumin included in the stimulus? If it does then the results of cells stimulated without ovalbumin should also be shown. If the figure shows responses without ovalbumin, it would be interesting to see the responses if ovalbumin is included in the stimulus

We thank the reviewer for this important comment. The data (now labeled Figure 3A) display the enhanced cytotoxic T cell activation upon immunization with EVs derived from B16.OVA cell cultures treated with oxaliplatin. Indeed, all splenic cell suspensions were restimulated with ovalbumin ex vivo. The new Figure 3B displays differences in IFN-γ-production upon restimulation with ovalbumin and steady-state cultures ex vivo. These data show that EV-induced immune responses are indeed - at least to some extent - tumor antigen-specific.

2) Although there is mention of using nanoparticle measuring device, it appears that the authors do not routinely count the number of EVs but use EVs based on isolation from 400,000 cells. Often treatment of cells with various reagent such as chemotherapy can result in increased or diminished production of EVs. If this is the case the altered number of EVs could potentially influence the immunogenicity. At a minimum it would be good to show that similar numbers of EVs are produced by the cells with or without treatment.

We completely agree with the reviewer that besides qualitative changes in the EV composition, an altered number of produced EVs may impact immunogenicity. However, this is exactly what we want to assess with this assay, taking in to account qualitative AND quantitative changes in EV production by tumor cells under certain conditions.

We have included a statement in the revised manuscript, that – depending on their scientific question – readers may normalize their stimulation experiments to the number of EV-producing tumor cells or, alternatively to a specific concentration of produced EVs.

Regarding oxaliplatin, the quantitative release of EVs is similar between treated and untreated B16.OVA cells, based on nanoparticle tracking analysis (data not shown).

Minor Concerns:
Have the authors tested this approach using human peripheral blood mononuclear cells?

Yes, EVs derived from human tumor cells have been successfully used to stimulate PBMCs from healthy donors. However, these data are incluced in another manuscript in preparation.

Reviewer #4:

Manuscript Summary:
This manuscript describes an interesting in vivo system for the screening of the immunogenic properties of tumor-derived EVs after cancer treatment using a flow cytometry approach for the study of splenic TCD8 immune response. The experimental approach comprises the assessment of in vivo antigen-specific TCD8 response elicited after immunization of mice with

EVs secreted from B16.OVA murine melanoma cells after its treatment with Oxaliplatin, an anticancer drug. The described methodology is accurately presented and described and have potential to be used as a system for the study of tumor-derived EVs antigenic potential in different cancer therapeutic contexts and also contribute with the biological knowledge of EVs in cancer immunology.

I recommend this article for publication after minor revision.

Major Concerns:
No major concerns

Minor Concerns:
1. The title of the manuscript although it describes the work done, it contain to much detailed information. I suggest removing the specification about the purification and in vitro generation of EVs. (Ex: In vivo immunogenicity screening of tumor-derived extracellular vesicles by flow cytometry analysis of splenic T cells)

We thank the reviewer for this helpful suggestion. The title has been adapted.

2. Line 39: In the sentence: "The protocol is based on flow cytometry…." Include the word "analysis" after "cytometry"

The word "analysis" has been added.

3. Line 43: T cell response has been measured in the spleen of vaccinated mice. Considering that immune cells in this secondary lymphoid organ can be circulating, specifically recruited (in a disease-specific manner) and even differentiated in the spleen by extramedullary hematopoiesis (in specific contexts) (See reference PMCID: PMC3912742), I suggest replacing the word "systemic" by "splenic" throughout the manuscript when referring to splenic response. To measure of a systemic T cells response authors could have analyzed PBMCs.

Indeed, the spleen not only hosts circulating lymphocytes activated in the periphery. "Diverse splenic populations not only trap and remove blood-borne antigens but also initiate innate and adaptive immune responses against pathogens."[9] Nevertheless, we do not believe that after subcutaneous injection, a significant number of EVs enter the circulation to be captured in the spleen. Nevertheless, we agree to change the term "systemic" to the more descriptive term "splenic."

4. Line 136: Please state the time in which EVs-containing culture supernatant can be keep at 4ºC. This is a crucial step for maintaining the stability of EVs. Also, please indicate if the conditioned media can be stored at -80ºC for longer period before isolation.

With EVs derived from B16.OVA, storing cell culture supernatants at 4 °C for one day did not affect our results. However, we strongly recommend adhering to the prescribed timetable. Theoretically, storing the cell culture supernatant, instead of the final EV isolates, at -80 °C might still yield similar results. However, we have not tested this. Therefore, it is up to the readers to test this with their specific EVs, applying the quantification and characterization methods listed in the manuscript.

5. Line 137: The note about assessing the therapeutic effect of the anticancer drug by measuring cell viability should be placed right after the drug treatment step (before collecting the supernatant for EVs isolation). Please consider indicate that this as a fundamental step of the protocol. If so, indicate the cell viability assay that authors have used.

We agree with the reviewer that a cell-viability assay to titrate the dosing at the first use of a substance is an essential step. We have included this in the revised protocol.

6. Line 150. It is unclear the instruction "avoid touching the pellet" just after indicating the resuspension of EVs in PBS through pipetting. Please clarify.

Indeed, this step needed clarification. Here, physically scratching the pellet from the wall with the tip must be avoided.

7. Line 168: The EVs suspension volume injected to the mice (15 ul EV suspension + 57 cold PBS) = 72 ul. Please correct the indicated volume of 60 ul.

We apologize for this mistake. The volumes have been corrected.

8. Line 188: Please indicate the RPMI volume used for the preparation of splenic cell suspension. "some" is not appropriate description in a scientific protocol.

5-10 mL cRPMI has been used. This has been clarified.

9. Line 210. It would be important indicate which negative controls are used in the flow cytometry experiments (Ex: isotypes, unstained cells, FMO)

FMO has been used. We have integrated this into the flow cytometry part of the protocol.

11. Line 254. Please indicates in the legend of figure 3 if these results correspond to cells stimulated with OVA in addition to PMA/ionomycine.

All splenic cell suspensions in Figure 3A have been restimulated with ovalbumin. Please see our comment to reviewer #3.

12. Line: 271: EVs used in vaccination assays have been semiquatified based on the number of total EVs generating cells. As authors mentioned, adequate EVs quantification is an important aspect to consider in terms of the reproducibility of EVs functional studies. Although authors mentioned to have quantified EVs concentration by nanoparticle track analysis (data not shown), a more reliable quantification method is the EVs protein concentration. Adjusting EVs dosage by protein amount remove the bias associated to alterations in EVs number secreted due to drug effects on cell viability. Authors could mention this alternative strategy for EVs quantification in the discussion.

EV protein concentration (as a MISEV2018 referenced method2) has been mentioned in the revise manuscript.

13. Line 285. Authors mention the drawbacks of possible contamination when using precipitation techniques for EVs isolation. They highlighted other techniques like immunocapture as alternative EVs isolation approache to overcome contamination. Although immunocapture techniques has been proved an excellent method for isolating EVs, it has limitations for its downstream use in functional assays. Other methods such as size exclusion chromatography in combination with density gradient centrifugation are more suitable methodologies to generate highly pure EVs for functional assays. Authors could include these alternatives methodologies in its discussion (PMID: 30891621)

Size exclusion chromatography in combination with density gradient centrifugation has been mentioned as an alternative isolation method.

14. Authors could indicate in the discussion the potential of their flow cytometry-based analysis for delineating other immune cell populations also relevant for the control of tumours.

Please see our comment to reviewer #2 Line 211.

15. In general, an important aspect in the EVs research field to which a considerable amount of effort is devoted is the extensive molecular characterization of EVs after its isolation, specially previous to perform functional analysis. Given that, this publication is a methodological paper, I believe that the EVs characterization should be indicated as an important step of the protocol. Alternative a note can be added with references to previous B16.OVA melanoma EVs molecular description.

We agree that molecular characterization is an essential component in publications about EVs. Thus, we have included this as an individual step in the protocol and thoroughly addressed it in the Discussion section.

1 Kapasi, Z. F., Murali-Krishna, K., McRae, M. L., Ahmed, R. Defective generation but normal maintenance of memory T cells in old mice. *European journal of immunology.* **32** (6), 1567-1573, (2002).
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