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Corresponding Author:	Andrean Burnett UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	andrean-simons@uiowa.edu
Order of Authors:	MM Hasibuzzaman
	Kathleen Ross
	Aliasger Salem
	Balaji Narasimhan
	Andrean Burnett
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TITLE:

Evaluation of the *In Vivo* Antitumor Activity of Polyanhydride IL-1α Nanoparticles

AUTHORS AND AFFILIATIONS

M. M. Hasibuzzaman[1,2], Kathleen Ross[3,4], Aliasger Salem[1,4,5,6], Balaji Narasimhan[3,4], Andrean L. Simons[1,2,4,5,6,7]

[1]Interdisciplinary Graduate Program in Human Toxicology, University of Iowa, Iowa City, IA.
[2]Department of Pathology, University of Iowa, Iowa City, IA.
[3]Department of Chemical and Biological Engineering, College of Engineering, Iowa State University, Ames, IA.
[4]Nanovaccine Institute, Iowa State University, Ames, IA.
[5]Division of Pharmaceutics and Translational Therapeutics, College of Pharmacy, University of Iowa, Iowa City, IA.
[6]Holden Comprehensive Cancer Center, University of Iowa, Iowa City, IA.
[7]Department of Oral Pathology, Radiology and Medicine, College of Dentistry, University of Iowa, Iowa City, IA.

Email Addresses of Co-authors:
M. M. Hasibuzzaman (mm-hasibuzzaman@uiowa.edu)
Kathleen Ross (kaross09@iastate.edu)
Aliasger Salem (aliasger-salem@uiowa.edu)
Balaji Narasimhan (nbalaji@iastate.edu)
Andrean L. Simons (andrean-simons@uiowa.edu)

Corresponding author:
Andrean L. Simons (andrean-simons@uiowa.edu)

KEYWORDS:

Interleukin-1, nanoparticles, HNSCC syngeneic mouse model, cytokines, multiplex, single-cell preparation, flow cytometry

SUMMARY:

A standard protocol is described to study the antitumor activity and associated toxicity of IL-1α in a syngeneic mouse model of HNSCC.

ABSTRACT:

Cytokine therapy is a promising immunotherapeutic strategy that can produce robust antitumor immune responses in cancer patients. The proinflammatory cytokine interleukin-1 alpha (IL-1α) has been evaluated as an anticancer agent in several preclinical and clinical studies. However, dose-limiting toxicities, including flu-like symptoms and hypotension, have dampened the enthusiasm for this therapeutic strategy. Polyanhydride nanoparticle (NP)-based delivery of IL-1α would represent an effective approach in this context since this may allow for a slow and controlled release of IL-1α systemically while reducing toxic side effects. Here an analysis of the

antitumor activity of IL-1α-loaded polyanhydride NPs in a head and neck squamous cell carcinoma (HNSCC) syngeneic mouse model is described. Murine oropharyngeal epithelial cells stably expressing HPV16 E6/E7 together with hRAS and luciferase (mEERL) cells were injected subcutaneously into the right flank of C57BL/6J mice. Once tumors reached 3–4 mm in any direction, a 1.5% 20:80 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane:1,6-bis(p-carboxyphenoxy)hexane (CPTEG: CPH) nanoparticle (IL-1α-NP) formulation was administered to mice intraperitoneally. Tumor size and body weight were continuously measured until tumor size or weight loss reached euthanasia criteria. Blood samples were taken to evaluate antitumor immune responses by submandibular venipuncture, and inflammatory cytokines were measured through cytokine multiplex assays. Tumor and inguinal lymph nodes were resected and homogenized into a single-cell suspension to analyze various immune cells through multicolor flow cytometry. These standard methods will allow investigators to study the antitumor immune response and potential mechanism of immunostimulatory NPs and other immunotherapy agents for cancer treatment.

INTRODUCTION:

One of the emerging areas of cancer immunotherapy is the use of inflammatory cytokines to activate patients' immune system against their tumor cells. Several proinflammatory cytokines (i.e., interferon-alpha (IFNα), interleukin-2 (IL-2), and interleukin-1 (IL-1)) can mount significant antitumor immunity, which has generated interest in exploring the antitumor properties as well as the safety of cytokine-based drugs. Interleukin-1 alpha (IL-1α) in particular, is a proinflammatory cytokine known as the master cytokine of inflammation[1]. Since the discovery of this cytokine in the late 1970s, it has been investigated as an anticancer agent as well as a hematopoietic drug to treat the negative effects of chemotherapy[2]. During the late 1980s, several preclinical and clinical studies were conducted to determine the anticancer effects of IL-1α[3–6]. These studies found promising antitumor activity of recombinant IL-1α (rIL-1α) against melanoma, renal cell carcinoma, and ovarian carcinoma. However, toxicities, including fever, nausea, vomiting, flu-like symptoms, and most severely dose-limiting hypotension were commonly observed. Unfortunately, these dose-related toxicities dampened the enthusiasm for further clinical use of rIL-1α.

To attempt to address the critical issue of IL-1α-mediated toxicities, polyanhydride nanoparticle (NP) formulations that allow for the controlled release of IL-1α by surface erosion kinetics will be investigated. These NP formulations are intended to reap the benefits of the antitumor properties of IL-1α while reducing dose-limiting side effects[7]. Polyanhydrides are FDA-approved polymers that degrade through surface erosion resulting in nearly zero-order release of encapsulated agents[8–12]. Amphiphilic polyanhydride copolymers containing 1,8-bis-(*p*-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis-(*p*-carboxyphenoxy) hexane (CPH), have been reported to be excellent delivery systems for various payloads in oncology and immunology-based research[8,12]. In the following protocol 20:80 CPTEG:CPH NPs loaded with 1.5 wt.% rIL-1α (IL-1α-NPs) will be used to study the antitumor activity and toxicity of this cytokine in a mouse model of HNSCC.

The overall goal of the following procedures is to assess the antitumor activity of IL-1α-NPs on

HNSCCs. The procedures described, including assessing tumor growth and survival, can be applied to any immune-modulatory agent of interest. These procedures should be performed in a syngeneic mouse model with an intact immune system[13] to maximize clinical relevancy. IL-1α-NP toxicity will also be assessed by measuring changes in circulating levels of proinflammatory cytokines and animal weight. There are many methods to determine *in vivo* drug toxicity; however, the most widely used methods involve the measurement of serum enzymes for organ toxicity and histological changes in those organs. However, to perform histological analyses, the animal needs to be sacrificed, which will affect the survival curves of the experiment. Therefore, this protocol will include a protocol for the collection of blood from live mice for the measurement of cytokines in serum samples. The collected serum can be used for the measurement of any desired serum analytes for organ toxicity. Multicolor flow cytometry will be used to understand the changes in the immune cell population in the tumor microenvironment and immune cell migration to the lymph node. Other methods can be utilized to identify immune cells, including immunohistochemistry and/or immunofluorescence of preserved sections[14]. However, these techniques can be time-consuming and tedious to perform on a large number of animals. Overall, the following methods will allow investigators to study the antitumor immune response and potential mechanisms of immunostimulatory agents for cancer treatment.

PROTOCOL:

All the *in vivo* procedures used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Iowa.

1. Preparation and maintenance of HNSCC cell line

NOTE: In this study, the murine oropharyngeal epithelial cell line stably transformed with HPV E6 and E7 together with hRas and luciferase (mEERL) will be used. This cell line was developed from C57BL/6J mouse strain and was a gift from Dr. Paola D. Vermeer (Department of Surgery, University of South Dakota Sanford School of Medicine, South Dakota, USA).

1.1. Thaw a frozen vial of mEERL cells in a pre-heated (37 °C) water bath and then transfer to a 15 mL conical tube containing warm culture media (Dulbecco's Modified Eagle Medium [DMEM] supplemented with 40.5% 1:1 DMEM/Hams F12, 10% Fetal Bovine Serum [FBS], 0.1% gentamicin, 0.005% hydrocortisone, 0.05% transferrin, 0.05% insulin, 0.0014% tri-iodothyronine, and 0.005% epidermal growth factor).

1.2. Centrifuge the conical tube at 277 x *g* for 5 min at 25 °C to remove the media. Then, resuspend the cell pellet in 3–5 mL fresh media and transfer it to a T-25 cell culture flask. For optimal recovery from frozen storage, plate cells at high density.

NOTE: T-25 flasks were used because of their smaller size, results in faster recovery times from frozen storage when cells are in close proximity compared to T-75 flasks.

1.3. Let the cells grow in a humidified incubator at 37 °C and 5% CO_2, expand to larger flasks (i.e., T-75 or T-150), and passage every 3 days. When there are enough cells for the desired

implantation into all mice, remove the flasks, discard the media, and gently rinse the cells with phosphate buffered saline (PBS). Then, add 4 mL (if using T150 flasks) of 0.25% trypsin-EDTA, and incubate at 37 °C for 2 min. Scale the amount of trypsin up or down depending on the dish/flask size being used.

NOTE: The type of cell line and degree of confluency may affect trypsinization times. Long trypsinization periods can damage cells resulting in low viability. Use the minimum amount of time needed for trypsinization.

1.4. Under the microscope, the detached cells on trypsinization move freely. If some cells are still attached, very gently tap the flask to mobilize the remaining adherent cells. Add fresh media (scale amount of media as desired) to stop the trypsin reaction and collect the cell suspension in 50 mL conical tubes. Centrifuge at 277 x *g* for 5 min at 25 °C to remove the media.

1.5. Resuspend the cells in fresh media and count the cells. Centrifuge once more (as described above), and then add cold PBS to the cells to make a final concentration of 10×10^6 cells/mL. Keep the cell suspension(s) on ice before injection to mice.

2. Tumor implantation, drug treatment, and measurement

NOTE: The experimental animals were kept in the Animal Care Facility at the University of Iowa and followed appropriate aseptic procedures to handle them.

2.1. Anesthetize C57Bl/6 mice with ketamine (80 mg/kg) and xylazine (10 mg/kg) mix. Carefully shave the flank area or desired injection site with an electric razor.

NOTE: Remember to document the use of controlled substances as required by institutional (or other) rules and regulations.

2.2. Disinfect the flank area with an ethanol pad and slowly inject 100 µL (containing 1×10^6 cells) of the cell suspension subcutaneously using a 25–28 G syringe. Anesthetize the mice before injection to prevent sudden movements and cell loss. Before each injection, gently mix the cell suspension to prevent cells from settling to the bottom of the vial or conical tube.

2.3. After taking up the cell suspension into the syringe, remove all the bubbles and dead space from the top. Inject the cell suspension in a slow and steady manner. Do not use the same needle on multiple mice. Always make extra cell suspensions to account for accidental loss.

2.4. Place the animal in their respective cages and monitor them until they recover from anesthesia. During anesthesia, animals are at risk of hypothermia; therefore, provide supplemental heat or place the mice close to each other to keep warm (if housed in the same cage).

2.5. When tumors reach 3 mm in any direction, randomize the mice (by tumor size and/or

weight) in the treatment groups and then start the drug treatment. Inject the mice intraperitoneally (i.p.) with NPs[15] containing 3.75 µg rIL-1α/mouse on Days 4 and 9. Measure and record the tumor volume and the mouse weight ((length x width^2) / 2) daily or every other day until the tumor size or the mice reach euthanasia criteria.

NOTE: Even with an experienced researcher, it is difficult to implant the same-sized tumor in all mice. Randomize animals into experimental groups based on the tumor size and mouse weight.

3. Blood collection and serum separation

NOTE: Blood collection from a submandibular vein is an easy and effective technique that allows blood collection from conscious animals or animals under anesthesia. For this study, blood was collected from the animals when they were under anesthesia.

3.1. Anesthetize the mice with an injection of ketamine/xylazine mix as mentioned above.

3.2. Grasp the loose skin over the shoulder using the non-dominant hand and puncture the submandibular vein with an 18 G needle or lancet, slightly behind the mandible (a white spot at that area).

3.3. Puncture the vein to ensure blood flow immediately. Collect 200–300 µL of blood (depending on mouse weight) into a 1.5 mL polypropylene microcentrifuge tube or serum separator tube. After blood collection, apply gentle pressure to the puncture site until the bleeding has stopped. Return the mice to their respective cages and observe until they recover from anesthesia.

NOTE: Do not collect more than 1% of the mouse body weight in one collection or over a 24 h period.

3.4. Let the collected blood clot at room temperature for 20–30 min. Place the tubes on ice until they are ready to be centrifuged.

3.5. Centrifuge the clotted blood at 1540 x *g* for 15 min at 4 °C.

3.6. Collect the upper layer (serum) without disturbing the red blood cells. Store at -80 °C until use.

4. Multiplexing of collected serum

4.1. Thaw the serum or plasma samples while keeping them on ice.

4.2. Centrifuge the samples at 1540 x *g* for 5 min at 4 °C to sediment any cell debris and carefully collect the serum layer from the top.

4.3. Bring out the multiplex kit at room temperature.

NOTE: There are several commercially available multiplex kits that are designed for specific cytokines, chemokines, or growth factors. Also, it is possible to customize the kit based on the protein of interest.

4.4. Perform the assay as per the manufacturer's protocol to detect cytokines.

NOTE: Most of the multiplex kits use magnetic beads to bind the captured antibody. So, it is essential to use an automated or handheld magnetic washer during washing. Otherwise, the magnetic beads will wash off from the plate, and one will not have enough events to take the reading.

5. Collection of tumor and inguinal lymph node and preparation of single-cell suspension

5.1. Anesthetize the mice with ketamine/xylazine mix. To ensure complete sedation, use pedal reflex (firm toe pinch). If the mice are not responsive, euthanize the mice by cervical dislocation.

5.2. Lay each mouse on its back and spray 70% ethanol on the abdominal area skin. Use forceps and scissors to cut the tumor out from the left side of the mice and the lymph node from the right side. If the tumor is big, cut into small pieces and take 500–600 mg of the tissue. For the lymph node, collect the whole organ.

NOTE: There are two lymph nodes on both sides of the inguinal region. Depending on the experimental goals, the lymph node and the tumor can be isolated from the same side. However, if the tumor becomes very large, it will not be easy to collect the lymph node from the same side.

5.3. Place the tissues onto the respective dissociator tubes containing 3–5 mL of RPMI media. Homogenize the tissue using an automated dissociator.

NOTE: Other automated or handheld homogenizers can be used.

5.4. After homogenization, transfer the cell suspension through a 70 µm filter into a 50 mL conical tube. Centrifuge at 277 x *g* for 5 min at 25 °C to remove the media. Wash and resuspend the cells in 1–2 mL of cold PBS.

6. FACS staining of single-cell suspension

6.1. Use 0.4% trypan blue staining to count the viable cells in a hemocytometer. Calculate the volume required to get 2–3 million cells and transfer them to the respective FACS tube.

6.2. Use viability dye to gate on live cells; otherwise, nonspecific binding of the antibody with dead cells may occur that can yield a false-positive result.

NOTE: Zombie dye was used for live and dead cell staining in this experiment. Several fixable and non-fixable dyes (e.g., propidium iodide) are commercially available.

6.3. Centrifuge (277 x *g* for 5 min at 25 °C) the counted cells. Decant the supernatant and resuspend the cells into 300 µL of PBS. Add 0.5–1 µL of zombie dye/tube. Incubate at room temperature for 20 min in the dark.

6.4. Centrifuge (277 x *g* for 5 min at 25 °C) and wash the cells with 1–2 mL of FACS buffer and resuspend them into 200 µL of FACS buffer.

6.5. Add Fc-receptor blocker (2 µL per 200 µL or according to the manufacturer's protocol) and incubate the cells for 10 min.

6.6. Centrifuge (277 x *g* for 5 min at 25 °C) and wash the cells with 1–2 mL of FACS buffer. Add the antibody cocktail and incubate for 30 min at 4 °C in the dark.

6.7. After the incubation, centrifuge (277 x *g* for 5 min at 25 °C) and wash the cells with 1–2 mL FACS buffer. Add 300–400 µL of 2% paraformaldehyde solution and resuspend for fixation. Store the cells at this step for a couple of days at 4 °C or analyze immediately by multicolor flow cytometer.

REPRESENTATIVE RESULTS:

In this study, the antitumor activity of polyanhydride IL-1α in a syngeneic mouse model of HNSCC was investigated. Recombinant IL-1α (rIL-1α) significantly slowed mEERL tumor growth (**Figure 1A**), although weight loss was observed in the treated mice, which was restored after treatment withdrawal (**Figure 1B**). IL-1α-NPs did not induce a significant antitumor effect compared to saline or blank-NPs (**Figure 1A**) and was accompanied by some weight loss, although not as prominent as rIL-1α (**Figure 1B**). Mice treated with rIL-1α survived significantly longer than the other treatment groups (**Figure 1C**). Additionally, circulating levels of IL-1α, IL-1β, and IFN-γ were higher in rIL-1α-treated mice compared to the other treatment groups (**Figure 2A–C**). These results suggest that improvements in the IL-1α-NP with regard to antitumor efficacy are warranted.

FIGURE AND TABLE LEGENDS:

Figure 1: Effect of rIL-1α on tumor growth, survival, and body weight. Male C57BL/6J mice (n = 10–11 mice/treatment group) bearing mEERL HNSCC tumors were treated i.p. on Day 1 and Day 5 with rIL-1α (3.75 µg of rIL-1α), IL-1α-NP (0.25 mg of NPs containing 3.75 µg of rIL-1α), Blank-NP (0.25 mg of NPs), and 100 µL of saline solution (CON). Shown are changes in average tumor volume (**A**), normalized body weights (**B**), and survival curves (**C**). Error bars represent the standard error of the mean. *$p < 0.05$ vs. other treatment groups.

Figure 2: Effect of rIL-1α on circulating cytokines. Blood samples were collected from a subset of mice (n = 4 mice/treatment group) after the second drug administration and analyzed for

circulating cytokine levels by multiplex assay. Shown are circulating levels of IL-1α (**A**), IL-1β (**B**), and IFN-γ (**C**). Error bars represent the standard error of the mean.

DISCUSSION:

This protocol will allow any investigator to study the antitumor activity and some of the underlying mechanisms of immunomodulatory drugs in an *in vivo* tumor mouse model system. Here, a syngeneic subcutaneous tumor model was used, which has several advantages over orthotopic models, including its technically straightforward protocol, easy monitoring of tumor growth, less animal morbidity, and higher producibility. Subcutaneous tumor models can also be modified to a bilateral tumor model by injecting tumor cells on both the left and the right flank. In this bilateral tumor model, radiotherapy or drugs can be administered to one tumor intratumorally, and abscopal responses can be monitored. Orthotopic HNSCC mouse models, while more clinically relevant, are technically challenging to generate, difficult to monitor tumor growth, and the tumor burden in the oral cavity often results in premature euthanasia due to the inability of the mice to eat and drink.

The preparation of cells is an important step for the formation of symmetrical and similar-sized tumors in all mice. Poor preparation of cells results in reduced cell viability and greatly affects tumor generation in mice. The tumor cells are recommended to be at an early passage number and within 80%–90% confluency. Higher passage number and confluency affect cell viability and thus tumor generation. Cells should also be injected as soon after preparation as possible since viability is reduced if kept in PBS beyond 20–30 min. If a large number of mice need to be implanted with tumors, it is recommended to make a stock solution of cells kept in media and prepare injectable cell suspensions in PBS for a smaller group of animals.

There are several critical steps in the protocol that needs to be carefully maintained after preparing tumor cells for injection. Injecting tumor cells to the subdermal space could produce different tumor growth patterns and sizes compared to the subcutaneous space. Therefore, careful attention should be placed on needle placement for consistent tumor formation. Needle selection is also important. If the needle is smaller than the cancer cell, smaller needles could stress the cells resulting in less viability. If the needle is very big, it could hurt the animal and result in cell leakage from the injected site. Even for experienced researchers with the correct needle size, there may be cell leakage at the injected site resulting in a small or no tumor. It is important that researchers use the correct technique for tumor injection and optimal needle size in order to reduce tumor cell loss and increase accuracy and precision during tumor cell implantation. Tumor measurements should be carefully done using Vernier calipers (manual or electronic). The best practice is to be consistent with the direction of tumor length and width measurement to reduce variability. Tumor measurement by the same researcher throughout the study can reduce variability.

As expected, mice receiving rIL-1α lost weight during treatment, which supports previous findings[15,16]. Although weight loss is a simple and straightforward way of assessing toxicity, there are other toxicological endpoints that can be utilized. Assessment of blood cell counts (white blood cells, red blood cells, and platelet counts) and liver enzyme levels (aspartate transaminase,

alanine aminotransferase, and alkaline phosphatase) provide valuable information about drug toxicity. Additionally, a subset of mice can be sacrificed, and histopathological analysis of organs (liver, kidneys, pancreas, lung, etc.) can be performed. Systemic inflammation is often used as an indicator of toxicity. Here, a number of circulating proinflammatory cytokines were analyzed in the mice after drug treatment by submandibular venipuncture using an 18 G needle. Submandibular venipuncture on mice requires a skill that comes from many repetitions of the procedure. If the puncture is too deep, it may cause bleeding from the ear and internal tissue damage. Whereas, if the needle is not penetrated far enough, an insufficient amount of blood may be collected. Alternatives to needles are the use of disposable bleeding lancets. There are different kinds of bleeding lancets that are commercially available that differ in their length. Researchers should use a suitable lancet size to ensure optimal blood collection and humane treatment of animals. For this procedure, results for three cytokines are shown (**Figure 2A–C**). It is likely that an increase in circulating proinflammatory cytokines including IL-1α, IL-1β, and IFN-γ observed in rIL-1α-treated mice may be associated with acute weight loss (**Figure 1B**) observed in this treatment group.

Lastly, a protocol for isolation and preparation of single-cell suspensions of mice tumor and lymph nodes is described. This method is useful for those seeking to detect changes in immune cell activation and recruitment due to drug treatment. During dissection of tumors, adipose tissue, skin, hair, and other debris should be eliminated as much as possible. Usually, the tumor volume should be greater than 30 mm^3 to have enough cells for flow cytometry. However, if the tumor is very large, it may be difficult to prepare single-cell suspensions. Large tumors should be cut into small pieces before placing into the dissociator tube. The process should be done quickly to get optimal viable cells. Additionally, large tumors make finding the inguinal lymph node on the tumor side difficult. In this case, the inguinal lymph node can be collected from the opposite site. Once single-cell suspensions are obtained, they can be stained with different antibodies and analyzed by multicolor flow cytometry.

Overall, these protocols provide an effective way to study the antitumor activity of immune-modulatory drugs, and the associated changes in the circulating cytokines and immune cell populations.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

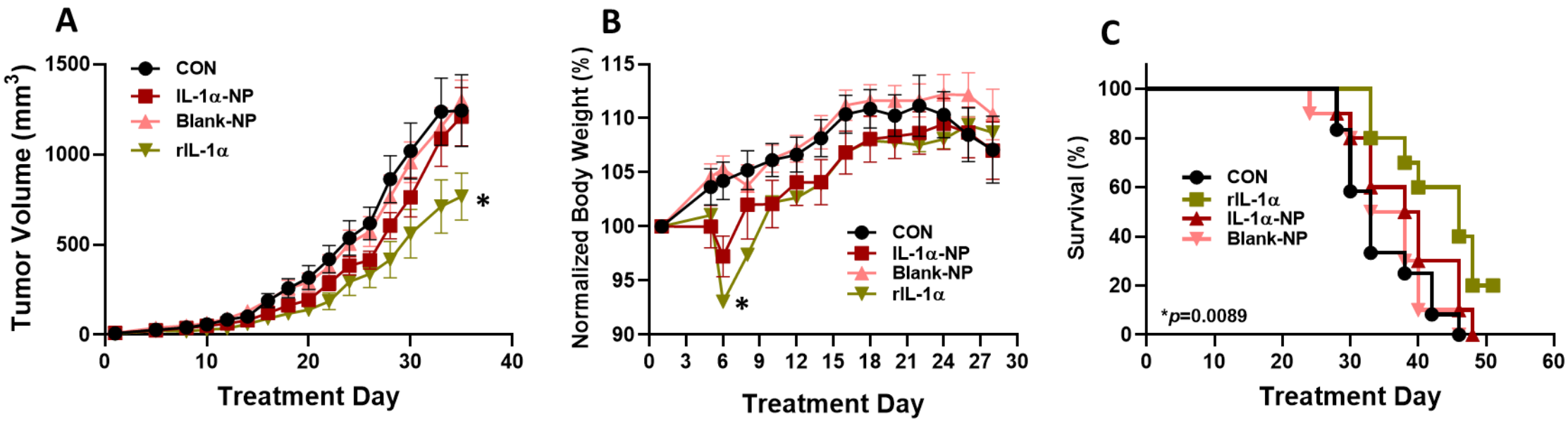

Figure 2

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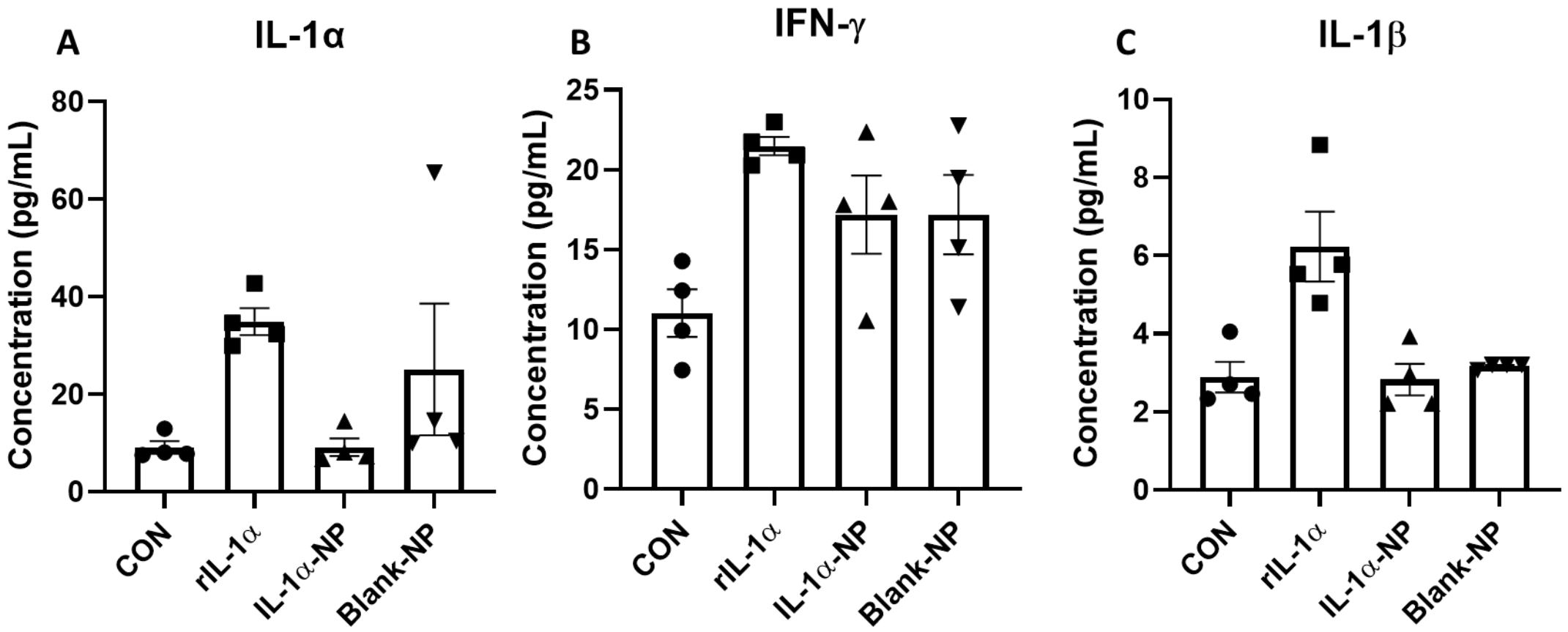

Table of Materials

Name of Material/Equipment	Company	Catalog Number
Bio-Plex 200 Systems	Bio-Rad	
Bio-Plex Pro Mouse Cytokine 23-plex Assay	Bio-Rad	M60009RDPD
C57BL/6J Mice	Jakson Labs	664
DMEM (Dulbecco's Modified Eagle Medium)	Thermo Fisher Scientific	11965092
DMEM/Hams F12 (Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12)	Thermo Fisher Scientific	11320033
EGF	Millipore Sigma	SRP3196-500UG
Fetal Bovine Serum	Millipore Sigma	12103C-500ML
Gentamycin sulfate solution	IBI Scientific	IB02030
gentleMACS Dissociator	Miltenyi biotec	
Hand-Held Magnetic Plate Washer	Thermo Fisher Scientific	EPX-55555-000
Hydrocortisone	Millipore Sigma	H6909-10ML
Insulin	Millipore Sigma	I0516-5ML
Ketamine/xylazine		
MEERL cell line		
Portable Balances	Ohaus	
Scienceware Digi-Max slide caliper	Millipore Sigma	Z503576-1EA
Sterile alcohol prep pad (70% isopropyl alcohol)	Cardinal	COV5110.PMP
Transferrin Human	Millipore Sigma	T8158-100MG
Tri-iodothyronin	Millipore Sigma	T5516-1MG

Comments/Description

The system was provided from the Flow Cytometry Facility University of IOWA Health Care

4 to 6 weeks old

Injectable anesthesia

Murine oropharyngeal epithelial cells stably expressing HPV16 E6/E7 together with hRAS and luciferase (mEERL) cells

Response to Reviewer Comments

We thank the reviewers for the comments received, which led to significant improvements in our manuscript. We have responded to all of the points raised by making changes to the text of our manuscript. These edits are shown in the manuscript in blue font. In addition, below please find our responses in blue font to each of the points made by the reviewer. We kindly request that you consider the revised manuscript for publication in the JoVe. Thank you for your consideration of our work.

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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. **Response:** The manuscript has been proofread.

2. Please revise the following lines to avoid previously published work: 68-70. **Response:** These lines have been revised.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.). **Response:** We have removed these.

4. 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, gentleMACS, LSR Violet, BD Biosciences, etc. **Response:** The trademark symbol and the commercial name have removed accordingly.

5. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method. **Response:** Done.
b) The rationale behind the development and/or use of this technique **Response:** Done.
c) The advantages over alternative techniques with applicable references to previous studies **Response:** Done.
d) A description of the context of the technique in the wider body of literature **Response:** Done.
e) Information to help readers to determine whether the method is appropriate for their application **Response:** Done.

6. Line 89/106/152/157/182: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm). **Response:** Done.

7. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution. **Response:** Done.

8. Line 130: Please replace the unit “ug” with “µg”. **Response:** Done.

9. Line 191/193/196/198: Please include the details of centrifugation. At what speed and temperature is the centrifugation performed? **Response:** Done.

10. Please include a one-line space between each protocol step and then 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. **Response:** Done.

11. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s). **Response:** Done.

12. Please do not use abbreviations for the journal and book titles in the references. 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. **Response:** Done.

13. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Sort the table in alphabetical order. **Response:** Done.

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Reviewers' comments:

Reviewer #1:

1. The figures are missing a legend as well as a description (also of the statistics behind). **Response:** The figure legends are included in the manuscript file as per journal instructions.

2. The nanoparticles alone appear to be releasing IL1a, why is that? This must be interpreted in the MS. **Response:** Thank you for this observation. In many of our immunotherapy experiments involving nanoparticle formulations, we observe cytokine release from blank/empty nanoparticles which is sometimes accompanied by slight anti-tumor immune responses. We believe that the nanoparticles themselves may be of sufficient size and structure that they can either be taken up by dendritic cells and stimulate their activity or they can trigger toll-like receptors on various immune cell types. As per journal instructions we were asked to focus the discussion section in the manuscript on the protocols described and not the discussion/interpretation of experimental results.

Reviewer #2:

1. Introduction and discussion should be focused more around the observations and novelty of this study. More concluding remarks must be also added. **Response:** As per journal instructions we were asked to focus the discussion section in the manuscript on the protocols described and not the discussion/interpretation of experimental results. This is the reason why we did not expand on the novelty of the study.

2. More details for polyanhydride IL-1α nanoparticles preparation. **Response:** Details can be found in reference #15. We have added this reference in the manuscript.

3. The references should be updated **Response:** Done.