

# Quantifying Antibody Responses Induced by Antigen-Agnostic Immunotherapies

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As the development and clinical application of cancer immunotherapies continue to expand, so does the need for novel methods to dissect their mechanisms of action. Antibodies are important effector molecules in cancer therapies due to their potential to bind directly to surface-expressed antigens and facilitate Fc receptor-mediated uptake of antigens by antigen-presenting cells. Quantifying antibodies that are specific for defined antigens is straightforward. However, we describe herein a preclinical method to evaluate tumor-associated and virus-specific antibody responses to antigen-agnostic immunotherapies. This method uses autologous tumor cells as reservoirs of bulk tumor antigens, which can be bound by antibodies from the serum or plasma of tumor-bearing mice. These antibodies can then be detected and quantified using isotype-specific secondary antibodies conjugated to a fluorochrome. Alternatively, virus-infected cells can be used as a source of viral antigens. This method will enable researchers to assess antibody responses following immunotherapies without requiring pre-defined antigens. Alternatively, total virus-specific antibody responses could be studied as an alternative to more limited virus-neutralizing antibody assays. Therefore, this method can facilitate studying the role of humoral responses in the context of immunotherapies, including those that rely on the use of viral vectors.

## INTRODUCTION

### Background

Immunotherapy has become a leading paradigm for the treatment of cancers. Cancer immunotherapy aims to empower a patient's own immune system to target and eliminate their own cancer.<sup>1</sup> Recently, the US Food and Drug Administration has approved several immunotherapies for the treatment of malignancies, including programmed death receptor-1 and cytotoxic T lymphocyte antigen-4 immune checkpoint blockades<sup>2</sup> and the oncolytic virus (OV) T-vec.<sup>3</sup> The current focus of these cancer immunotherapies is to unlock the killing potential of a patient's existing anti-cancer cytotoxic T cells. However, studies are continuing to reveal the contributions of other immunological pathways, both innate and adaptive, in driving potent anti-cancer immune responses.

Cancer therapy has benefitted enormously from the discovery and development of monoclonal antibodies.<sup>4</sup> For treating cancers, several monoclonal antibodies targeting cancer-related antigens have been clinically tested and used, including anti-human epidermal growth factor receptor-2<sup>5,6</sup> and anti-epidermal growth factor receptor,<sup>7,8</sup> for breast and colorectal cancers, respectively. The limited success achieved with cancer-targeting monoclonal antibodies is due to the requirement that patient tumors overexpress the target. However, many tumors can escape from antibodies by selection and amplification of clones that downregulate targets.<sup>9,10</sup> Despite these results, the clinical use of monoclonal antibodies highlights the potential for tumor antigen-targeting antibodies to contribute to cancer therapies and warrants the

investigation of their roles in cancer therapies currently under development.

Antigen-agnostic immunotherapies focus on driving potent immune responses against an array of tumor antigens represented in a patient's tumor, without the requirement to define them. A patient's specific tumor neoantigen catalog is therefore targeted by their existing T cell repertoire, and the efficacy of therapy is proportional to both the tumor neoantigen load and the existence of T cell clones able to recognize them.<sup>11–15</sup> We recently developed a method to detect tumor-specific T cell responses to antigen-agnostic immunotherapies.<sup>16</sup> This method relies on the presentation of bulk tumor antigens in the context of major histocompatibility complexes on cancer cells to T cells *ex vivo*, which can be quantified by flow cytometry. We reasoned that the principle of using cancer cells as targets to detect tumor antigen-specific T cells following the use of antigen-agnostic cancer immunotherapies could be extended to the detection of tumor-associated antibodies.

We describe herein a method similar to the in-cell western blot, which uses autologous tumor cells as reservoirs of bulk tumor

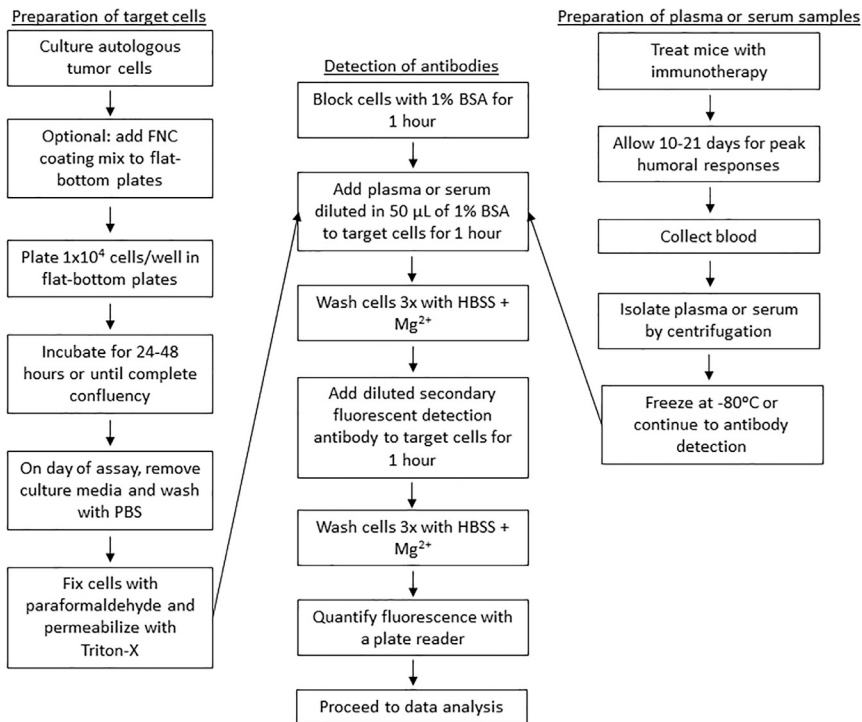
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**Figure 1. Experiment Workflow for the In-Cell Western Blotting Assay, from Preparing Target Tumor Cells, Collecting Plasma from Mice and Detecting Therapy-Induced Antibodies**

- 0.25% EDTA (Corning, NY, USA, reference number [Ref#]25-052-CI)
- PBS (Fisher Scientific, Cat#SH30256.01)
- Cell culture-treated flasks and plates, including 96-well flat-bottom plates
- Fibronectin-collagen (FNC) coating mix (AthenaES, MD, USA, Cat#0407)
- Sample Processing
  - HBSS (Fisher Scientific, Cat#SH3003103)
  - 4% Paraformaldehyde (Fisher Scientific, Cat#J19943-K2)
  - 0.2% Triton X-100 (Sigma-Aldrich, MA, USA, Cat#T8787)
  - BSA (Fisher Scientific, Cat#BP1600100)
- Antibody
  - Goat anti-mouse IgG (H+L)-Alexa Fluor 488 (Fisher Scientific, Cat#A28175). An antibody bound to a different fluorochrome could be used, as long as it can

be detected by a plate reader. Also, a different isotype-specific antibody could be used, depending on the isotype(s) of interest.

antigens to bind to serum- or plasma-derived antibodies that can subsequently be detected using species- and isotype-specific fluorochrome-conjugated antibodies for quantification via assessment of relative fluorescence intensity. This method can also be applied to detecting virus-specific antibodies, which is relevant to any treatment that relies on the use of viruses, such as oncolytic virotherapy. This provides a valuable method that can be added to the toolbox of preclinical cancer researchers to evaluate the role of endogenous antibodies induced by antigen-agnostic immunotherapies and can help inform the design of future cancer therapies.

## MATERIALS

### Reagents

- Retro-Orbital Blood Draw
  - Heparinized microhematocrit capillary tubes (Fisher Scientific, MA, USA, catalog number [Cat#]22-362-566). This allows for a separate assessment of cells, such as T cell responses; alternatively, clotted blood can be used, but this would restrict analysis to serum-derived factors only.
  - 1.5 mL microtubes
  - Gauze pads
  - Eye lubricant
  - Container filled with ice pellets
- Cell Culture
  - Complete DMEM (Fisher Scientific, Cat#SH30022.01) or media specific to the tumor cell line of interest
  - 10% fetal bovine serum (VWR, PA, USA, Cat#97068-085)
  - Penicillin streptomycin cocktail (Fisher Scientific, Cat#SV30010)

### Equipment

- Plate reader capable of detecting fluorescence at a wavelength of 490 nm.

## PROCEDURE

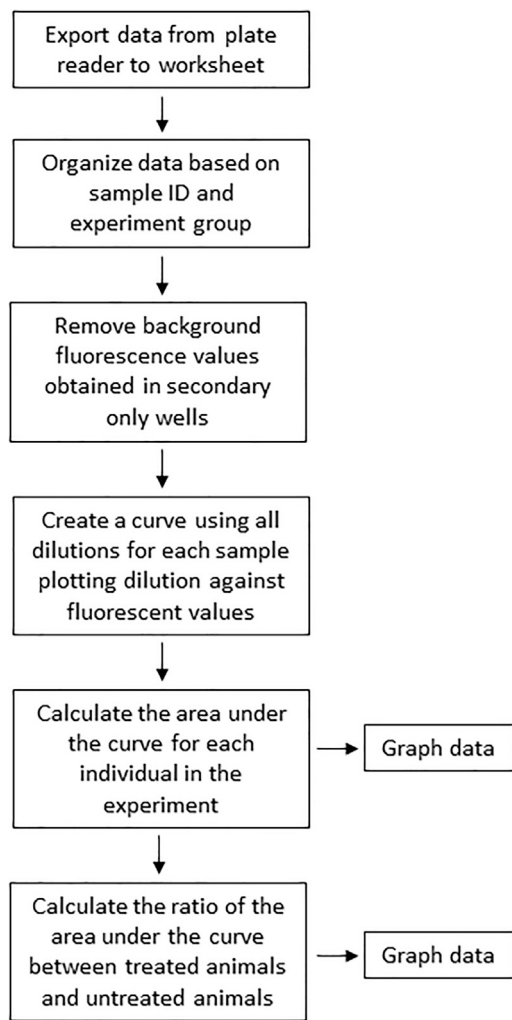
Refer to [Figure 1](#).

### 1. Cell Preparation

- Seed 10,000 healthy target tumor cells per well in a 96-well plate.
  - Target cells should be 100% confluent in each well upon starting the assay. This ensures a high quality signal and prevents binding of the secondary detection antibody to the surface of the plate.
- Incubate target cells overnight at 37°C, 5% CO<sub>2</sub>, and 21% O<sub>2</sub>.

### 2. Collection of Plasma

- Collect blood from mice in 1.5-mL microfuge tubes.
  - Collecting ~200 µL blood will yield ~50 µL plasma for analysis.
  - Maximizing the blood volume will maximize the sensitivity of the assay but adhere to institutional guidelines. The work presented here was approved by the University of Guelph Animal Care Committee and adhered to the policies published by the Canadian Council on Animal Care.
- Place tube containing blood on ice until sample collection is complete.
- Centrifuge blood at 500 × g for 10 min at 4°C.



**Figure 2. Data Analysis Workflow, from Data Export to Graphical Representation**

- Centrifuged samples will separate plasma or serum to the top layer. Collect clear plasma or serum without disturbing the cellular component beneath and aliquot into new tubes.
  - *Pause point*: plasma or serum samples can be stored long-term at  $-80^{\circ}\text{C}$  or can continue to assay.
3. In-Cell Western Assay
- Remove media from 96-well plate and wash cells two times with  $100\ \mu\text{L}$  HBSS+ $\text{Mg}^{2+}$ .
  - Fix cells with  $25\ \mu\text{L}$  3.7% paraformaldehyde, incubating for 10–15 minutes at room temperature.
  - Wash cells three times in  $50\ \mu\text{L}$  HBSS+ $\text{Mg}^{2+}$ .
  - Permeabilize cells with  $50\ \mu\text{L}$  0.2% Triton X-100, incubating for 10 min at room temperature.
    - This step can be skipped if the researcher only wants to target antibodies against surface antigens.
  - Wash cells three times in  $50\ \mu\text{L}$  HBSS+ $\text{Mg}^{2+}$ .

- Block cells with 1% BSA in  $50\ \mu\text{L}$  total volume of PBS, incubating for 1 h at room temperature.
- *Pause point*: blocking can be done overnight at  $4^{\circ}\text{C}$ .
- Begin thawing plasma or serum *on ice* 1 h before intended use.
- Prepare dilutions of plasma or serum samples in 1% BSA-PBS in a 96-well plate for easy transfer.
  - Recommended dilutions: 1/10, 1/100, 1/200, 1/500, and 1/1,000.
  - A range of dilutions should be tested for each experiment to identify one that is optimal (the range will vary depending on the concentration of antibodies induced by a given therapy, with more potent therapies requiring a greater dilution range).
- Remove the blocking solution.
- Add  $50\ \mu\text{L}$  diluted plasma or serum samples to target cells.
  - Do not add plasma or serum to one row of target cells. This will serve as a secondary antibody-only background control.
- Incubate for 1 h at room temperature.
- *Pause point*: incubation with plasma or serum can be extended to overnight at  $4^{\circ}\text{C}$ .
- Remove plasma or serum from wells and wash three times with  $50\ \mu\text{L}$  HBSS+ $\text{Mg}^{2+}$  to remove unbound antibodies.
- Dilute the secondary antibody, Alexafluor-488-conjugated goat anti-mouse IgG(H+L), to 1/2,000 in 1% BSA-PBS.
- Add  $50\ \mu\text{L}$  secondary detection antibody to each well (including the row of control wells that get treated with the secondary antibody only) and incubate for 1 h at room temperature in the dark.
- Remove the secondary detection antibody and wash three times with  $50\ \mu\text{L}$  HBSS+ $\text{Mg}^{2+}$ .
- Quantify fluorescence using a plate reader with a 490 nm blue filter.
- Optional (but recommended): confirm the quality of antibody detection using a fluorescent microscope.
  - Secondary-only controls should have little to no fluorescent background.
  - Test samples for which antibody responses are expected should have an intact monolayer of fixed cells with variable fluorescence.

#### 4. Data Analysis

- Refer to [Figure 2](#).
- Data can be plotted as fluorescent units (FU) and then used to calculate the area under the curve formed after plotting the results from the five-dilution series on a x-y graph.
- The mean FU from control wells treated with the secondary antibody only define the background signal and can be subtracted from the fluorescence intensity of experimental samples.
- Data can be expressed as area under the curve following subtraction of the background or as the fold-change in area under the curve of treated mice compared to untreated mice.

#### Timing

##### **Approximate Time Based on an Experiment with 20 Mice**

- Typical time to detectable antibody response: 10–21 days.
- Kinetics of antibody responses can be determined using this method by sampling blood on multiple days; we chose

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1A (1:10)	Sample 1B (1:10)	Sample 2A (1:10)	Sample 2B (1:10)	Sample 3A (1:10)	Sample 3B (1:10)	Sample 4A (1:10)	Sample 4B (1:10)	Sample 5A (1:10)	Sample 5B (1:10)	Secondary Only	Secondary Only
B	Sample 1A (1:100)	Sample 1B (1:100)	Sample 2A (1:100)	Sample 2B (1:100)	Sample 3A (1:100)	Sample 3B (1:100)	Sample 4A (1:100)	Sample 4B (1:100)	Sample 5A (1:100)	Sample 5B (1:100)	Secondary Only	Secondary Only
C	Sample 1A (1:200)	Sample 1B (1:200)	Sample 2A (1:200)	Sample 2B (1:200)	Sample 3A (1:200)	Sample 3B (1:200)	Sample 4A (1:200)	Sample 4B (1:200)	Sample 5A (1:200)	Sample 5B (1:200)	Secondary Only	Secondary Only
D	Sample 1A (1:500)	Sample 1B (1:500)	Sample 2A (1:500)	Sample 2B (1:500)	Sample 3A (1:500)	Sample 3B (1:500)	Sample 4A (1:500)	Sample 4B (1:500)	Sample 5A (1:500)	Sample 5B (1:500)	Secondary Only	Secondary Only
E	Sample 6A (1:10)	Sample 6B (1:10)	Sample 7A (1:10)	Sample 7B (1:10)	Sample 8A (1:10)	Sample 8B (1:10)	Sample 9A (1:10)	Sample 9B (1:10)	Sample 10A (1:10)	Sample 10B (1:10)	Blank	Blank
F	Sample 6A (1:100)	Sample 6B (1:100)	Sample 7A (1:100)	Sample 7B (1:100)	Sample 8A (1:100)	Sample 8B (1:100)	Sample 9A (1:100)	Sample 9B (1:100)	Sample 10A (1:100)	Sample 10B (1:100)	Blank	Blank
G	Sample 6A (1:200)	Sample 6B (1:200)	Sample 7A (1:200)	Sample 7B (1:200)	Sample 8A (1:200)	Sample 8B (1:200)	Sample 9A (1:200)	Sample 9B (1:200)	Sample 10A (1:200)	Sample 10B (1:200)	Blank	Blank
H	Sample 6A (1:500)	Sample 6B (1:500)	Sample 7A (1:500)	Sample 7B (1:500)	Sample 8A (1:500)	Sample 8B (1:500)	Sample 9A (1:500)	Sample 9B (1:500)	Sample 10A (1:500)	Sample 10B (1:500)	Blank	Blank

**Figure 3. Example of a Standard Plate Setup, Including Recommended Plasma Dilutions and Control Wells**

days 10 (pre-peak) and 21 (approximate peak of response) post-treatment.

- Plating target cells: 12–24 h to achieve 100% confluency (seeding density should be optimized)
- Blood sampling: 1 h
- Plasma or serum collection: 20 min
- Cell fixation and permeabilization: 30 min
- Blocking and binding of plasma or serum-derived antibodies: 1.5 h (or overnight)
- Detection with a secondary antibody: 1 h
- Data collection: 20 min
- Total time from plasma or serum collection to the end of data acquisition: 4 h
- Note: this experimental protocol contains a total of three potential pause points. However, pause points during the in-cell western assay portion should be avoided for optimal results.

### Troubleshooting

#### Complete Target Cell Monolayers

The outcome of this assay depends on the use of cells that serve as reservoirs of target antigens. The antibodies are derived from mouse plasma or serum and bind to antigens in or on permeabilized tumor cells. Antibodies bound to antigens that are retained after washing are detected by an anti-mouse immunoglobulin G (IgG) secondary antibody conjugated to a fluorochrome (this antibody could be switched to look for other isotypes). For adherent tumor cells, each well must consist of a confluent monolayer to ensure a consistent number of available targets for all samples and to minimize background fluorescence due to non-specific binding of secondary antibodies to the plate. Incomplete monolayers or loss of cells during the experimental procedure can result in variability and artificially low on-target signals due to a reduction in the quantity of target antigens.

Adherent tumor cell lines vary in their ability to adhere to commercially available polymer-coated culture plates. To maximize adhesion

of target tumor cells, we pre-coat cell culture plates with FNC coating mix. Additionally, all washing steps are performed with HBSS containing  $Mg^{2+}$ , which retains ions critical for cellular adhesion. Before beginning the assay, wells should be visualized by brightfield microscopy to ensure confluency, and any that do not meet quality control criteria should be excluded. The cell monolayers should be inspected again just prior to or just after quantification of fluorescence as a final quality control check.

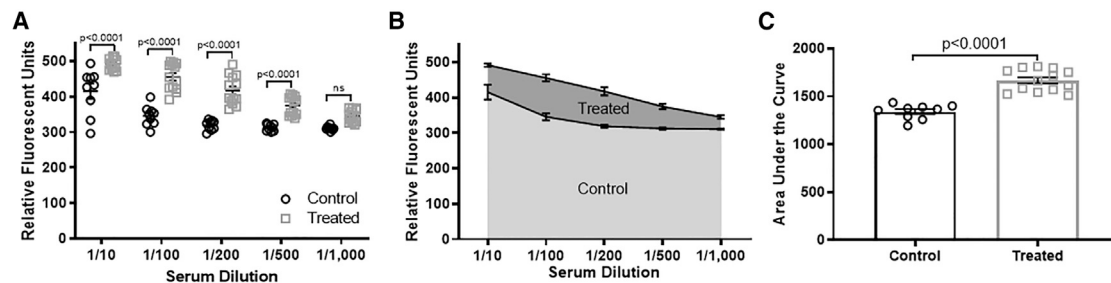
Tumor cells differ in their growth kinetics and response to contact-inhibition. It is important to evaluate each target cell line for the optimal initial seeding density and growth time to achieve complete confluency at the time of the assay.

#### Dilution of Plasma or Serum Samples and the Secondary Detection Antibody

The sensitivity of this assay relies on optimizing the dilution of plasma or serum samples. At extremely high plasma or serum concentrations, there is an increased risk of non-specific binding of antibodies to target cells, leading to a plateau in terms of how much fluorescence a plate reader can detect. To resolve these issues, it is recommended that a range of plasma or serum dilutions are included for each sample in each experiment, especially if relatively high-magnitude secondary responses are being assessed. This can be easily accommodated in the 96-well plate format (Figure 3). Figure 4 shows results using an ideal dilution range for plasma samples, thereby facilitating relative quantification of antibodies by calculating areas under the curves. This harnesses the power of the dynamic range of the assay. To confirm the selection of proper dilutions, samples should be visualized by fluorescent microscopy.

Similarly, the fluorochrome-conjugated secondary antibody should be tested at a range of dilutions to ensure optimal detection of plasma- or serum-derived antibodies bound to target cells, without producing a substantial signal in target cells that have been treated with only the





**Figure 4. Immunotherapy-Induced Antibody Responses Detected in the Plasma of Mice Treated with an Oncolytic Virus**

Plasma was collected from C57BL/6 mice bearing orthotopic, syngeneic ID8 ovarian carcinomas 10 days following treatment with oncolytic OrfV or immune checkpoint blockade (anti-PD-1). Control mice were untreated. ID8 cells were used as targets for the in-cell western blotting assay, in which plasma was diluted across a range from 1:10 to 1:1,000 before detection using AlexaFluor488-conjugated rabbit anti-mouse IgG diluted 1/2,000. Fluorescence was quantified with a plate reader using excitation and emission wavelengths of 490 nm and 525 nm, respectively. (A) Immunotherapy-induced antibody responses across the full range of plasma dilutions represented as relative fluorescent units following removal of secondary antibody-only control values. To capture the effects of the dynamic range of this assay, these data were used to calculate (B) areas under the curve, which could also be shown as (C) bar graphs. Data were from a single representative *in vivo* experiment in which archived aliquots of plasma were tested at three independent times using the same assay. Technical duplicates were included in each test. The data points represent the mean fluorescence across all three tests. Means and SEs are shown, with p values determined by (A) two-way analysis of variance using Tukey's multiple comparisons test and (C) Student's unpaired two-tailed t test.

secondary antibody. We have identified a 1:2,000 dilution as optimal in our experiments. However, this should be tested for every secondary antibody that is used. This can be easily accommodated in the 96-well plate format. Proper dilutions of secondary antibodies can be confirmed by fluorescent microscopy.

#### Preparation of Assay Controls

**Secondary Antibody-Only Background Controls.** Non-specific binding of secondary antibodies can occur, especially when they are used at high concentrations. We recommend testing a series of dilutions of the secondary antibody with target cells to determine a dilution that fails to yield a significant fluorescent signal on the plate reader. This can be confirmed by fluorescent microscopy and should yield results similar to wells with target cells that were not treated with the secondary antibody. When quantifying immunotherapy-induced antibody responses, secondary antibody-only controls are required to remove background cellular auto-fluorescence from all experimental data.

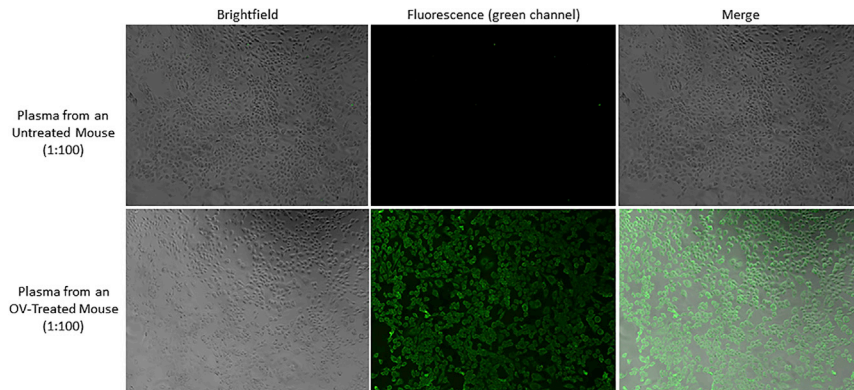
**Off-Target Cell Controls.** In-cell western blotting can be conducted with tumor cells that are different from those used for tumor implantation. This would serve to identify antibody responses against antigens shared between different cancer cells. Alternatively, or in addition, normal cells could be used to determine if antibodies are detecting antigens that are not cancer-specific. Note that, in many cases, antibody responses to "normal cell" controls would be expected. For example, if treating melanomas, off-target responses to normal melanocytes that share immunodominant antigens (e.g., enzymes associated with melanogenesis) could be substantial. Also, antibody responses to so-called "universal" tumor-associated antigens, such as telomerase reverse transcriptase or survivin, would be expected to cross-react with many normal cells that also express these, albeit typically at lower levels. Therefore, it is important to include a sham-treated control group in each experiment to prove that antibody responses are truly therapy-induced.

**Uninfected Off-Target Cell Controls for Virus-Specific Antibody Detection.** This control is critical for reducing background when applying this technique to detecting virus-specific antibodies following OV therapy. Oncolytic virotherapy could induce antibody responses against conserved cellular antigens that are not tumor-specific. Although utilizing cells from a different species mitigates this risk, the inclusion of uninfected off-target cell controls allows for background fluorescence readings from these wells to be subtracted from the values obtained from test wells.

#### Anticipated Results

##### Detecting Immunotherapy-Induced Antibody Responses in Mice Treated with OVs

To test the in-cell western blotting protocol for detecting immunotherapy-induced antibodies, we used a C57BL/6 murine model of orthotopic, syngeneic ID8 epithelial ovarian carcinomas, as has been previously described.<sup>17</sup> Sixty days following tumor implantation, we treated mice with an OV known as Orf virus (OrfV<sup>18</sup>) that was injected into the peritoneal cavity. Ten days following treatment, blood was collected and plasma harvested for the in-cell western blotting assay. ID8 cells were used as targets and plasma samples were diluted following the format shown in Figure 3. Plasma samples were run through the in-cell western blotting protocol in three independent assays, with each assay containing intra-assay secondary antibody-only controls. Plasma samples collected from tumor-bearing mice that were untreated yielded relatively low fluorescent signals following removal of background (Figure 4). In contrast, mice treated with the OV had evidence of therapy-induced antibody responses. Greater fluorescent signaling in test wells compared to control wells was confirmed by fluorescent microscopy (Figure 5). These significant differences between treated and untreated mice demonstrated that the in-cell western blotting assay could detect treatment-induced changes in the antibody repertoire following administration of an immunotherapy expected to induce a humoral response. It is important



**Figure 5. Visualization of Immunotherapy-Induced Antibody Responses by Fluorescent Microscopy**

Plasma from an untreated tumor-bearing mouse (top panels) and serum from a tumor-bearing mouse that received oncolytic virotherapy (OV; bottom panels) were analyzed using the in-cell western blotting protocol. Plasma samples were diluted 1:100 and added to permeabilized and fixed tumor cells in plates. Therapy-induced antibodies bound to cell-derived antigens were detected by a rabbit anti-mouse IgG conjugated to AlexaFluor488 diluted 1/2,000. Brightfield and fluorescent images were captured with a camera mounted on a microscope at 20 $\times$  magnification, with identical exposures for untreated and OV-treated samples.

to note that these antibody responses were “therapy-induced” or “tumor-associated” and not necessarily tumor-specific, as some responses against antigens shared with off-target normal cells is common with many antigen-agnostic approaches.

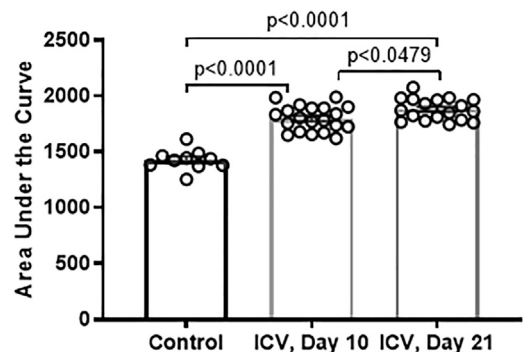
#### **Evaluating the Kinetics of a Tumor-Associated Antibody Response Induced by OV Therapy**

To demonstrate the potential of using the in-cell western blotting assay to assess the kinetics of an antibody response, BALB/c mice were challenged intravenously with syngeneic K7M2 osteosarcoma cells to establish lung metastases. Four days following tumor challenge, mice were treated with an immunotherapy in the form of an OrfV-infected K7M2 cell vaccine (ICV). Since blood collection is a non-terminal procedure, plasma could be obtained from mice 10 and 21 days following treatment. K7M2 cells were used as target cells, and plasma samples were analyzed in three separate in-cell western blotting assays. Mice treated with the ICV had significantly higher magnitude tumor-associated plasma antibody responses compared to untreated control mice at day 10 (Figure 6), which significantly increased by 21 days following treatment. Untreated tumor-bearing mice had reached endpoint prior to the second blood collection, therefore precluding our ability to include these controls at the second time point. This confirmed the in-cell western blotting assay could be applied to analyze the kinetics of antibody responses induced by cancer immunotherapies.

#### **Detecting OV-Specific Antibodies**

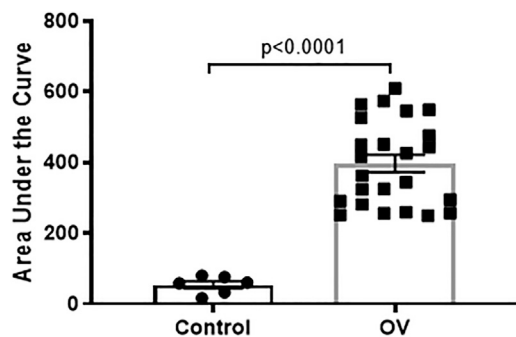
Due to the immunogenic nature of viruses, OV therapies induce potent virus-specific immune responses. We reasoned that using off-target cells infected with the OV would enable the in-cell western protocol to be applied to detecting virus-specific antibodies. C57BL/6 mice bearing orthotopic ID8 ovarian cancers were treated with OrfV 60 days following tumor challenge, with plasma collected 10 days following initiation of treatment. Vero African monkey kidney cells were used as off-target cells, as they should share relatively few antigens with murine tumor cells. Vero cells were plated and allowed to adhere before infection with OrfV at an MOI of 10 for 12 h. We reasoned that this would expose every cell to the virus to maximize production of viral proteins, without killing a substantial number of cells. The in-cell western blotting assay was then conducted as previ-

ously described, except for the addition of uninfected Vero cells as a negative control. Mice treated with OrfV had potent virus-specific antibody responses, as depicted by an overall increase in fluorescent values compared to plasma from untreated animals (Figure 7). A gold standard for quantifying virus-specific antibody responses is by virus neutralization assays, wherein infection is inhibited by pre-treatment of permissive cells with antibodies present in serum or plasma. Virus neutralization can be analyzed by plaque assays or flow cytometry.<sup>19,20</sup> However, the importance of non-neutralizing antibodies in resistance to viral infections, which, by definition, would not be detected by classical virus-neutralization assays, is well described.<sup>21–24</sup> The in-cell western blotting technique is not limited to detecting neutralizing antibodies, as both structural and non-structural virus proteins would be expressed by infected target cells. The application of this technique to detecting virus-specific



**Figure 6. Kinetics of Immunotherapy-Induced Antibody Responses Induced by an Oncolytic Virus**

BALB/c mice bearing syngeneic K7M2 osteosarcoma lung metastases were treated with an Orf virus-infected K7M2 cell vaccine (ICV) 4 days following tumor challenge. Plasma was collected 10 and 21 days following therapy. In-cell western blotting was performed using K7M2 cells as targets, with plasma diluted across a range from 1:10 to 1:1,000 and detected by rabbit anti-mouse IgG conjugated to AlexaFluor488 diluted 1/2,000. Tumor-induced antibody responses were represented as areas under the curves after determination of relative fluorescent units and removal of secondary antibody-only control values. Means and SEs are shown, with p values determined by one-way analysis of variance using Tukey’s multiple comparisons test.



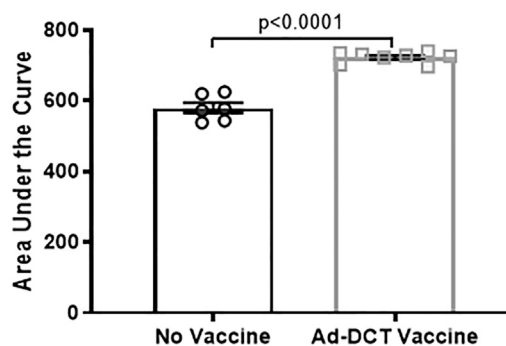
**Figure 7. Detection of Virus-Specific Antibodies**

Plasma was collected from C57BL/6 mice bearing orthotopic, syngeneic ID8 ovarian carcinomas 10 days following treatment with oncolytic OrV. Control mice were untreated. Vero African monkey kidney cells infected with the oncolytic virus were used as target cells for the in-cell western blotting assay, and plasma was diluted across a range from 1:10 to 1:1,000 before addition to the cells. Plasma-derived antibodies were detected with rabbit anti-mouse IgG conjugated to AlexaFluor488 diluted 1/2,000. Fluorescence was quantified with a plate reader at excitation and emission wavelengths of 490 nm and 525 nm, respectively. Oncolytic virus-specific antibodies were quantified as areas under the curves after calculating relative fluorescent units and subtracting mean background values determined from control cells. Means and SEs are shown, with p values determined using an unpaired two-tailed Student's t test.

antibodies will endow researchers with a tool to model antibody responses to the entire repertoire of viral antigens, which can be combined with traditional assays to identify neutralizing antibodies.

In conclusion, this protocol provides a detailed methodology for detecting tumor-associated antibody responses following antigen-agnostic immunotherapies. In theory, the in-cell western blotting method could be applied to any antigen-agnostic therapy capable of inducing tumor-associated antibodies, including, but not limited to, chemo- and radiation therapies that induce immunogenic cancer cell death, and should be amenable to any transplantable murine tumor model. Autologous tumor cells serve as reservoirs of target antigens and, theoretically, every relevant tumor antigen not generated *de novo* in the animal is represented. Therefore, this method could also be potentially useful for assessing antigen spreading following antigen-specific immunotherapies. Further, we demonstrated that this method can also serve as an alternative to other techniques for assessing antibody responses to pre-defined tumor antigens by expressing the known target antigen in a cell line that normally lacks expression of the protein (Figure 8).

The experiments presented herein apply the antibody detection method to primary responses to tolerized tumor-antigens, which are expected to be of low magnitude. This method would also be useful for detecting secondary antibody responses, for example, in patients that have pre-existing tumor-associated antibodies at the time of initial treatment or that receive multiple rounds of treatment. Blood collection is not terminal, so therapy-induced antibody responses can be monitored over time in the same animal and correlated to clinically relevant outcomes, such as survival. The protocol



**Figure 8. Using the In-Cell Western Blotting Assay to Quantify Antibody Responses Induced by a Vaccine Targeting a Defined Antigen**

Tumor-free female C57BL/6 mice were vaccinated intramuscularly with  $1 \times 10^8$  infectious units of a recombinant human serotype 5 adenovirus expressing the human melanoma-associated antigen dopachrome tautomerase (DCT). Plasma-derived IgG isotype antibodies specific for human DCT were quantified 33 days post-vaccination using the in-cell western blotting assay, in which Vero cells infected with vesicular stomatitis virus expressing human DCT were used as a source of bulk antigens. Mean areas under the curves were calculated following determination of relative fluorescent units for all five plasma dilutions that were tested. These were plotted, along with SEs and compared using Student's unpaired two-tailed t test.

presented here has been optimized for adherent tumor cell lines, but, in principle, could be applied to cancer cells that grow in suspension, extending the application to hematological malignancies such as leukemias if flow cytometry were to be used to quantify fluorescence. More detailed studies of tumor-associated or virus-specific antibody responses can be performed using secondary detection antibodies against other immunoglobulin isotypes. The studies described herein focused on detection of IgG, but detection of other isotypes, such as IgM or IgA, could be easily included and used to analyze class-switching and type 1 versus type 2 immune response biases throughout the course of the antibody response. Since the only major prerequisite for this protocol is the existence of a target tumor cell line, we envision that this method could be applied clinically. For example, tumor cells could be collected from patients and cultured to generate polyclonal cell lines *ex vivo*. Samples to facilitate this would be acquired anyway for therapies such as infected cell vaccines. Patient-derived tumor cell lines would then be applied in the method presented herein to monitor therapy-induced antibody responses. Indeed, we conducted proof-of-principle studies to demonstrate the translational potential of this method. Specifically, we successfully generated polyclonal cancer cell lines from osteosarcoma metastases excised from two dogs. In both cases, this was accomplished in less than 3 weeks. This timing would facilitate the assessment of most primary antibody responses at or near their peak.

#### AUTHOR CONTRIBUTIONS

Conception and Design, J.P.v.V., K.K., G.M., J.J.P., S.K.W., and B.W.B.; Development of Methodology, J.P.v.V., E.M.K., and B.W.B.; Acquisition of Data: J.P.v.V., E.M.K., and B.W.B.; Analysis and Interpretation of Data, J.P.v.V., E.M.K., K.K., G.M., J.J.P., S.K.W., and

B.W.B.; Writing, Review, and/or Revision of the Manuscript, J.P.v.V., E.M.K., K.K., G.M., J.J.P., S.K.W., and B.W.B.

## CONFLICTS OF INTEREST

The authors declare no competing interests.

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## REFERENCES

- Mellman, I., Coukos, G., and Dranoff, G. (2011). Cancer immunotherapy comes of age. *Nature* 480, 480–489.
- Topalian, S.L., Drake, C.G., and Pardoll, D.M. (2015). Immune checkpoint blockade: a common denominator approach to cancer therapy. *Cancer Cell* 27, 450–461.
- Pol, J., Kroemer, G., and Galluzzi, L. (2015). First oncolytic virus approved for melanoma immunotherapy. *OncoImmunology* 5, e1115641.
- Weiner, L.M., Surana, R., and Wang, S. (2010). Monoclonal antibodies: versatile platforms for cancer immunotherapy. *Nat. Rev. Immunol.* 10, 317–327.
- Vogel, C.L., Cobleigh, M.A., Tripathy, D., Gutheil, J.C., Harris, L.N., Fehrenbacher, L., Slamon, D.J., Murphy, M., Novotny, W.F., Burchmore, M., et al. (2002). Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J. Clin. Oncol.* 20, 719–726.
- Viani, G.A., Afonso, S.L., Stefano, E.J., De Fendi, L.I., and Soares, F.V. (2007). Adjuvant trastuzumab in the treatment of her-2-positive early breast cancer: a meta-analysis of published randomized trials. *BMC Cancer* 7, 153.
- Rowland, A., Dias, M.M., Wiese, M.D., Kichenadasse, G., McKinnon, R.A., Karapetis, C.S., and Sorich, M.J. (2015). Meta-analysis of BRAF mutation as a predictive biomarker of benefit from anti-EGFR monoclonal antibody therapy for RAS wild-type metastatic colorectal cancer. *Br. J. Cancer* 112, 1888–1894.
- Sorich, M.J., Wiese, M.D., Rowland, A., Kichenadasse, G., McKinnon, R.A., and Karapetis, C.S. (2015). Extended RAS mutations and anti-EGFR monoclonal antibody survival benefit in metastatic colorectal cancer: a meta-analysis of randomized, controlled trials. *Ann. Oncol.* 26, 13–21.
- Ferris, R.L., Jaffee, E.M., and Ferrone, S. (2010). Tumor antigen-targeted, monoclonal antibody-based immunotherapy: clinical response, cellular immunity, and immunoscape. *J. Clin. Oncol.* 28, 4390–4399.
- Garrett, J.T., Olivares, M.G., Rinehart, C., Granja-Ingram, N.D., Sánchez, V., Chakrabarty, A., Dave, B., Cook, R.S., Pao, W., McKinley, E., et al. (2011). Transcriptional and posttranslational up-regulation of HER3 (ErbB3) compensates for inhibition of the HER2 tyrosine kinase. *Proc. Natl. Acad. Sci. USA* 108, 5021–5026.
- Gubin, M.M., Artyomov, M.N., Mardis, E.R., and Schreiber, R.D. (2015). Tumor neoantigens: building a framework for personalized cancer immunotherapy. *J. Clin. Invest.* 125, 3413–3421.
- Schumacher, T.N., and Schreiber, R.D. (2015). Neoantigens in cancer immunotherapy. *Science* 348, 69–74.
- McGranahan, N., Furness, A.J., Rosenthal, R., Ramskov, S., Lyngaa, R., Saini, S.K., Jamal-Hanjani, M., Wilson, G.A., Birkbak, N.J., Hiley, C.T., et al. (2016). Clonal neoantigens elicit T cell immunoreactivity and sensitivity to immune checkpoint blockade. *Science* 351, 1463–1469.
- Ott, P.A., Hu, Z., Keskin, D.B., Shukla, S.A., Sun, J., Bozym, D.J., Zhang, W., Luoma, A., Giobbie-Hurder, A., Peter, L., et al. (2017). An immunogenic personal neoantigen vaccine for patients with melanoma. *Nature* 547, 217–221.
- Snyder, A., Makarov, V., Merghoub, T., Yuan, J., Zaretsky, J.M., Desrichard, A., Walsh, L.A., Postow, M.A., Wong, P., Ho, T.S., et al. (2014). Genetic basis for clinical response to CTLA-4 blockade in melanoma. *N. Engl. J. Med.* 371, 2189–2199.
- van Vloten, J.P., Santry, L.A., McAusland, T.M., Karimi, K., McFadden, G., Petrik, J.J., Wootton, S.K., and Bridle, B.W. (2019). Quantifying Antigen-Specific T Cell Responses When Using Antigen-Agnostic Immunotherapies. *Mol. Ther. Methods Clin. Dev.* 13, 154–166.
- Matuszewska, K., Santry, L.A., van Vloten, J.P., AuYeung, A.W.K., Major, P.P., Lawler, J., Wootton, S.K., Bridle, B.W., and Petrik, J. (2019). Combining Vascular Normalization with an Oncolytic Virus Enhances Immunotherapy in a Preclinical Model of Advanced-Stage Ovarian Cancer. *Clin Cancer Res* 25, 1624–1638.
- Rintoul, J.L., Lemay, C.G., Tai, L.H., Stanford, M.M., Falls, T.J., de Souza, C.T., Bridle, B.W., Daneshmand, M., Ohashi, P.S., Wan, Y., et al. (2012). ORFV: a novel oncolytic and immune stimulating parapoxvirus therapeutic. *Mol Ther* 20, 1148–1157.
- Earl, P.L., Americo, J.L., and Moss, B. (2003). Development and use of a vaccinia virus neutralization assay based on flow cytometric detection of green fluorescent protein. *J. Virol.* 77, 10684–10688.
- Kraus, A.A., Messer, W., Haymore, L.B., and de Silva, A.M. (2007). Comparison of plaque- and flow cytometry-based methods for measuring dengue virus neutralization. *J. Clin. Microbiol.* 45, 3777–3780.
- Laidlaw, B.J., Decman, V., Ali, M.A., Abt, M.C., Wolf, A.I., Monticelli, L.A., Mozdzanowska, K., Angelosanto, J.M., Artis, D., Erikson, J., and Wherry, E.J. (2013). Cooperativity between CD8+ T cells, non-neutralizing antibodies, and alveolar macrophages is important for heterosubtypic influenza virus immunity. *PLoS Pathog.* 9, e1003207.
- DiLillo, D.J., Palese, P., Wilson, P.C., and Ravetch, J.V. (2016). Broadly neutralizing anti-influenza antibodies require Fc receptor engagement for in vivo protection. *J. Clin. Invest.* 126, 605–610.
- Frey, G., Chen, J., Rits-Volloch, S., Freeman, M.M., Zolla-Pazner, S., and Chen, B. (2010). Distinct conformational states of HIV-1 gp41 are recognized by neutralizing and non-neutralizing antibodies. *Nat. Struct. Mol. Biol.* 17, 1486–1491.
- Carragher, D.M., Kaminski, D.A., Moquin, A., Hartson, L., and Randall, T.D. (2008). A novel role for non-neutralizing antibodies against nucleoprotein in facilitating resistance to influenza virus. *J. Immunol.* 181, 4168–4176.