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THERAPY

Quantifying Antigen-Specific T Cell Responses When Using Antigen-Agnostic Immunotherapies

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Immunotherapies are at the forefront of the fight against cancers, and researchers continue to develop and test novel immunotherapeutic modalities. Ideal cancer immunotherapies induce a patient's immune system to kill their own cancer and develop long-lasting immunity. Research has demonstrated a critical requirement for CD8^{+} and CD4^{+} T cells in achieving durable responses. In the path to the clinic, researchers require robust tools to effectively evaluate the capacity for immunotherapies to generate adaptive anti-tumor responses. To study functional tumor-specific T cells, researchers have relied on targeting tumor-associated antigens (TAAs) or the inclusion of surrogate transgenes in pre-clinical models, which facilitate detection of T cells by using the targeted antigen(s) in peptide re-stimulation or tetramer-staining assays. Unfortunately, many pre-clinical models lack a defined TAA, and epitope mapping of TAAs is costly. Surrogate transgenes can alter tumor engraftment and influence the immunogenicity of tumors, making them less relevant to clinical tumors. Further, some researchers prefer to develop therapies that do not rely on pre-defined TAAs. Here, we describe a method to exploit major histocompatibility complex expression on murine cancer cell lines in a co-culture assay to detect T cells responding to bulk, undefined, tumor antigens. This is a tool to support the preclinical evaluation of novel, antigen-agnostic immunotherapies.

INTRODUCTION

Immunotherapies for the treatment of cancers rely on unlocking the potential of a patient's immune system to kill neoplastic cells. The strategies to accomplish this are diverse, but generally rely on activating T cell clones capable of targeting tumor-associated antigens (TAAs). Notably, conventional T cells are emphasized as key effectors because high numbers of these infiltrating the tumor microenvironment correlates with improved prognosis.[1] One way to induce tumor-specific T cells is with oncolytic virotherapy, highlighted by US Food and Drug Administration (FDA) approval of the recombinant herpesvirus talimogene laherparepvec (T-Vec).[2] Oncolytic viruses (OVs) are multimodal anticancer agents that can directly target and kill tumor cells in an immunogenic fashion, culminating in the release of tumor antigens and danger signals that promote inflammation, recruit immunological effector cells, and stimulate anticancer immunity.[3] Elucidating the mechanisms by which OVs induce antitumor immune responses, particularly T cell responses, is of considerable interest to researchers who aim to provide durable cures and induce immunological memory. Moving forward, it is critical that researchers possess a comprehensive toolbox for evaluating tumor-specific T cell responses in pre-clinical models of immunotherapies that are destined for the clinic.

Assessment of functional tumor-specific T cell responses currently relies on techniques centered around defined target antigens. For some preclinical models, antigens have been well-characterized, such as dopachrome tautomerase (DCT; tyrosinase-related protein-2) for melanomas.[4] For models where no tumor antigen has been defined, exogenous surrogate antigens like ovalbumin[5,6] can be stably introduced to tumor cell lines and used to evaluate T cell responses through *ex vivo* peptide re-stimulation or tetramer staining. Despite their usefulness in this regard, exogenous antigens can alter immunogenicity of cancer cell lines, which impacts engraftment and immunoediting as tumors develop. In addition, surrogate antigens should not be expected to engage the T cell compartment in the same way as endogenous tumor antigens.

Both techniques of either directly targeting a defined tumor antigen or introducing a model antigen enable researchers to monitor T cells responding to those antigens in circulation. Blood sampling is nonlethal and, therefore, T cell responses can be examined during the course of treatment and correlated with important outcomes such as tumor growth and survival. For tumor models that lack defined tumor antigens or surrogate antigens, researchers often sacrifice animals and enumerate T cells directly in tumor tissues by flow cytometry.[7] Also, many researchers are concerned about antigen-directed therapies being limited to patients with cancers that express the target(s). To circumvent this, many prefer the concept of antigen-agnostic immunotherapies that allow each patient's immune system to determine its own antigen specificities.[8]

Detecting primary tumor-specific T cell responses following immunotherapy is challenging because they are generally of low magnitude since many tumor antigens are self-derived. Tumor neoantigens are developed through multiple mechanisms, including the accumulation of mutations left unchecked by abnormal DNA repair machinery in cancer cells, and represent altered-self proteins that can be recognized by T cells that escaped negative selection in the thymus.[9,10] Cancers that have a high neoantigen load have been shown to respond better to immunotherapies, including checkpoint inhibitors, providing strong evidence that T cell responses against neoantigens are functional.[11–13]

We reasoned that tumor cell lines used to generate preclinical transplantable tumor models in mice would contain relevant tumor antigens and/or neoantigens capable of engaging the T cell compartment. Autologous tumor cells would thus serve as sources of antigens for detecting tumor-specific T cells in circulation following immunotherapy, such as oncolytic virotherapy and infected cell vaccine strategies, without the need to previously define a target tumor antigen.

Recognition of tumor antigens by T cells relies on efficient loading of major histocompatibility complex (MHC) molecules with antigens and their transport to the cell surface. CD8$^+$ and CD4$^+$ T cells recognize antigens in the context of MHC class I and II, respectively. Tumors frequently downregulate expression of MHC molecules to escape elimination by the immune system.[14,15] It has been demonstrated that interferon (IFN)γ is a driver of MHC class I expression and subsequent antigen recognition.[16] IFNγ-mediated upregulation of MHC class I is critical for recognition of targets by CD8$^+$ T cells,[17] and increasing attention is being placed on mechanisms to induce MHC class I expression on deficient tumors to improve immunotherapies.[14] Accordingly, recombinant (r)IFNγ has been used to increase expression of MHC class I on melanomas in a phase II trial.[18] Interestingly, although expression of MHC class II is typically associated with antigen-presenting cells, some cancer cells can also upregulate expression of this molecule in response to IFNγ.[18]

We developed a method to detect tumor-specific T cell responses in murine preclinical models of cancers with undefined target antigens. rIFNγ was used to induce expression of MHC molecules on murine cancer cell lines, rendering them capable of presenting bulk tumor antigens to T cells *ex vivo* for quantification by flow cytometry.

MATERIALS
Reagents
Retro-Orbital Blood Draw
Heparinized microhematocrit capillary tubes (Fisher Scientific, MA, USA, catalog [Cat]# 22-362-566)

1.5 mL microtubes

Heparin (3 μg/mL; Sigma-Aldrich MA, USA, Cat# H3149) in Hank's balanced salt solution (HBSS; HyClone UT, USA, Cat# SH3026802)

Gauze pads

Eye lubricant

Container filled with ice

Equipment
Retro-Orbital Blood Draw
Anesthetic machine

Flow Cytometry
A flow cytometer capable of detecting a minimum of three colors (for CD4, CD8, and IFNγ) is required; detection of up to seven colors is optimal. A three-laser, eight-color FACS Canto II (BD Biosciences, ON, Canada) was used to generate the data shown here.

Neubauer improved cell counting chamber

Cell Culture
DMEM (Hyclone, Cat# SH30022.01) or media specific to the tumor cell line of interest, containing 10% fetal bovine serum (VWR, PA, USA, Cat# 97068-085) and penicillin/streptomycin cocktail (Fisher Scientific, Cat# SV30010) (cDMEM)

0.25% Trypsin-EDTA (Corning, NY, USA, reference [Ref]# 25-052-CI)

PBS (Hyclone, Cat# SH30256.01)

Cell culture-treated flasks or plates, including 96-well U-bottom (Fisher Scientific, Cat# 12-565-65)

Murine rIFNγ (eBioscience, SD, USA, Cat# 14-8311-63)

Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Cat# P1585)

Ionomycin (Sigma-Aldrich, Cat# I9657)

Blood Processing and Flow Cytometry
Fluorescence-activated cell sorting (FACS) tubes (Falcon round-bottom polystyrene tubes, Corning, Cat# 14-959-5)

Ammonium chloride potassium (ACK) lysing buffer (see reagent setup)

NH$_4$Cl, KHCO$_3$, Na$_2$EDTA (Sigma-Aldrich, Cat# A9434, 237205, and 324503, respectively), and HCl (Fisher Scientific, Cat# SA481)

HBSS

FACS buffer (PBS + 0.5% BSA [Fisher Scientific, Cat# BP1600-100])

RPMI-1640 with L-glutamine (Hyclone, Cat# SH3002701) containing 10% fetal bovine serum, penicillin/streptomycin cocktail, and 0.01% 2-mercaptoethanol (GIBCO, MD, USA, Cat# 21-985-023) (complete RPMI [cRPMI])

Brefeldin A (eBioscience, Ref# 00-4506-51)

Fixation buffer (BioLegend, CA, USA, Cat# 420801)

Intracellular staining permeabilization wash buffer (BioLegend, Cat# 421002)

Preparation of tumor cells

```
┌─────────────────────┐
│ Culture autologous  │
│ tumor cells         │
└─────────────────────┘
          ↓
┌─────────────────────┐
│ Plate 1x10⁵ cells/well │
│ in U-bottom plates  │
└─────────────────────┘
          ↓
┌─────────────────────┐
│ Add 50-500U/well of │
│ rIFNγ               │
└─────────────────────┘
          ↓
┌─────────────────────┐
│ Incubate for 48 hours │
└─────────────────────┘
          ↓
┌─────────────────────┐
│ Pellet tumor cells by │
│ centrifugation and re- │
│ suspend in complete │
│ medium              │
└─────────────────────┘
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Preparation of blood samples

```
┌─────────────────────┐
│ Treat mice with     │
│ immunotherapy       │
└─────────────────────┘
          ↓
┌─────────────────────┐
│ Allow 7-12 days for │
│ peak adaptive       │
│ responses           │
└─────────────────────┘
          ↓
┌─────────────────────┐
│ Collect blood in    │
│ heparinized tubes   │
└─────────────────────┘
          ↓
┌─────────────────────┐
│ Isolate leukocytes  │
└─────────────────────┘
          ↓
┌─────────────────────┐
│ Re-suspend in complete │
│ media               │
└─────────────────────┘
```

Co-culture

```
┌─────────────────────┐
│ Plate leukocytes with │
│ tumor cells, plus the │
│ appropriate controls │
└─────────────────────┘
          ↓
┌─────────────────────┐
│ Incubate 1 hour, then │
│ add brefeldin A     │
└─────────────────────┘
          ↓
┌─────────────────────┐
│ Incubate 4 hours, then │
│ proceed to staining for │
│ flow cytometry      │
└─────────────────────┘
```
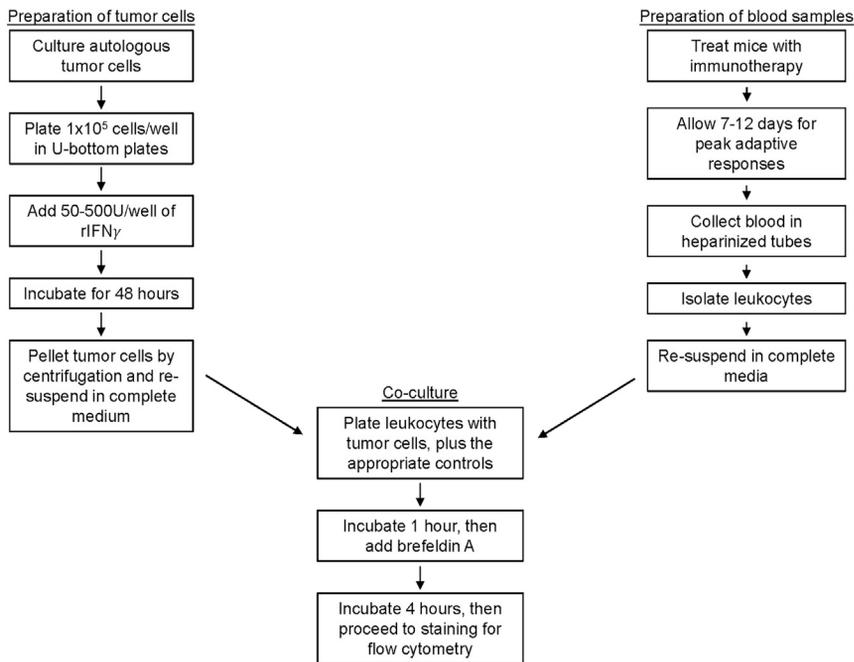
Figure 1. Tumor Cell-Leukocyte Co-culture Assay Workflow Showing the Steps from *In Vivo* Vaccination to *In Vitro* Tumor Cell Preparation and Co-culture Setup

Fixable viability dyes:

Zombie NIR fixable viability kit (BioLegend, Cat# 423105)

7-amino-actinomycin D (7-AAD) (BioLegend, Cat# 420404)

Antibodies against murine:

CD16/32 (Fc block, clone: 93; BioLegend, Cat# 101320)

CD3ε-BV421 (clone: 145-2C11; BioLegend, Cat# 100336)

CD8a-BV510 (clone: 53-6.7; BioLegend, Cat# 100752)

CD4-fluorescein isothiocyanate (FITC) (clone: RM4-4; eBioscience, Ref# 11-0043085)

IFNγ-allophycocyanin (APC) (clone: XMG1.2; BioLegend, Cat# 505810)

TNF-α-phycoerythrin (PE) (clone: MP6-XT22; eBioscience, Ref# 12-7321-82)

CD107a-peridinin chlorophyll protein complex (PerCP)-Cy5.5 (optional; clone: 1D4B; BioLegend, Cat# 121625)

MHC class I (H-2k^b)-PE (clone: AF6-88.5; BD Biosciences, Cat# 561072)

MHC class I (H-2k^d)-PE (clone: 34-1-25; BioLegend, Cat# 114708)

MHC class II (I-A/I-E)-APC780 (clone: M5/114.15.2; eBioscience, Ref# 47-5321-82)

Reagent Setup

For ACK lysing buffer, add the following to H_2O:

NH_4Cl (0.15 M)

$KHCO_3$ (10.0 mM)

Na_2EDTA (0.1 mM)

Adjust pH to 7.2–7.4 with 1N HCl

Filter-sterilize through a syringe-tip filter with a 0.2 μm cut-off and store at room temperature

Equipment Setup

Flow cytometry: manufacturer recommendations should be followed to set up a flow cytometer for multi-color analysis.

PROCEDURE

For an experimental workflow, see Figure 1.

Timing

To maximize the sensitivity of this assay, blood should be collected from mice at the peak of the T cell response, which is often ≈7–12 days following treatment, depending on the therapy.

Seeding Tumor Cells and Pre-treatment with IFNγ

Culture tumor cells in flasks or plates to 80%–90% confluency

Tumor cell lines for this research were obtained directly from American Type Culture Collection (ATCC). To assure reproducibility, cell lines were expanded in isolation from other cell lines immediately upon arrival, and many aliquots were frozen to create a low-passage lab stock from which project-specific stocks were made.

All cell lines were confirmed mycoplasma-free using the MycoAlert PLUS Mycoplasma Detection Kit (Lonza, Basel, Switzerland, Cat# LT07-705)

Detach cells using trypsin, re-suspend in cDMEM, and enumerate using a counting chamber

Seed 1×10^5 cells/well of a U-bottom 96-well tissue culture plate in 180 μL complete medium

Note: plate one well of target tumor cells per blood sample, plus two extra wells (one with and one without rIFNγ) to confirm expression of MHC

Dilute rIFNγ in complete medium such that 50 U in 20 μL can be added to each well (which will have a final volume of 200 μL)

Active units (usually provided in U/mg) of rIFNγ should be listed on the associated product data sheet and can vary between lots

Different cancer cell lines may require varying concentrations of rIFNγ to maximally upregulate MHC. A pilot flow cytometry experiment is recommended to optimize this for each cell line.

Incubate tumor cells at 37°C, 5% CO_2 for 48 h

Duration of pre-treatment with rIFNγ will vary depending on the cell line and should be optimized to maximize MHC expression. Some cell lines may require longer pre-treatment (up to 72 h), and others require no pre-treatment if high levels of MHC molecules are constitutively expressed.

Blood Collection and Processing

Collect blood (100–200 μL is recommended) from mice in 1.5 mL microfuge tubes containing 5 μL heparin (3 μg/mL of HBSS) to prevent clotting

The amount of blood that is collected correlates with the sensitivity of the assay, and the number or proportion of antigen-specific T cells can be low with many immunotherapies. Therefore, it is recommended that the maximum volume of blood allowed by the institutional animal care committee guidelines be acquired. The work presented here was approved by the University of Guelph Animal Care Committee (Animal Utilization Protocol #3807) and adhered to the policies published by the Canadian Council on Animal Care.

Immediately put blood samples on ice

Transfer blood to FACS tubes and record the blood volume from each sample

Add 2 mL ACK lysing buffer and incubate for 5 min at room temperature to remove erythrocytes

Add 2 mL HBSS to stop lysis

Centrifuge at $500 \times g$ for 5min

Remove supernatant and re-suspend cell pellet in 1 mL ACK lysing buffer; incubate for 5 min at room temperature to remove residual erythrocytes

Add 2 mL HBSS to stop lysis

Centrifuge at $500 \times g$ for 5 min

Re-suspend in 300 uL of cRPMI containing 2-mercaptoethanol and antibiotics

Co-culturing Tumor Cells and Blood-Derived Leukocytes

Centrifuge the 96-well U-bottom plate containing tumor cells at $500 \times g$ for 5 min and remove supernatant

Transfer 150 μL blood-derived leukocytes to wells containing tumor cells

Transfer remaining 150 μL processed leukocytes to an empty well

This serves as a negative control for each blood sample and is used to remove background during analysis

Additional controls:

Positive control: include one extra blood sample stimulated with a combination of PMA (10 ng/mL) and ionomycin (1.5 μg/mL) at the time of plating. This serves as a control to ensure the downstream staining protocol worked.

Confirmation of MHC expression: additional tumor cells with and without rIFNγ-mediated re-stimulation should be stained with MHC class I and II-specific antibodies to confirm MHC expression in each experiment.

Optional: add anti-CD107a to each well to assess degranulation

Incubate at 37°C, 5% CO_2 for 1 h

Add 4×Brefeldin A in 50 μL cRPMI/well for a total volume of 200 μL/well

Continue incubation for 4 h

Staining for Surface and Intracellular Markers

Centrifuge plate at $500 \times g$ for 5 min, remove supernatant by rapid inversion of the plate followed by blotting on absorbent paper, and then re-suspend the cells by gently tapping the side of the upright plate.

Add anti-CD16/32 at a 1/200 dilution in 50 μL FACS buffer/well to block Fc receptors

Incubate for 20 min at 4°C

Add 150 μL FACS buffer

Centrifuge plate at $500 \times g$ for 5 min, remove supernatant, and re-suspend cells

Add antibodies for surface markers (anti-CD3ε, -CD4, and -CD8a) at 1/200 dilutions in 50 μL FACS buffer/well

Incubate for 20 min at 4°C in the dark

Add 150 μL PBS/well

Centrifuge plate at $500 \times g$ for 5 min, remove supernatant, and re-suspend cells

Add 200 μL PBS

Centrifuge plate at $500 \times g$ for 5 min

Remove supernatant and re-suspend cells

Make a 1/1,000 dilution of fixable viability dye in PBS and add 100 μL per well

Incubate for 30 min at 4°C in the dark

Add 100 μL PBS

Centrifuge plate at $500 \times g$ for 5min, remove supernatant and re-suspend cells

Add 50 μL/well of fixation buffer

Incubate for 20 min at 4°C in the dark

Add 150 μL/well of 1× permeabilization buffer

Centrifuge plate at $500 \times g$ for 5 min, remove supernatant, and re-suspend cells

Add 200 μL/well of 1× permeabilization buffer

Centrifuge plate at $500 \times g$ for 5min, remove supernatant, and re-suspend cells
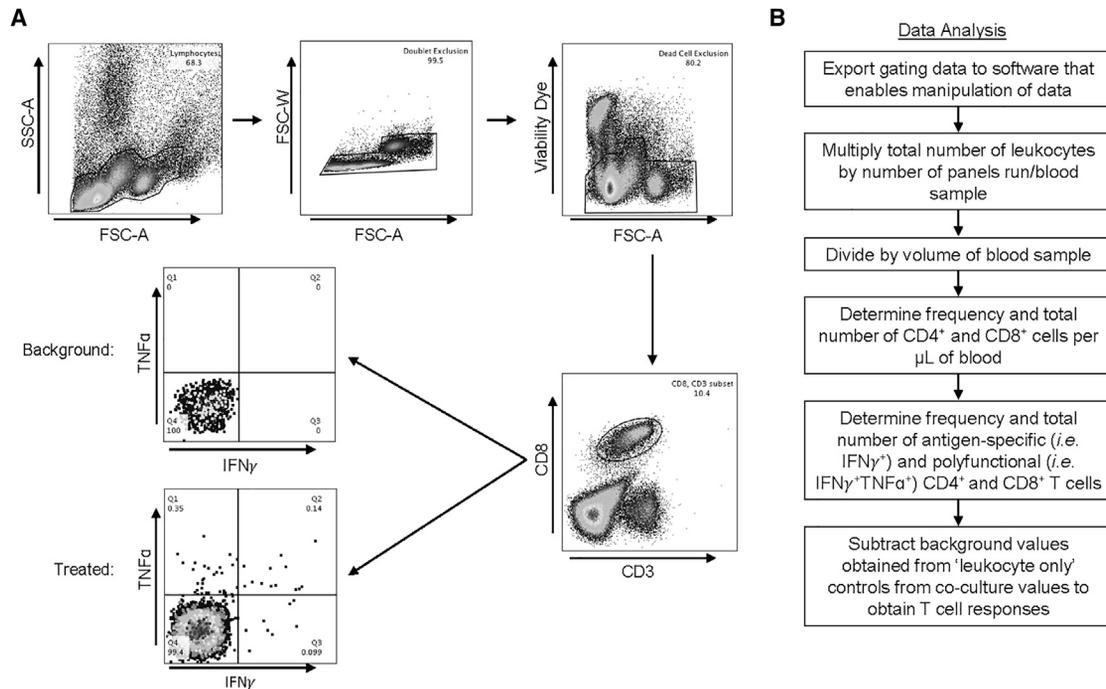
Figure 2. Overview of Flow Cytometry Gating Strategy and Data Analysis

(A) Example of the gating strategy used to assess CD8$^+$ T cells by flow cytometry. Total lymphocytes are gated by forward scatter-area (FSC-A) and side scatter-area (SSC-A) characteristics. Doublets are then excluded by FSC-A and FSC-width (FSC-W). Viable cells are defined as those excluding a viability dye. CD3$^+$CD8$^+$ T cells are gated and then analyzed for expression of IFNγ and TNF-α. (B) An example of the data analysis workflow following gating and export from flow cytometry analysis software. Total percentage and number of IFNγ^+ and IFNγ^+ TNF-α^+ T cells from the non-co-cultured controls are subtracted from the co-culture data to remove background.

Add cytokine-specific antibodies (anti-IFNγ and anti-TNF-α) at a 1/200 dilution in 1$\times$ permeabilization buffer

Incubate for 20 min at 4°C in the dark

Add 150 μL/well of 1$\times$ permeabilization buffer

Centrifuge plate at 500 $\times$ g for 5min, remove supernatant, and re-suspend cells

Add 200 μL/well diluted permeabilization buffer

Centrifuge plate at 500 $\times$ g for 5 min, remove supernatant, and re-suspend cells

Re-suspend samples in 200 μL FACS buffer for analysis on a flow cytometer

Pause point: stained and fixed samples can be kept at 4°C in the dark for up to 24 h prior to flow cytometry analysis

Flow Cytometry Gating
Refer to Figure 2A.

Data Analysis
Refer to Figure 2B.

Timeline
Approximate time based on an experiment with 20 mice

Typical time to the peak of a primary vaccine-induced T cell response: 7–12 days

Plating tumor cells and treating them with rIFNγ: up to 3 days

Blood sampling: 1 h

Sample processing, plating, and incubation: 6 h

Flow cytometry staining: 3 h

Running samples on a flow cytometer: 1 h

Total time for blood sampling to data analysis: 11 h

Troubleshooting
Expression of MHC Class I and II on Cancer Cell Lines
This method relies on the ability of cancer cell lines to present tumor antigens in the context of MHC class I and II to CD8$^+$ and CD4$^+$ T cells, respectively. Therefore, it is critical to determine the capacity for the cancer cell line of interest to express MHC molecules either at baseline or in response to stimulation with rIFNγ. We analyzed expression of MHC class I and class II on several transplantable tumor cell lines from BALB/c and C57BL/6 mouse strains by flow cytometry, with or without stimulation with rIFNγ at several doses and time points.

Cancer cell lines from the C57BL/6 background, including RM9 prostate cancer, ID8 ovarian carcinoma, and B16-F10 melanoma, were
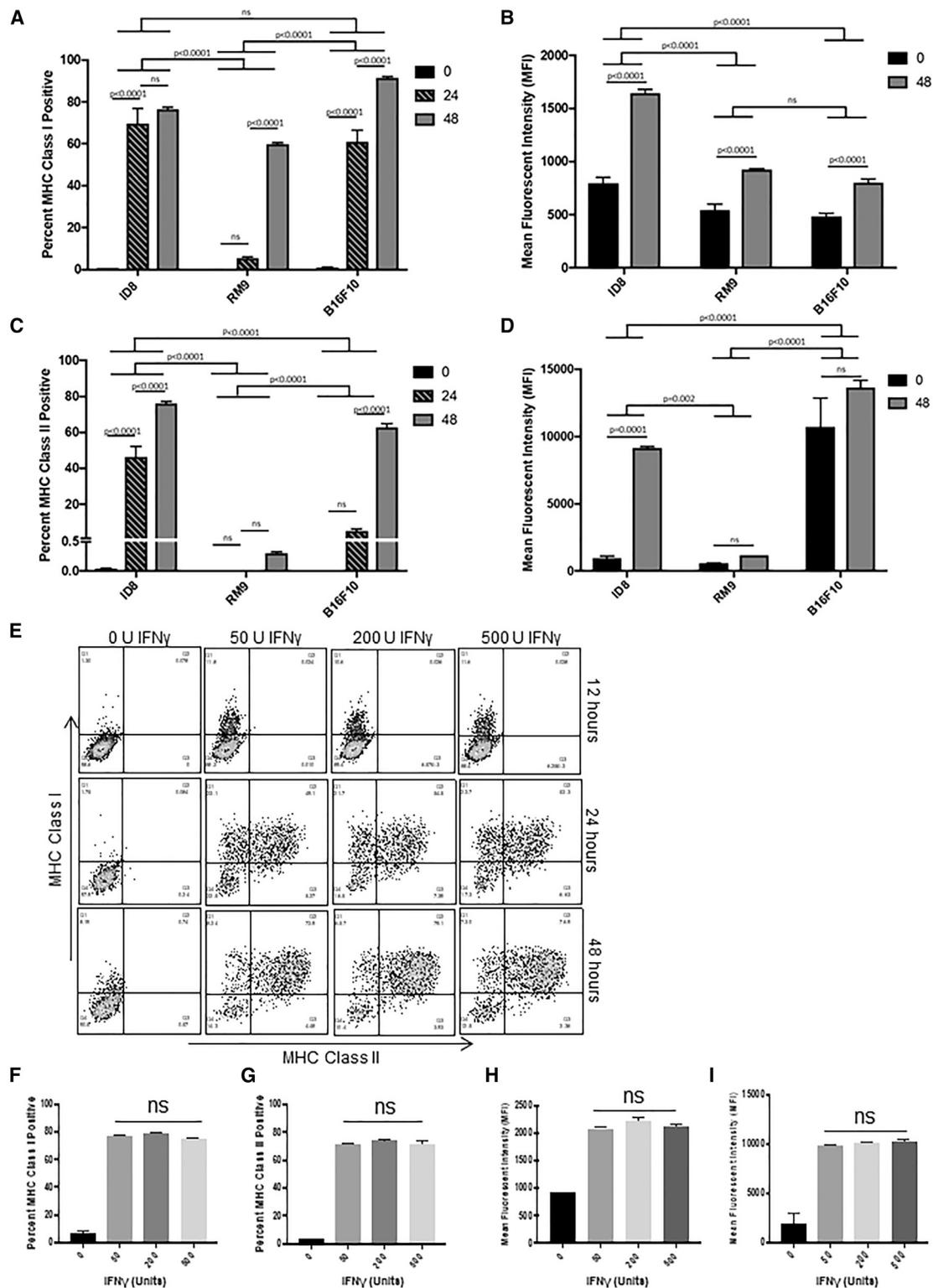
Figure 3. Induced Expression of MHC Molecules on Cancer Cell Lines from C57BL/6 Mice

Both the (A) percentage and (B) amount expressed per cell, as determined by geometric mean fluorescent intensity (MFI), of MHC class I were significantly increased on C57BL/6 mouse-derived ovarian cancer (ID8), prostate cancer (RM9), and melanoma (B16-F10) cancer cell lines after treatment with 50 U rIFNγ. This occurred in

(legend continued on next page)

treated with 50 U of rIFNγ for 24 or 48 h prior to quantification of surface-expressed MHC class I and class II by flow cytometry. All three cell lines expressed little or no MHC prior to stimulation with rIFNγ (Figure 3A, 0 h). Following 24 h of stimulation with rIFNγ, the frequency of ID8, RM9, and B16-F10 cells expressing MHC class I increased dramatically relative to baseline levels (Figure 3A). Extending stimulation to 48 h further increased the percentage of MHC class I+ RM9 and B16-F10 cells, but not ID8 cells. In all three cell lines, the amount of MHC class I expressed per cell, as determined by geometric mean fluorescence intensity (MFI), was significantly increased after 48 h (Figure 3B). ID8 and B16-F10 cells had the highest capacity to upregulate MHC class I, whereas ID8 cells had the greatest expression level per cell following rIFNγ stimulation (Figures 3A and 3B).

MHC class II expression was very low prior to stimulation with rIFNγ in all three C57BL/6 cancer cell lines tested (Figures 3C and 3D). Following stimulation with rIFNγ, the frequency of ID8 and B16-F10 cancer cell lines expressing MHC class II significantly increased (Figure 3C), again in a time-dependent manner. The MFI of MHC class II also significantly increased in ID8 and B16-F10 cancer cell lines (Figure 3D). Despite being capable of upregulating MHC class I, we were unable to induce MHC class II expression on RM9 cells (Figures 3C and 3D).

To investigate whether the dose of rIFNγ could affect MHC class I and class II upregulation, ID8 cells were treated with 0, 50, 200, or 500 U of rIFNγ and analyzed by flow cytometry. MHC class I was upregulated as early as 12 h post-stimulation (Figure 3E), with MHC class II upregulation requiring up to 24 h. The dose of rIFNγ did not alter the frequency of MHC class I or II-expressing cells (Figures 3F and 3G) or the MFI of MHC class I or II (Figures 3H and 3I) on ID8 cells, suggesting that the duration of stimulation with rIFNγ is most critical for upregulating MHC molecules on tumor cells from the C57BL/6 background.

We conducted similar analyses on CT26-CL25 and CT26 colorectal carcinoma and K7M2 osteosarcoma cancer cell lines from the BALB/c background. In contrast to C57BL/6-derived cell lines, all three lines from the BALB/c background constitutively expressed high levels of MHC class I prior to stimulation (Figure 4). Stimulation with up to 200 U rIFNγ failed to induce expression of MHC class II in K7M2, CT26-CL25 (Figure 4A), or CT26 cells (data not shown). The percentage of cells positive for MHC class I following stimulation with 50 U rIFNγ did not increase over time in any tested cell line (Figure 4B). Likewise, the MFI for MHC class I was not increased over time, even in CT26 cells that had the lowest MFI compared to both CT26.CL25 and K7M2 (Figure 4C).

These findings demonstrate key differences in both the baseline expression and capacity for upregulation of MHC molecules on tumor cell lines from different mouse strains following stimulation with rIFNγ. Due to these differences, each transplantable tumor model should be tested to determine baseline and induced expression of MHC molecules when optimizing the co-culture assay.

Detection of Rare Populations of Tumor-Specific T Cells

Tumor-specific T cell responses induced by antigen-agnostic immunotherapies could be detected with our co-culture method in cancer-bearing BALB/c and C57BL/6 mice (Figure 5). However, primary T cell responses to immunotherapies can often be of low magnitude. Notably, our method could resolve responses that averaged only one tumor-specific T cell/10 μL of blood (Figure 6A).

Induction of low numbers of tumor-specific T cells is expected from many immunotherapies as they attempt to reactivate the immune system against self-derived, weakly immunogenic cancer antigens. This can be particularly problematic for cancer vaccines that use OVs to express defined TAAs, where the most robust responses were to virus backbone-derived antigens.[19]

Acquiring maximal volumes of blood from experimental animals will maximize the chance of detecting rare tumor-specific CD8+ or CD4+ T cells. To further facilitate detecting low-magnitude responses, we recommend aliquoting processed leukocytes such that two-thirds get co-cultured with cancer cells (i.e., test sample) and only one-third get cultured alone (i.e., negative control).

Preparation of Internal Experimental Controls

Controls must be included in each experiment to support interpretation of results.

The inclusion of a *positive control* is critical to ensure that technical aspects of staining and flow cytometric assessments were performed correctly. For each experiment, include one sample of processed leukocytes that are stimulated with PMA (10 ng/mL) and ionomycin (1.5 μg/mL) in parallel with test samples. PMA and ionomycin non-specifically activate T cells.[20] Aside from acting as a positive control for staining, this sample can be used to assist with gating during flow cytometry analysis.

Negative Control. Each blood sample needs to have an aliquot that is not co-cultured with autologous tumor cells. During data analysis, CD8+ or CD4+ T cells positive for IFNγ from these leukocyte-only negative controls are categorized as "background" that is independent of the immunotherapy, and this background is subtracted from values acquired for co-cultured test samples. An additional negative control

a time-dependent manner, increasing over a 48-h period. Similarly, expression of MHC class II, as measured by (C) frequency and (D) MFI, could be induced in a time-dependent fashion. (E) Typical flow cytometry dot plots for surface expression of MHC class I versus II on ID8 cells are shown after treatment with various doses of rIFNγ for various lengths of time. The frequency of ID8 cells expressing (F) MHC class I or (G) MHC class II molecules and the relative amount of (H) MHC class I or (I) MHC class II expressed per cell (as measured by geometric MFI) did not correlate with the dose of rIFNγ. The p values from Tukey's multiple comparison test are shown (one- or two-way ANOVAs); errors bars represent standard errors with a minimum of n = 3 experimental replicates per treatment; ns, not significant.
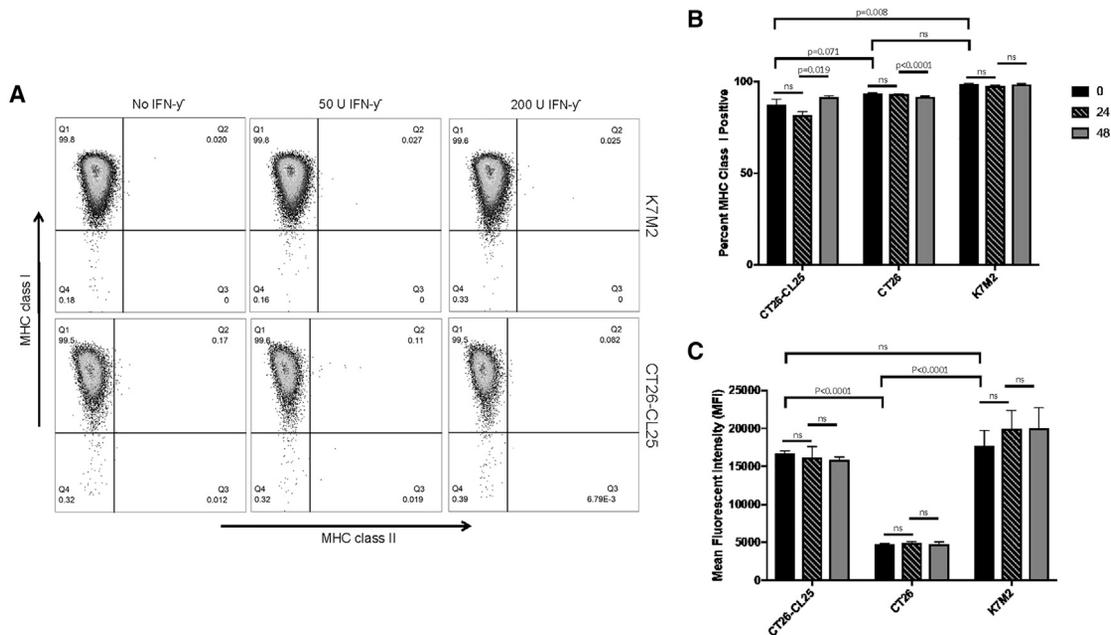
Figure 4. Constitutive Expression of MHC Molecules on Cancer Cell Lines from Balb/c Mice

(A) The CT26-CL25 and K7M2 tumor cell lines from the BALB/c background constitutively expressed high levels of MHC class I but not MHC class II. MHC class I and class II were not upregulated by increasing the dose of rIFNγ from 50 to 200 U. (B) An increase in MHC class I⁺ cells following stimulation with 50 U rIFNγ for 24 or 48 h was observed on CT26.CL25 cells but not CT26 or K7M2 cells. (C) The geometric mean fluorescence intensity of MHC class I on cells was not significantly increased by longer duration stimulation with rIFNγ. However, CT26.CL25 and K7M2 cells both expressed significantly more MHC class I per cell compared to CT26 cells. The p values from Tukey's multiple comparison test are shown (two-way ANOVA); errors bars represent standard errors with a minimum of n = 3 experimental replicates per treatment; ns, not significant.

could be considered to help prove the specificity of T cell responses. Specifically, leukocytes could be co-cultured with a different cancer cell line from the same mouse strain. Responses detected using this control would be indicative of either non-tumor-specific T cells or T cells responding to tumor antigens conserved among the target and off-target tumor cell lines. Another alternative would be use an immortalized but non-malignant cell line that would have less chance of sharing tumor antigens. As with the target cell line, any other control cells would need to be tested for baseline and rIFNγ-inducible expression of MHC.

Determination of the level of expression of MHC on cancer cells: this co-culture method relies on the expression of MHC molecules on autologous tumor cells. Therefore, seeding two wells with tumor cells only is important. One sample should have been unstimulated and the other treated with rIFNγ. These controls can be stained with viability dye and anti-MHC class I and class II to help interpret results from test samples. Our data suggest that this control is especially critical for applications involving tumor cell lines from the C57BL/6 mouse strain (Figure 3).

Anticipated Results

Detecting Tumor-Specific CD8⁺ T Cells in Transplantable Tumor Models in BALB/c Mice

BALB/c mice were challenged intravenously with syngeneic K7M2 osteosarcoma cells to establish lung metastases. Mice were treated with an infected cell vaccine immunotherapy after challenge. Ten days later, blood-derived leukocytes were co-cultured with K7M2 cells that had not been pre-treated with rIFNγ because they constitutively expressed high levels of MHC class I. Following co-culture, CD8⁺ T cells were analyzed by flow cytometry for intracellular expression of IFNγ and TNF-α. Tumor-bearing mice that were untreated had either undetectable or very low numbers of tumor-specific (IFNγ⁺) CD8⁺ T cells (Figure 6B, top panel). In contrast, mice treated with the immunotherapy had detectable tumor-specific and polyfunctional (IFNγ⁺ TNF-α⁺) CD8⁺ T cells in circulation (Figure 6B, bottom panel). The mice receiving immunotherapy had a significantly higher total number of tumor-specific CD8⁺ T cells (Figure 6A) compared to untreated mice.

To further examine tumor-specific CD8⁺ T cell responses in BALB/c mice using our co-culture method, we pooled data from multiple experiments using transplantable tumor models in which mice were treated with antigen-agnostic immunotherapies (i.e., oncolytic virotherapy or an infected cell vaccine). Blood was sampled 10 days following the immunotherapeutic intervention. Mice treated with immunotherapy had a significant increase in the total number of CD8⁺ T cells in circulation (Figure S1A). The frequency of tumor-specific blood-derived CD8⁺ T cells of untreated mice and mice receiving the immunotherapy were assessed using the co-culture assay (Figure 5A). The background frequency of responding IFNγ⁺ CD8⁺ T cells from untreated tumor-bearing mice was very low

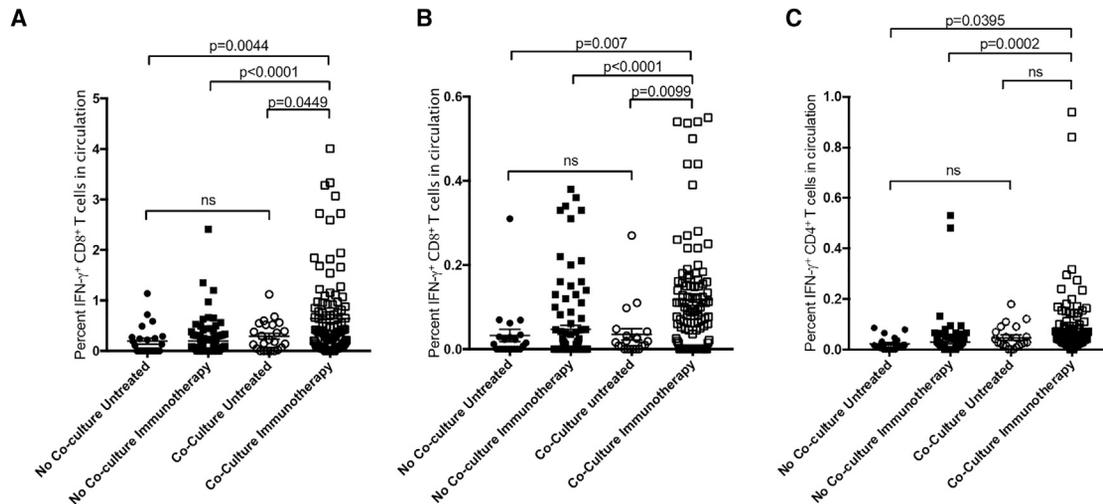**Figure 5. Quantification of T Cell Responses Induced by Antigen-Agnostic Immunotherapies**

The frequencies of tumor-specific (i.e., IFNγ+) CD8+ T cells in circulation in response to antigen-agnostic immunotherapies, which included oncolytic virus monotherapy and infected cell vaccines, were pooled from (A) BALB/c or (B) C57BL/6 mice to demonstrate the overall potential of the cancer cell-leukocyte co-culture method to detect responses. Cancer cells from C57BL/6 mice that were used in the co-culture method had been pre-treated with rIFNγ to maximize expression of major histocompatibility molecules. (C) Data for tumor-specific CD4+ T cells in circulation in response to antigen-agnostic immunotherapies were pooled from C57BL/6 mice to demonstrate the overall potential of the cancer cell-leukocyte co-culture method to detect responses. Tumor-specific CD4+ T cells were detected only in mice treated with immunotherapy and analyzed by co-culture. The p values were determined by two-way ANOVA; standard errors are shown; ns, not significant.

regardless of whether or not these T cells were co-cultured with the target tumor cells. The frequency of these cells from mice receiving an immunotherapy was equivalently low when they were not co-cultured with the target cancer cells. However, the percentage of IFNγ+ CD8+ T cells from mice that received an immunotherapy was significantly higher than the background when they were co-cultured with the target tumor cells. This demonstrated that the assay could detect tumor-specific CD8+ T cells induced by this antigen-agnostic therapy. These data indicate that co-culture of leukocytes with cancer cells is required for detection of tumor-specific CD8+ T cell responses.

Detection of Tumor-Specific CD8+ and CD4+ T Cells from Transplantable Tumor Systems in the C57BL/6 Background

To determine if the co-culture method could be used to detect cancer-specific CD8+ and CD4+ T cells after antigen-agnostic immunotherapies in C57BL/6 mice with transplantable tumors, we challenged mice with ID8 ovarian carcinoma cells in the ovarian bursa.[21] Sixty days post challenge, we treated mice with OV monotherapy and analyzed blood for circulating tumor-specific CD8+ and CD4+ T cells 10 days later. Since stimulation with rIFNγ is required for expression of MHC class I and class II on ID8 cells (Figures 3 and 7A), we compared the co-culture assay with or without pre-stimulation of ID8 target cells with rIFNγ.

Samples analyzed without any co-culture yielded low numbers of IFNγ+ CD8+ and CD4+ T cells (Figures 7B and 7C, respectively). Samples analyzed by co-culture without prior stimulation of ID8 cells with rIFNγ also yielded low numbers of IFNγ+ CD8+ and CD4+

T cells in circulation and was not significantly different from samples analyzed without co-culture. In contrast, when samples were analyzed by co-culture with prior stimulation of ID8 cells with rIFNγ, a significantly higher number of tumor-specific IFNγ+ CD8+ T cells were detected compared with co-culture without pre-stimulation. Co-culture with stimulation also detected a significantly higher number of tumor-specific IFNγ+ CD4+ T cells in circulation compared to co-culture without pre-stimulation. These data indicate that CD8+ and CD4+ tumor-specific T cell responses were detectable using the co-culture assay, but there was a strict requirement for stimulation with rIFNγ to upregulate MHC molecules for presentation of cognate cancer cell-derived antigens to T cells.

With the knowledge that tumor-specific CD8+ T cell responses can be detected by co-culture with rIFNγ, we pooled data from multiple experiments to determine the expected tumor-specific CD8+ T cell responses to OV monotherapy in transplantable tumor models from the C57BL/6 background. In all experiments, tumor-bearing mice were treated with an OV monotherapy and blood-derived tumor-specific CD8+ T cell responses were quantified using the co-culture assay using autologous tumor cells pre-stimulated with rIFNγ. Mice treated with immunotherapy had a significant increase in the total number of CD8+ T cells in circulation (Figure S1B). Pooled analysis of samples from untreated mice yielded equivalently low frequencies of tumor-specific IFNγ+ CD8+ T cells whether or not they were co-cultured with cancer cells (Figure 5B). Tumor-specific CD8+ T cells were not detected in mice treated with antigen-agnostic immunotherapies when analyzed without co-culture and were not significantly different from untreated mice analyzed with or without co-culture. In contrast,
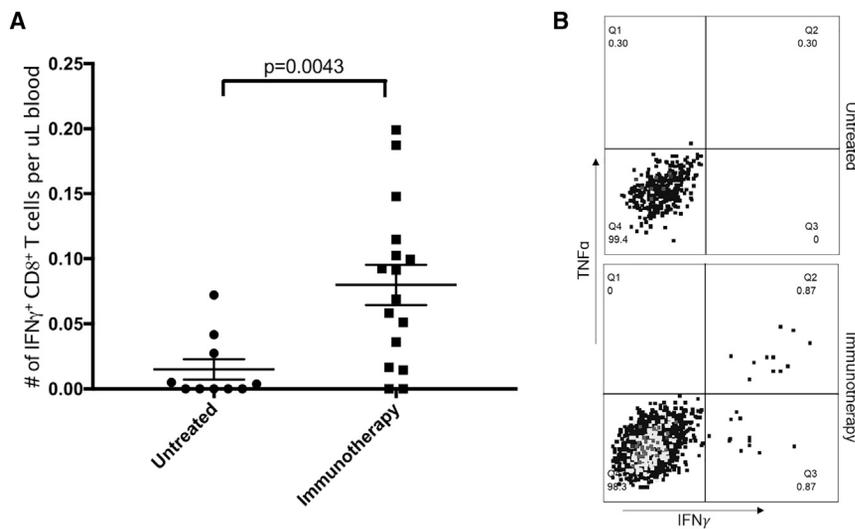
Figure 6. BALB/c Mice that Had Been Challenged Intravenously with 1 × 10⁵ K7M2 Cells to Establish Pulmonary Osteosarcoma Tumors Were Treated with an Antigen-Agnostic Infected Cell Vaccine Immunotherapy

At the expected peak of the T cell response, blood-derived leukocytes were co-cultured with K7M2 cells that had not been pre-treated with rIFNγ since it was shown to have no effect on expression of major histocompatibility molecules. (A) K7M2 tumor-bearing mice that received immunotherapy had a significantly higher absolute number of tumor-specific CD8⁺ T cells (n = 16) as detected by the cancer cell-leukocyte co-culture assay compared to untreated tumor-bearing mice (n = 10). (B) Mice treated with the immunotherapy had evidence of significantly more K7M2-specific (IFNγ⁺) CD8⁺ T cells, of which many were polyfunctional (IFNγ⁺TNF-α⁺) (bottom panel) compared to tumor-bearing mice that had not received the therapy (top panel).

the co-culture method unveiled tumor-specific CD8⁺ T cells in the circulation of mice treated with antigen-agnostic immunotherapies.

We also pooled data from multiple experiments wherein C57BL/6 mice were treated with OV monotherapy and analyzed the overall capability to detect tumor-specific CD4⁺ T cells in circulation. Only co-culture of leukocytes from treated animals with rIFNγ-stimulated cancer cells revealed tumor-specific CD4⁺ T cell responses (Figure 5C). These data demonstrate that the co-culture method can be used to detect both tumor-specific T cells from the circulation of C57BL/6 tumor-bearing mice.

Direct Comparison of Ex Vivo Re-stimulation with Peptides versus Co-culturing with Cancer Cells for Detecting CD8⁺ T Cell Responses

To validate the co-culture method for detecting tumor-specific CD8⁺ T cells, we directly compared it to the method of *ex vivo* re-stimulation with peptide, which can be considered a gold standard.[22] B16-F10 melanoma cells express high levels of the TAA DCT, which is a component of the melanogenesis pathway.[23] The immunodominant CD8⁺ T cell epitope for DCT (DCT$_{180-188}$) is well defined in C57BL/6 mice. For re-stimulation of T cells, DCT$_{180-188}$ peptides were introduced into the *ex vivo* culture of blood-derived leukocytes of mice that had been vaccinated with a previously described replication-deficient adenovirus (Ad) expressing the defined antigen DCT.[24] When added at a high concentration, this peptide can directly bind to MHC class I molecules on the blood-derived leukocytes. Any T cells expressing cognate T cell receptors would become activated and begin expressing IFNγ and TNF-α, which could be quantified by flow cytometry. We knew we would be able to readily detect DCT-specific CD8⁺ T cell responses with the *ex vivo* peptide re-stimulation assay using mice vaccinated with the Ad-DCT vaccine. We hypothesized that we would also be able to detect these responses using the leukocyte-cancer cell co-culture assay when using B16-F10 cells pre-stimulated with rIFNγ as the source of DCT.

C57BL/6 mice were vaccinated with 1 × 10⁸ PFU Ad-DCT in the semitendinosus muscle of both hind limbs (n = 8) or left unvaccinated (n = 3) and were sacrificed 11 days later. Splenocytes were harvested to assess the frequency of DCT$_{180-188}$-specific CD8⁺ T cells by re-stimulation with peptides or tumor-specific T cells via the co-culture assay with B16-F10 cells pre-stimulated with rIFNγ. Mice vaccinated with Ad-DCT had detectable splenic CD8⁺ T cell responses when analyzed by both methods (Figure 8). The highest responses were detected in vaccinated mice by re-stimulation with peptides. Although mean responses were slightly lower using the co-culture method, they were significantly higher than unvaccinated mice and did not differ significantly from the mean response determined by the peptide re-stimulation method. These data show that the antigen-agnostic co-culture method can reliably detect tumor-specific T cell responses against a defined target antigen and that the sensitivity matches that achieved by *ex vivo* re-stimulation with peptides. This is impactful because the co-culture assay depends on the endogenous expression level of DCT in B16-F10 cells and the induced expression level of MHC class I, which can exceed 90% following 48 h of stimulation with rIFNγ. Further, these data extend the utility of the co-culture method beyond blood-derived leukocytes, to include splenic T cells.

Detecting Virus-Specific T Cells by Co-culture

Since cancer cells can be induced to express MHC molecules, we hypothesized that the co-culture system could be manipulated to detect T cell responses to undefined exogenous antigens, including viral antigens, by using permissive infected cells to present antigens. To test this, we vaccinated C57BL/6 mice intramuscularly with 5 × 10⁸ PFU of Ad-DCT. Ten days later, mice were sacrificed and splenocytes were processed to analyze Ad-specific CD8⁺ T cell responses with the co-culture method. To detect viral antigens by co-culture, we pre-stimulated ID8 cells with 50 U rIFNγ to upregulate expression of MHC class I. To present viral antigens to T cells, we infected the pre-stimulated ID8 cells with Ad-DCT at a multiplicity of infection of 10 12 h before initiating the co-culture. To assess the requirement for rIFNγ
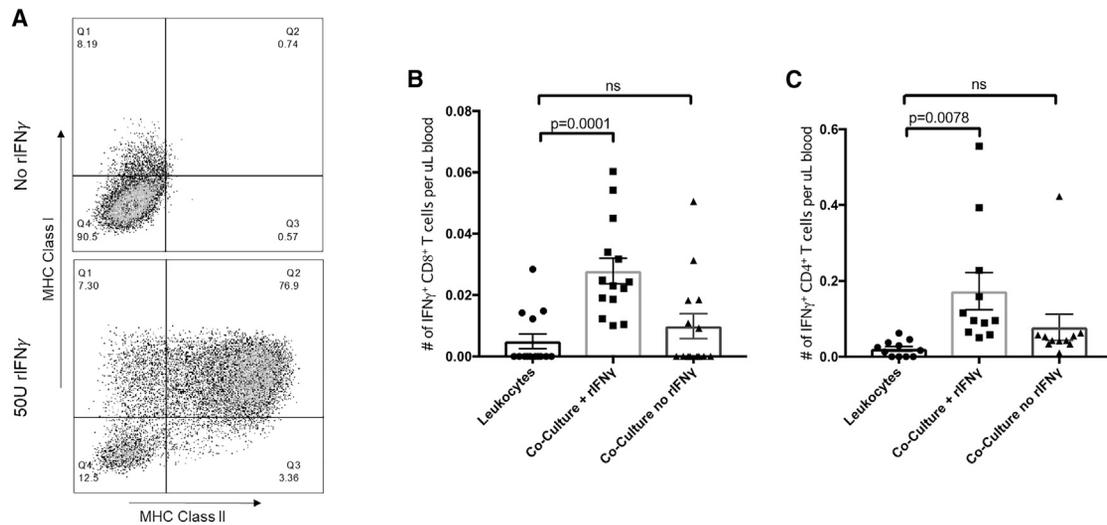
Figure 7. Induction of Surface-Expressed MHC on ID8 Cancer Cells Is Required to Detect T Cell Responses following Antigen-Agnostic Immunotherapy

(A) C57BL/6 mouse-derived ID8 ovarian cancer cells did not express MHC class I or class II (top panel) unless stimulated with 50 U rIFNγ for 48 h (bottom panel). (B) Pre-stimulation of ID8 cells with rIFNγ was required for the detection of tumor-specific CD8$^+$ T cells in the cancer cell-leukocyte co-culture assay (one-way ANOVA with Dunnett's multiple comparison test; n = 14/group). (C) Pre-stimulation of ID8 cells with rIFNγ was also needed to detect tumor-specific CD4$^+$ T cells (one-way ANOVA with Dunnett's multiple comparison test; n = 11/group). Co-culture with unstimulated ID8 tumor cells failed to result in IFNγ expression from either CD8$^+$ or CD4$^+$ T cells. ns, not significant.

stimulation, we included an unstimulated ID8 cell co-culture control for both vaccinated and naive mice.

The frequency of IFNγ$^+$ CD8$^+$ T cells in the spleen was low in samples from naive mice, with no significant difference between those co-cultured with infected ID8 cells with or without pre-stimulation with rIFNγ (Figure S2). In contrast, IFNγ$^+$ CD8$^+$ T cells were readily detected in the spleens of Ad-DCT-vaccinated mice, but only when they were co-cultured with ID8 cells pre-stimulated with rIFNγ and infected with Ad-DCT. The detection of Ad-specific CD8$^+$ T cells required pre-stimulation of ID8 cells with rIFNγ, as co-culture with unstimulated but infected ID8 cells was significantly
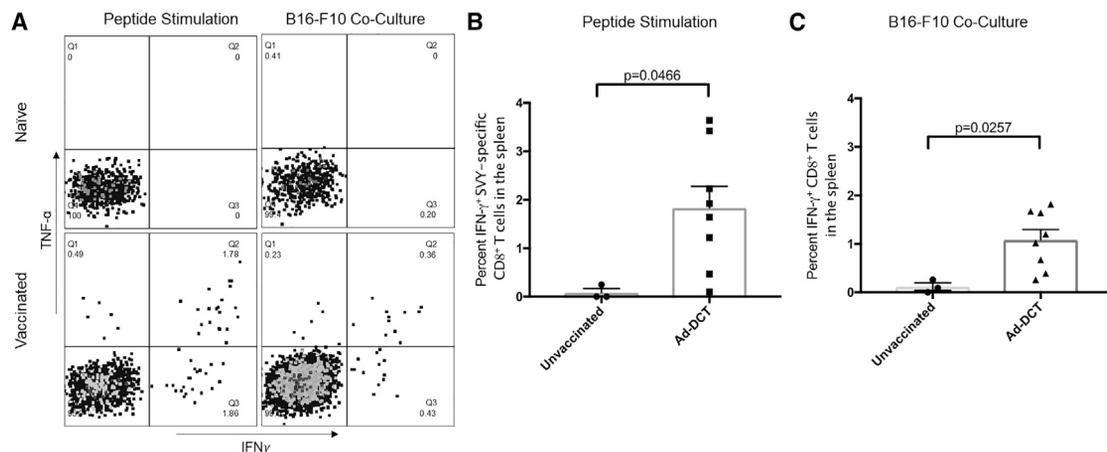

Figure 8. Head-to-Head Comparison of CD8$^+$ T Cell Responses Detected by *Ex Vivo* Re-stimulation with Peptides versus the Cancer Cell-Leukocyte Co-culture Method

C57BL/6 mice (n = 8) were vaccinated intramuscularly with 1 × 10^8 PFU of a replication-deficient human serotype 5 adenovirus carrying a transgene encoding full-length dopachome tautomerase (DCT), which is a melanoma-associated antigen; controls were unvaccinated (n = 3). Mice were sacrificed 11 days later, and the percentage of splenic DCT-specific CD8$^+$ T cells were quantified after re-stimulation with the immunodominant epitope of DCT (i.e., DCT$_{180-188}$) or with the co-culture assay in which leukocytes were co-incubated with B16-F10 melanoma cells, which express DCT. (A) Typical flow cytometry dot plots demonstrated that both (B) the peptide re-stimulation method and (C) the co-culture method detected IFNγ$^+$ CD8$^+$ T cells above background and were significantly higher than unvaccinated mice. Means and standard errors are shown and data were analyzed via two-tailed t tests.

lower and did not differ from the same co-culture technique with naive mice.

These data suggest that the co-culture method can be expanded to allow for the detection of T cell responses to additional classes of undefined antigens, as long as the antigens can be delivered to cell lines that express MHC molecules and for which the host has no pre-existing immunity. As we have shown here, this can include detecting responses to viral vectors. One can envision expressing a vaccine target from cancer cells by transfection or stable integration and using the co-culture method to detect vaccine-induced T cell responses against that target.

In conclusion, the leukocyte-cancer cell co-culture method presented here can be used to detect tumor-specific T cell responses to antigen-agnostic immunotherapies. It is applicable to transplantable tumor models in multiple strains of mice but requires expression of high levels of MHC class I and/or II on the cancer cells that are targeted in the assay. We demonstrated that the tumor cell lines CT26.CL25, CT26, and K7M2 from the BALB/c background constitutively express MHC class I and do not need to be stimulated with rIFNγ to detect CD8^{+} T cell responses. However, these tumor cell lines are refractory to expression of MHC class II, even following stimulation with high doses of rIFNγ, making detection of CD4^{+} T cell responses unlikely. In contrast, the tumor cell lines ID8, RM9, and B16 from the C57BL/6 background require stimulation with rIFNγ to induce MHC class I and class II. In addition to IFNγ, type 1 IFNs are capable of modulating expression of MHC class I and class II on cancer cells[25–27] and could be a useful alternative pre-treatment for cancer cells with disrupted type 2 IFN signaling. In vivo, we were able to detect primary tumor-specific CD8^{+} and CD4^{+} T cell responses to an antigen-agnostic immunotherapy in C57BL/6 mice. We recommend this co-culture method as an affordable tool for detecting tumor-specific blood-derived T cell responses when target antigens are undefined. This method should be broadly applicable to the assessment of antigen-agnostic vaccines and antiviral responses in a variety of tissues. However, when using blood, this technique allows for correlation analysis between tumor-specific T cell responses and efficacy, and, because blood sampling is not a terminal procedure, it facilitates determination of response kinetics.

SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures and can be found online at https://doi.org/10.1016/j.omtm.2019.01.012.

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., L.A.S., T.M.M., S.K.W., and B.W.B.; acquisition of data: J.P.v.V., L.A.S., and T.M.M.; analysis and interpretation of data: J.P.v.V., 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., L.A.S., T.M.M., 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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