Chapter 12

Methods to Evaluate the Antitumor Activity of Immune Checkpoint Inhibitors in Preclinical Studies

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Abstract

Immune checkpoint inhibitors (ICI) are a new class of drugs characterized by their ability to enhance antitumor immune responses through the blockade of critical cell surface receptors involved in the maintenance of peripheral tolerance. The recent approval of ICI targeting CTLA-4 or PD-1 for the treatment of cancer constitutes a major breakthrough in the field of oncology and demonstrates the potential of immune-mediated therapies in achieving durable cancer remissions. The identification of new immune regulatory pathways that could be targeted to reactivate or boost antitumor immunity is now a very active field of research. In this context, the use of syngeneic mouse models and immune monitoring techniques are the cornerstone of proof-of-concept studies. In this chapter, we describe the general methodology to evaluate antitumor activity of ICI in immunocompetent mice. We outline protocols to reliably establish tumors in mice and generate lung metastasis through tail vein injections with the aim of testing the efficacy of ICI. We also present methods to analyze the composition of the tumor immune-infiltrate by multicolor flow cytometry.

Key words Immune-checkpoint, PD-1, PD-L1, Immuno-oncology, Cancer

1 Introduction

Over the last decade, the discovery of the main pathways involved in the control and in the resolution of immune responses has enabled a better understanding of the chronic inflammation process occurring in auto-immune disorders, infectious diseases and in cancer. In the case of cancers, the activation of immune inhibitory receptors is a critical mechanism by which tumors evade immunosurveillance [1]. As a consequence, the targeted blockade of pivotal components of these inhibitory pathways, commonly referred as immune checkpoints, has emerged as a ground-breaking approach for cancer therapy [2–4]. The first generation of biological therapeutics capable of blocking immune checkpoints, also called immune checkpoint inhibitors or ICI, have been recently approved for the treatment of metastatic melanoma or non-small-cell lung cancers (NSCLC).

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The first ICI, ipilimumab (Yervoy), was marketed in 2011 and consists of a monoclonal antibody (mAb) targeting the inhibitory receptor CTLA-4 [5, 6]. CTLA-4 is expressed at the surface of T lymphocytes and transmits inhibitory signals when interacting with CD80 or CD86 expressed by antigen presenting cells (APC). The binding of ipilimumab on CTLA-4 blocks its interaction with CD80 and CD86 which prevents immunosuppressive signaling and promotes antitumor immune responses [7]. Ipilimumab is currently approved for the treatment of metastatic melanoma and several clinical trials are ongoing to evaluate its clinical efficacy in patients with prostate cancer, NSCLC and bladder cancers. While the exact mechanism of action of ipilimumab is still unclear, there are evidence that it blocks the interaction of CTLA-4 with CD80 and CD86 and that it can deplete CTLA-4-expressing T regulatory cells (Tregs) through antibody-dependent cellular cytotoxicity (ADCC) [8–10]. Notably, transcriptome analysis of tumor-specific CD8+ T cells following CTLA-4 blockade revealed increased T cell proliferation and effector memory function [11].

The second ICI that has been developed and approved in cancer patients targets the PD-1/PD-L1 pathway [12]. PD-1 is an inhibitory receptor mainly expressed by activated and exhausted lymphocytes. Engagement of PD-1 by its ligand PD-L1 or PD-L2 expressed on APC, myeloid cells or tumor cells inhibits T cell activation [13] and restrains their antitumor functions [14]. In both preclinical models and cancer patients, PD-1/PD-L1 blockade promotes antitumor immunity [12, 14–16]. From a mechanistic point of view, blocking PD-1 promotes antitumor effector functions and T cell metabolism [11]. Notably, PD-1/PD-L1 blockade modulates glucose availability in the tumor microenvironment, thereby restoring glucose metabolism in activated T cells present in the tumor microenvironment, thus favoring differentiation into effector T cells [11, 17]. A recent report also suggested that anti-PD-1/PD-L1 mAb therapies could be modulated by Fc receptor function [18].

In the past 2 years, anti-PD-1 and anti-PD-L1 mAbs have benefited from accelerated evaluation by the FDA through the "breakthrough therapy" designation. This has led to the approval of two anti-PD-1 mAbs, pembrolizumab (Keytruda™, Merck) and nivolumab (Opdivo™, Bristol-Myers Squibb), for the treatment of melanoma and NSCLC. Importantly, both pembrolizumab and nivolumab showed greater clinical benefit and less side-effects than ipilimumab alone in melanoma patients, and this observation may apply to other types of cancers [19]. With this favorable efficacy and toxicity profile, inhibitors of PD-1/PD-L1 are now the backbone for clinical trials involving immune-mediated therapies [20].

Given the clinical successes of ICI, the search for new immune checkpoints contributing to tumor immune evasion is rising and the list of immunomodulatory targets with antitumor activities is rapidly expanding [20]. Many of these "second-generation" immune checkpoints are currently evaluated in preclinical and clinical trials [20].

Preclinical testing of new compounds is a critical step in the drug development process. It enables the demonstration of therapeutic activity in vivo and the selection of lead compounds that will be evaluated in phase I clinical trial. Hence, choosing the right preclinical models to test a drug candidate is of outmost importance [21]. In the field of oncology, a large variety of preclinical cancer models are available and can be divided into two categories: immune-deficient and immune-competent models. For preclinical testing of ICI, immune-competent models are required as treatments are meant to stimulate the immune system to attack tumors in a MHC-matched manner. The most common immunocompetent models involve subcutaneous transplantation of syngeneic tumor cells in mice. Although these models present clear limitations, they are still important tools to test the antitumor activity of drug candidates. Intrinsic limitations of these models can be circumvented, at least in part, by testing compounds against multiple tumor cell lines to better recapitulate tumor heterogeneity in patients or by performing orthotopic injections. In this chapter, we describe the methods [22–32] we reliably use in syngeneic transplantable mouse models of cancer to test the antitumor activity of ICI.

2 Materials

2.1 Cell Culture Before the Injection of Tumor Cells

1. Tumor cell lines (*see* American Type Culture Collection website).
2. Cell culture medium; usually RPMI 1640 or DMEM, supplemented with 5% FBS.
3. Fetal bovine serum.
4. Antibiotics for cell culture: penicillin/streptomycin.
5. Trypsin 0.25%.
6. 1× phosphate buffer saline, without calcium and magnesium (PBS).
7. Trypan blue solution.
8. Sterile cell culture plastics: 100 mm petri dishes, 15 mL or 50 mL polypropylene tubes, pipets.
9. Sterile filter tips.
10. Sterile 1.5 mL microtubes.
11. 40 μm cell strainers.
12. Hemocytometer.
13. Tabletop centrifuge.

2.2 Subcutaneous Injections

1. Syngeneic mice; C57BL/6 or Balb/c.
2. Small animal clipper.
3. Ear tags.
4. Ear tags applier.
5. Sterile 1 mL syringe.

6. Sterile 26G 5/8 or 26G 3/8 needles.
7. Alcohol pads.
8. Digital caliper.
9. Immune checkpoint blocking mAbs (BioXcell; anti-PD1, clone RPM4-14; anti-CTLA4, clone 9H10).
10. Euthanyl.
11. Dissection instruments and dissection board.
12. Precision scale.

2.3 4T1 Tumor Model

1. 4T1 cell line (ATCC # CRL-2539).
2. Balb/c mice.
3. RPMI 1640 medium supplemented with 5% FBS.
4. Same material as in Subheading 2.2.
5. 10 mL syringes.
6. Pipet tips of 200 and 10 μL.
7. India ink.
8. 1× phosphate buffer saline.
9. Fekete's solution: 100 mL 70% ethanol, 10 mL formalin, 5 mL glacial acetic acid.

2.4 Experimental Metastasis Through Tail Vein Injections

1. Tumor cell line.
2. Syngeneic mice.
3. Ear tags.
4. Ear tags applier.
5. Heating lamp.
6. Tail vein restrainer (Braintree Scientific).
7. Alcohol pads.
8. 0.5 mL 28G ½ needles.
9. Dissection instruments and dissection board.
10. 10 mL syringes.
11. Pipet tips of 200 and 10 μL.
12. India ink.
13. 1× phosphate buffer saline.
14. Fekete's solution.
15. Bouin's solution (if using the B16F10 tumor model).

2.5 Analysis of Tumor Immune Infiltrate by Flow Cytometry

1. EDTA solution.
2. Collagenase IV.
3. DNAse I.
4. 1× PBS.
5. RPMI 1640.

6. Dissection instruments.
7. Incubator.
8. Tabletop centrifuge.
9. 40 μm cell strainers.
10. Strainer-cap FACS tubes.
11. Percoll Solution.
12. FACS buffer: 1× PBS containing 2% FBS and 5 mM EDTA.
13. Digestion buffer: RPMI 1640 containing 2% FBS, 1 mg/mL collagenase IV, 20 μg/mL DNAse I.
14. Fluorochrome-labeled antibodies compatible with cytometry (*see* Table 1).

Table 1
List of fluorochrome-labeled antibodies compatible with cytometry

Panel 1		Panel 2	
Antibody	**Dilution**	**Antibody**	**Dilution**
Anti-CD16/CD32 (BD)	1/100	Anti-CD16/CD32	1/100
Viability dye eFluor 506 (eBioscience)	1/500	Viability dye eFluor 506	1/500
Anti-CD45-BUV395 (BD)	1/100	Anti-CD45-BUV737 (BD)	1/200
Anti-CD3-BV786 (BD)	1/500	Anti-CD11b-BUV395 (BD)	1/500
Anti-CD4-BUV737 (BD)	1/1000	Anti-CD11c-V450 (BD)	1/200
Anti-CD8-BV650 (BD)	1/500	Anti-F4/80-BV605 (BioLegend)	1/400
Anti-NK1.1-Alexa 700 (BD)	1/200	Anti-merTK-biotin (Miltenyi)	1/25
Anti-CD19-PerCPCy5.5 (Tonbo)	1/500	Anti-Ly6C-BV711 (BioLegend)	1/500
Anti-CD44-APC-Cy7 (BD)	1/100	Anti-Ly6G-APC-Cy7 (BD)	1/500
Anti-CD62L-PECF594 (BD)	1/100	Anti-SiglecF-PE (BD)	1/200
Anti-ckit-BB515 (BD)	1/200	Anti-MHCII-BV650 (BD)	1/1000
Anti-FcERIa-PECy7 (eBioscience)	1/100	Anti-B220-BV786 (BD)	1/500
Anti-CD49b-BV421 (BD)	1/400	Anti-PDCA1-FITC (BioLegend)	1/100
Anti-Foxp3-PE (eBioscience)	1/400	Anti-CD19-PerCPCy5.5	1/500
Anti-Eomes-eFluor 660 (eBioscience)	1/400	Anti-Nkp46-PerCPCy5.5 (BD)	1/200
		Anti-CD3-PerCPCy5.5 (Tonbo)	1/200
		Anti-CD206-PECy7 (BioLegend)	1/100
		Anti-NOS2-eFluor 660 (eBioscience)	1/400

3 Methods

3.1 Preparation of the Tumor Cells Before Subcutaneous or Intravenous Injections

1. Tumor cells are thawed rapidly by adding 1 mL of warm medium to the cryovial and resuspended carefully. Alternately, the cryovial can be incubated in a 37 °C water bath for 1–2 min to rapidly thaw the cells.
2. The cell suspension is then transferred into a 15 mL tube containing 10 mL of warm medium, homogenized and dispensed into a 100 mm petri dish (*see* **Note 1**).
3. Cells are incubated overnight in an incubator at 37 °C with 5 % CO_2.
4. The following day, cells are rinsed with PBS and fed with fresh medium.
5. When the cells reach 80–90 % confluency, the cells are detached using trypsin and split into several petri dishes for expansion (*see* **Note 2**).
6. The day before the in vivo injection, cells are rinsed with PBS and fresh medium is added.
7. The day of the injection, the cells are rinsed with PBS and detached by adding 3 mL of trypsin per 100 mm petri dish and incubating for 2 min at 37 °C (*see* **Note 3**).
8. When the cells are all floating, trypsin is neutralized by adding 7 mL of medium containing FBS.
9. The cell suspension is then transferred into a 15 mL tube and centrifuged at 280 × *g* for 5 min. If several petri dishes/flasks are used, cells can be pooled in one/several 50 mL tubes.
10. The supernatant is discarded and the cell pellet is resuspended carefully with a micropipette in 1 mL of PBS. Then, 9 mL of PBS (19 mL for a 50 mL tube) are added and the suspension is homogenized with a 10 mL pipette.
11. The cell suspension is then passed through a 40 μm cell strainer adapted on a 50 mL tube. The cell strainer is wetted with 5 mL PBS before dispensing the cell suspension. The strainer is rinsed with 10 mL PBS after passing the cell suspension (*see* **Note 4**).
12. The cells are centrifuge at 1200 rpm for 5 min.
13. Repeat **step 10** and **12**.
14. The supernatant is discarded and the cell pellet is resuspended in 1 mL of PBS per petri dish/T75 flask used at the beginning of the procedure (increase the volume of PBS accordingly if larger dishes are used). Measure the exact volume of the cell suspension.

15. Collect 10 μL of the cell suspension and dilute it in 1 m of PBS containing 20% (v/v) of trypan blue to count viable and dead cells. During the counting step, place the cell suspension on ice to maintain a good viability.
16. Cells are counted with a Malassez's hemocytometer using 10 μL of the cell solution diluted in trypan blue (*see* **Note 5**).
17. Adjust the volume of the cell suspension with PBS to obtain the desired concentration of cells for injections. If the cells are too diluted, centrifuge and resuspend in a smaller volume.
18. Split the cell suspension in several tubes (one tube par cage to be injected). Add more cells than needed as there will be some loss in the syringe (three doses more than needed is enough).
19. Until the injection, keep the cells on ice.

3.2 Subcutaneous Injections of Syngeneic Tumor Cell Lines, Monitoring of Tumor Growth and Treatment with Immune Checkpoint Inhibitors

1. Before performing a large experiment with many mice and many treatments, use the injection procedure described below (**steps 2–13**) to determine the optimal number of tumor cells to inject in vivo (*see* **Note 6**).
2. One day before the injections, shave the mice on their right flank and identify them with an ear-tag or an ear-punch.
3. Prepare the cells as described in Subheading 3.1 and keep them on ice until the injection.
4. We usually prepare one tube of cells per cage; a cage contains five mice.
5. At the animal facility, under a laminar flow hood, make sure the cell suspension is homogeneous by flicking and inverting the tube of cells.
6. Using a needle-free 1 mL syringe, aspirate 0.7 mL of the cell suspension (*see* **Note 7**).
7. Attach a 26G 5/8 needle to the syringe and get rid of air bubbles; adjust the syringe volume to 0.5 mL to inject five mice (*see* **Note 8**).
8. Wipe the needle tip with an alcohol pad.
9. Grab a mouse and make sure it is well restrained (*see* **Note 9**).
10. Insert the needle under the skin, on the flank, and locate the tip of needle between the third and fourth mammary gland (as shown in Fig. 1, *see* **Note 10**).
11. Slowly inject 100 μL of the cell suspension; a bump should appear under the skin (*see* **Note 11**).

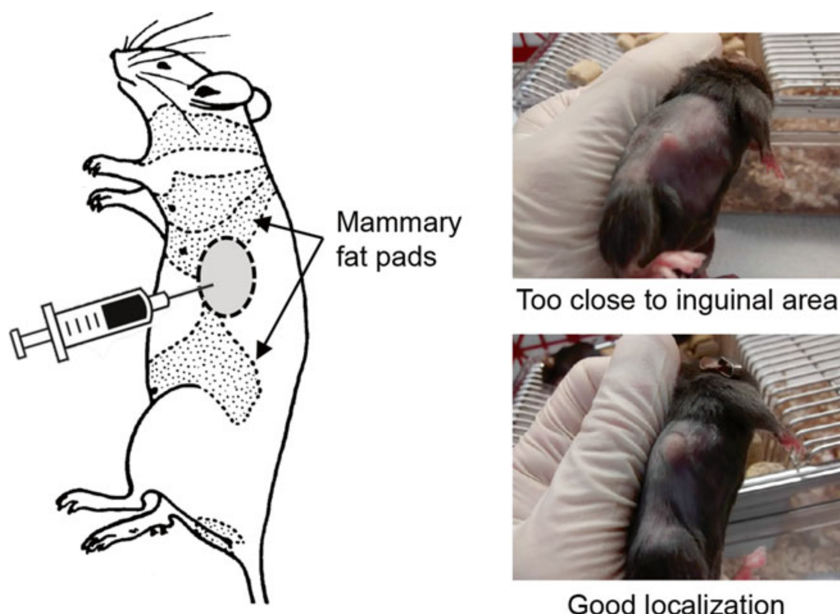

Fig. 1 Technique for subcutaneous injection of cancer cells. *Left Hand Panel*: Schematic diagram illustrating the location of the third and fourth mammary glands in rodents along with the optimal area for subcutaneous injection between both glands. *Right Hand Panel*: A representative example of proper versus improper injection techniques based on the distance from the inguinal (fourth) mammary gland

12. Slowly withdraw the needle and replace the mouse in its cage.
13. Repeat this procedure to inject the other mice (wipe the needle with an alcohol pad for each mouse, *see* **Note 12**).
14. 3 days after the injection, check the presence of a visible tumor mass in mice. Sometimes nothing is visible before day 7. On the contrary, with some tumor models, it is already possible to perform a first measurement of tumor size after 3 days.
15. Monitor tumor growth, two or three times per week, by measuring the size of the tumors using a digital caliper (*see* **Note 13**).
16. When the average tumor size reaches 20–30 mm^2, start the first treatment with immune checkpoint inhibitors (*see* **Note 14**).
17. Treat mice with immune checkpoint inhibitors (e.g., 100 μg twice per week i.p. for a total of 4–6 injections), while continuing to monitor tumor size.
18. Sacrifice mice at the end of the experiment using CO_2 or a lethal injection of pentobarbital (*see* **Note 15**).
19. Dissect subcutaneous tumors and weigh them.

3.3 Spontaneous Lung Metastasis Model Using the 4T1 Tumor Cell Line

3.3.1 Tumor Growth and Treatment with Immune Checkpoint Blockers

1. Prepare the 4T1 cell line as described in Subheading 3.1 (*see* **Note 16**).
2. Inject mice subcutaneously as described in Subheading 3.2 (*see* **Note 17**); subcutaneously inject 1×10^5 cells per mouse in 100 μL PBS.
3. Small tumors should be visible and measurable within 3–5 days.
4. When tumors reach 20–30 mm^2 (7–10 days after tumor inoculation), start treatments with immune checkpoint inhibitors as described in **steps 16** and **17** of Subheading 3.2.
5. Sacrifice mice at the end of the experiment (3–4 weeks after tumor inoculation) using CO_2 or a lethal injection of pentobarbital (*see* **Note 18**).
6. Dissect subcutaneous tumors and weigh them.

3.3.2 Evaluation of Lung Metastasis Tumor Burden

1. After collecting and weighing the subcutaneous tumors, open the thoracic cavity being careful not to damage the lungs; completely remove the rib cage to expose the heart and the lungs (Fig. 2a).

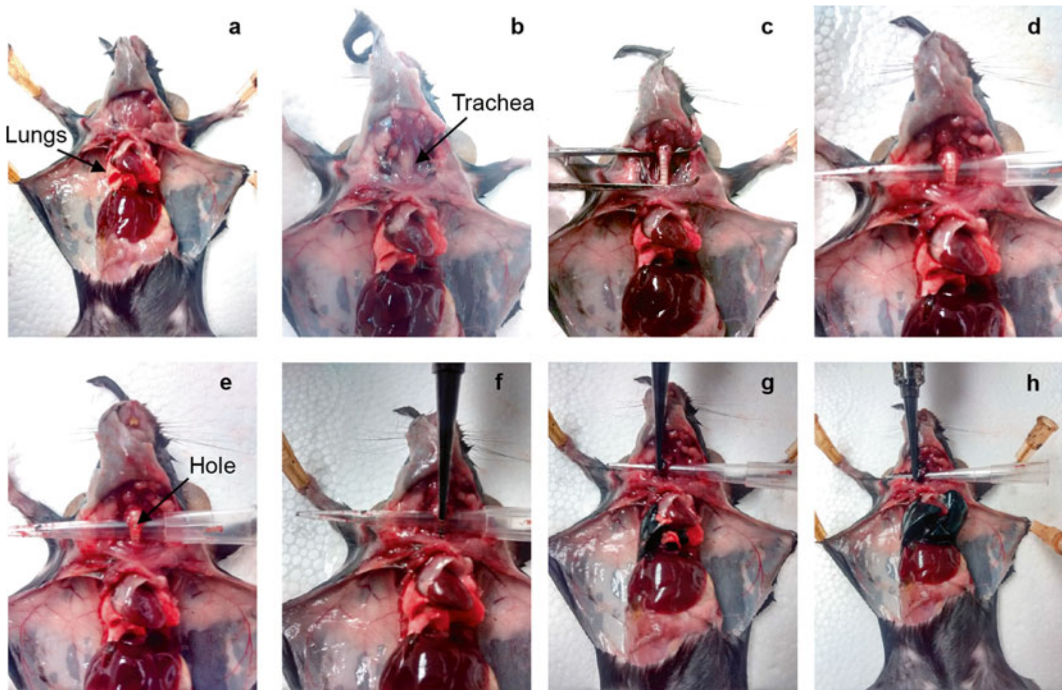

Fig. 2 Method to evaluate lung metastatic tumor burden in mice. (**a**) Open the thoracic cavity being careful not to damage the lungs; completely remove the rib cage to expose the heart and the lungs. (**b**) Expose the trachea over 1 cm, by removing the salivary glands and the muscle layer surrounding the trachea. (**c**) Gently insert the tip of a curved tweezer under the trachea to detach it from the underlying tissues. (**d**) Insert a 200 μL pipet tip under the trachea through the space created with the tweezer. (**e**) Partially cut the trachea so that a small hole is visible. (**f**, **g**) Perfuse the lungs, through the trachea, with 2–3 mL of the 20 % India ink solution. Lungs should swell progressively and turn black. (**h**) When all the pulmonary lobes are black, remove the syringe and dissect out the lungs keeping the heart attached to them

2. Expose the trachea over 1 cm, by removing the salivary glands and the muscle layer surrounding the trachea (Fig. 2b).
3. Gently insert the tip of a curved tweezer under the trachea to detach it from the underlying tissues and create a small space under the trachea (Fig. 2c).
4. Insert a 200 μL pipet tip under the trachea through the space created with the tweezer (Fig. 2d).
5. Using a small and sharp pair of scissors half-cut the trachea; be careful not to completely cut the trachea; a small hole should be visible (Fig. 2e).
6. Insert the tip of a 5 mL syringe adapted onto a 10 μL pipet tip, and loaded with a 20% India ink solution in PBS, into the trachea (Fig. 2f).
7. Perfuse the lungs, through the trachea, with 2–3 mL of the 20% India ink solution. Lungs should swell progressively and turn black (Fig. 2g).
8. When all the pulmonary lobes are black, remove the syringe and dissect out the lungs keeping the heart attached to them.
9. Wash the lungs in a beaker containing PBS to remove excess ink.
10. Fix the lungs immersing them in Feketes's solution. At this step, metastatic foci should appear as white dots on the lung surface (Fig. 2h). Lungs can remain in this solution until the tumor nodules are enumerated.
11. Enumerate macroscopic metastatic nodules on the lung surface.

3.4 Immune Checkpoint Inhibitors Testing in Experimental Lung Metastasis Models

3.4.1 Tail Vein Injections

1. Perform a preliminary experiment to select the optimal number of cells to inject (*see* **Note 19**).
2. One day before the injections identify the mice with an ear-tag or an ear-punch.
3. Prepare the cells as described in Subheading 3.1; one tube of cells per cage with an excess volume of three doses (one dose is usually 100 μL for intravenous injections).
4. At the animal facility, under a laminar flow hood, place one cage of five mice under a heating lamp for 5 min (*see* **Note 20**).
5. Make sure the cell suspension is homogeneous by flicking and inverting the tube of cells.
6. Using a 0.5 mL syringe with 28G ½ needle, slowly aspirate 0.4 mL of the cell suspension (*see* **Note 21**).
7. Get rid of air bubbles (*see* **Note 8**); adjust the syringe volume to 0.3 mL to inject three mice in a row (*see* **Note 22**).
8. Wipe the needle tip with an alcohol pad.

9. Grab a mouse and place it in a tail-vein injection restrainer (*see* **Note 23**).
10. Keep the tail out of the restrainer and clean the injection site with an alcohol pad.
11. Gently pull the tail and localize the lateral tail veins (located on each side of the tail).
12. With the bevel of the needle facing upward and the needle almost parallel to the vein, slide the needle into the tail vein (*see* **Note 24**).
13. Slowly press the plunger to inject the cell suspension (*see* **Note 25**).
14. Remove the needle and apply firm pressure to the injection site to prevent backflow of the injected cell suspension and/or blood.
15. At the same time rapidly remove the plug from the restrainer and extract the animal from the restrainer.
16. Keep pressure on the injection site for 15–20 s and release the animal in its cage.
17. Inject the following animals.

3.4.2 Evaluation of Lung Metastasis Tumor Burden

1. 14–21 days after tumor cell inoculation, sacrifice the animals using an overdose of pentobarbital.
2. Follow **steps 2–9** of the Subheading 3.3.2, except for the B16F10 model.
3. If using the B16F10 melanoma model, do not perfuse lungs with India ink. Directly collect the lungs and fix them in Bouin's solution (*see* **Note 26**) before counting the tumor nodules.

3.5 Analysis of the Tumor Immune Infiltrate by Flow Cytometry

3.5.1 Tumor Disaggregation and Isolation of Tumor Infiltrating Leukocytes

1. Euthanize mice by CO_2 asphyxiation or with an overdose of pentobarbital.
2. Dissect subcutaneous tumors and place them in a 1.5 mL microtube filled with 0.5 mL of medium containing 2% of FBS.
3. Keep the samples on ice while collecting the tumors.
4. With a small and sharp dissection scissor, finely cut the tumor directly in the microtube.
5. Using a 1 mL pipet, transfer the tumor homogenate into a 15 mL tube filled with 5 mL of digestion buffer.
6. Incubate the tumor homogenate at 37 °C with vigorous agitation for 30–60 min (*see* **Note 27**).
7. After 30 min, check the digestion efficiency; if there are no tumor pieces left proceed to next step; otherwise, incubate the sample for 15–30 min more.

8. Once tumor pieces are completely digested, place the tubes on ice and add 5 mL of FACS buffer containing EDTA to stop the digestion process.
9. Pipet the cell suspension up and down with a 10 mL pipette and pass it through a 40 μm cell strainer placed on a 50 mL falcon tube. Gently mash remaining aggregates with the plunger of a 5 mL syringe. Keep the tubes on ice.
10. Add 10 mL of FACS buffer through the filter to rinse it.
11. Spin 5 min at 1200 rpm and discard supernatant.
12. Resuspend the pellet in 2.5 mL of FACS buffer and pass through a 40 μm strainer capped FACS tube (*see* **Note 28**).
13. Rinse the strainer with 1 mL of FACS buffer.
14. Spin 5 min at 1200 rpm, discard supernatant.
15. Resuspend the pellet in 2 mL of PBS containing 30% of Percoll.
16. Gently layer the cell suspension onto a 70% Percoll solution (2 ml in a FACS tube).
17. Centrifuge at 1200 rpm, at 4 °C for 30 min with no brake.
18. Collect the tumor infiltrating leukocytes located at the interface.
19. Rinse twice with a large excess of FACS buffer (*see* **Note 29**).

3.5.2 Staining for Flow Cytometry Analysis

1. Resuspend in 50 μL of FACS buffer containing Fc blocking antibodies (anti-CD16/CD32) and the fixable viability dye. Incubate on ice for 15 min (*see* **Note 30**).
2. Add 50 μL of the cell surface antibody cocktail (*see* Table 1) and incubate for 30 min on ice, protected from light.
3. Rinse twice with FACS buffer.
4. If no intracellular staining is performed, samples can be analyzed directly on the flow cytometer or fixed with formaldehyde and acquired later on (*see* **Note 31**).
5. If performing intracellular stainings, fix and permeabilize the samples using the fix/perm kit.
6. Add 100–200 μL of the fix/perm solution and incubate samples on ice for 30 min.
7. Rinse twice using the perm/wash buffer (*see* **Note 32**).
8. Add 100 μL of the intracellular antibody mix (diluted in perm/wash buffer) and incubate for 30–60 min on ice.
9. Rinse twice with perm/wash buffer.
10. Resuspend samples in FACS buffer before acquisition.

4 Notes

1. At this step, cells can be directly plated or centrifuged once to completely remove the DMSO contained in the freezing medium. For most cells, diluting the freezing medium at least ten times with complete medium and plating the cells immediately will not affect cell viability, even if some DMSO is remaining (1 % or less depending on the concentration used in the freezing medium). Depending on the number of cells contained in the cryovial, the appropriate dish is used. It is common to freeze 1–10 million cells/cryovial/mL. If the cell recovery after thawing is good (over 70 %), plating the cells in a 100 mm petri dish or T75 flask should be OK to have a confluent dish a few days after thawing.
2. The number of flask/petri dish to use for expansion depends on the number of cells needed for the experiment. Always plan to have more cells than needed, as a significant fraction of the cells will be lost during the procedure and during the injections.
3. On the day of injection, the cells should be in the log phase of their proliferation curve (confluency around 70 %). Cells can be detached with trypsin 0.25 %, trypsin 0.05 % or with nonenzymatic solutions such as Versene buffer (PBS—8 mM EDTA) or Accutase. Most of the cells will support trypsin treatment very well and the time required to detach the cells should be optimized for each cell type (usually a few minutes).
4. The cells are passed through the 40 μm cell strainer to remove cell aggregates and to have a uniform single-cell suspension. Some cells are lost during this step, but this will improve the reproducibility of tumor growth in vivo. This step is very important for intravenous injection as it will prevent mice dying from a pulmonary embolism that could arise from big cell aggregates.
5. Be sure that the cell suspensions were well homogenized before pipetting the 10 μL needed for the dilution in trypan blue and for loading the hemocytometer cell. Using the resuspension volume and the dilution mentioned in **step 14** and **15** respectively; there should be more than 100 and less than 500 cells to count. Do not count less than 100 cells. Count the bright refringent cells (viable) and also the ones colored in blue (dead cells) located on the grid. If there are not many cells (100–250), count the cells present on the whole grid (10 columns divided in 10 rows = 100 small squares). If there are a lot of cells, just count 10 or 20 small squares amongst the 100. To obtain the concentration in your cell suspension, divide the number of cells counted by the number of columns included in the count (if you counted the whole grid divide by 10; if you counted 20

small squares, which represents 2 columns, divide by 2) and multiply by the dilution factor used in **step 15** (here 100) and by 10,000. The number obtained will be your number of cells per mL of solution. Do this calculation for both viable and dead cells. The dead cells should not represent more than 10% of the cells. If there are too many dead cells, the tumor growth could be altered. A high number of dead cells could result from an over-incubation with trypsin. Some cell lines are more sensitive to trypsin; the use of a diluted (0.05%) trypsin or a nonenzymatic detaching solution could improve cell viability.

6. Before starting an experiment to test the antitumor efficacy of immune checkpoint inhibitors (or other antitumor compounds), it is recommended to titrate the dose of tumor cells to inject in vivo. We have frequently tried to use the same dose as described in reference papers and found that the number of cells injected was too high or too low. Tumor growth is affected by numerous factors, including the housing temperature of the mouse facility, microbiota of the mice, and the in vitro conditions used to culture tumor cell lines. As a consequence, variations in the growth of the same tumor cell line in vivo can be observed from lab to lab and titrating the dose of tumor cells to inject when testing a new model is highly recommended. To perform a titration experiment, inject three groups of five mice with three different concentrations of tumor cells according to doses reported in the literature. Select the cell concentration that generates similar size tumors in every mouse with the following properties: (1) tumors should be visible, measurable with a caliper within 3–7 days, (2) 7 days after tumor injection the average tumor size should not exceed 30 mm^2, (3) ideally, tumors should reach a size of 20–30 mm^2 within 7–12 days after inoculation. To observe the antitumor activity of immune checkpoint inhibitors or immunomodulatory compounds, an immune response against the tumor is often required. In mice, it takes approximately 7–10 days to elicit a cellular immune response against an antigen; therefore, ICI treatments such as anti-PD1 or anti-CTLA4 are routinely initiated within 1 or 2 weeks after tumor cell inoculation (7 days at the earliest for subcutaneous tumors) [22–24]. Furthermore, those treatments are far less effective in vivo when tumors are too big (over 40 mm^2).
7. Avoid aspirating cells with the needle attached to the syringe as it could damage the cells. If the syringe needs to be reloaded remove the needle.
8. To eliminate air bubbles attached to the plunger, maintain the syringe vertically, needle up, and flick it once to make air bubbles going up just below the needle entry. Still maintaining the syringe vertically, slowly expulse air bubbles through the nee-

dle. If the cell suspension is precious, collect the droplets of liquid expulsed during the procedure by placing a tube close to the needle exit. Experienced manipulators can inject five mice in less than 2 min which means that the cell suspension usually remains homogeneous in the syringe along the procedure. If it takes more time to inject, or if the cell suspension is highly concentrated (more than 10^7 cells/mL), it is better to reload the syringe between each mouse or each two mice to be sure that the cells are not pelleting in the syringe during the injections.

9. Some strains such as Balb/c mice are calm and usually do not move once restrained. On the contrary, C57Bl/6 are more nervous and a really good contention is required; in addition, when restraining C57Bl/6 mice, we usually hold the back leg with one finger as those mice most often try to kick out the needle.
10. The needle should be visible under the skin, easy to insert and the tip should be able to move. If there is resistance, the needle may be located in the dermis instead of the subcutaneous space (which corresponds to the hypodermis). Try not to inject too close to the fourth mammary fat pad as the tumor may be difficult to measure and to discriminate from the bump created by the inguinal lymph node once inflamed. If using a 26G 5/8 needle, do not insert the needle completely (insert a little more than half of the length) as tumor cells could implant in the needle path once the needle is removed and generate elongated tumors or several tumor nodules that will be more difficult to measure.
11. If there is resistance during the injection, you are probably located in the dermis rather than in the subcutaneous space. We observed that tumors growing too close to the skin surface are more susceptible to ulceration.
12. If you have more than 40 mice to inject, make two groups and inject in two sessions.
13. Usually, the tumors have an ellipsoid shape. Measure the largest diameter of the tumor (L) and the larger distance perpendicular to L (W). Then calculate tumor area multiplying L by W. Alternately, tumor volume can be calculated using the following formula: $V = (L \times W^2)/2$.
14. Prepare treatments in a physiological solution (usually PBS for immune checkpoint blocking monoclonal antibodies) and administer treatments in the peritoneal cavity. Use a 26G 3/8 needle for intraperitoneal injections and inject 100 or 200 μL of treatment (for anti-PD-1 and anti-CTLA4 mAbs the dose is usually 5 or 10 mg/kg, which corresponds to 100 or 200 μg per mouse (an 8–12 week old female C57Bl/6 or Balb/c mouse weighing approximately 20 g). Alternately, depending on the compound, treatment can be administered by oral gavage, by intra-tumor injections or by intravenous injections.

15. Before starting an experiment with animals, submit a protocol to your local animal ethic committee and wait for approval. Determine end points beyond which animals should be treated or euthanized. For subcutaneous tumor models, tumor size exceeding 2500 mm^3 and ulceration of the tumor are end-points requiring euthanasia of animals.
16. The 4T1 cell line is cultured is RPMI supplemented with 5 % serum. We routinely inject 1×10^5 cells per mouse in 100 μL of PBS.
17. The 4T1 cell line is syngeneic to Balb/c mice. When injected subcutaneously into Balb/c mice, 4T1 cells spontaneously metastasize to the lung, liver, lymph nodes and brain while the primary tumor is still growing in situ. Increased metastatic potential can be achieved by injecting the 4T1 cells orthotopically in the mammary fat pad. The primary tumor does not have to be removed to induce the growth of the metastatic lesions. The metastatic spread of 4T1 cells in Balb/c mice closely mimic what can be observed with human breast cancer. The 4T1 model is a relevant animal model for stage IV human breast cancer and triple negative breast cancer. Subclones of the 4T1 parental line, with specific metastatic tropism have been isolated (the 4T1.2 subclone is used as a model of breast cancer metastasis to bones).
18. Tumors should be around 150 mm^2 in size to observe a significant number of lung metastatic nodules (average of 20–30 nodules per lung) [22].
19. Inject three groups of five mice with three different doses of cells according to the literature. In experimental lung metastasis models the number of cells to inject usually range between 1 and 5×10^5 cells. Select a dose of cells that will generate at least 100 and less than 400 tumor nodules per lung in 2–3 weeks. With such a tumor burden, mice should not present clinical signs during the experiment (but this latter point depends on each tumor model).
20. Placing mice under a heating lamp is required to dilate the tail vein before the injections. Be very careful with heating lamp as different heating capacities can be observed depending on the lamp. Overheating mice could kill or severely injure them so make sure that the temperature is not too high.
21. The syringes we use for i.v. injections have permanent needles attached. Therefore the cell suspension has to be pipetted with the needle attached to the syringe. To limit the pressure applied to cells passing through the needle and thus limit cell damage, a slow pipetting is required.
22. We usually inject a cage in two steps; three mice in a row and then two mice. This prevents cells from pelleting into the

syringe due to the time required for injections and limits the pipetting of the cells with the needle to twice. An experimented person can inject 30 mice/h.

23. Be careful not to restrain mice too much with the plug as it could prevent them from breathing properly.
24. When inserting the needle in the tail vein there should be no resistance. Moreover, you should be able to see the needle penetrating in the vein as lateral tail vein are superficial. Do not insert the needle too proximal as you will not be able to try a second time immediately after a failure. If the first trial fails, it is also possible to try with the second lateral tail vein.
25. If the needle is correctly inserted in the vein there should be no resistance while injecting the cell suspension and the vein will blanch temporarily. If the needle is not in the vein, the fluid will be difficult to inject and will cause blanching around the vein or a subcutaneous bleb.
26. The Bouin's solution will color the lungs in yellow making black B16F10 tumor nodules easier to visualize and count.
27. Place the tubes horizontally to have a good agitation of the tumor pieces.
28. If there is a lot of blood in the pellet, lyse red blood cells resuspending the pellet in 2 mL of ACK buffer for 1 min at room temperature; then add 1 mL of FACS buffer to stop the lysis and pass the cell suspension through a 40 μm strainer-capped FACS tube.
29. Add a large excess of FACS buffer to dilute the Percoll and to efficiently pellet the cells.
30. At this step, samples can be transferred in a V-shaped 96-well plate.
31. Cell viability is decreasing over time even if samples are kept on ice. Hence, if you have a lot of samples, viability of the cells can be negatively affected due to the acquisition time. To avoid this problem, samples can be fixed in 1 or 2% paraformaldehyde for 15 min at room temperature prior the acquisition. If fixing the samples, be careful of overfixation as it could damage the fluorochromes used to stain the cells.
32. It is critical to use the perm/wash buffer for rinsing and diluting intracellular antibodies. The kit used here contains saponin as permeabilizing agent which means that permeabilization is not permanent unless saponin-containing buffer are used. After rinsing the fix/perm solution, cells can be stored overnight in perm/wash buffer and intracellular stainings performed the following day.

Conflict of Interest

J. Stagg was a paid consultant for MedImmune, Palobiofarma, and Surface Oncology, has received research grants from MedImmune, Palobiofarma, and Surface Oncology, and is a member of the Scientific Advisory Board of Surface Oncology.

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