Video Article

Studying the Role of Alveolar Macrophages in Breast Cancer Metastasis

Surya Kumari Vadrevu[1], Sharad Sharma[1,2], Navin Chintala[1,3], Jalpa Patel[1], Magdalena Karbowniczek[1], Maciej Markiewski[1]

[1]Department of Immunotherapeutics and Biotechnology, School of Pharmacy, Texas Tech University Health Science Center

[2]Merck Research Labs

[3]Department of Surgery, Memorial Sloan Kettering Cancer Center

Correspondence to: Magdalena Karbowniczek at magdalena.karbowniczek@ttuhsc.edu, Maciej Markiewski at maciej.markiewski@ttuhsc.edu

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Abstract

This paper describes the application of the syngeneic model of breast cancer (4T1) to the studies on a role of pulmonary alveolar macrophages in cancer metastasis. The 4T1 cells expressing GFP in combination with imaging and confocal microscopy are used to monitor tumor growth, track metastasizing tumor cells, and quantify the metastatic burden. These approaches are supplemented by digital histopathology that allows the automated and unbiased quantification of metastases. In this method the routinely prepared histological lung sections, which are stained with hematoxylin and eosin, are scanned and converted to the digital slides that are then analyzed by the self-trained pattern recognition software. In addition, we describe the flow cytometry approaches with the use of multiple cell surface markers to identify alveolar macrophages in the lungs. To determine impact of alveolar macrophages on metastases and antitumor immunity these cells are depleted with the clodronate-containing liposomes administrated intranasally to tumor-bearing mice. This approach leads to the specific and efficient depletion of this cell population as confirmed by flow cytometry. Tumor volumes and lung metastases are evaluated in mice depleted of alveolar macrophages, to determine the role of these cells in the metastatic progression of breast cancer.

Video Link

The video component of this article can be found at https://www.jove.com/video/54306/

Introduction

The premetastatic niche is an important process in cancer metastasis defined as a set of alterations that occur in the organs that are targets for metastases prior to arrival of tumor cells[1,2]. Therefore, therapeutic targeting of this step of cancer progression may prevent metastases to the vital organs that cause approximately 90% of cancer-associated deaths. Although the concept of the premetastatic niche, also known as the "seed and soil" theory, was introduced more than a century ago, no experimental proof has been provided until recently, when the bone marrow-derived cells were demonstrated to contribute to the premetastatic soil[1,3-7]. Despite these developments, the premetastatic niche remains a largely understudied aspect of cancer pathophysiology and further research to identify cellular players and mechanisms involved is needed.

Here we report the *in vivo* approaches to study the role of alveolar macrophages in breast cancer metastases and the lung premetastatic niche. The alveolar macrophages arrive to the lungs early during the embryonic development and self-renew there during adulthood[8]. They also have important immunomodulatory and homeostatic functions including the protection of this organ from undesired inflammatory responses to the environmental innocuous antigens[9]. Therefore, we hypothesize that tumors exploit this physiological immunosuppression, imposed by alveolar macrophages, and, consequently, alveolar macrophages contribute to the lung premetastatic niche by suppressing antitumor immunity. This hypothesis is supported by our recent report demonstrating that the specific depletion of these cells reduces lung metastases and enhances antitumor T cell responses[10].

For these studies we apply a well-established syngeneic model of breast cancer (4T1), which mimics stage IV metastatic breast cancer[11]; and has been previously reported in studies of the premetastatic niche[6]. To track metastasizing tumor cells *in vivo* we use 4T1 cells expressing GFP (4T1-GFP) in conjunction with animal imaging and confocal microscopy. We focus on the lung premetastatic niche, since this organ is one of the most common targets of hematogenous metastases of human malignancies[12]. To investigate functions of alveolar macrophages in the premetastatic niche, we use clodronate liposomes to deplete these cells[13]; and evaluate impact of this depletion on lung metastases. Of note, this method specifically depletes alveolar macrophages but no other phagocytic cells in the lungs or in circulation[10].

Protocol

All animal studies have been approved by Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center and followed the guidelines outlined in the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health. Use

eight to twelve week old female BALB/c mice that are commercially available. Inject 1 x 10^5 4T1 or 1 x 10^5 4T1 cells expressing GFP, which can be purchased from various vendors, into the mammary fat pad.

1. Culture of 4T1 and 4T1-GFP Cells & Preparation of Tumor Cell Suspension for Injections[14]

1. **4T1 and 4T1-GFP Cell Culture**
   NOTE: Perform all steps using sterile solutions in a laminar airflow (LAF) bio-safety cabinet unless specified otherwise.
   1. Maintain 4T1 and 4T1-GFP cells in RPMI medium supplemented with 10% heat inactivated fetal bovine serum and 100 U/ml penicillin G and 100 µg/ml streptomycin sulfate.
      NOTE: Determine a passage number by counting every trypsinization and plating of cells. It is important to use cells with the same passage number to all mouse experiments, as the number of passages affects cell tumorigenicity and metastatic potential.
   2. Prior to cell injections remove the T75 cm^2 flask, containing tumor cells at 80 % confluency, from an incubator and aspirate medium. Add 10 ml of PBS and rotate the flask gently to wash out remaining medium, and then aspirate PBS.
   3. Add 2 ml of 0.25 % trypsin with EDTA to the flask and tilt it to distribute the solution uniformly on the surface and incubate in a humidified incubator for 3 min at 37 ˚C with 5 % CO_2.
   4. Tap the flask to facilitate detachment of cells and add 8 ml of fresh medium. Pipette up and down several times to disrupt clumps and get a single cell suspension.
   5. Centrifuge cells for 5 min. at 500 x g at RT. Aspirate the supernatant and wash cells in 10 ml of PBS.
   6. Pipette up and down several times to disrupt clumps and get a single cell suspension. Take 100 µl of a cell suspension and count the cells using the cell viability analyzer as per manufacturer's instructions.
   7. Calculate number of cells required for all injections using formula: 10^5 cells per mouse x # of mice used in an experiment (always prepare extra cells to be on a safe side).
   8. Centrifuge cells as in 1.1.5. Remove the supernatant through aspiration and resuspend cells in the amount of fresh PBS required to obtain a final cell concentration of 1 x 10^6 cells/ml. Keep the cell suspension on ice until ready for injection.

2. **4T1 or 4T1-GFP Cell Injection-mouse Procedures**
   1. The day before tumor cell injection, anesthetize mice in an induction chamber with 3 % isoflurane. Once mice are asleep and breathe regularly, place the mouse on a surgical pad with nose inside the nose cone, and connect it to the isoflurane vaporizer. Maintain anesthesia with 2.5 % isoflurane. Pinch the mouse toe to ensure that the mouse is anesthetized.
   2. Apply hair removal cream using a cotton swab to the injection site (right pectoral mammary fat pad) and wait for 2 min. Clean the site using a wet paper towel, place the mouse back into the cage and monitor an animal until it regains sufficient consciousness to maintain the sternal recumbency.
   3. On the day of injection, anesthetize the mice as in 1.2.2 and place on the surgical pad.
   4. Vortex the tube containing tumor cells to get uniform cell suspension. Aspirate 100 µl of a cell suspension, containing 1 x 10^5 tumor cells, into a 0.5 ml insulin syringe (29 G, 12.7 mm-needle length).
   5. Lift the skin using the thumb and finger index near the 2nd and 3rd nipple, insert the needle of the syringe into the mammary fat pad just below the third nipple under the skin between the fingers and inject cells slowly to form a bubble (subcutaneous injection).
   6. Place the mouse back into the cage after injection and monitor as in 1.2.2.

3. **Monitoring Tumor Growth**
   1. **Caliper Measurements**[14]
      NOTE: Injected 4T1 or 4T1-GFP cells form aggressive breast tumor. Usually the palpable tumors appear ~ at day 4 - 5 after cell inoculation. 4T1-GFP cells form tumors that grow slower and give metastases later. Measure the tumor two to three times a week. If clinical status of animals deteriorates, animals lose more than 10 % body weight, tumors exceed 10 % of body weight or become ulcerated, euthanize mice immediately.
      1. Anesthetize a mouse as in 1.2.1., weigh, place on the surgical pad and wet the area with 70 % ethanol.
      2. Palpate tumor in the site of injection (4 - 5 days after cell inoculation).
      3. Measure the largest diameter (D) and smaller diameter (d1) with a caliper.
      4. Calculate tumor volume using formula Volume= (D X d1 X d1)/2 mm^3. Monitor mouse recovery from anesthesia as in 1.2.2.

   2. **Imaging (Only for 4T1 GFP Cells)**
      1. Anesthetize the mouse as in 1.2.1. Place the mouse on a movable stage inside the imaging instrument. Maintain anesthesia through the nose cone.
      2. Turn on the imaging instrument and the light source as per manufacturer's instructions.
      3. Under the tab "Acquisition" go to "Lighting" and switch to the empty position (no filter) for white light. Ensure that the light engine is ON and select 'Trans' in the light table.
      4. Under the tab "Microscope", select "Clear" emission filter and 0.5X magnification. In the "Camera" tab, make sure that binning for capture is '1 x 1' and preview '4 x 4'.
      5. Click on preview to locate the tumor in white light, focus to get clear image, and click capture.
      6. Go to "Acquisition" tab and change lighting to filter 1 (filter for GFP as per manufacturer's instruction). In the "Microscope" tab, change emission filter to "530/20 GF". Preview and capture image in a green channel. Adjust exposure time to get the appropriate brightness.
      7. Apply pseudocolor to the image and save in appropriate folder (**Figure 1**).

2. Intranasal Administration of Liposomes[10]

NOTE: Perform all steps using sterile solutions in a laminar airflow (LAF) Bio-safety cabinet unless specified otherwise.

1. On day 6 after tumor cell injection anesthetize mice as in 1.2.1.
2. Vortex the clodronate or control (PBS) liposome suspension obtained from the manufacturer. Take 60 µl liposome suspension using sterile pipette tip.
3. Slowly release liposome solution (5 µl each time) near the nostrils allowing the mouse to breathe in the solution, repeat until the entire dose of 60 µl is administered.
4. Let the mouse regain consciousness before placing it back in the cage.
5. Repeat liposome administration every 3 days until the mice are sacrificed. Do not administer liposomes on a day of sacrifice.

3. Mouse Sacrifice and Tissue Collection

NOTE: Use autoclaved and sterile instruments for mouse dissection. Euthanize mice while under anesthesia through the exsanguination and removal of the vital organs.

1. On day 22 (for 4T1 cells) or day 26 (for 4T1-GFP cells) after tumor cell injection anesthetize the mouse as in 1.2.1. and place on the surgical pad with a nose cone connected to vaporizer, maintain anesthesia as in 1.2.1. Pinch one of the toes to ensure that the mouse is unconscious.
2. Pin the toes using needles to the dissection board. Spray ethanol on the mouse skin.
3. Lift the skin using forceps and make an incision with the surgical scissors. Slowly expose the peritoneum and continue cutting skin through the thorax until the neck.
4. Using the surgical scissors make an incision in the peritoneum and expose carefully the organs with cotton tips avoiding damage to the blood vessels.
5. Move the organs to one side and expose the inferior vena cava. Puncture the vein and collect blood with the 29 G insulin syringe. Place collected blood into the tube and leave on ice for further processing.
6. Cut the diaphragm to expose the rib cage, lungs and heart. Slowly cut the rib cage on both sides using the bone cutter and lift the top of ribcage. Grab the heart with forceps and cut the connective tissue connecting the heart and lungs to the chest cavity.
7. Place the lungs in small amount of PBS in the 60 mm Petri dish on ice until ready for imaging and further processing to frozen sections for immunofluorescent microscopy or routine histology [including hematoxylin and eosin (H & E) staining].

4. Lung Metastases Evaluation

1. **Counting and Scoring Surface Metastases**
    1. Place the Petri dish with the lungs under the dissection microscope. Focus to get a clear view of the lung surface.
    2. Using a dissection microscope observe the lung surface. Count metastases on the anterior and posterior sides of both lungs.
    NOTE: The normal lung is whitish or pinkish and porous and has a sponge like structure. 4T1 metastases are solid and nonporous, sometimes appear to be similar to a drop of liquid (**Figure 2A**).

2. **Lung imaging**
    1. Place the Petri dish with lungs on the movable stage inside the imaging instrument.
    2. Turn on the instrument and the biolite multispectral source. Open the software. Under the tab, "Acquisition", go to "Lighting", and switch to empty position (no filter) for white light. Light Engine--> ON, Light table--> Epi. Under the tab "Microscope" Select 'Clear' emission filter and 1.66X magnification. In the "Camera" tab, make sure that binning for capture is '1 x 1' and preview '4 x 4'.
    3. Click on preview and focus to get a clear picture of lungs in white light and click capture.
    4. Go to "Acquisition" tab and change to the filter 1 (filter for GFP as per manufacturer's instruction). In the "Microscope" tab, change emission filter to "530/20 GF". Preview and capture image in a green channel. Adjust exposure time to get the appropriate brightness.
    5. Apply pseudocolor to the image and save in appropriate folder.
    6. Flip the lungs to get image of the other side in a similar way. This procedure generates images of GFP-positive metastases that can be further quantified using appropriate software (**Figure 2B**)[14].

3. **Immunofluorescent Microscopy**
    1. Fix the lungs in 4% paraformaldehyde, at 4 °C for 4 hr in dark.
    2. Wash the tissue twice with 10 ml PBS for 5 min to completely remove the fixative. Transfer to 18 % sucrose in PBS and incubate O/N at 4 °C.
    3. Remove the tissue from the sucrose and dab off any excess.
    4. Take a cryomold, cover the bottom layer with OCT medium. Place the tissue on OCT medium. Fill the mold with OCT medium to completely cover the tissue and place on dry ice.
    5. Store block at -80 °C until sectioning.
    6. Prepare 5 µm thick sections with a cryostat and place on charged slides.
    7. Store unused slides in -80 °C until further usage.
    8. Air dry the slides for 7 min. and wash with PBS to remove OCT. Wipe the slide with tissue or paper towel to remove excess of water, mount cover slips with mounting medium containing DAPI and visualize with GFP filter under microscope.
    9. Quantify GFP metastases (**Figure 3A**) with the use of appropriate software[14].

4. **Histology and Digital Pathology**
    1. Click the manual load button under the start tab and wait for the slide holding rack to eject from the scanner.
    2. Take the H&E stained tissue section, clean it with tissue to remove any dirt, and place it securely in the slide holding rack.

3. Enter the slide description in the popped-up window.
4. Select the Scan Area tab on the SS imaging console window and adjust the green square to define the region of interest (ROI) to be scanned.
5. Drag the blue calibration diamond to a clear portion of the slide, where tissue is absent, however, within the ROI.
6. Next, select the Focus Points tab and click the auto select button to obtain several focus points (yellow plus signs) on the tissue in the ROI.
7. If necessary, place additional focus points manually by double-clicking within the ROI.
8. Proceed to the calibrate tab and select the calibrate button to begin slide calibration at the blue calibration diamond point. After the calibration is completed the image from the calibration point will be displayed.
9. Inspect this image to ensure that it is clear and free of dirt or artifacts.
10. Proceed to the Scan tab and select Start Scan to begin scanning of the ROI. Scanned histological section is converted to a digital slide (**Figure 3B and 4A**)
11. Quantify metastatic burden as previously described (**Figure 4B**)[14].

5. Flow Cytometry (FACS) Analysis of Alveolar Macrophages in the Lungs

1. **Preparation of Single Cell Suspension**
   1. After imaging and counting of metastases, wash the lungs with sterile PBS and place to the Petri dish. Make 1 - 2 mm pieces of the lungs using surgical scissors and forceps.
   2. Transfer the lung pieces to the 15 ml conical tube with 3 ml of the digestion buffer containing 1 mg/ml collagenase P, 0.04mg/ml DNase I, and 10 µg/ml trypsin inhibitor dissolved RPMI medium (sterile).
   3. Incubate the conical tube on a rotating shaker in the incubator at 37 °C for 40 min.
   4. Remove the tube conical from the incubator and pipette the solution up and down to dissociate the tissue.
   5. Pass the solution through the 40 µm strainer to avoid clumps and get a single cell suspension. If needed, to disintegrate better the digested tissue press tissue against the cell strainer with the help of a syringe plunger.
   6. When a single cell suspension has been achieved, collect the cells by centrifugation and lyse red blood cells with 2 ml of ACK buffer for 10 min at RT. Then, wash a pellet two times in 8 ml of RPMI.
   7. Resuspend cells in 3 ml of the complete RPMI medium and proceed to cell counting using the cell viability analyzer as per manufacturer's instructions.

2. **Staining of Lung Cells for Surface Markers**
   NOTE: Perform all incubations at 4 °C. Centrifuge plate at 500 x g and 4 °C.
   1. Pipette 1 x 10^6 cells in 100 µl of FACS buffer (1% FBS in PBS) to individual wells of a V-bottom 96-well plate. Using a V-bottom 96 well plate facilitates handing of multiple samples.
   2. Pre-incubate the cell suspension with 1 µg Mouse Fc Block purified anti-mouse CD16/CD32 in 100 µl of FACS buffer for 15 min. Centrifuge the V-bottom 96 well plate to pellet the cells and remove the supernatant by flipping the plate and letting the solution drain.
   3. For surface staining, in advance, dilute the antibodies to murine antigens in one tube (master mix): BV605 CD45 (30-F11), PE CD11b (M1/70), PE/Cy7 F4/80 (BM8), APC/Cy7 CD11c (N418), PerCPCy5.5 I^A/I^E (MHCII) (M5/114.152), PE CD80 (16-10A1), AF 647 CD86 (GL-1) to a final concentration of 2.5 µg/ml in FACS buffer containing 10 µg/ml of Fc block CD16/CD32 antibody.
      NOTE: This set of antibodies is useful for the identification and the evaluation of a functional status of alveolar macrophages and dendritic cells.
   4. Add 100 µl of diluted antibodies (master mix) to the wells of the V-bottom 96 well plate and incubate at 4 ˚C for 30 min.
   5. Centrifuge the plate to pellet cells and remove supernatant as in 5.2.2. Wash cells with 200 µl of FACS buffer, centrifuge at 500 g for 5 min. Remove the supernatant as in 5.2.2. and resuspend cells in 200 µl of PBS.
   6. Centrifuge the plate to pellet cells and add viability dye diluted in PBS (1:1,000). Incubate for 20 min at 4 ˚C.
   7. Centrifuge the plate to pellet cells and remove the supernatant as in 5.2.2. Wash with 200 µl of PBS and centrifuge the plate again.
   8. Resuspend cells in 100 µl of 1% paraformaldehyde and store at 4 °C until the acquisition by FACS instrument. Prior to acquisition transfer cell suspensions to the polypropylene FACS tubes.

6. Analysis of FACS Data: Gating Strategies and the Identification of Cell Subpopulations

1. Perform FACS analysis as previously reported[10]. Gate acquired cells/events based on forward-(FSC) and side-scatter (SSC); and then gate live and $CD45^+$ cells. For alveolar macrophage identification, gate CD11b-negative and then $CD11c^+F4/80^+$ cells. (**Figure 5A**).

Representative Results

The injection of 4T1-GFP tumor cells into the mammary fat pad leads to the formation of mouse tumors (**Figure 1A**) that recapitulate the metastatic spread of human breast cancer, as metastases are rapidly formed in the lungs (**Figure 2**), liver, bones and brain of mice[11]. The stable transfection of 4T1 cells with GFP facilitates monitoring of tumor growth (**Figure 1B**), tracking metastasizing tumor cells and quantifying the metastatic burden (**Figure 2B, 3B**). In addition, imaging of tumors provides the additional information regarding several pathological features such as tumor cell death and tumor vasculature. The bright green fluorescence (**Figure 1B-the middle panels**) indicate the high GFP expression, which is usually associated with the viable tumor parenchyma. Whereas the lack of GFP in some tumor areas may indicate tumor cell death (**Figure 1B-the right panel**). GFP is also absent from the tortuous aberrant tumor blood vessels (**Figure 1B-the right panel**).

Since the majority of lung metastases are located on the lung surface, they can be observed under the regular dissection microscope. The metastatic lesions are usually clearly distinct from the surrounding parenchyma (**Figure 2A**). To verify gross observations and evaluate metastases that are deeper within the lung parenchyma, fluorescent imaging of lungs can be performed (**Figure 2B**), if GFP expressing cells were used for injection. Although lung autofluorescence is clearly visible in the images, bright GFP-derived fluorescence facilitates distinguishing metastases from surrounding lung parenchyma. Alternatively, routine histology (**Figure 3A and Figure 4A**) in conjunction with digital pathology algorithms (**Figure 4B**) provide good tools to verify and quantify metastases.

Multicolor FACS provides opportunity to characterize even rare cell populations through the use of multipole cell surface and/or cytoplasmic markers simultaneously. Alveolar macrophages are characterized by the lack of CD11b expression and expression of F4/80 and CD11c (**Figure 5**). The typical approach to identify these cells by flow cytometry involves: (i) selection of cell population based on morphology depicted by forward and side scatter properties, (ii) selection of viable $CD45^{+}$ cells followed by (iii) gating of $CD11b^{negative}$ cells, and (iv) gating of cells coexpressing F4/80 and CD11c (**Figure 5A,B**).

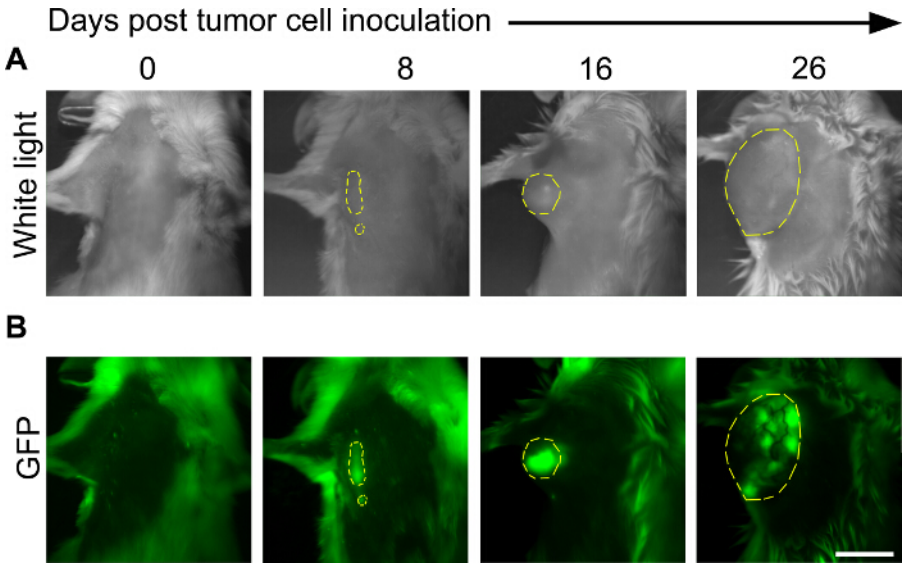

Figure 1. Monitoring Tumor Growth Through Animal Imaging. (**A**)White light images of the mouse injected with GFP expressing cells into the mammary fat pad at different time points after tumor cell injection. **(B**) Corresponding fluorescent images obtained through the use of GFP filter. Dashed lines mark tumor area. Note the lack of bright green fluorescence in some tumor areas on day 26 that may result from tissue necrosis or developing neovasculature. Scale bar, 10 mm. Please click here to view a larger version of this figure.

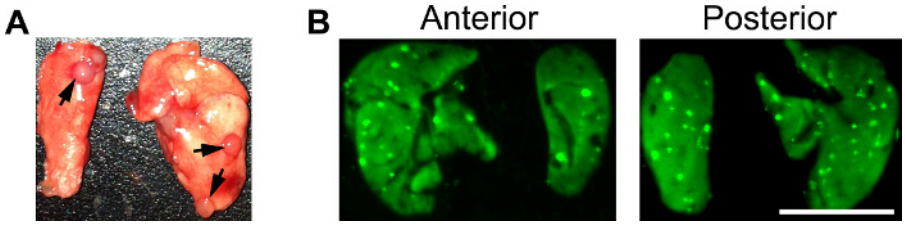

Figure 2. Evaluation of Lung Metastatic Burden. (**A**) Images of the lungs of mice injected with 4T1 cells into the mammary fat pad with the surface metastases (arrows). (**B**) Images of GFP^{+} lung metastases (bright green fluorescence) obtained through use of imaging microscope. Scale bar, 10 mm. Please click here to view a larger version of this figure.

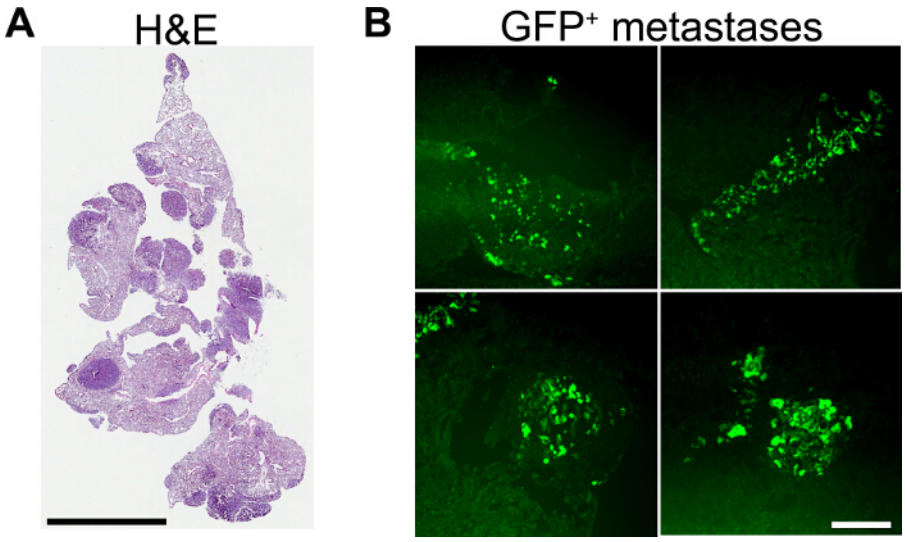

Figure 3. Digital Histopathology and Confocal Microscopy in Imaging of Lung Metastases. (**A**) The scan of the hematoxylin and eosin (H&E) stained lung section from mice injected with 4T1 cells into the mammary fat pad (dark blue tissue-metastases). Scale bar, 4 mm. (**B**) Confocal microscopy of GFP^+ lung metastases (bright green fluorescence). Scale bar, 200 µm. Please click here to view a larger version of this figure.

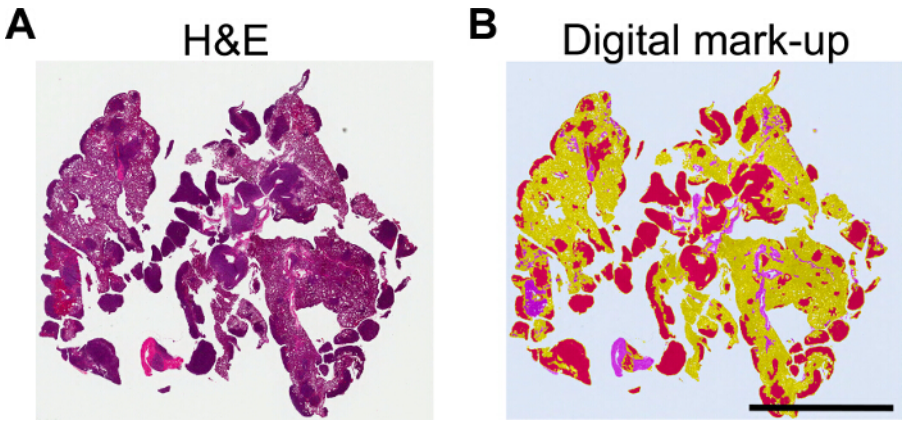

Figure 4. Quantification of Lung Metastatic Burden by Digital Histopathology. (**A**)The scan of hematoxylin and eosin (H&E) stained lung section (dark blue tissue-metastases). **(B**) The image of the lungs shown in (**A**) generated by a trainable histomorphology image analysis tool (software-"Genie"). Red color indicates the lung areas identified by "Genie" as metastases. Scale bar, 5 mm. Please click here to view a larger version of this figure.

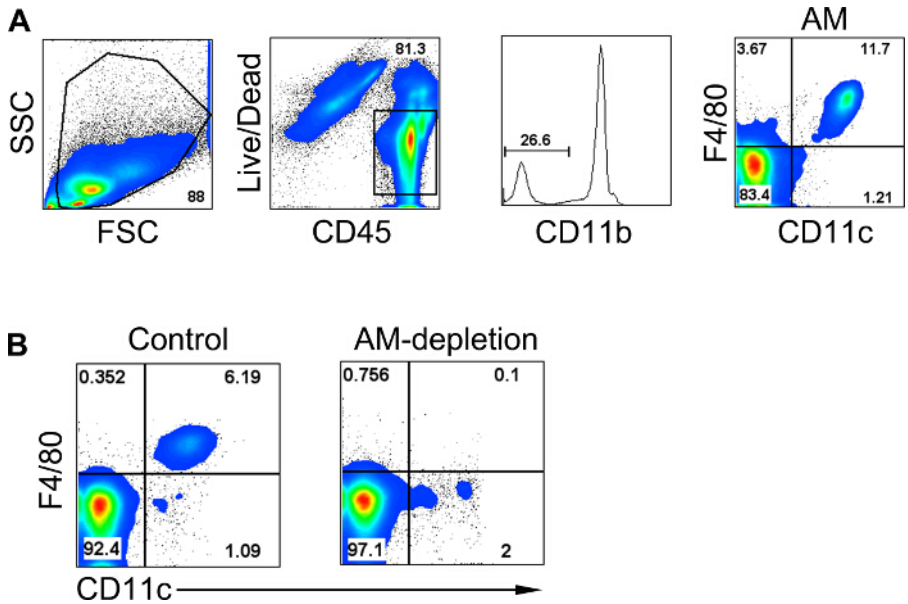

Figure 5. Flow Cytometry Approaches for Identifying Alveolar Macrophages in the Lungs. (**A**)The representativeflow cytometrydot plotsillustrating the gating strategy to identify $CD45^{+}CD11b^{negative}F4/80^{+}CD11c^{+}$ alveolar macrophages. (**B**) Representative flow cytometry dot plotsillustrating effect of control or clodronate liposomes on lung alveolar macrophages. Numbers in plots represent percentages of gated cells. Please click here to view a larger version of this figure.

Discussion

The recent insights into cancer biology and causative factors involved in carcinogenesis and tumor progression lead to development of genetically engineered mouse (GEM) models of cancer, in which tumors grow spontaneously, usually over a period of several months[15]. Although these tumor models appear to reflect better the natural history of human malignancies than xenografts or syngeneic models, much time required for tumor development and various degrees of malignant phenotype penetrance limit the use of these approaches in mechanistic studies. Therefore, the models utilizing immunocompetent mice injected orthotopically with syngeneic cells, although not perfect, are still helpful in addressing mechanistic questions pertaining to the tumor microenvironment and a role of the immune system in cancer. The 4T1 model has been extensively used for these purposes, as it recapitulates stage IV metastatic breast cancer in humans[11].

The use of 4T1 tumor cells expressing fluorescent proteins offers opportunity to monitor tumor growth, changes in the tumor cell viability, and metastasis through animal imaging[14]. Although this is an elegant approach to track metastasizing cells, some concerns related to the potential immunogenicity of 4T1 cells expressing GFP, and the generation of antibodies specifically recognizing 4T1-GFP cells have been raised[16]. However, these antibodies were found to be present in mouse plasma three weeks or later after the implantation of tumor cells[16]. Therefore, considering rapid growth of tumors in this model and the fact that mice are usually sacrificed before a humoral response develops or shortly after, 4T1-GFP model appears to be a useful tool for tracking circulating tumor cells in short term studies. In addition, it has been noted that low GFP expression, which characterize cells that we used for our studies, induces tolerance to GFP peptides instead of causing lymphocyte activation[17].

The potential problem associated with the low level of GFP expression is long exposure time required to image organs, which are target for metastases, or tissue sections, to detect endogenous GFP expression. For immunofluorescent microscopy this problem can be, however, overcome by staining sections with appropriate GFP antibodies

The critical aspect of these studies is also an appropriate technique of the orthotopic cell injection, as any leakage of the solution containing tumor cells reduces the number of cells injected and, consequently, affects the pace of tumor growth and metastatic potential. By adjusting the number of injected cells, one can modify speed of tumor growth and time interval between injection of tumor cells and metastatic progression of disease. In addition, it is important to consider high variability of results obtained through the majority of *in vivo* approaches including those discussed here. Therefore, a sufficient number of mice, individually determined through a power analysis, needs to be used. In our laboratory, we typically use ten mice per cohort.

The use of clodronate liposomes to deplete different macrophage populations has been extensively explored[18]. However, several concerns related to specificity of this approach are repeatedly raised. Although intravenous injections of clodronate-liposomes depletes several different type of phagocytes in different organs, intratracheal or intranasal administration affects mainly lung macrophages[18]. Furthermore, our recent study has demonstrated that intranasal instillation of clodronate-liposomes, according to the protocol established in our laboratory, leads only to the depletion of alveolar macrophages, typically located in the lumen of lung alveoli, but does not affect myeloid-derived suppressor cells and interstitial macrophages[10].

Disclosures

The authors declare that they have no competing financial interests.

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