A Tumor-admixture Model to Interrogate Immune Cell–dependent Tumorigenesis

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Abstract

A rigorous determination of effector contributions of tumor-infiltrating immune cells is critical for identifying targetable molecular mechanisms for the development of novel cancer immunotherapies. A tumor/immune cell–admixture model is an advantageous strategy to study tumor immunology as the fundamental methodology is relatively straightforward, while also being adaptable to scale to address increasingly complex research queries. Ultimately, this method can provide robust experimental information to complement more traditional murine models of tumor immunology. Here, we describe a tumor/macrophage-admixture model using bone marrow–derived macrophages to investigate macrophage-dependent tumorigenesis. Additionally, we provide commentary on potential branch points for optimization with other immune cells, experimental techniques, and cancer types.

Keywords: Cancer, Immunology, Immunotherapy, Macrophage, Mouse models

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Background

Evasion of anti-tumor immunity is now a well-established hallmark of cancer progression (Hanahan and Weinberg, 2011). Translation of fundamental concepts in tumor immunity has resulted in the development of immune checkpoint inhibitors that are highly efficacious in select oncological types (Xin et al., 2019; Robert, 2020). However, several types of malignancies do not respond, have intrinsic resistance, or develop secondary adaptive resistance to these therapies (Sharma et al., 2017; Jenkins et al., 2018; Schoenfeld and Hellmann, 2020). These issues have led to an outpouring of basic science and translational projects aimed at identifying additional molecular mechanisms by which cancer cells or infiltrating immune cells evade anti-tumor immunity, with the goal of developing novel immunotherapeutics (Fares et al., 2019; Karasarides et al., 2022). Here, we detail a published tumor/immune cell–admixture model (Liu et al., 2020; Noe et al., 2021), using tumor-associated macrophages as an example, that can serve as a useful tool to investigate the relative contributions of tumor-infiltrating immune cells in promoting cancer progression.

Tumor-associated macrophages (TAMs) are myeloid-derived immune cells that infiltrate into the tumor microenvironment to affect tumorigenesis (Mantovani et al., 1992; Noy and Pollard, 2014; Pan et al., 2020; Pittet et al., 2022). TAMs have both pro- and anti-tumor effector functions—their phagocytosis of cancer cells (Kamber et al., 2021), presentation of tumor-associated antigens (Asano et al., 2011), and activation of anti-tumor adaptive immunity play a critical role in the elimination of cancer cells (Modak et al., 2022). In contrast, TAMs can also promote tumorigenesis by suppressing phagocytic and antigenic innate immune responses (Barkal et al., 2019; Demaria et al., 2019; Zhou et al., 2020), as well as by inducing adaptive immune cell exhaustion (DeNardo and Ruffell, 2019; Singhal et al., 2019). Understanding the TAM-dependent contribution to tumor progression or regression is critical for identifying novel tumor immunotherapeutic strategies.

Several different tumor models have been developed to interrogate the effects of specific immune cell types in tumorigenesis. These protocols traditionally include the targeted depletion (Xiao and Jiang, 2014) or transgenic manipulation (McCubbrey et al., 2017) of the desired immune cell in the context of orthotopic or heterotopic tumors in xenograft or allograft models. The tumor-admixture model described herein is a heterotopic, allograft murine model using ex vivo differentiated/polarized bone marrow–derived macrophages (BMDMs) injected with tumor cell lines into congenic recipient mice. This admixture model has been shown to be an advantageous strategy to relatively easily determine gene-specific contributions to TAM-dependent tumor progression (Liu et al., 2020; Noe et al., 2021).

While this protocol was developed to investigate the ability of ex vivo interleukin-4 (IL-4) polarized pro-tumor TAMs to suppress anti-tumor immunity leading to enhanced tumor growth (Liu et al., 2020; Noe et al., 2021), this model can be used with other TAM phenotypes, different immune cell types, and additional experimental manipulations. A potential critique of this model includes the relative contribution of host-derived, tumor-infiltrating TAMs, which can be addressed by incorporating a targeted depletion strategy (Xiao, and Jiang, 2014). Adapting this model using anti-tumor TAMs (Liu et al., 2020; Noe et al., 2021) offers an interesting approach to understanding the mechanisms by which TAMs promote tumor regression. With rigorous optimization, other immune cell types that are critical regulators of tumorigenesis, such as neutrophils (Shaul, and Fridlender, 2019), dendritic cells (Wculek et al., 2020), or myeloid-derived suppressor cells (Veglia et al., 2021), could be adapted for use. Lastly, this model is highly amendable to the post-injection administration of small molecules or biologic inhibitors to further validate their importance as a clinically viable therapeutic (Zanoni et al., 2012; Noe and Mitchell, 2020). Altogether, this tumor-admixture model is a relatively straightforward, highly adaptable protocol to investigate immune cell–dependent cancer progression.

Materials and Reagents

1.   0.70 μm filter
2.   2 mL syringe
3.   28 gauge needle
4.   Falcon conical centrifuge tubes, 15 mL (Corning, catalog number: 352095)
5.   Microcentrifuge tubes, 1.5 mL (Sigma Aldrich, catalog number: HS4323)
6.   C57BL/6J mice, CD45.2$^+$ donor (Jackson Laboratory, strain #: 000664)
7.   B6.SJL mice, CD45.1$^+$ congenic recipient (Jackson Laboratory, strain #: 002014)
8.   Matrigel matrix basement membrane (Corning, catalog number: 354234)
9.   CellStripper dissociation solution (Corning, catalog number: 25-056-CI)
10. Trypsin-EDTA, 0.05% (Gibco, catalog number: 25300062)
11. Phosphate-buffered saline (PBS) (Corning, catalog number: 21-040)
12. RPMI 1640 medium (Gibco, catalog number: 11-875-093)
13. Fetal bovine serum (FBS), heat-inactivated (Gibco, catalog number: 10-082-147)
14. 10 cm tissue culture–treated plates (Corning, catalog number: CLS430167)
15. Trypan Blue solution (Gibco, catalog number: 15250061)
16. Lewis lung carcinoma cells (ATCC, catalog number: CRL-1642)

Note: As an example, we engrafted Lewis lung carcinoma cells; see Procedure section B for more information. Please utilize the previously described Bio-Protocol manuscripts for BMDM differentiation and culture of C57BL/6J CD45.2$^+$ donor mice: Zanoni et al. (2012), Haag and Murthy (2021).

Equipment

1.   Tabletop centrifuge (e.g., Kendro Sorval legend RT)
2.   Microcentrifuge (e.g., Eppendorf refrigerated microcentrifuge 5415R)
3.   Water bath at 37 °C (e.g., Thermo Scientific PRECISION water bath)
4.   Incubator at 37 °C with 5% CO_2 (e.g., Thermo Scientific HERACell 150i CO_2 incubator)
5.   Laminar flow hood (e.g., The Baker Company, SterilGARD class II biological safety cabinet)
6.   Cell lifter (VWR, catalog number: 76036-006)
7.   Digital calipers (VWR, catalog number: 62379-531)

Procedure

A.   Preparation

1.   Pre-warm RPMI 1640 medium, PBS, FBS, trypsin-EDTA, and CellStripper dissociation solution in a water bath set to 37 °C.
2.   Thaw Matrigel matrix basement membrane on ice.
     Tip: Matrigel can prematurely solidify at room temperature, so care must be taken to ensure it is maintained in a liquid state until immediately before use.
3.   Make wash media: RPMI 1640 medium + 5% FBS.
4.   Make injection solution: PBS + 5% FBS.

B.   Tumor cell line collection

Note: Most murine tumor cell lines are likely amendable to this model, but this should be predetermined by optimization studies using B6.SJL recipient mice. On the day of injection, tumor cells should be in 10 cm tissue

culture plates and in log-growth phase (approximately 5.0×10^6–7.0×10^6). Although 5.0×10^5 tumor cells per injection is our recommended baseline, this number should be optimized based upon the cell line's in vivo proliferation rate and desired time of tumor growth.

1. Wash plates twice with 10 mL of PBS and aspirate PBS after each wash.
2. Add 3 mL of trypsin-EDTA to each plate and place in incubator for 3–5 min.
3. Transfer cells to fresh Falcon tubes.
4. Wash plate twice with 5 mL of wash media and add wash to Falcon tubes.
5. Centrifuge at $350 \times g$ for 5 min and aspirate supernatant.
6. Resuspend cell pellet with 10 mL of wash solution.
7. Repeat steps B5 and B6.
8. Filter cell suspension through a 0.70 μm filter into a fresh Falcon tube.
9. Count cells in Trypan Blue solution using a cell counter or other standard method.
10. Centrifuge at $350 \times g$ for 5 min and aspirate supernatant.
11. Resuspend cells to 4×10^6 cells/mL in the Falcon tubes and place tumor cells on ice.
    Tip: The tumor cell collection can be performed simultaneously with the BMDM collection (Section C) to increase efficiency and reduce the length of time the tumor cells are kept on ice.

C. Bone marrow–derived macrophage (BMDM) collection

Note: BMDMs need to be derived from C57BL/6J CD45.2+ donor mice. The length of BMDM polarization prior to injection should be optimized based upon polarization method and experimental investigations (i.e., M1 vs. M2 polarization, small molecule inhibitor treatments, or transgenic manipulations). For example: we have used a transgenic model of BMDMs from C57BL/6J CD45.2+ donor mice that were polarized with IL-4 for 24 h (Noe et al., 2021). On the day of injection, BMDMs should have high viability (>90%), as determined by Trypan Blue exclusion, and 2.0×10^5 BMDMs per injection is needed.

1. In a laminar flow hood, wash plates twice with 10 mL of PBS and aspirate PBS after each wash.
2. Add 3 mL of CellStripper dissociation solution to each plate and place in the incubator for 3–5 min.
3. Gently lift BMDMs with a cell lifter and transfer cells to Falcon tubes.
   Tip: Avoid aggressive scraping of BMDMs or using trypsin instead of cell stripper, as this causes reduced BMDM viability.
4. Wash plate twice with 5 mL of wash media and add wash to Falcon tubes.
5. Centrifuge at $350 \times g$ for 5 min and aspirate supernatant.
6. Resuspend cell pellet with 10 mL of wash solution.
7. Repeat steps C5 and C6.
8. Filter cell suspension through a 0.70 μm filter into a fresh Falcon tube.
9. Count cells in Trypan Blue using a cell counter or other standard method.
10. Centrifuge at $350 \times g$ for 5 min and aspirate supernatant.
11. Resuspend cells to 1.6×10^6 cells/mL in the Falcon tubes and place BMDMs on ice.

D. Tumor-BMDM admixture

Note: A limited optimization study should be performed beforehand to determine the preferred tumor cell:BMDM ratio. As a baseline, we have found that a 2.5:1 ratio provides meaningful data (Liu et al., 2020; Noe et al., 2021), but results may vary depending on tumorigenic potential, immunogenicity, and other experimental conditions. Each 2 mL tube can hold three tumor injections (1.5 mL injection + 0.5 mL slop; the required volume needed for tumor injections should be determined beforehand). For best results, the following steps should be performed immediately prior to injections. Currently, we describe a heterotopic model utilizing subcutaneous injections. In some cancer subtypes, different treatment responses occur depending on whether an orthotopic or heterotopic model is used (Erstad et al., 2018). We anticipate that this admixture model could be adapted to an orthotopic approach, but this will likely require thorough optimization beforehand.

Per 2 mL microcentrifuge tube: three injections (500 μL/injection) + 500 μL extra volume:

1. Mix tumor cell suspension, aliquot 500 μL to fresh microcentrifuge tube, and place on ice.
2. Mix BMDM suspension, aliquot 500 μL to the same microcentrifuge tube, mix well, and place on ice.
3. Immediately prior to injection, aliquot 1 mL Matrigel to the microcentrifuge tube, mix well, and place on ice.
4. Draw 500 μL of the admixture into a 2 mL syringe and place a 28 gauge needle.
   Tip: Again, Matrigel can prematurely solidify at room temperature. Pre-chilling syringes on ice before drawing admixture and placing syringe on ice in between injections may be needed.
5. Administer a 500 μL subcutaneous injection into the flank of a B6.SJL recipient mouse.
   Tip: The flank can be shaved prior to injection to increase visibility during injection or subsequent measurements. Following injection, leave the needle in place for 1–3 s to prevent leakage from the insertion site. Any significant leakage should be noted and may represent a failed injection.
6. Using a new syringe, repeat steps D4 and D5 for each sequential mouse.

E. Downstream analyses

Note: Following injections, several experimental manipulations and/or downstream analyses are possible. The effect of a small molecule inhibitor or a biologic can be interrogated through the administration of these agents at predetermined time points. Once tumor endpoints are achieved, immunophenotyping with flow cytometry is possible by obtaining the spleen, blood, and tumor through previously described protocols (Almeida et al., 2021). At a minimum, tumor growth and end-point weight should be measured to determine gross changes in tumorigenesis, which are described below.

1. Monitor mice for tumor outgrowth to indicate a successful injection.
2. Once tumors are palpable (approximately day 5–7), measure the tumor length and width every 2–3 days with external calipers.
3. Record the length and width to calculate the tumor volume using the following formula: $(length \times width^2)/2$.
4. Once the tumor endpoint is reached, euthanize mice, resect tumors, and measure weight (Figure 1).
5. Proceed with downstream analyses, if desired.

Figure 1. Tumor-admixture model enhances tumor outgrowth. Gross dissection of tumors from mice 16 days post-injection with Lewis lung carcinoma (LLC) cells plus M2-polarized bone marrow–derived macrophages (BMDMs) (above) or LLC cells alone (below). Size differences represent variability of tumor size within each cohort. Please refer to Noe et al. (2021) for a more comprehensive analysis of tumor growth curves and endpoint tumor weight.

Acknowledgments

Competing interests

The authors declare no competing interests.

Ethics

Animals were maintained under specific pathogen-free conditions and handled in accordance with the Association for Assessment and Accreditation of Laboratory Animals Care international guidelines. The Institutional Animal Care and Use Committee at the University of Louisville approved the experiments.

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