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Studying the Effects of Tumor-Secreted Paracrine Ligands on Macrophage Activation Through the Use of Transwell Co-Culture

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Phillip Steindel, PhD
Review Editor
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Response to the Review of Manuscript JoVE60453, entitled "Studying the Effects of Tumor-Secreted Paracrine Ligands on Macrophage Activation Through the Use of Transwell Co-Culture"

Dear Editor and Review Committee:

We thank the reviewers and editors for both their positive comments and insightful critiques, which we have used to make our manuscript even stronger. We have taken the one-week response period to significantly improve the text, incorporating editorial and reviewer comments, and are now resubmitting our revised manuscript. We have also followed the instructions provided for manuscript formatting.

Thanks in advance for your consideration of our revised manuscript.

Sincerely,

Eric S. Ubil

Eric Ubil, PhD, MBA
Assistant Professor
UAB Department of Microbiology and O'Neal Comprehensive Cancer Center

TITLE:

Studying the Effects of Tumor-Secreted Paracrine Ligands on Macrophage Activation using Co-Culture with Permeable Membrane Supports

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KEYWORDS:

cancer, paracrine signaling, ligand, receptor, permeable membrane supports, co-culture

SUMMARY:

Here, we present a method using permeable membrane supports to facilitate the study of non-contact paracrine signaling used by tumor cells to suppress the immune response. The system is amenable to studying the role of tumor-secreted factors in dampening macrophage activation.

ABSTRACT:

Tumor-derived paracrine signaling is an overlooked component of local immunosuppression and can lead to a permissive environment for continued cancer growth and metastasis. Paracrine signals can involve cell-cell contact between different cell types, such as PD-L1 expressed on the surface of tumors interacting directly with PD-1 on the surface of T cells, or the secretion of ligands by a tumor cell to affect an immune cell. Here we describe a co-culture method to interrogate the effects of tumor-secreted ligands on immune cell (macrophage) activation. This straightforward procedure utilizes commercially available 0.4 µm polycarbonate membrane permeable supports and standard tissue culture plates. In the process described, macrophages are cultured in the upper chamber and tumor cells in the lower chamber. The presence of the 0.4 µm barrier allows for the study of intercellular signaling without the confounding variable of physical contact, because the two cell types share the same medium and exposure to paracrine ligands. This approach can be combined with others, such as genetic alterations of the macrophage (e.g., isolation from genetic knock-out mice) or tumor (e.g., CRISPR-mediated alterations) to study the role of specific secreted factors and receptors. The approach also lends itself to standard molecular biological analyses such as quantitative reverse transcription polymerase chain reaction (qRT-PCR) or Western blot analysis, without the need for flow sorting to separate the two cell populations. Enzyme-linked immunosorbent assays (ELISAs) can similarly

be utilized to measure secreted ligands to better understand the dynamic interaction of cell signaling in the multiple cell type context. Duration of co-culture can also be varied for the study of temporally regulated events. This co-culture method is a robust tool that facilitates the study of tumor-secreted signals in the immune context.

INTRODUCTION:

Recent studies have focused on the ability of cancer cells to avoid detection by immune cells, suppress local immune activation, or to produce a tolerogenic tumor-permissive milieu in the tumor microenvironment. Two broad classes of tumor and immune cell interactions have been described that facilitate these effects: contact-mediated interactions or tumor-secreted ligands. One of the most well-studied and clinically tractable mechanisms of contact-mediated immune inhibition utilized by tumors is the expression of PD-L1, which interacts with PD-1 on T cells to inhibit their activation and function[1,2]. In response to interferon-gamma (IFNγ), which is expressed by a number of activated immune cells, tumor cells can increase expression of PD-L1 to induce exhaustion of PD-1–expressing activated T cells, thereby preventing them from effectively eradicating tumor cells[3]. The use of antibodies to block the interaction between PD-L1 and PD-1 is currently used to treat multiple cancer types in humans[4]. In light of this clinical success and others, the identification and targeting of tumor-derived immunosuppressive mechanisms has received increasing attention.

Beyond suppression of adaptive immunity, tumors are also known to secrete factors that suppress the pro-inflammatory responses of innate immune cells. Tumor-derived or tumor-induced secretions, including IL-6, IL-10, VEGF, IL-23, and colony stimulating factor (CSF-1), have been shown to inhibit antitumor responses of natural killer (NK) cells, granulocytes, and dendritic cells in the tumor microenvironment[5-7]. Tumor cells can also secrete factors that skew the recruitment and differentiation of myeloid-derived cells in the tumor microenvironment to promote suppression of T cell activation[8,9].

One type of innate immune cell that has a profound effect on tumor progression is the macrophage. For many years, the presence of tumor-associated macrophages (TAMs) has been used as a negative prognostic of patient survival[10]. The concept that immunosuppressive TAMs dampen immune cell-mediated clearance of tumors was introduced more than 40 years ago[11]. More recently, it has been shown that the macrophage pro-inflammatory response can be downregulated while a pro-tumor phenotype can be induced in the tumor microenvironment. These immunosuppressive macrophages can contribute to a tolerogenic response, driving tumor progression and resistance to chemo- and immunotherapy[12]. Given that macrophages are often one of the most abundant leukocytes with the tumor, restoration of their tumor-specific immune activity represents a potential target for anticancer therapeutics[13].

While contact-mediated interactions between tumor cells and macrophages can be modeled through direct coculture, the use of permeable membrane supports can elucidate which tumor-secreted factors are immunomodulatory without the potentially confounding influence of tumor-immune cell-cell contact. Using somewhat similar methods, others have demonstrated the potential of identifying secreted factors in microglia/neuronal interactions[14] as well as tumor cells

with mesothelial cells[15]. We have also successfully used this co-culture technique to characterize the role of a tumor-secreted protein, Pros1, as a suppressor of pro-inflammatory gene expression after the stimulation of peritoneal macrophages with LPS and interferon-gamma[16]. Here we describe a straightforward methodology that can be used to interrogate how tumor-secreted factors can affect macrophage activation.

PROTOCOL:

All procedures related to the harvest and use of murine peritoneal macrophages were conducted at the University of North Carolina at Chapel Hill (UNC) and were approved by the UNC Institutional Animal Care and Use Committee (IACUC).

1. Macrophage culture

NOTE: This procedure can utilize primary peritoneal macrophages (described in detail below), bone marrow-derived macrophages, or macrophage cell lines such as J774 (ATCC) or RAW264 (ATCC).

1.1. Harvest peritoneal macrophages as previously described[16,17] and plate directly into the upper chamber(s) of a 0.4 µm polyester membrane insert co-culture 6 well plate (**Figure 1A**).

NOTE: The approximate yield of macrophages from each isolation is 1 x 10^6 cells total, so the average number of cells per well is ~1.5–1.6 x 10^5 cells in a 6 well plate.

1.2. Culture the harvested macrophages in Dulbecco's Modified Eagle Medium (DMEM)/F12, 10% Fetal Bovine Serum (FBS), 1x penicillin/streptomycin, 20 ng/mL macrophage colony stimulating factor (M-CSF) for 3 days at 37 °C, 5% CO_2.

NOTE**:** The upper chamber contains 1 mL of culture medium while the lower chamber is filled with 1.5 mL. The medium must be added to each chamber.

2. Coculture of tumor cells with macrophages

2.1. Prior to use, culture commercially available tumor cells in their respective medium following ATCC-recommended tissue culture methods.

2.2. Wash adherent tumor cells once with phosphate buffered saline (PBS), add 0.05% trypsin + ethylenediaminetetraacetic acid (EDTA), and incubate at 37 °C until the cells detach. Resuspend the cells in FBS containing medium, quantitate the total number of cells using a hemocytometer or cell counter, and then centrifuge for 5 min at 220 x *g* to pellet.

2.3. During centrifugation, aspirate the medium from the upper and lower chambers of the macrophage-containing permeable membrane support plates and replace with fresh medium.

2.3.1. For lower chambers where tumor cells will be plated, fill with 1 mL of medium instead of 1.5 mL to allow for sufficient volume for cell addition.

2.4. Aspirate medium from pelleted tumor cells and resuspend cells in DMEM/F12 with 10% FBS, 1x penicillin/streptomycin, and 20 ng/mL M-CSF at a concentration of 3 x 10^5 cells/mL.

2.5. Add 0.5 mL of 3 x 10^5 cells/mL tumor cells to the lower chamber of desired wells (**Figure 1B**).

NOTE: Cells can be treated immediately.

3. Treatment of co-cultured cells

3.1. To induce macrophage pro-inflammatory gene expression, treat singly or co-cultured macrophages by adding 100 ng/mL IFNγ and 50 ng/mL LPS.

3.1.1. Vary the duration of treatment times in culture as needed. Macrophage activation occurs within 2 h and some tumor-mediated suppression occurs by 8 h. Co-culture for 24 h yields robust and consistent tumor-derived suppression.

NOTE: Alternatively, macrophages can be induced to adopt a pro-wound healing phenotype through the addition of factors such as interleukin-10 (IL10), and the effect of the tumor-secreted ligand assessed.

3.2. As a negative control, culture macrophages singly and leave untreated. As a positive control, treat singly cultured macrophages with 100 ng/mL IFNγ and 50 ng/mL LPS.

4. Downstream analysis of co-cultured cells

4.1. After desired incubation time has elapsed, isolate the cell lysate or conditioned culture medium as desired, depending on testing needs.

4.2. To isolate the cell lysate for quantitative polymerase chain reaction (qPCR) analysis, aspirate the media from both chambers of the well and wash once with 2 mL of PBS. Apply RNA lysis buffer to the top chamber containing the macrophages. Gently scrape the membrane to release the cell lysate, and transfer to a collection tube for further processing according to the RNA isolation kit manufacturer's protocol.

REPRESENTATIVE RESULTS:

To determine the effect of tumor-secreted ligands on macrophage polarization, the procedure described was utilized. Peritoneal macrophages cultured in the absence of tumor cells were used as negative (untreated = far left) and positive (IFNγ and LPS stimulated = 2nd from left) controls (**Figure 2A**). Alternatively, peritoneal macrophages were co-cultured with B16F10 melanoma tumor cells (ATCC) (**Figure 1A**). Immediately after plating, cells were either treated with IFNγ and

LPS or left untreated. After 24 h of culture, macrophages were harvested, RNA prepared, and qRT-PCR performed to measure the expression of pro-inflammatory genes. Using the co-culture system described here, we show that peritoneal macrophages co-cultured in the presence of B16F10 tumor cells, but without activating stimuli (LPS + IFNγ), did not increase expression of pro-inflammatory–associated genes (**Figure 2A**, B16F10 membrane/untreated). This implies that tumor-secreted ligands are 1) not sufficient by themselves to induce pro-inflammatory gene expression or 2) if there is immune activation by tumor secretions, paracrine ligands are sufficient to suppress it to naive levels. This co-culture method illustrates that when macrophages polarized by IFNγ and LPS are cultured in the presence of tumor cells, suppression of inflammation-associated gene expression was reduced by as much as 60% (**Figure 2A**, B16F10 Membrane/IFNγ+LPS). A comparable level of macrophage pro-inflammatory gene suppression was observed when the murine macrophage cell line J774 was substituted for peritoneal macrophages (**Figure 2B**).

Our previous work identified Pros1 as a tumor-secreted factor that can inhibit macrophage activation[16]. Using the permeable membrane support co-culture model in conjunction with ELISA, we assayed the concentration of Pros1 in conditioned medium after 24 h. We observed that in conditioned medium from IFNγ and LPS treated B16F10 melanoma cells Pros1 was expressed at 475 ng/mL ± 120 ng/mL (**Figure 3**). Peritoneal macrophages treated in the same conditions expressed Pros1 at 61 ng/mL ± 5 ng/mL (**Figure 3**). Interestingly, when co-cultured, the Pros1 within the IFNγ and LPS treated well was 86 ng/mL ± 15 ng/mL. This suggests that 1) macrophages consume tumor-secreted Pros1 or 2) the amount of Pros1 secreted by B16F10 cells is substantially decreased when in the presence of macrophages. Results from both **Figure 2** and **Figure 3** highlight profound changes of macrophage activation and paracrine signaling when macrophages are co-cultured with tumor cells.

FIGURE LEGENDS:

Figure 1. Schematic for permeable membrane support co-culture of tumor cells with macrophages. Positive and negative treatment controls can be applied to singly cultured macrophage wells **(A)**. To determine the effects of tumor-secreted signals on macrophage activation, macrophages are cultured in the upper chamber of permeable membrane support co-culture plates and tumor cells cultured in the lower chamber **(B)**.

Figure 2. Tumor paracrine signals suppress macrophage pro-inflammatory polarization. Macrophage expression of inflammation-associated genes was assayed by qRT-PCR in untreated or IFNγ and LPS stimulated macrophages in the presence or absence of tumor cells. Expression of pro-inflammatory genes was decreased in peritoneal macrophages **(A)** or J774 macrophage cell line **(B)** when cultured in the presence of tumor cells separated by a permeable membrane support so that the effect was transmitted by a paracrine soluble ligand. $*p < 0.05$ relative to untreated, $^{\dagger}p < 0.05$ relative to IFNγ and LPS stimulated. Data are mean ± SEM; *p* values calculated by two-tailed Student's t test.

Figure 3. Coculture of tumor cells and macrophages leads to changes in the amount of tumor-secreted paracrine ligands found in conditioned medium. ELISA was used to determine the concentration of Pros1 in tumor alone, macrophage alone, or co-cultured conditioned medium after 24 h. Co-culture leads to a decrease in the amount of Pros1 relative to tumor cell only controls. *$p < 0.05$ relative to untreated. *p* values calculated by two-tailed Student's t test.

DISCUSSION:

The co-culture assay presented here is a modification of previously established assays that allows for the study of tumor-secreted factors on immune cell activation. While cell-cell contact is known to induce changes in immune activity, the ability of tumor-secreted ligands to modulate immune activation is less well understood. We describe a method which, unlike direct co-culture, can be used to interrogate how tumor-derived secreted factors impact immune cell activation without the confounding nature of contact-mediated signaling. Given the potential clinical importance of tumor-secreted ligands in immunosuppression, this method offers an easy to use tool to study these mechanisms in ways not addressed by direct co-culture.

Essentially, the co-culture method described here involves the culture of macrophages (primary cells: resident, bone marrow-derived, or a macrophage cell line) with tumor cells (cell line or primary cells) without physical contact. It is critical that the macrophages be cultured on a 0.4 µm pore size polyester membrane insert to allow free permeability of tumor-secreted ligands while preventing possible macrophage cell migration through the filter. It is also important to add the described amount of culture medium to the upper and lower chambers to ensure proper cell coverage. Experimental design, such as inclusion of one cell type only controls, is another key factor when planning co-culture experiments. Several other factors, described in more detail later, can also have effects on the results of the assay and it is important to keep each in mind during study design.

Two useful modifications to the outlined protocol to consider are the duration of co-culture or the relative ratio of tumor cells to macrophages. In the method described, macrophages and tumor cells are plated at roughly equal concentrations. An important point to consider is the relative proportion of macrophages to tumor cells that naturally occurs in the tumor microenvironment. To better mimic the tumor microenvironment, the relative proportion of macrophages could be altered to mirror what is found within the tumor in vivo, though preliminary work may be necessary to establish the correct ratio.

Another critical point, and possible limitation of the system, is that treatments applied to stimulate one cell type in the assay may have unexpected or unintended effects on the other cell type. As described here, addition of LPS and IFNγ is intended to stimulate pro-inflammatory gene expression in macrophages. However, in Ubil et al. we showed that IFNγ also induces the expression and secretion of immunosuppressive Pros1[16], and others have shown the effects of LPS on tumor cells[18]. It is therefore essential to include the appropriate controls to monitor potential off-target or unintended effects on the other cell type to verify experimental outcomes. One method by which this may be achieved is by treating individual cell types with the agents of interest and monitoring effects using standard molecular biology assays.

When designing permeable membrane support experiments, it is also important to consider possible diffusion gradients of secreted ligands. The relative rate of ligand secretion, the duration of co-culture and whether the culture plate is maintained in a stationary position can all have effects on the results. In addition, it is possible for some secreted ligands to adhere to the surface of culture plates.

While the representative results shown are characteristic of this system, the degree of pro-inflammatory gene suppression observed when co-culturing other tumor lines may vary dramatically. In Ubil et al., we show that some human tumor lines can almost completely suppress the pro-inflammatory gene expression of a human macrophage cell line[16]. Conversely, other tumor cell types or cell lines may vary substantially in their immunosuppressive capabilities. The cause for these variances is unclear but is an area for further study.

Permeable membrane support co-culture is a robust methodology that can be easily modified to address a variety of questions and can be adapted to a range of molecular biology readouts including qRT-PCR, Western blot, and ELISA. The system can be used to interrogate individual gene functions, such as ligands or receptors, when genetic alterations like those from gene-deleted mice or CRISPR edits are made to either macrophages or the tumor cells. The system is also amenable to the study of pharmacological activators or inhibitors and their effects on paracrine signaling. Also, while not discussed here, the system can be used to study the effects of immune activation on tumor cell gene expression.

This method has been used successfully in the discovery and characterization of a novel function for an immunosuppressive, tumor-secreted ligand. This robust tool can be utilized to interrogate the much broader subset of non-contact tumor/immune interactions in the hopes of discovering new therapeutic targets.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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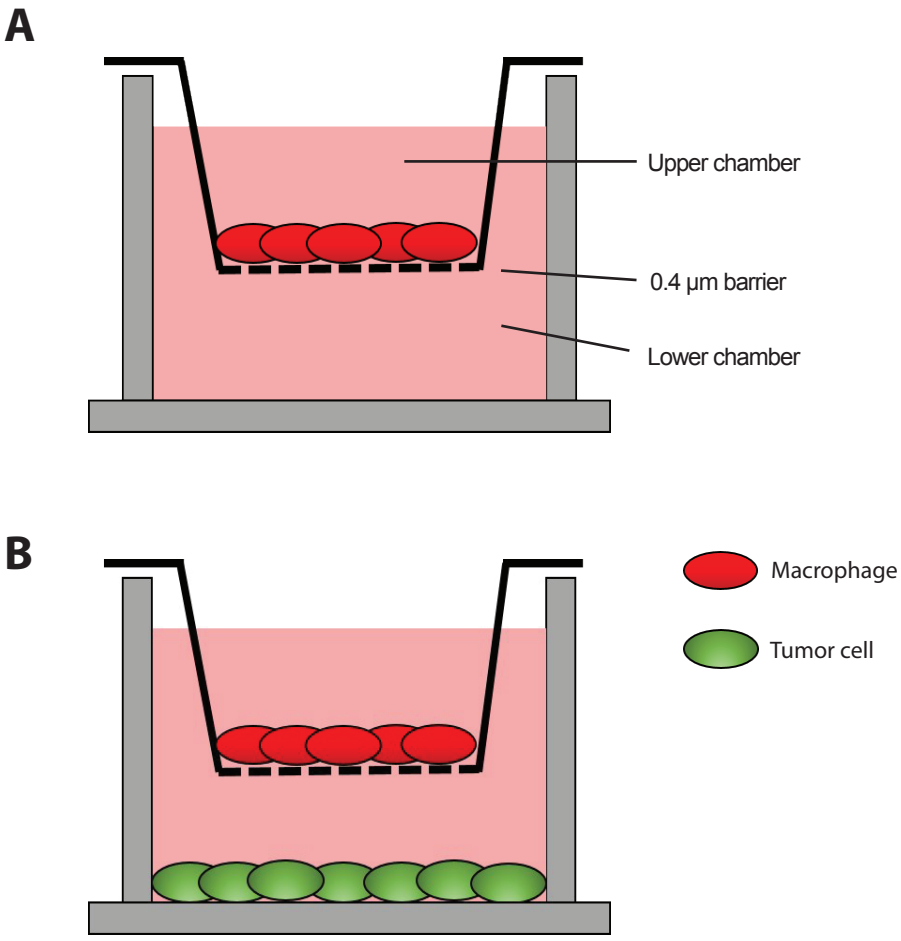

Figure 2

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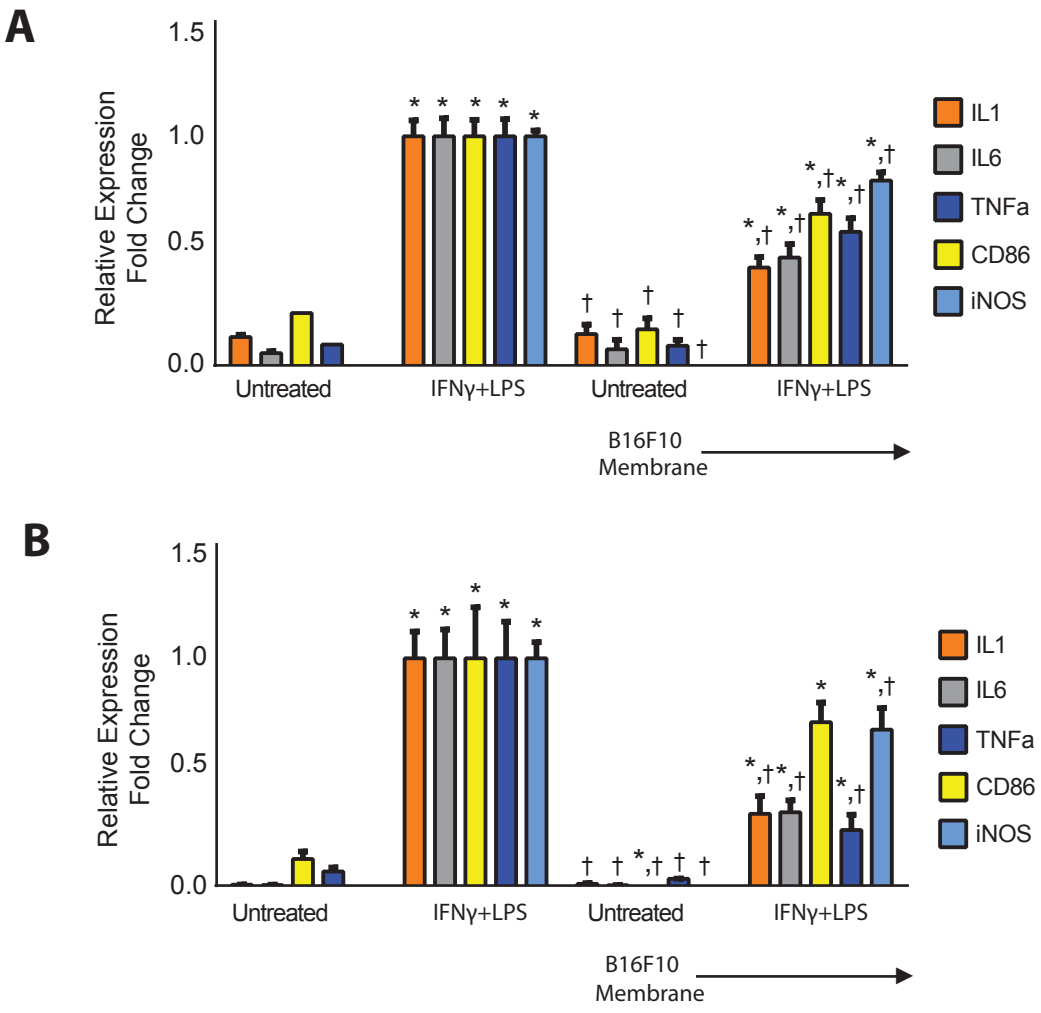

Figure 3

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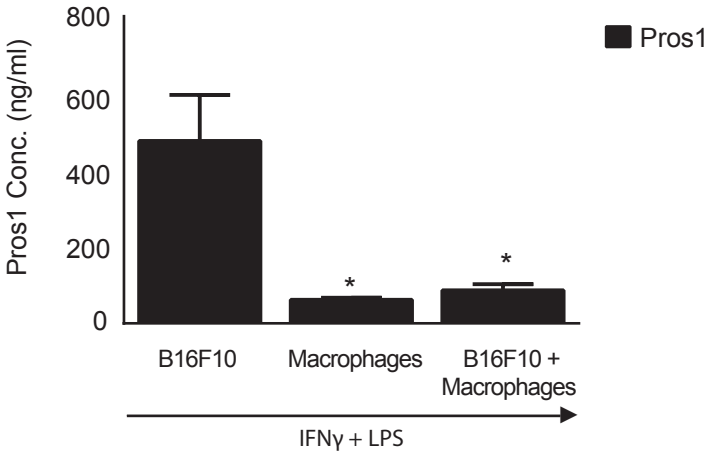

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
B16-F10	ATCC	ATCC CRL-6475	
cDNA synthesis kit	Promega	A3500	
DMEM/F12 media	ThermoFisher Scientific- Gibco	11320033	
Fetal Bovine Serum	Millipore	TMS-013-B	
J774A.1	ATCC	ATCC TIB-67	
Lipopolysaccharides from *Escherichia coli* O111:B4	Sigma-Aldrich	L5293-2ML	
Murine M-CSF	Prospec	CYT-439	
Penicillin-Streptomycin (10,000 U/mL)	ThermoFisher Scientific- Gibco	15140122	
Pros1 ELISA	MyBioSource	MBS2886720	
RAW264.7	ATCC	ATCC TIB-71	
Recombinant Mouse IFNγ	BioLegend	575302	
Sensimix SYBR Low-ROX kit	Bioline	QT625-05	
Transwell permeable supports	Fisher Scientific	07-200-170	
Trypsin-EDTA	ThermoFisher Scientific- Gibco	25200056	

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We thank the editor and reviewers for their positive comments and critiques. We believe we have addressed each comment fully. In several instances, we revised and expanded the text to incorporate points made by reviewers that increase the rigor of the manuscript. In bold below are line-by-line responses to individual editorial comments. We have also provided summarized responses to comments made by Reviewers 1 and 2.

Editor:

General:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been proofread.

2. Please provide email addresses for all authors within the manuscript itself.

E-mail addresses for all authors have been included in the manuscript.

3. Please define all abbreviations before use; e.g., DMEM, FBS, M-CSF.

All abbreviations have been defined upon first use.

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Commercial language has been removed from the manuscript, though commercial products are fully referenced in the Table of Material and Reagents.

Protocol:
1. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Additional detail has been included in a number of the protocol steps to address the "how" question.

Specific Protocol steps:
1. 2.1: Where do tumor cells come from? Is this procedure generally done with, e.g., commercially available cell lines?

Additional language stating that work was performed with commercially available cell lines has been included. A comment was also made in the manuscript that the method should be amenable to use with primary tumor cells as well.

2. 2.3: Please express centrifuge speeds as 'x'g' instead of rpm.

The substitution of x g has been made.

3. 4.1: How exactly is cell lysate isolated?

The text has been altered to state that cell lysate is isolated per the instructions of the RNA preparation kit vendor.

Figures:
1. Please upload each Figure individually to your Editorial Manager account (i.e., 3 separate files).

The initial combined figures have been separated out for individual loading.

2. Figures 2 and 3: Please explain what the error bars represent in the appropriate figure legend. Please also explain what the stars (*) and crosses (†) represent, including statistical test used, if applicable.

Additional language has been included to describe the indications of statistical significance and the tests used.

Discussion:
1. Please revise the Discussion to explicitly cover the following:
a) Critical steps within the protocol
b) Any limitations of the technique
c) The significance with respect to existing methods

Each point has been addressed within the discussion section. The terms "critical" and "important" have been added to more clearly indicate essential steps. Further commentary on limitations, including a point made by a reviewer, have been incorporated and significance has been stressed, in regard to the ability to use the method to study cancer immune suppression.

References:
1. Please include references within the main text of the manuscript, not as footnotes.

References have been incorporated within the text and as endnotes.

2. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

References have been modified to fit the above format.

3. Please do not abbreviate journal titles.

Full journal titles have been substituted for abbreviations.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Materials used have been fully referenced.

Reviewer 1:

We thank Reviewer 1 for their positive comments on our manuscript and for suggestions to improve the scientific rigor of the writing.

Major concerns
Comment #1. Reviewer 1 points out that while the focus of the article is on the macrophage/cancer cell interaction, we spend considerable time discussing the interactions of T cells and cancer cells. We have since revised the introduction to provide a broader overview of the types of tumor/immune signaling interactions and have highlighted relevant tumor/macrophage interactions.

Comment #2. We added additional references and commentary about the use of transwell assays in the context of tumor/macrophage interactions, particularly in the introduction.

Comment #3. Rightfully, the reviewer comments how the M1/M2 paradigm has increasingly become outdated, especially in light of current single-cell sequencing findings. We have since removed mention of M1/M2 polarization.

Minor concerns
Comment #1. We initially mentioned peritoneal macrophages and macrophage cells lines but have since expanded our writing to include bone marrow derived macrophages as well.

Comment #2. Reviewer 2's comments that reagent additions will have consequences on both cell types is absolutely correct. We have since expanded the discussion to address this point, and included references demonstrating potential off-target or unintended effects and some appropriate controls.

Reviewer 2:

We also thank Reviewer 2 for their positive comments.

Major concerns
Comment #1. No major concerns raised.

Minor concerns
Comment #1. Reviewer 2 mentions potential diffusion gradients and the possibility that soluble secreted factors may adhere to cell culture dishes. We have expanded the discussion to address these concerns and highlighted how the added variable may affect overall outcomes.