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Assessment of phagocytic activity in live macrophages-tumor cells co-cultures by Confocal and Nomarski Microscopy

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Abstract

Macrophages have been recognized as the main inflammatory component of the tumor microenvironment. Although often considered as beneficial for tumor growth and disease progression, tumor-associated macrophages have also been shown to be detrimental to the tumor depending on the tumor microenvironment. Therefore, understanding the molecular interactions between macrophages and tumor cells in relation to macrophages functional activities such as phagocytosis is critical for a better comprehension of their tumor-modulating action. Still, the characterization of these molecular mechanisms *in vivo* remains complicated due to the extraordinary complexity of the tumor microenvironment and the broad range of tumor-associated macrophage functions. Thus, there is an increasing demand for *in vitro* methodologies to study the role of cell–cell interactions in the tumor microenvironment. In the present study, we have developed live co-cultures of macrophages and human prostate tumor cells to assess the phagocytic activity of macrophages using a combination of Confocal and Nomarski Microscopy. Using this model, we have emphasized that this is a sensitive, measurable, and highly reproducible functional assay. We have also highlighted that this assay can be applied to multiple cancer cell types and used as a selection tool for a variety of different types of phagocytosis agonists. Finally, combining with other studies such as gain/loss of function or signaling studies remains possible. A better understanding of the interactions between tumor cells and macrophages may lead to the identification of new therapeutic targets against cancer.

Keywords: tumor biology; RAW264.7 macrophages (ATCC Cat# TIB-71, RRID:CVCL_0493); THP-1 monocytes (ATCC Cat#TIB-202, RRID:CVCL_0006); CL-1 and PC3 (ATCC Cat# CRL-7934, RRID:CVCL_0035) prostate cancer cells; phagocytosis; cell imaging

Introduction

Macrophages are innate immune cells that play critical roles in the clearance of pathogens and the maintenance of tissue homeostasis [1]. While macrophages modes of action in nonpathological situations have been widely described, accumulating evidence yet suggests that macrophages also affect cancer initiation, development, and progression [2]. Tumor-associated macrophages (TAMs) are the main population of inflammatory cells present in many

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human solid tumors. TAMs are derived from circulating monocytes and their mobilization toward tumor tissues is regulated through multiple microenvironmental signals such as cytokines, chemokines, extracellular matrix (ECM) components, and hypoxia [2, 3–5]. In response to environmental stimuli, TAMs can appear with a range of different phenotypes and exhibit both beneficial and detrimental roles in various functions such as regulation of tumor growth, angiogenesis, metastasis, matrix remodeling, and immune evasion through the release of different cytokines [2, 6, 7]. In this context, two main subsets designated as M1 and M2 have been identified [8, 9]. While the M2 (alternatively activated) macrophages, closely resembling TAMs, induce an anti-inflammatory response, wound healing, and pro-tumorigenic properties, M1 (classically activated) macrophages are involved in the inflammatory response, pathogen clearance, and anti-tumor immunity. Recent studies have highlighted the role of TAMs in resistance to chemotherapy and protection against radiotherapy emphasizing the therapeutic importance of a better understanding of the role of TAMs in the tumor microenvironment [8–11]. Still, the functional role of macrophages in tumor progression and their prognosis value remain conflicting [12]. Further investigation regarding macrophages functional activities in the context of tumor progression are therefore urgently needed.

Phagocytosis, from Ancient Greek $\phi\alpha\gamma\epsilon\tilde{\imath}\nu$ (phagein), meaning "to devour," $\kappa\acute{\upsilon}\tau o\varsigma$ (kytos), meaning "cell," and -osis, meaning "process," is a receptor-mediated process by which phagocytes, such as macrophages, neutrophils, and monocytes, engulf and kill invading pathogens, remove foreign particles, and clear cell debris (>0.5μm in diameter). Phagocytosis is important in fighting infections and maintaining tissues homeostasis [13, 14]. On the other hand, resistance to phagocytosis has also been recognized for its role in the growth and metastasis of human solid tumors [9]. Surprisingly, the detailed mechanisms of tumor cell phagocytosis by macrophages remain incompletely understood urging for more studies. While observing cell behavior in their native environment may represent the best option to attain increased level of knowledge, *in vivo* data may be also extremely challenging to obtain because of the complexity of the model and of the native environment [15]. In contrast, a two-dimensional (2D) co-culture system is a cell culture method in which only two or more different populations of cells are grown on planar surfaces allowing some levels of cell contact [16]. Investigating these cellular interactions could provide insight into cell functions and mechanisms that are only apparent when another population of cells is present. Therefore, the aim of the present study was to develop a 2D *in vitro* live co-culture model to functionally assess and quantify the phagocytosis of tumor cells by macrophages using a combination of Confocal and Nomarski imaging microscopy. While the proposed co-culture model was specifically applied to prostate tumor cells in the present study, we emphasized that this sensitive, measurable, and highly reproducible model can also be used with multiple types of cancer.

Materials and methods

In the present method, we developed an uncomplicated assay to measure and characterize phagocytosis in live human castration-refractory prostate cancer (CRPC) cells (PC3 and CL-1) and macrophages co-cultures (RAW264.7 and THP-1) using Confocal and Nomarski Microscopy.

Cells and cell culture

Murine macrophage cell line RAW264.7 (ATCC Cat# TIB-71, RRID:CVCL_0493) and human monocytic cell line THP-1 (ATCC Cat#TIB-202, RRID:CVCL_0006) were purchased from the ATCC (Manassas, VA, USA). Both cell lines were cultured as recommended, between passages 1 and 12, to block gene loss and impair macrophage immune function [17]. Human CRPC CL-1 and PC3 DsRed Express cells were established as we described in [18]. Briefly, PC3 DsRed Express were derived from PC3 cells (ATCC Cat# CRL-7934, RRID:CVCL_0035) and cultured according to the ATCC recommended conditions. The CL-1 DsRed Express cells were derived from the CL-1 CRPC cell line (kind gift from Dr A. Belldegrun; University of California, LA, CA, USA) [19]. CL-1 cells were derived from the parental androgen-sensitive LNCaP cells grown under androgen deprivation to develop androgen-independent variant. As observed in CRPC patients, CL-1 cells express androgen receptors but lost transcriptional regulation of prostate-specific genes in response to the androgen deprivation, therefore representing an excellent experimental model to study CRPC. CL-1 DsRed Express cells were grown, between passages 4 and 12, in RPMI1640 supplemented with 5% heat-inactivated charcoal-dextran-treated serum (androgen deprivation to keep the expression of the androgen receptor up), 2 mM L-Gln, 1× NEAA acids and 1 mM NaP. Prostate cancer cells with similar expression level of the DsRed Express fluorescent protein (whole, uncloned population) were selected by fluorescence-activated cell sorting to reach a purity >96%. The expression and homogeneity level of the DsRed Express fluorescent protein were then verified by fluorescence microscopy as we described in [18]. Importantly, we demonstrated that both CL-1 and PC3 DsRed expressing cells maintain the characteristics of the parental cell lines CL-1 and PC3, respectively, therefore validating DsRed expressing cells suitability [18].

Co-culture of CRPC cells and macrophages

1. Five days before imaging, gently scrape the monocytes/macrophages from the maintaining culture flask. After centrifugation, resuspend the cells in fresh complete medium and seed the 2.5×10^5 cells into a 10-cm cell culture dish in 10 ml of complete medium. After 24 h, remove the medium from the dish and treat for 48 h with 10 ml of fresh complete medium that contains 50 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich). As we previously demonstrated, PMA treatment activates RAW264.7 cells (reduced expression of PKCα), and induces the differentiation into macrophages and the adherence of THP-1 cells [20]. As a result of PMA treatment, both cell lines express $IL10_{High}$, $IL12_{Low}$, $TNF\alpha_{Low}$, $iNOS_{Low}$, and $Arginase\text{-}1_{High}$ suggesting a M2/alternatively activated pathway. Note 1: before seeding the RAW264.7 macrophages, make sure no differentiation has occurred (dendrite-like processes and/or increased body size). If such cell morphology signs are visible, disregard the maintaining flask and thaw a new cell vial from an earlier passage. Note 2: the number of cells should be decided according to the cells used. Over-confluent cells may not be sensitive to PMA action leading to flaws in macrophage activation/differentiation.
2. Split the tumor cells (purified DsRed Express fluorescent cells) 24 h after monocytes/macrophages were treated with PMA. Before seeding the tumor cells, place 25 mm microscope sterile glass coverslips into wells of 6-well plate. The tumor cells should be 80% confluent before splitting. Seed 2.5×10^4 cells into each well of a 6-well plate, containing a coverslip, in 2 ml of complete medium and place the 6-well dish into a CO_2 (5%) incubator at 37°C for 24 h. Note 3: alternatively, non-fluorescent tumor cells may be dyed using the

Cell Tracker Red CMTPX dye (Life Technologies) as recommended by the manufacturer.

3. One day after the prostate cancer cells have been seeded into the 6-well plate, aspirate the medium from the 10-cm cell culture dishes containing the differentiated/activated macrophages. Rinse the macrophages with sterile PBS, then gently scrape the cells from the dishes and transfer the cell suspension into a single conical tube. Count the cells, transfer the desired amount of cells (putting into account an extra well) into a fresh conical tube. Centrifuge the cell suspension for 8 min at 100 × *g* at 4°C. Carefully aspirate the supernatant. Resuspend the cell pellet in RPMI-1640 (serum-free) containing 1% (v/v) penicillin/streptomycin, 50 nM PMA, with or without the agonist of phagocytosis designated as the Pigment Epithelium-Derived Factor (PEDF; 10 nM), for a final concentration of 3.5–7.5 × 10^5 cells/2 ml medium [21, 22]. Finally, aspirate the complete medium from the 6-well plate containing the cancer cells and add 2 ml of the macrophages suspension into each 6 well. Place the 6-well plate into a CO_2 (5%) incubator at 37°C, and image live cells after 48 h. Note 4: our experience demonstrated that when the cells in the co-cultures are too confluent (>70%), phagocytosis is drastically reduced. Thus, the ratio macrophages: tumor cells needs to be adjusted depending on the aggressiveness of the tumor cells and the origin (cell lines or primary cells) of the macrophages. Note 5: the incubation time before imaging may vary depending on the phagocytosis agonist used in this assay. A preliminary kinetics study is therefore strongly recommended. Note 6: to confirm that the fluorescent endocytosis vesicles are targeted to the endosome/phagosomes/phagolysosomes/lysosomes degradation pathway, the CellLight Endosome/Early Phagosome-GFP (targeting Rab5a – Ras-related protein 5a, a small GTPase associated with early phagosomes) and Late Phagosome/Lysosomes-GFP (targeting LAMP-1 – lysosomal-associated membrane protein 1, a protein that is utilized to show phagosomes after lysosomal fusion; all from Invitrogen) were used as recommended by the manufacturer [23]. Alternatively, other markers such as Mac1, transferrin receptor can be used [23–25].

Nomarski and Confocal microscopy imaging

Before imaging, prepare the cell suspension buffer (CSB): 0.35 mM Na_2HPO_4, 110 mM NaCl, 0.44 mM KH_2PO_4, 5.4 mM KCl, 1 mM $MgSO_4$, 1.3 mM $CaCl_3$, 25 mM HEPES, pH: 7.4 [26]. Carefully remove the 25 mm microscope cover glass using forceps and place it into the bottom of an Attofluor® Cell Chamber (Life Technologies, A-7816). Adjust the cover glass in the center of the chamber and gently screw on the top chamber. Finally, add 1 ml CSB to the top chamber.

Nomarski/DIC images and confocal images were obtained using the Nikon T1-E microscope with A1 confocal and STORM super-resolution with a 60× objective (N.A. 1.4; oil; Z-stack). After imaging, Z-stacks were merged. Percent phagocytosis was determined by counting the number of DsRed Express positive macrophages over the total number of macrophages. More than 150 macrophages per condition treatment were investigated to control statistical significance within a single experiment. The experiment was repeated at least five times to verify reproducibility.

Note 7. To validate that the red fluorescent present within the cytoplasm of macrophage does come from the DsRed Express-positive tumor cells, spectral imaging microscopy can be used.

Results

In the present assay, we analyzed the phagocytosis of DsRed Express fluorescent tumor cells (PC3 and CL-1) by RAW264.7 (Fig. 1A) or THP-1 (Fig. 2) macrophages through confocal microscopy using the Nikon T1-E microscope (60×) in combination with the appropriate filters. After imaging, the Z-stacks were merged using the NIS-Elements AR 4.00.03. The number of DsRed Express-positive macrophages, as well as the total number of macrophages was assessed (~150 macrophages per condition). Percent phagocytosis was then calculated and the data were represented on a boxplot graph using the IBM SPSS Statistics 23 software (Figs 1B, 2). No agonist (-PEDF) was used as a control. As expected, no significant phagocytosis occurred in the absence of the agonist (Figs 1A–B, 2). In contrast, we showed that under stimulatory treatment (in the presence of a phagocytosis agonist/PEDF), the engulfment of cell material was markedly induced in both macrophages cell lines tested (Fig. 1A-2, white arrows). Stimulation of phagocytosis was demonstrated by an increased number of fluorescent macrophages and fluorescent vesicles within the cytoplasm of macrophages, but also by an elevated level of fluorescence in each phagocytosis vesicle when compared with the control (Fig. 2). The experiment was repeated 20× to verify reproducibility. Specificity of the endosome/phagosome/phagolysosome/lysosome degradation pathway was controlled using the Rab5a and LAMP-1 markers (Invitrogen). Figure 3 shows that both markers overlaid with the DsRed Express-positive cell debris suggesting that the degradation of ingested cellular material involves phagosome maturation through fusion of early endosomes, late endosomes, and lysosomes. In this model, we also used Nomarski/DIC (Differential Interference Contrast) to investigate the macrophage cell morphology associated with phagocytosis activity. Pronounced differentiation of the macrophages (increased body size level and dendritic processes length) was observed in the presence of the agonist suggesting the present method as possible selection tool for phagocytosis agonists.

In conclusion, our data indicate that the presented *in vitro* model in correlation with Confocal and Nomarski imaging may be used to characterize functionally and quantify the phagocytosis of tumor cells by macrophages in live co-cultures.

Discussion

Macrophages have been acknowledged as the major inflammatory cell type present in the microenvironment of most human solid tumors including prostate tumors. Furthermore, interactions between cancer cells and macrophages are important in allowing cancer cells to avoid immunological surveillance and creating a favorable environment for tumor growth and dissemination. While TAMs are usually considered beneficial for tumor cell migration, invasion, and metastasis, depending on the tumor microenvironment it is now recognized that macrophages can also be detrimental to the tumor through cytotoxic and/or phagocytic activities [6]. Since macrophages are particularly important for tumor development, analyses of the molecular interactions between macrophages and tumor cells, and quantification of the phagocytic activity of macrophages are essential for the determination of their functional activities. Still, the characterization of these molecular mechanisms *in vivo* remains complicated and requires expensive high-technology equipment. There is therefore an increasing demand for *in vitro* methodologies to study the role of cell–cell interactions in the tumor microenvironment. Using cell co-cultures has been recognized as a suitable model to study

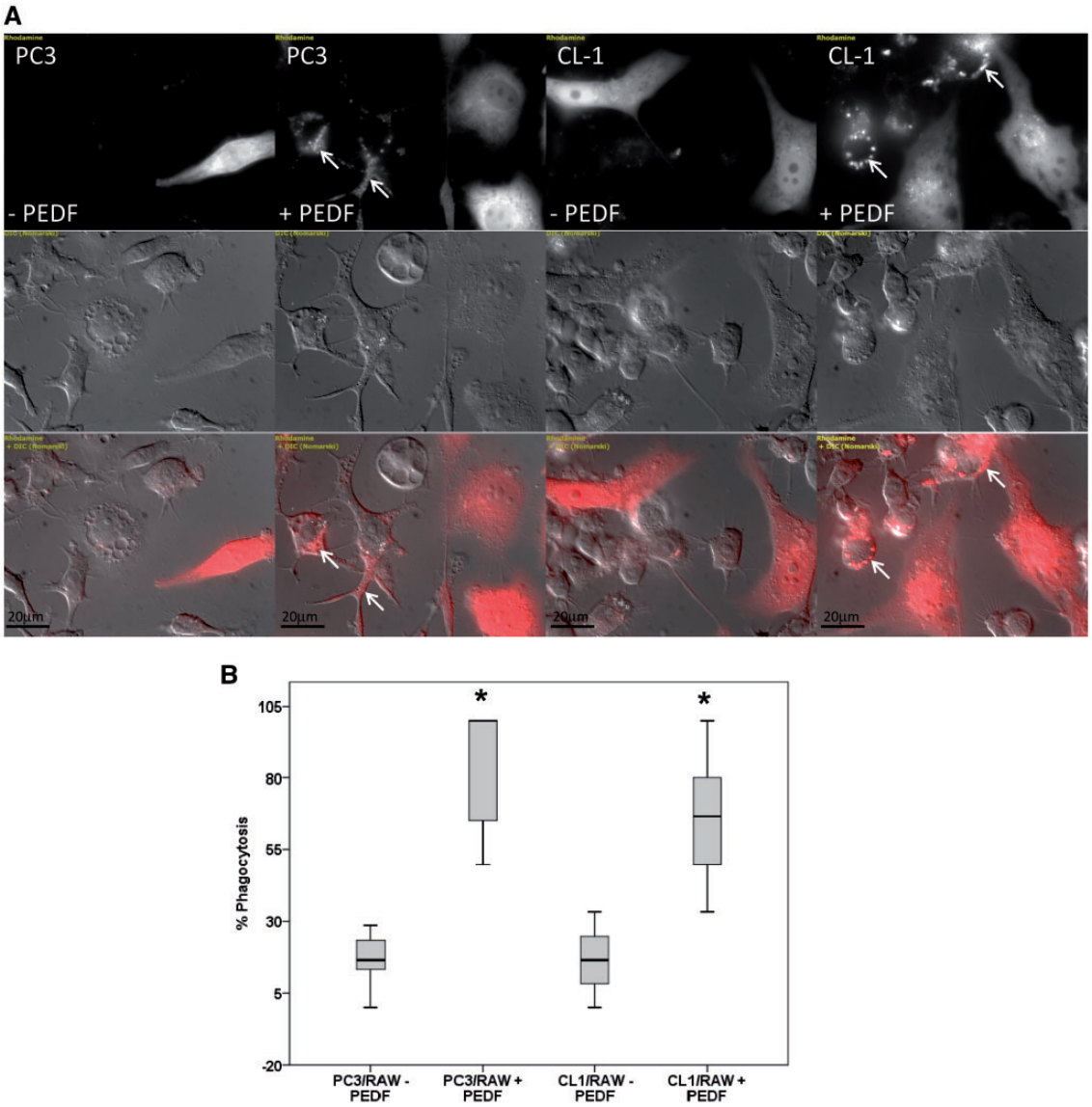

Figure 1: The phagocytosis agonist PEDF increases the engulfment of tumor cells by RAW264.7 macrophages. DsRed Express fluorescent prostate cancer cells (PC3 or CL-1) and PMA-activated macrophages were co-cultured as described in the "Materials and methods" section. After 24 h, the co-cultures were treated with 50 nM PMA in the presence or absence of PEDF (10 nM). Phagocytosis was analyzed by Confocal and Nomarski/DIC microscopy 48 h after the initiation of the treatment. (**A**) Representative pictures of prostate tumor cells (Red) – macrophages (unstained) co-cultures showing an increase in the number of DsRed Express-positive macrophages, as well as in the number of DsRed Express-positive vesicles within the macrophages treated with PEDF. Top panel: Red fluorescence; middle panel: Nomarski; bottom panel: overlay Red fluorescence/Nomarski. White arrows: intracellular vesicles located within the cytoplasm of macrophage which contains DsRed Express fluorescent tumor cell debris. (**B**) Quantification of % Phagocytosis. Number of macrophages positive for the DsRed Express protein and the total number of macrophages (>150 per treatment condition) were counted. Percent phagocytosis was then calculated. Data were represented using a boxplot graph showing the median, inter-quartile range, upper and lower quartiles, and whiskers. Statistical analysis was performed using one-way ANOVA followed by Games–Howell post-hoc test. *$P < 0.05$. Graphs and statistical analysis were performed using the IBM SPSS Statistics 23 software.

the tumor microenvironment and as such, the impact of this study using a combination of Nomarski and Confocal microscopy is highly significant [16].

There are currently a number of assays available to assess phagocytic activity *in vitro*. Most of these assays include using fluorescence labeled latex beads, fluorescent polystyrene microspheres [27], zymosan, IgG-opsonized or IgM/complement component 3-opsonized erythrocytes [28] or *Escherichia coli* particles [29], and microscopes or flow cytometers [17] to measure the phagocytic activity of particular phagocytes. The present protocol describes the materials and methods, and notes for the quantification and characterization of the phagocytosis of prostate tumor cells by macrophages using Confocal and Nomarski microscopy. The present protocol presents multiple advantages compared to other pre-existing *in vitro* assays. First, this assay is easy to perform with no required specialization in cell culture. While we recognize the equipment price and availability may be limiting (similarly to other technologies such as intravital multiphoton imaging), this protocol remains inexpensive without requiring the purchase of additional kits, specific supplies, reagents, or mice. This assay does not involve any processing of the samples (single-step protocol) allowing the researcher to study functionally live cells. A second advantage of working with live cells may be a possible combination with time

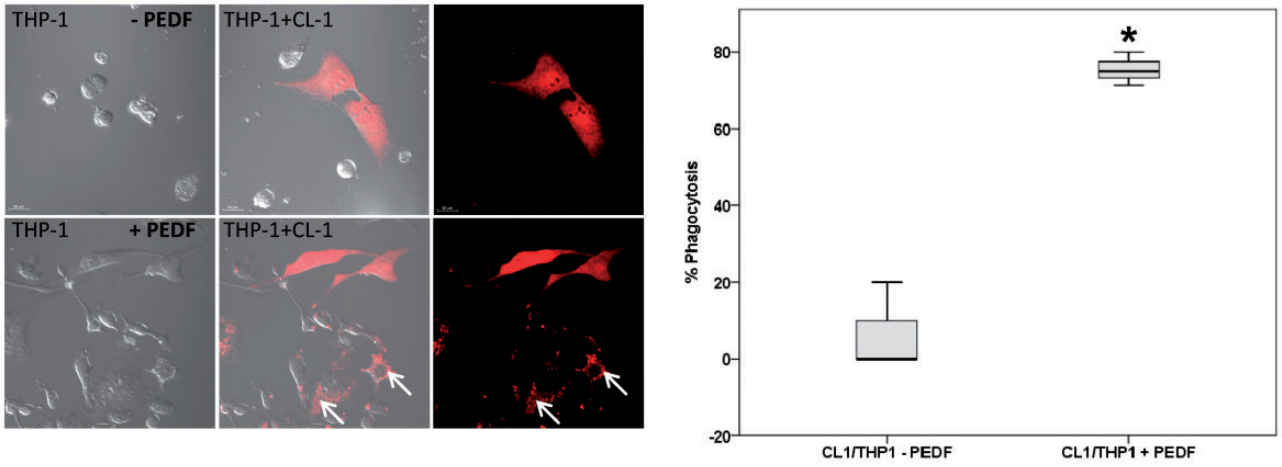

Figure 2: Stimulation of the phagocytosis of CL-1 prostate tumor cells by THP-1 macrophages. PMA-differentiated THP-1 were cultured alone (left panel) or with DsRed Express-fluorescent CL-1 cells (center and right panels) +/− a phagocytosis agonist (10 nM PEDF) as described in the "Materials and methods" section. Cells were imaged using Nomarski (left and center panels) or Confocal (right panel) microscopy. Number of macrophages positive for the DsRed Express protein and the total number of macrophages (>150 per treatment condition) were counted. Percent phagocytosis was showed using a boxplot graph. Data were represented using a boxplot graph showing the median, inter-quartile range, upper and lower quartiles, and whiskers. Statistical analysis of the data was performed using the Student's *t*-test. $^{*}P < 0.05$. Graphs and statistical analysis were performed using the IBM SPSS Statistics 23 software.

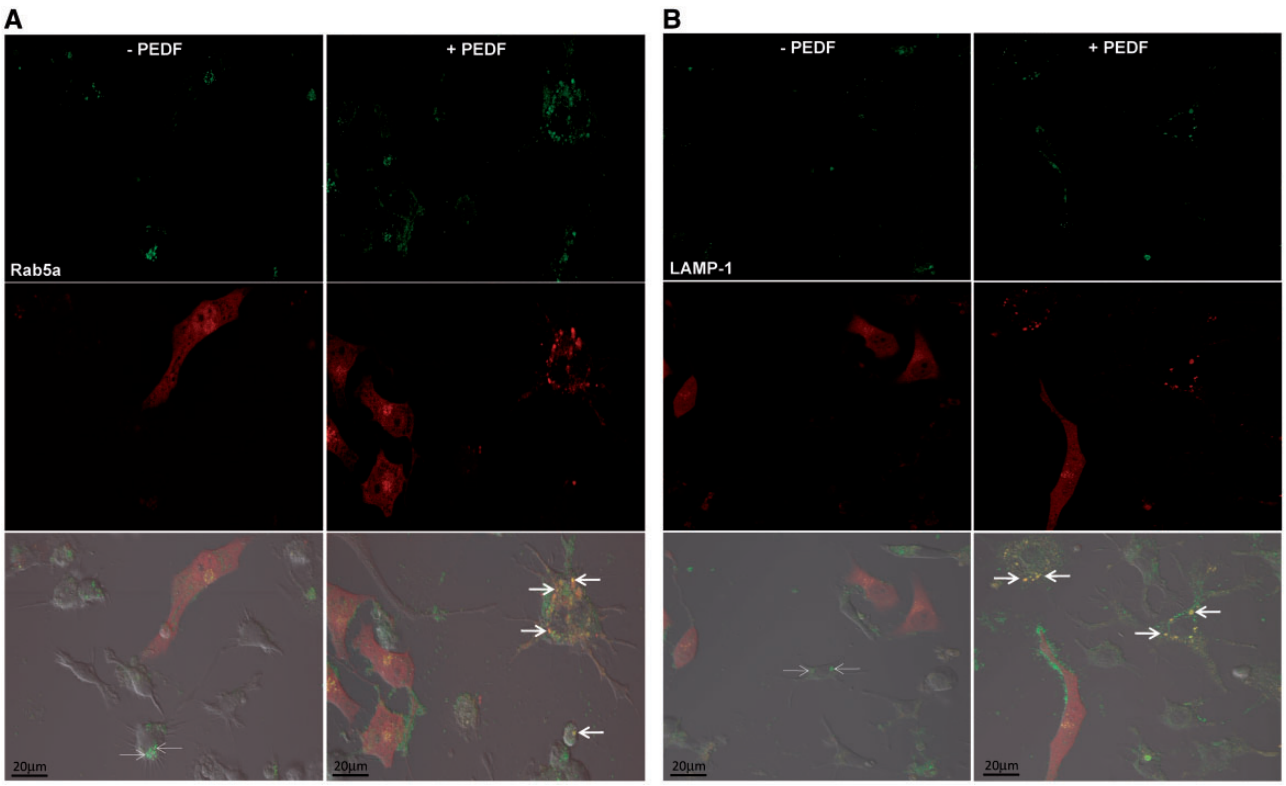

Figure 3: DsRed Express-positive macrophagic vesicles co-localized with markers of the Phagosome/phagolysosome/lysosome degradation pathway. RAW264.7 – CL-1 co-cultures were incubated +/− a phagocytosis agonist in the presence of the CellLight Endosome/Early Phagosome-GFP (Rab5a, A) or Late Phagosome/Lysosomes-GFP (LAMP-1, B; both green). Co-cultures were then imaged by Nomarski (left panels) or Confocal (right panels) microscopy. Thin white arrows show Rab5a and LAMP-1 location within the cytoplasm of unstimulated macrophages. In contrast, thick white arrows show that DsRed Express-positive vesicles within the stimulated macrophages (+ phagocytosis agonist) were also positive for Rab5a and LAMP-1, therefore identifying these vesicles as part of the endosome/early phagosome and late phagosome/lysosomes, respectively.

lapse microscopy. To further characterize this assay, we have applied this technology to different tumor cell experimental models including human and mouse melanoma cell lines (Plebaneck *et al.*, 2016, submitted for publication). Different types of phagocytosis agonists such as recombinant protein, antibodies, cell conditioned media, or chemotherapeutic drugs have been extensively tested by our laboratory (Martinez-Marin *et al.*, 2016, submitted for publication; Jarvis *et al.*, 2016, manuscript in revision). For all of these agonists, we were able to functionally detect and quantify the phagocytosis of tumor cells

in a highly reproducible manner. Furthermore, the sensitivity of the assay was high with the detection of phagocytosis for doses of chemotherapeutic drugs significantly inferior to their serum concentration. Finally, the use of the present assay in combination with other tests such as competition assays using phagocytosis agonist and antagonist, gain/loss of function studies using mammalian expression plasmid, siRNA, shRNA or either pathways activators or inhibitors make it a good tool to select and characterize new TAMs targeting drugs for anti-neoplasic treatments.

For this study, we chose to use RAW264.7 (most commonly used macrophage line) macrophages and THP-1 monocytic cell lines. Guo and collaborators recently demonstrated in a murine phagosome proteomics study that several phagosomal functions such as acidification and proteolysis are significantly reduced in the RAW264.7 cell line compared to Bone Marrow-Derived Macrophages (BMDMs) from C57/BL6 mice [30]. In two other studies, Berghaus *et al.* and Chamberlain *et al.* agreed that RAW264.7 cells most closely mimic BMDMs, when compared to splenic macrophages and bone marrow dendritic cells, in terms of cell surface receptors and response to microbial ligands that initiate cellular activation via Toll-like receptors 3 and 4/TLR3-4 [31, 32]. Interestingly, TLR3-4 has been recently identified as important player in tumor cell phagocytosis [22]. The totality of these findings therefore emphasize on the fact that cautions must be applied when extrapolating findings obtained with RAW264.7 or other monocytes cells to those of primary macrophage-lineage cells. It is also important to recognize that monocyte-derived cells exist as a highly plastic population of cells with varied functional capabilities [32].

Current studies on TAM-targeting cancer therapy have focused on: (i) inhibiting macrophages recruitment into the tumor, (ii) converting TAMs or pro-tumorigenic macrophages to the anti-tumor phenotype, and (iii) suppressing TAM survival, emphasizing on the promising role of macrophages in anticancer therapy [33, 34]. *In vivo* study models using intravital multiphoton imaging recently provided a true breakthrough in the understanding of TAMs function [11, 21, 35–37]. However, the use of this technology to study specific organs such as the prostate gland still remains difficult because of the location of the gland deep in the pelvic cavity. As an alternative, Abedinpour developed a pseudo orthotopic model in which fluorescent TRAMP prostate tumor spheroids are co-implanted with prostate tissue in mice using the dorsal window chamber model [38]. On the other hand, the extraordinary complexity and range of TAMs functions forced researchers to develop more controlled *in vitro* systems that recreate the tumor microenvironment for the elucidation of mechanisms of crosstalk between macrophages and carcinoma cells [16, 39, 40]. A 2D coculture system is a cell culture method in which two or more different populations of cells are grown on planar surfaces allowing some levels of cell contact [16]. Investigating these cellular interactions can provide insight into cell functions and mechanisms that are only apparent when another population of cells is present, as well as could complement *in vivo* intravital imaging data. All together, these findings may lead to a better understanding of cell–cell communication, signaling, and tumor microenvironment and as well support the development of new therapeutic targets for cancer.

In summary, our study describes the combination of two imaging strategies to visualize and quantify the phagocytosis of tumor cells by macrophage cells. We emphasize on the uncomplicatedness, sensitivity, and high reproducibility level of the present technology and also insist that this model may be used to complement *in vivo* studies using primary macrophage lineage cells.

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Conflict of interest statement. None declared.

References

1. Gordon S. The macrophage: past, present and future. *Eur J Immunol* 2007;**37(Suppl. 1)**:S9–17.
2. Chanmee T, Ontong P, Konno K *et al*. Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers* 2014;**6**:1670–90.
3. Mizutani K, Sud S, McGregor NA *et al*. The chemokine CCL2 increases prostate tumor growth and bone metastasis through macrophage and osteoclast recruitment. *Neoplasia* 2009; **11**:1235–42.
4. Qian BZ, Li J, Zhang H *et al*. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* 2011; **475**:222–25.
5. Su S, Liu Q, Chen J *et al*. A positive feedback loop between mesenchymal-like cancer cells and macrophages is essential to breast cancer metastasis. *Cancer cell* 2014;**25**:605–20.
6. Condeelis J, Pollard JW. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell* 2006; **124**:263–66.
7. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. *Cancer Res* 2006;**66**:605–12.
8. Mantovani A, Biswas SK, Galdiero MR *et al*. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol* 2013;**229**:176–85.
9. Mantovani A, Locati M. Tumor-associated macrophages as a paradigm of macrophage plasticity, diversity, and polarization: lessons and open questions. *Arterioscler Thromb Vasc Biol* 2013;**33**:1478–83.
10. De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell* 2013;**23**:277–86.
11. Jinushi M. Immune regulation of therapy-resistant niches: emerging targets for improving anticancer drug responses. *Cancer Metastasis Rev* 2014;**33**:737–45.
12. Takeya M, Komohara Y. Role of tumor-associated macrophages in human malignancies: friend or foe? *Pathol Int* 2016;**66**:491–505.
13. Flannagan RS, Jaumouille V, et Grinstein S. The cell biology of phagocytosis. *Annual review of pathology* 2012;**7**:61–98.
14. Underhill DM, Goodridge HS. Information processing during phagocytosis. *Nat Rev Immunol* 2012;**12**:492–502.
15. Palmer GM, Fontanella AN, Shan S *et al*. In vivo optical molecular imaging and analysis in mice using dorsal window chamber models applied to hypoxia, vasculature and fluorescent reporters. *Nat Protoc* 2011;**6**:1355–66.
16. Dovas A, Patsialou A, Harney AS *et al*. Imaging interactions between macrophages and tumour cells that are involved in metastasis in vivo and in vitro. *J Microsc* 2013;**251**:261–69.

17. Tseng D, Volkmer JP, Willingham SB *et al.* Anti-CD47 antibody-mediated phagocytosis of cancer by macrophages primes an effective antitumor T-cell response. *Proc Natl Acad Sci USA* 2013;**10**:11103–08.
18. Nelius T, Martinez-Marin D, Hirsch J *et al.* Pigment epithelium-derived factor expression prolongs survival and enhances the cytotoxicity of low-dose chemotherapy in castration-refractory prostate cancer. *Cell Death Dis* 2014;**5**:e1210.
19. Tso CL, McBride WH, Sun J *et al.* Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells. *Cancer J* 2000;**6**:220–33.
20. Nelius T, Samathanam C, Martinez-Marin D *et al.* Positive correlation between PEDF expression levels and macrophage density in the human prostate. *Prostate* 2013;**73**:549–61.
21. Gul N, Babes L, Siegmund K *et al.* Macrophages eliminate circulating tumor cells after monoclonal antibody therapy. *J Clin Investig* 2014;**124**:812–23.
22. Feng M, Chen JY, Weissman-Tsukamoto R *et al.* Macrophages eat cancer cells using their own calreticulin as a guide: roles of TLR and Btk. *Proc Natl Acad Sci USA* 2015;**112**:2145–50.
23. Wagner BJ, Lindau D, Ripper D *et al.* Phagocytosis of dying tumor cells by human peritoneal mesothelial cells. *J Cell Sci* 2011; **124**:1644–54.
24. Morrissette NS, Gold ES, Guo J *et al.* Isolation and characterization of monoclonal antibodies directed against novel components of macrophage phagosomes. *J Cell Sci* 1999;**112**:4705–13.
25. Huynh KK, Eskelinen EL, Scott CC *et al.* LAMP proteins are required for fusion of lysosomes with phagosomes. *EMBO J* 2007;**26**:313–24.
26. Allavena P, Sica A, Garlanda C *et al.* The Yin-Yang of tumor-associated macrophages in neoplastic progression and immune surveillance. *Immunol Rev* 2008;**222**:155–61.
27. Enomoto R, Imamori M, Seon A, Yoshida K, Furue A, Tsuruda H, et Lee-Hiraiwa E. Proposal for a new evaluation of phagocytosis using different sizes of fluorescent polystyrene microspheres. *Advances in Biological Chemistry* 2013;**3**(6):556–563.
28. Hamczyk MR, Villa-Bellosta R, et Andres V. In Vitro Macrophage Phagocytosis Assay. *Methods in molecular biology* 2015;**1339**:235–246.
29. Geraldo MM, Costa CR, Barbosa FM *et al.* In vivo and in vitro phagocytosis of Leishmania (Leishmania) amazonensis promastigotes by B-1 cells. *Parasite Immunology* 2016;**38**:365–76.
30. Guo M, Hartlova A, Dill BD *et al.* High-resolution quantitative proteome analysis reveals substantial differences between phagosomes of RAW 264.7 and bone marrow derived macrophages. *Proteomics* 2015;**15**:3169–74.
31. Chamberlain LM, Godek ML, Gonzalez-Juarrero M *et al.* Phenotypic non-equivalence of murine (monocyte-) macrophage cells in biomaterial and inflammatory models. *J Biomed Mater Res A* 2009;**88**:858–71.
32. Berghaus LJ, Moore JN, Hurley DJ *et al.* Innate immune responses of primary murine macrophage-lineage cells and RAW 264.7 cells to ligands of Toll-like receptors 2, 3, and 4. *Comp Immunol Microbiol Infect Dis* 2010;**33**(5):443–54.
33. Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. *Immunity* 2014;**41**:49–61.
34. Ostuni R, Kratochvill F, Murray PJ *et al.* Macrophages and cancer: from mechanisms to therapeutic implications. *Trends Immunol* 2015;**36**:229–39.
35. Gabrilovich D. Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nat Rev Immunol* 2004;**4**:941–52.
36. Rodriguez PC, Quiceno DG, Zabaleta J *et al.* Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res* 2004;**64**:5839–49.
37. Patsialou A, Bravo-Cordero JJ, Wang Y *et al.* Intravital multiphoton imaging reveals multicellular streaming as a crucial component of in vivo cell migration in human breast tumors. *Intravital* 2013;**2**:e25294.
38. Abedinpour P, Baron VT, Welsh J *et al.* Regression of prostate tumors upon combination of hormone ablation therapy and celecoxib in vivo. *Prostate* 2011;**71**:813–23.
39. Wyckoff JB, Wang Y, Lin EY *et al.* Direct visualization of macrophage-assisted tumor cell intravasation in mammary tumors. *Cancer Res* 2007;**67**:2649–56.
40. Egeblad M, Ewald AJ, Askautrud HA *et al.* Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy. *Dis Model Mech* 2008;**1**:155–67; discussion 165.