Research paper

An optimized protocol to determine the engulfment of cancer cells by phagocytes using flow cytometry and fluorescence microscopy

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ABSTRACT

The engulfment of cancer cells by macrophages is an important cellular process in innate cancer immunity. Antitumor immunotherapy that utilizes the enhanced engulfment of cancer cells by phagocytic cells has attracted much attention. Therefore, there is a growing demand for methods of measuring cancer cell phagocytosis. Quantifying the various stages of phagocytosis is invaluable for elucidating cancer-immune responses during this process. Here, we describe two phagocytosis assays, a flow cytometric assay and a fluorescent microscopic assay; the flow cytometric method utilizing CellTracker dye provides a simple, measurable, and highly reproducible functional assay to measure the phagocytosis efficiency of cancer cells by bone marrow-derived macrophages. As an alternative method of evaluating various states of cancer cell phagocytosis, a fluorescent microscopic method that employs a pH-sensitive dye (pHrodo-SE dye) is also described in this paper. Image-based analysis using this labeling approach enables researchers to measure phagocytic indices that indicate the number of cancer cells engulfed by each macrophage. We have highlighted that these assays can be applied to multiple tumor types and used as selection tools for a variety of phagocytosis agonist types. The results of this study may facilitate a better understanding of the interactions between tumor cells and phagocytes, which could lead to the identification of new therapeutic targets against cancer.

1. Introduction

Cancer immunotherapies have led to unprecedented rates of positive clinical responses in patients with various cancer types (Palucka and Banchereau, 2012). To induce antitumor immunity, innately immune cells such as macrophages or dendritic cells (DCs) must phagocytose tumor cells and process tumor specific antigens (Palucka and Banchereau, 2012). Professional antigen presenting cells (APCs) among phagocytes specialize in presenting antigens to T cells in antitumor immune responses. In particular, macrophages are highly efficient at internalizing cancer cell antigens, which represents a critical component of innate immunity against cancer.

The determination of the phagocytic activity of innately immune cells can be a reliable predictor for evaluating response rates to immunotherapy (Garg et al., 2016). However, the evaluation methods that

have been used thus far have not led to a well-established understanding of the process of phagocytosis of cancer cells. In addition, phagocytic activity cannot be measured clearly due to intercellular adhesion between cancer cells and macrophages, resulting in poor reproducibility and sensitivity of these methods (Miksa et al., 2009). Thus, there is an increasing need for the development of a precise and highly reproducible functional assay to evaluate the degree of phagocytosis of cancer cells by macrophages.

In this paper, we describe two *in vitro* phagocytosis methods that utilize fluorescent dyes to assess cancer cell engulfment by macrophages, which can easily be applied to other phagocyte processes (Fig. 1). The first is the flow cytometry–based phagocytosis assay, which is a simple and effective method for analyzing the phagocytic activity of macrophages using carboxyfluorescein succinimidyl ester (CFSE) or CellTracker dye (Willingham et al., 2012). CellTracker dye,
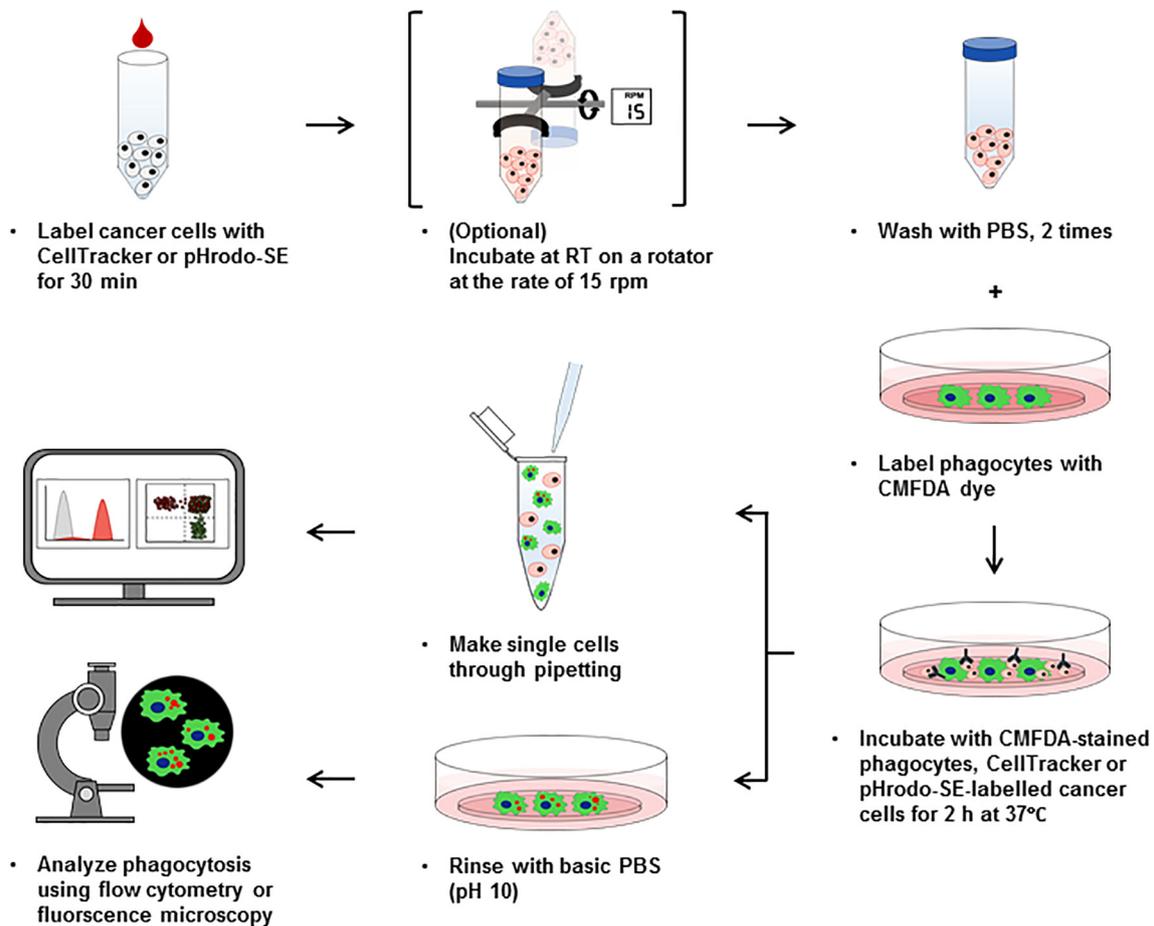
Fig. 1. The schematic method of cancer phagocytosis assay utilizing CellTracker or pHrodo-SE fluorescence dyes.

which can penetrate cell membranes, yields higher fluorescent signals at physiological pH. The degree of phagocytosis can be confirmed by tracing the co-localization of fluorescence detected from cancer cells and macrophages stained with distinguishable CellTracker dyes.

However, the flow cytometry–based phagocytosis assay provides limited information and may overestimate the actual potential of the phagocytosis of cancer cells as a result of the inappropriate detection of intercellular binding. Therefore, we suggest an optimized protocol to determine the phagocytosis of cancer cells based on fluorescence microscopy using pHrodo-succinimidyl ester (pHrodo-SE) dye. The pHrodo-SE dye utilized for the labeling of cancer cells reacts with the primary amines on cancer cells to yield covalently linked pH probes, which display increased fluorescence as the environmental pH becomes more acidic (Miksa et al., 2009). Due to the low pH of the phagolysosome, engulfed cancer cells can be visualized by pHrodo-SE staining, which can be distinguished from cancer cells merely adhering to the outer cell surface of the phagocytes. Hence this technique allows for the straightforward quantification of phagocytosis of cancer cells using fluorescence microscopy.

These phagocytosis assays can be applied to multiple types of tumors or phagocytes and be used to evaluate the efficacy of various phagocytosis enhancers. A better understanding of the interactions between tumor cells and phagocytes through *in vitro* phagocytosis assays may contribute to the identification of novel targets for cancer immunotherapy.

2. Materials and methods

2.1. Reagents and animals

Recombinant murine macrophage colony–stimulating factor (M-CSF) was purchased from Peprotech. CellTracker 5-Chloromethylfluorescein diacetate (CMFDA), CellTracker™ Deep Red, pHrodo™ Red SE, and β-mercaptoethanol were purchased from Thermo Fisher Scientific Inc. APC-anti-F4/80 (clone BM8, 123116, 1:100) and APC-rat IgG2a, κ Isotype Ctrl antibody (clone RTK2758, 400512, 1:100) were obtained from BioLegend.

Male BALB/c white mice (8 weeks old) were purchased from Orient Bio Inc. All mice were bred and maintained under specific pathogen-free conditions at the Korea Institute of Science and Technology (KIST). The study protocols were performed following guidelines of the Institutional Animal Care and Use Committee (IACUC) of the KIST.

2.2. Cell culture

Murine 4 T1-Luc breast cancer cells, murine CT26.CL25 colon adenocarcinoma, and human HT29 colon adenocarcinoma were cultured in RPMI-1640 (Hyclone) containing 10% fetal bovine serum (Atlas) and 1% antibiotic-antimycotic (Thermo). Murine B16F10-Ova melanoma cells were cultured in DMEM (Hyclone) containing 10% fetal bovine serum (Atlas) and 1% antibiotic-antimycotic (Thermo). All cell lines were maintained in 5% CO_2 at 37 °C.

To generate bone marrow-derived macrophages (BMDMs), bone marrow cells were isolated from the leg bones of BALB/c mice. Isolated bone marrow cells were grown in RPMI-1640 medium (Welgene) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic-
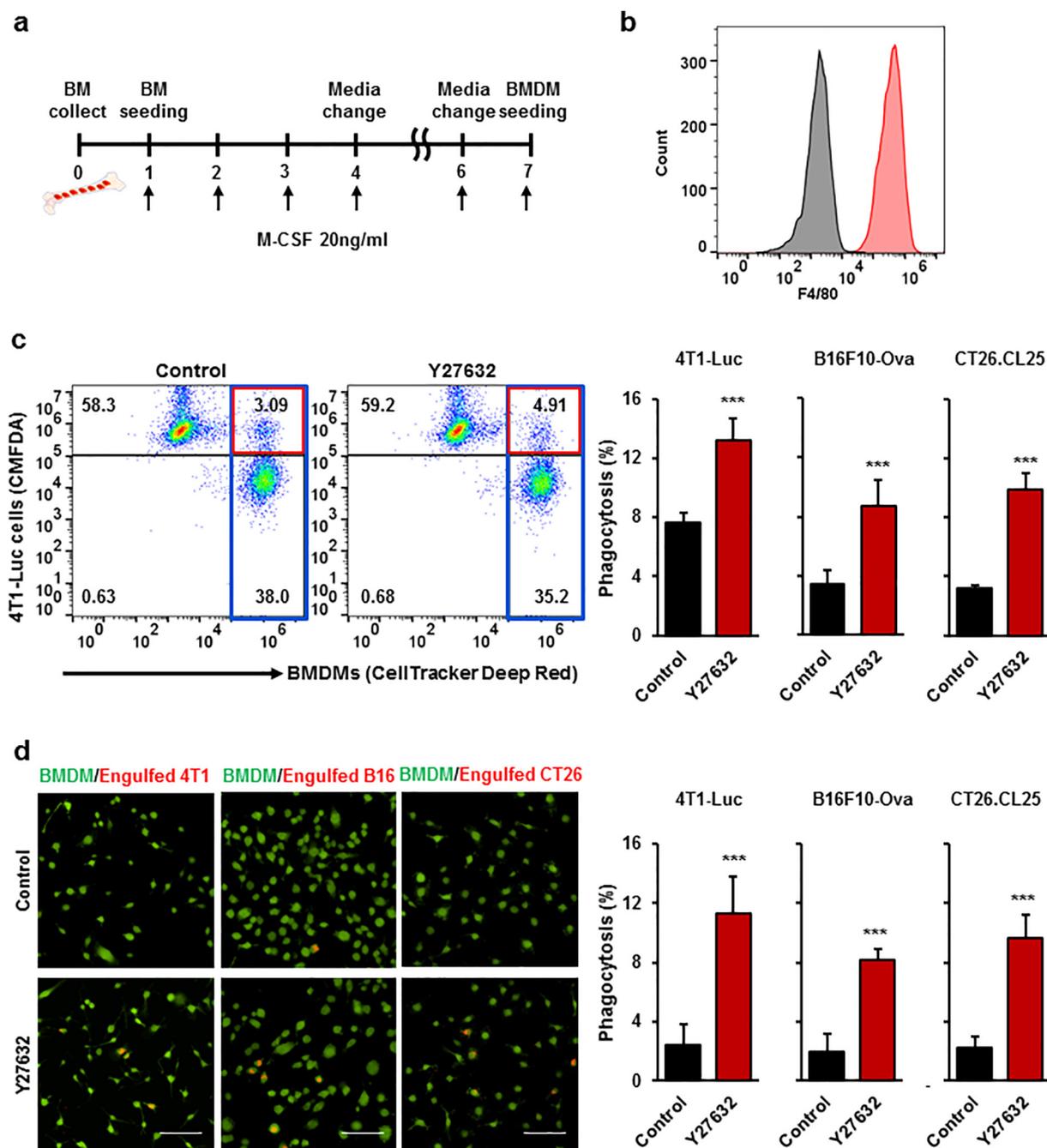
Fig. 2. Phagocytosis assay using CellTracker dye and pHrodo-SE. (a) Schematic diagram of experiments for differentiation of BMDMs. (b) Representative histograms showing the differentiation of BMDMs with anti-F4/80 antibody for phagocytosis assays. (c) *Left*: Representative FACS plots showing the phagocytosis of a range of 4 T1-Luc cells stained with CellTracker by BMDMs. *Right*: Phagocytosis was calculated as the percentage of double-positive BMDMs among CellTracker Deep Red-positive BMDMs, assessed by FACS analysis. Data are presented as means ± S.D. ($n = 5$–6). (d) *Left*: Representative microscopic images of pHrodo-SE phagocytosis assays performed using CMFDA-stained BMDMs (green) against 4 T1-Luc, B16F10-Ova or CT26.CL25 cells labeled with pHrodo-SE. Scale bars: 100 μm. *Right*: Phagocytosis was calculated from fluorescence microscopic images. Data are presented as means ± S.D. ($n = 6$–9). The phagocytosis percentage indicates the percentage of BMDMs containing cancer cells per total BMDMs. ***$p < .001$ by Student's *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

antimycotic (Thremo). The detailed schedule for the differentiation of BMDMs is shown in Fig. 2a. Bone marrow cells were extracted from the hind leg and seeded in a culture dish overnight (Day 0). After the floating bone marrow cells were isolated, they were seeded with 20 ng/ml murine macrophage colony-stimulating factor (M-CSF) at 3.5×10^6 per 100 mm petri dish (Day 1). Then, M-CSF was added daily (Day 2–3) and replaced with fresh media containing 20 ng/ml M-CSF (Day 4 and Day 6). Differentiation of BMDMs was confirmed by flow cytometry (Accuri C6; BD Biosciences) using anti-F4/80 antibodies (Fig. 2b).

2.3. BMDMs and cancer cell labeling with CellTracker dye

BMDMs and cancer cells were washed with DPBS and re-suspended in culture media at a concentration of 10^6 cells/ml. CellTracker Green (CMFDA) or CellTracker Deep Red dye was added to 1 ml of cell suspension to a final concentration of 1 μM. Note that CFSE (Carboxyfluorescein succinimidyl ester) dye can be used as an alternative to CellTracker dyes in experimental assays. After incubating for 30 min at room temperature, the cells were washed with PBS and re-

suspended in the culture media.

2.4. Cancer cell labeling with pHrodo-SE

4 T1-Luc, B16F10-Ova, CT26.CL25, and HT29 cancer cells were washed twice with DPBS and re-suspended in DPBS at a concentration of 10^7 cells per 50 ml. pHrodo-SE dye was added to 50 ml of cell suspension to a final concentration of 120 ng/ml. After incubating for 30 min on a rotator (15 rpm) at room temperature, the cells were washed twice with PBS and re-suspended in the culture media.

2.5. Flow cytometry–based phagocytosis assay

For flow cytometry analyses, CellTracker Deep Red–stained BMDMs were plated at a density of 2×10^5 cells per 35 mm petri dish (Falcon). The next day, the stained BMDMs were pretreated with 30 μM Y27632 for 1 h. Then, 4T1-Luc, B16F10-Ova, and CT26.CL25 cells were stained with 1 μM CMFDA and were then co-cultured with syngeneic BMDMs at a ratio of 1:2 in the presence or absence of 30 μM Y27632. After incubation for 2 h at 37 °C, the collected cells were pipetted repetitively with PBS to suspend the pellets as single cells and analyzed by flow cytometry (Accuri C6; BD Biosciences) using the FlowJo (v10) software (Fig. 1). The phagocytosis (%) was calculated as the percentage of $CMFDA^+$ cells within Deep Red^+ macrophages according to the following formula: the number of BMDMs phagocytosing cancer cells (right-upper quadrant, double positive)/total number of BMDMs (right quadrants, green) $\times$ 100.

2.6. Fluorescence microscopy–based phagocytosis assay

For microscopic analyses, BMDMs (2×10^5) labeled with CMFDA were cultured in 35-mm glass-bottom confocal dishes (Corning) along with various cancer cells (4 T1-Luc, B16F10-Ova, and CT26.CL25 cells) labeled with pHrodo-SE at a ratio ranging from 1:1 to 1:4 in the presence of phagocytosis agonists (30 μM Y27632 or 10 μg/ml anti-CD47 antibody). Notably, CMFDA-stained BMDMs were pretreated with a ROCK inhibitor, Y27632 (30 μM) for 1 h before phagocytic incubation.

After co-incubation for 2 h at 37 °C, cells were washed several times with basic PBS (pH 10) to remove unengulfed pHrodo-labeled tumor cells. The degree of the phagocytosis of tumor cells was measured by fluorescence microscopy (Nikon) and analyzed by randomly selected six or more microscopic fields per assays (Fig. 1).

Phagocytosis (%) was calculated according to the following formula: the number of BMDMs phagocytosing cancer cells/total number of BMDMs $\times$ 100.

The phagocytosis index (PI) is defined as the following formula based on microscopy images: the number of engulfed cancer cells (pHrodo-SE, red)/total number of BMDMs (green).

The phagocytosis capacity (PC) is defined as the following formula: the number of engulfed cancer cells/the number of BMDMs phagocytosing cancer cells.

2.7. Statistical analyses

Data are expressed as means ± standard deviation (SD) for control and experimental samples. The sample size was chosen based on the typical size used in this field. Comparisons between two groups were performed with the Student's *t*-test using Sigma Plot 10.0 software. *P*-values < .05 were considered to be statistically significant; individual *p*-values are indicated in the figure legends.

3. Results

3.1. Comparison of assay methods for the detection of phagocytosis

To quantify the phagocytosis of cancer cells by macrophages

requires that targeted cells are labeled with a fluorescent dye, such as CellTracker, which emits fluorescence at different wavelengths. This phagocytosis assay can be characterized by its sensitivity as well as its simplicity and countability. To determine the phagocytic function of macrophages, fluorescence-labeled target cells were co-incubated for 2 h, and they were dislodged from the plates with harsh pipetting. The extent of phagocytosis was easily quantified by tracing the co-localization of fluorescence using flow cytometry; the percentage of double-stained macrophages (red square) out of the whole macrophage population (blue square) measures the fraction of the macrophage population involved in the phagocytosis of target cells (phagocytosis %; Fig. 2c). This phagocytosis assay that utilizes CellTracker dye can also be performed using fluorescent microscopy.

In this study, Y27632 was used to enhance phagocytosis, which was previously known as the Rho kinase inhibitor that blocks RhoA/ROCK signaling (Nam et al., 2018). In agreement with the previous report, the result of the phagocytosis assay using CellTracker dye showed that the percentage (%) of the double positive signal (deep red and green) per the total number of macrophages, which indicates that the extent of phagocytosis increased in the Y27632-treated group compared to the control group(Fig. 2c).

Next, we performed an alternative image-based phagocytosis assay using a pHrodo-SE dye that enabled the measurement of the fluorescence of engulfed cancer cells (Fig. 2d). The pH sensitive, pHrodo-SE dye emits weak fluorescence at natural pH but changes to bright fluorescence at acidic pH. The pHrodo-SE-stained non-engulfed cancer cells emit light at a low fluorescence, while cancer cells engulfed by BMDMs emit bright florescence at a lower phagosome pH, which can easily be detected through fluorescence microscopy.

To analyze the extent of phagocytosis, targeted cells (CMFDA-stained BMDMs and cancer cells labeled with pHrodo-SE dye) were co-incubated for 2 h, followed by washing with basic PBS (pH 10) for removing unengulfed tumor cells. The percentage of phagocytosis was calculated by the number of BMDMs that phagocytized cancer cells (green containing red) per total number of BMDMs (green). Similar to the results from the flow cytometry–based phagocytosis assay, treatment of BMDMs with Y27632 led to a significant increase in the phagocytosis of cancer cells (Fig. 2d).

Note that it is difficult to exclude the possibility of cell-to-cell binding in the double positive signal of flow cytometric results utilizing CellTracker dyes. As shown in Fig. 2, the measured phagocytosis of 4 T1-Luc cells determined by the flow cytometry–based method was slightly higher than that determined by the fluorescence microscopy–method (Fig. 2c-d). This result indicates that the CellTracker-method might overestimate the baseline of cancer phagocytosis. Therefore, the appropriate methods should be selected to measure the degree of phagocytosis of cancer cells with strong aggregation such as 4 T1-Luc cells.

3.2. Evaluation varied states of phagocytosis

Current methods that utilize CellTracker dyes to verify phagocytosis are largely limited to flow cytometry– and manual image–based assays, providing limited information in spite of varying phagocytosis status. However, more detailed information beyond the measured phagocytosis percentage can be successfully assessed by a fluorescent microscopic method employing pHrodo-SE dye; the phagocytic index (PI) is calculated as the ratio of engulfed tumor cells (red) per total BMDMs (green), and the phagocytic capacity (PC) indicates engulfed cancer cells among double-positive BMDMs.

To confirm whether pHrodo-based microscopy images comprise an advantageous method with which phagocytosis can be accurately analyzed, we used anti-CD47 antibodies as a phagocytosis agonist. In agreement with a previous study (Lee et al., 2018), phagocytosis was significantly enhanced by anti-CD47 antibodies that block the interaction between CD47-SIRPα (Fig. 3a, b-left). Notably, the PI and PC were
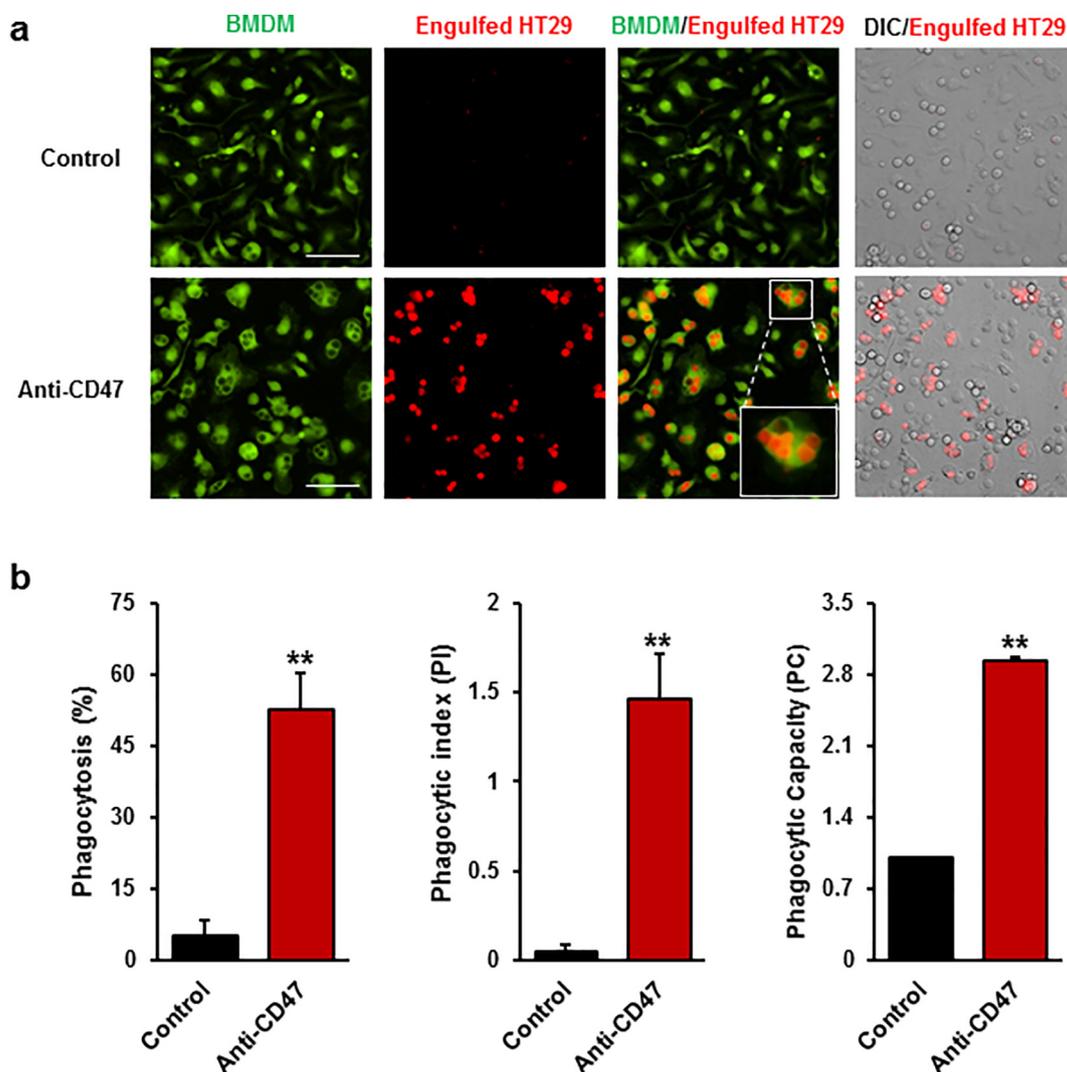
Fig. 3. Several methods of analyzing phagocytosis of cancer cells stained with pHrodo-SE through fluorescent images. (a) Representative microscopic images of pHrodo-SE phagocytosis assays performed using CMFDA-stained BMDMs (green) against engulfed HT29 cells (red), the enlarged image of which represents BMDMs' phagocytosis of multiple cancer cells (insets). Scale bars: 100 μm. (b) *Left*: Phagocytosis calculated as the percentage of BMDMs containing cancer cells per total BMDMs. *Middle*: PI calculated as the ratio of engulfed cancer cells (pHrodo-SE, red) per total BMDMs (green). *Right*: PC calculated as the ratio of engulfed cancer cells per BMDMs containing cancer cells. Data are presented as means ± S.D. (n = 3–7). **$p < .01$ by Student's t-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

considerably escalated in the anti-CD47-treated group compared with the untreated control (Fig. 3a, b). Remarkably, macrophages exposed to anti-CD47 exhibit enhanced phagocytic ability for simultaneous engulfment of multiple cells (Fig. 3a, *inset*). These results indicate that the proposed fluorescence microscopy-based method using pHrodo-SE is capable of representing the degree of phagocytosis, *e.g.*, the number of engulfed cancer cells by a single macrophage.

4. Discussion

Macrophages, which are specialized phagocytes, are particularly active in the phagocytosis process (Chimini and Chavrier, 2000; Poon et al., 2014). Of late, the phagocytic function of macrophages has become increasingly recognized as an attractive target in cancer immunotherapy (Brown et al., 2017). Given that an understanding the molecular interactions between phagocytes and tumor cells is critical for a better comprehension of their tumor-modulating action, there is an increasing demand for *in vitro* methods to study the role of macrophages in tumor cell phagocytosis.

The most widely used approaches for flow cytometry-based phagocytosis studies involve the use of CellTracker and CFSE dyes due to their staining efficiency and ease of use. However, the significant deviations in data caused by cell-cell aggregation may limit the use of these conventional methods. To overcome this limitation, we present optimized methods of analyzing cancer phagocytosis by utilizing CellTracker or pHrodo-SE fluorescence dyes. The former suggests a more precise evaluation protocol than the conventional method, which exhibited no significant difference than the latter suggested method. Note that to eradicate the feasibility of detection of cancer cells bound to the surface of the phagocytes require pHrodo-SE staining. As well, the fluorescence microscopy–based method that utilizes pHrodo-SE guarantees more detailed reporting of phagocytosis; this enables the assessment of the definite number of cancer cells engulfed by each phagocyte.

Furthermore, these *in vitro* phagocytosis assays can be used to analyze the role of DCs that play important roles in antitumor immunotherapy by evaluating engulfment, which is also the primary function of DCs. In order to initiate cancer immunity, it is important to uptake cancer cells and present antigens, which leads to the generation of tumor-specific T cells. Unlike phagocytes such as macrophages, DCs

can preserve antigenic peptides for a longer duration after phagocytosis (Lennon-Dumenil et al., 2002), and this plays a key role in the formation of adaptive immunity through effective antigen processing and presentation (Gordon, 2016).

The ability to reinforce innate immune responses is an important factor that affects the therapeutic efficacy of cancer immunotherapy. To effectively elicit tumor antigen-specific immunity, immunotherapeutic candidates must potentiate the function of antigen-presenting cells at the initial stages of the antitumor immunity cycle. Therefore, we highlighted that these methods for detecting phagocytic function are expected to be utilized for further investigations into regulating the activity of DCs in antitumor therapy. Furthermore, these optimized methods that determine the engulfment of cancer cells by phagocytes can be applied to multiple types of tumors and used as a screening assay for a variety of phagocytosis agonists.

Declaration of interest

The authors declare no competing financial interest.

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