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The nonopsonic allogeneic cell phagocytosis of macrophages detected by flow cytometry and two photon fluorescence microscope

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Abstract

Phagocytosis, one of the apparent functions for macrophages, represents an early and crucial event in triggering host defenses against invading pathogens as well as allo- or xenogeneic rejection. Now, some methods have been used in detecting the opsonic phagocytosis of macrophages in xenogeneic settings. Efficient nonopsonic phagocytosis analysis method has not been established yet. In the present studies, allogeneic lymphocytes pre-labeled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) or derived from green fluorescent protein transgeneic B6 mice (GFP-B6 mice) were co-incubated with primary murine peritoneal macrophages (PEMs) for 1–2 h or were injected into murine peritoneal cavity for 30 to 240 min. Assays by flow cytometry (FCM) and two photon laser scanning microscope (TPM) showed an efficient uptake of both allogeneic lymphocytes and xenogeneic chicken red blood cells. The continuing process of nonopsonic phagocytosis of allogeneic lymphocytes by PEMs was recorded by TPM. Furthermore, the phenotype differences of PEMs with or without phagocytosis of allogeneic cells were determined by three-color FCMs. Significantly upregulated expressions of CD11b, CD44, TLR2 and TLR4 on PEMs were observed as early as 6 h after phagocytosis of allogeneic cells. Our present data indicated that the FCM and TPM combined method is a practical approach to detect macrophage nonopsonic phagocytosis of allogeneic lymphocytes and to identify the phenotype alteration of macrophages after phagocytosis.

Keywords: Macrophage; Phagocytosis; Transplantation

1. Introduction

It has been reported that the innate immune processes including granulocytes and macrophages, are involved in the acute and chronic rejection of allografts and xenografts [1,2]. Phagocytosis, one of the apparent functions for macrophages, represents an early and crucial event in triggering host defenses against invading pathogens [3]. In addition to FcγR and CR3-mediated opsonic phagocytosis, macrophages can use another type of phagocytosis, the nonopsonic phagocytosis, to efficiently eradicate pathogens [1]. Moreover, the allogeneic cell nonopsonic phagocytosis of macrophages has played a great role in the rejection response of allografts [4]. Now, certain methods have been used in detecting the opsonic phagocytosis of macrophages in allogeneic immune response [5]. However, nonopsonic phagocytosis effects of macrophages have not been studied in allogeneic immune response due to the shortage of efficient phagocytosis analysis methods.

Flow cytometry (FCM) offers rapid multi-parameter measurements of single cells in suspension. FCM techniques have been used for a while to evaluate phagocyte cellular defects and opsonic phagocytosis during diseases. Recently, multi-parameter assays by FCM have been developed to quantitatively reveal the antigen-specificity of opsonic phagocytic responses. The

Abbreviations: Ab, antibody; CFSE, 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester; cRBC, chicken red blood cells; EDTA, ethylenediamine tetraacetic acid; FCM, flow cytometry; GFP, green fluorescent protein; PEMs, peritoneal exudate macrophages; PBS, phosphate buffered saline; TPM, Two photon laser scanning microscope; TLRs, Toll-like receptors.

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combined method of FCM and two photon laser scanning microscope (TPM) to evaluate the phagocytosis ability of macrophages is an elegant procedure, because it offers multi-parameter measurement of single cells [6,7]. In this study, we explored the possibility of using both FCM and TPM to evaluate the nonopsonic phagocytosis on allogeneic lymphocytes macrophages. Our results present the basic methodological principles for FCM and TPM assays to evaluate nonopsonic phagocytosis of macrophages.

2. Materials and methods

2.1. Animals

Five-to-seven week-old C57BL/6 (B6) (H-2^b), and Balb/c (H-2^d) mice were purchased from Beijing University Experimental Animal Center (Beijing, China). Green fluorescent protein (GFP)-transgenic B6 mice in which all lymphocytes express GFP were kindly offered by Dr. Q. Chen. All mice were maintained in specific pathogen-free facility and were housed in microisolator cages containing sterilized feed, autoclaved bedding, and water. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals.

2.2. Preparation of mouse peritoneal exudate macrophages (PEMs)

Balb/c mouse peritoneal exudate cells were obtained from the peritoneal exudates of mice. After washed twice with cold Hanks' solution, these cells were adjusted to 5×10^6 cells/ml in RPMI1640 medium (Gibco BRL, Grand Island, NY) and cultured in 2% gelatine (Sigma)-pretreated six-well plates (Costar, Cambridge, Mass.) for 1–2 h at 37 °C and 5% CO_2. The non-adherent cells were removed by washing with warm RPMI1640 medium. The adherent cells were harvested with 5 mM EDTA (Sigma) in ice-cold PBS (pH 7.2) and readjusted to 1×10^6 cells/ml. The cell viability was usually more than 95% as determined by trypan blue exclusion. The macrophage purity was analyzed by TPM LSM510 (Zeiss, Germany) and FCM (Becton Dickinson, Mountain View, CA) using macrophage marker F4/80. The adherent cells always constituted more than 90% of F4/80$^+$ macrophages (data not shown), as reported before [8,9].

2.3. Detection of macrophage phagocytosis by TPM and FCM

Naïve CD3$^+$T cells were purified by negative selection of mouse splenocytes of Balb/c and B6 mice using mouse CD3$^+$T lymphocyte enrichment set-DM (BD Biosciences Pharmingen). Following two washes with PBS, 1×10^7 cells/ml chicken red blood cell (cRBCs) or CD3$^+$T cells in PBS were labeled with 5.0 μM 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Inc. Eugene, OR) for 15 min at 37 °C [10]. These cells were then washed thoroughly and suspended with DMEM without serum at a concentration of 1×10^7 cells/ml [1]. Cell viability was determined by trypan blue exclusion. Cell viability was usually more than 95%. 1×10^6 peritoneal macrophages (F4/80$^+$ PEMs) were co-incubated with 1×10^7 CFSE-labeled cRBCs or allogeneic T cells in six-well plates (Costar, Cambridge, Mass.) that has been pre-treated with 2% gelatin (Sigma) at 37 °C and 5% CO_2 for 1–2 h [11]. Cell nuclei were stained with DAPI (eBioscience, San Diego, CA) and assessed by TPM [10]. In addition, some of the adherent cells were harvested with 5 mM EDTA (Sigma) in ice-cold PBS (pH 7.2) and adjusted to 1×10^6 cells/ml. Cells were blocked with anti-mouse FcγR mAb (clone 2.4G2) and stained with PE-conjugated anti-F4/80 mAb (BM8; eBioscience, San Diego, CA). Meanwhile, cells (1×10^6 cells/sample) were analyzed using a flow cytometry (BD Biosciences, San Diego, CA). Forward and side scatter gate was set to exclude debris. Data were analyzed using Cell Quest software (BD Biosciences) [12].

2.4. Murine PEM phagocytosis study in vivo

GFP-B6 mouse splenocytes were prepared, and red blood cells were lysed with ACK lysis buffer (Invitrogen, San Diego, CA) as described before [13]. After washed twice with cold Hanks' solution, these cells were adjusted to 5×10^6 cells/ml in RPMI1640 medium (Gibco BRL, Grand Island, NY) and cultured in six-well plates (Costar, Cambridge, Mass.) for 1–2 h at 37 °C and 5% CO_2. The non-adherent cells were harvested by washing with warm RPMI1640 medium and readjusted to 1×10^7 cells/ml. The cell viability was usually more

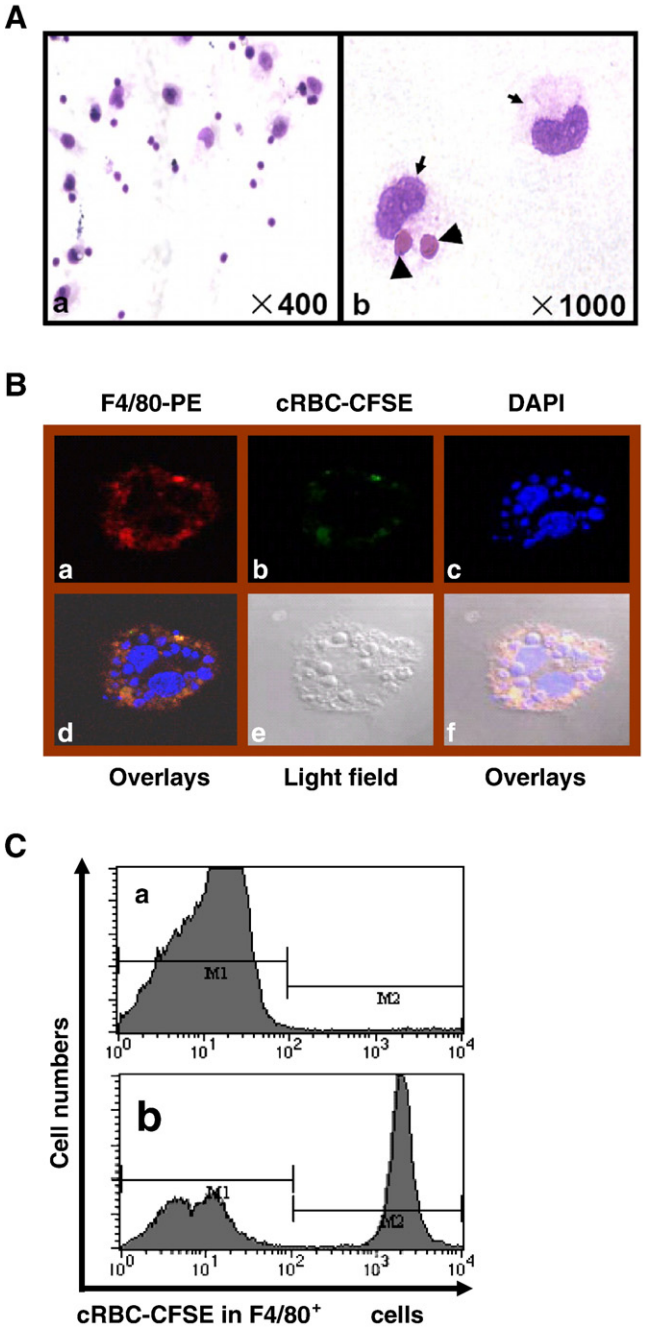

Fig. 1. Peritoneal macrophage phagocytosis effects of cRBC were detected by Giemsa staining, TPM and FCM methods. A. Giemsa staining was observed by light microscope with 400× (a) and 1000× (b). B. Peritoneal macrophage phagocytosis effects of cRBCs were observed by TPM. Peritoneal macrophages were stained with PE-anti-mouse F4/80 mAb and cRBCs were stained with CFSE in advance. Cell nuclei were stained with DAPI. One representative of five independent experiments is shown. C. Peritoneal macrophage phagocytosis effects of cRBCs were observed by FCM. Peritoneal macrophages were stained with PE-anti-mouse F4/80 mAb and cRBCs were stained with CFSE. Data is one representative of more than five independent experiments.