Journal of Immunological Methods 390 (2013) 9–17

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

# Development and validation of a flow cytometric method to evaluate phagocytosis of pHrodo™ BioParticles® by granulocytes in multiple species

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ARTICLE INFO

*Article history:*
Received 31 March 2011
Received in revised form 24 June 2011
Accepted 24 June 2011
Available online 13 July 2011

*Keywords:*
Phagocytosis
Granulocytes
pHrodo™ BioParticles®
Flow cytometry
Apoptotic cells

ABSTRACT

Phagocytosis plays a pivotal and essential role in host immune defense, both as a focal constituent of the innate immune response and a bridging element linking innate and adaptive immunity. Phagocytosis has been demonstrated to be critical in development, tissue remodeling, wound healing and resolution of inflammation through clearance of foreign organisms, apoptotic cells and the production of anti-inflammatory mediators. During pre-clinical investigations, therapeutic drug candidates may alter host resistance to infectious agents by modulating the immune system. The assessment of phagocytic function can be a critical parameter of immunotoxicology for this adverse effect. Utilizing pH-sensitive pHrodo™ BioParticles®, a flow cytometric phagocytosis method was developed and validated in rodent and non-human primate (NHP) species under rigorous GLP compliant procedures. Using species-specific granulocyte markers as well as appropriate temperature and pharmacologic controls, we have developed an *ex vivo* assay to measure phagocytic function. The method has been optimized to utilize minimal sample volume of whole blood. The assay represents a rapid and reliable tool that can be implemented to evaluate the immunotoxic and immunomodulatory effects of therapeutic candidates.



## 1. Introduction

Phagocytosis of foreign organisms and apoptotic cells plays a pivotal role in maintaining basal functions of host defense, development, remodeling, tissue homeostasis and resolution of inflammation. Phagocytosis of pathogens via engulfment and intracellular acidification results in pathogen killing and clearance, whereas phagocytosis of apoptotic cells prevents the release of toxic and immunogenic intracellular contents into the surrounding tissues. The anti-inflammatory cytokine, such as TGF-β, produced through apoptotic cell–phagocyte engagement further facilitate resolution of inflammation (Fadok et al., 1998; McDonald et al., 1999; Huynh et al., 2002; Xiao et al., 2002). Disruptions in phagocytic functions can be associated with infectious diseases, cancers, pulmonary diseases, Alzheimer's disease, and autoimmune diseases (Vanholder and Ringoir, 1993; Henson et al., 2006; Erwig and Henson, 2007; Hershkovitz et al., 2007; Cashman et al., 2008; Zhu et al., 2009).

The rapid growth of biotechnology-derived pharmaceutical products engineered to modulate the immune system and address autoimmune diseases, cancers, allergy and infectious diseases has highlighted safety concerns. The safety issue of the first-in-human phase 1 study of TGN1412, a novel humanized anti-CD28 monoclonal antibody was a prevalent example (Suntharalingam et al., 2006; Giezen et al., 2008). It is therefore not surprising that the study of phagocytic function has been an object of investigation in immunotoxicity studies (ICH Harmonised Tripartite Guideline S8, Immunotoxicity Studies for Human Pharmaceuticals). Various

*Abbreviations:* NHP, non-human primate;HBSS, Hank's Balanced Salt Solution;FBS, Fetal Bovine Serum;HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;DPBS, Dulbecco's phosphate buffered saline;MFI, mean fluorescence intensity;DMSO, dimethyl sulfoxide;PC, positive control;NC, negative control;IC, inhibitor control.

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doi:10.1016/j.jim.2011.06.027

techniques have been developed to assess phagocytosis, including microscopy-based and flow cytometry-based assays. Microscopy-based techniques are time consuming and widely used for qualitative rather than quantitative assessment. Flow cytometry-based methodology, on the other hand, offers rapid and reproducible measurements of single cells in suspension. However, distinguishing membrane-bound from ingested targets by flow cytometry has turned out to be an uneasy task. Efforts to address this issue have been made through the use of fluorescence quenching technique or fluorochrome-conjugated antibodies specific for detection of membrane-bound particles in multi-channel flow cytometry systems (Van Amersfoort and Van Strijp, 1994). In the present study, we have developed and validated a flow cytometric method using pHrodo™ BioParticles®, which are nonfluorescent at neutral pH but fluoresce bright red in acidic environments. This increase in fluorescence signal is directly correlated to engulfment of pH sensitive dye-conjugated bioparticles and occurs at low pH phagosomes. The process is optimal to identify ingested versus surface-bound targets without involving multi-step procedures or requiring the preparation of additional specific reagents. Validation of the current method was performed in non-human primates (NHP) as well as three strains of rats and mice, respectively. The methodology described herein may be readily adapted to the analysis of phagocytosis of apoptotic cells and used for evaluating potential immunotoxicity or immunomodulatory effects of pharmaceutical, biologic or environmental products in different species.

## 2. Experimental

### 2.1. Materials

#### 2.1.1. Animals

Ten male and ten female experimental cynomolgus macaque non-human primates (NHP) approximately 30–40 months of age at receipt, were provided from an MPI Research stock colony (originally received from Harlan Laboratories, Inc. or Covance Research Products, Inc). Eleven rats per sex per strain (Fisher 344; CD®; and Wistar Han), approximately 6–8 weeks old, were obtained from Charles River Laboratories. Ten mice per sex per strain (CD-1, Balb/c, and C57/BL6), approximately 8–10 weeks old, were also obtained from Charles River Laboratories. All the experiments were performed according to national guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee (IACUC) at MPI Research.

#### 2.1.2. Reagents

The pHrodo™ *Escherichia coli* BioParticles®, HBSS, FBS, HEPES and 1X DPBS were purchased from Invitrogen Corporation. Monoclonal anti-human CD14 PE-Cy7 antibody (Clone RMO52; Beckman Coulter), monoclonal anti-rat CD11b FITC antibody (Clone OX-42; Serotec), and monoclonal anti-mouse Ly-6G FITC antibody (Clone 1A8; BD Biosciences) were purchased for detection of species-specific granulocytes in NHP, rat, and mouse, respectively. Cytochalasin D, bafilomycin A1, chloroquine, tissue culture-grade DMSO, and sodium azide were procured from Sigma Aldrich. 1X RBC Lysis Buffer was purchased from eBioscience. IO Test® 3 Fixative Solution was purchased from Beckman Coulter.

### 2.2. Methods

#### 2.2.1. BioParticle preparation

Lyophilized pHrodo™ *E. coli* BioParticles® were reconstituted in Uptake Buffer (20 mM HEPES in HBSS, pH 7.4) to a concentration of 1 mg/mL. The vials were vortexed for a minimum of 60 seconds, sonicated for 10 minutes, and placed on ice for an additional 10 minutes prior to use.

#### 2.2.2. Phagocytosis assay

Peripheral whole blood samples of different species were collected into tubes containing sodium heparin, and kept at ambient temperature until analysis. The blood samples were aliquotted (100 µL/sample) into polypropylene 96-well deep square bottom plates or test tubes for the following procedures. All incubation steps were performed protected from light unless indicated otherwise.

Inhibitor controls, comprising of peripheral whole blood treated with pre-determined concentrations of cytochalasin D, bafilomycin A1, or chloroquine, were incubated at 37 °C with 5% $CO_2$ prior to the addition of 20 µL of 1 mg/mL pHrodo™ *E. coli* BioParticles® (pHrodo). pHrodo was added to each of the positive control (PC), inhibitor control (IC) and negative control (NC) samples. PC and IC samples were incubated at 37 °C with 5% $CO_2$, while NC samples were maintained on ice. The samples were incubated with the species-specific antibodies against granulocyte markers and placed on ice for 30 minutes. Red blood cells were lysed at room temperature for 10 minutes using 1 mL RBC Lysis Buffer. The samples were then centrifuged at 450 ×*g* for 5 minutes at 4 °C, washed twice with Staining Buffer (1% FBS and 0.09% sodium azide in 1X DPBS), resuspended in 0.5 mL Staining Buffer and fixed for 15 minutes at room temperature using 15 µL IOTest® 3 10× Fixative. The samples were kept at 4 °C until analysis with the Beckman Coulter FC500 MPL flow cytometer.

#### 2.2.3. Flow cytometry analysis

Detector settings and compensation levels were optimized using unlabeled samples as well as samples single-stained with pHrodo or with species-specific antibodies against granulocyte markers. A single parameter histogram illustrating the cell populations that were positive or negative for the granulocyte marker was used to identify the granulocyte population. Further gating techniques pinpointed the percentage of granulocytes that were "pHrodo Bright." In addition, the mean fluorescence intensity (MFI) of the whole granulocyte population was utilized in assessing shifts in pHrodo fluorescence attributable to increasing inhibitor concentrations. Percentage inhibition (% Inhibition) was calculated using the following equation:

$$\%\ \textbf{Inhibition} = 100 \times \left(\frac{\textbf{PC}-\textbf{IC}}{\textbf{PC}}\right)$$

#### 2.2.4. Data analysis and statistics

For the validation studies, PC samples for each animal were tested as triplicate wells, while the NC and IC samples