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Research paper

# An improved flow cytometry assay to monitor phagosome acidification

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ABSTRACT

Phago-lysosome formation is important for cell-autonomous immunity to intracellular pathogens, antigen presentation and metabolism. A hallmark feature of phago-lysosomal compartments is that they undergo progressive luminal acidification controlled by the activation of vacuolar V-ATPase. Acidification is required for many enzymatic processes taking place in phago-lysosomes, like proteolysis, and supports the microbicidal activity of macrophages. Here we present a new quantitative methodology to assess phagosome acidification by flow cytometry based on the use of bi-fluorescent particles. This method relies on the use of UV polystyrene beads labelled with the acid sensor pHrodo-succinimidyl ester (pHrodo™ SE red) and enables us to dissociate particle association with phagocytes from their engulfment in acidified compartments. This methodology is well suited to monitor the acidification of phagosomes formed in vivo after fluorescent bead administration.



## 1. Introduction

Particles are engulfed by phagocytes through the activation of signalling pathways leading to actin remodelling (Flannagan et al., 2012). Once sealed, phagocytic vesicles undergo a maturation process leading to the formation of phago-lysosomes (Steinberg and Grinstein, 2008; Fairn and Grinstein, 2012; Flannagan et al., 2012). One of the first events of this process is the acquisition of a functional vacuolar Adenosine TriPhosphatase (V-ATPase). This large enzyme complex acidifies the phagosomal lumen by transferring protons from the cytosol into the vesicle using energy from ATP hydrolysis (Lukacs et al., 1990, 1991; Hackam et al., 1997). Acidification itself is required for efficient phagolysosomal maturation (Gordon et al., 1980). Stepwise membrane fission and fusion events then culminate in the delivery of hydrolases to the phagosome lumen (Kielian and Cohn, 1980; Gabay et al., 1986; Desjardins et al., 1994, 1997). Lumenal acidification of phagosomes is required for the activity of digestive enzymes such as acid-dependent cathepsins D and L. Phagosome acidification is key for the control of various intracellular microbes. As a consequence, successful intracellular pathogens such as *Mycobacteria* have developed efficient escape mechanisms to prevent V-ATPase recruitment (Sturgill-Koszycki et al., 1994; Wong et al., 2011) and phagolysosomal maturation (Armstrong and Hart, 1971) (Chua et al., 2004; Vergne et al., 2004; Flannagan et al., 2009) and disrupt phagosomal function in infected cells (Podinovskaia et al., 2013a,b).

Phagosomal acidification and acid-dependent proteolysis are important parameters in antigen presentation by MHC

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molecules (Blum et al., 2013). Acidification generally favours the presentation of phagocytosed antigens by MHC II molecules since they need to undergo proteolytic digestion before being loaded onto MHC class II molecules whose maturation also depends on acid-dependent proteases (Trombetta et al., 2003; Trombetta and Mellman, 2005; Blum et al., 2013). Cross-presentation of phagosomal antigens by MHC class I, by contrast, is favoured by limited acidification of phagosomes (Accapezzato et al., 2005; Hotta et al., 2006; Savina et al., 2010), perhaps in relation with the access of undigested antigens to cytosolic proteasomal degradation (Kovacsovics-Bankowski and Rock, 1995; Norbury et al., 1997; Rodriguez et al., 1999).

Finally, acidification of phago-lysosomes is also important for lipid and amino-acid metabolism. For example, lysosomal acid lipase is a lysosomal enzyme the activity of which depends on acidification and plays an important role in the digestion of internalized lipoproteins and reverse cholesterol transport via the ABCA1/G1 pathway (Ouimet and Marcel, 2012).

Given the paramount importance of phago-lysosomes, many different techniques have been developed to monitor phago-lysosomal formation and maturation. Most of these techniques rely on the assessment of phagosome lumen acidification. pH-dependent fluorescent probes offer unique opportunities to monitor the onset of acidification. For example, fluorescein isothiocyanate (FITC) exhibits a pH-dependent quenching which has been used by Okhuma and Poole to set up an assay monitoring lysosomal pH (Ohkuma and Poole, 1978). This approach has been successfully used to monitor phagosomal acidification in vitro, often after the engulfment of pathogens (Geisow et al., 1981; Horwitz and Maxfield, 1984; Lukacs et al., 1991; Bouvier et al., 1994; Oh and Straubinger, 1996; Vergne et al., 1998; Kuehnel et al., 2001; Podinovskaia et al., 2013a, b; Tan et al., 2013). In most cases, particles could also be labelled with pH-insensitive reporter dyes.

Flow cytometry offers a convenient and versatile way to read and quantify fluorescence on large numbers of cells. To assess phagosomal pH by flow cytometry, we have developed a technique that involves the labelling of phagocytic particles (polystyrene latex beads) with pH sensitive (FITC) and pH-insensitive (alexa647) fluorochromes, distinguishing particle association with cells from phagosome acidification (Savina et al., 2006; Savina et al., 2010). However, this method was limited by the difficulty of achieving simultaneous bright labelling with both probes and also because acidification measurement is based on the loss of FITC fluorescence.

More recently, pHrodo™, a dye whose red emission is increased in acidic environments, became available. This led to the development of fluorescence-based methods to monitor phagocytosis and acidification using pHrodo-labelled particles that have an increased fluorescence in acidic compartments (Miksa et al., 2009; Fabbrini et al., 2012; Toda et al., 2012; Neaga et al., 2013; Tan et al., 2013). However, the use of biodegradable particles such as bacteria or dead cells does not allow precise monitoring of the efficiency of particle association and engulfment by cells. Here we have developed a new flow cytometry method consisting of polystyrene beads fluorescently labelled with an UV dye insensitive to pH changes (BB beads, Polysciences) coated with pHrodo™ (Invitrogen) at their surface. We show that this simple technique enables precise tracking of the process of phagocytosis, from association of particles with phagocytes through the progressive acidification of phago-lysosomes. Importantly, this method enables the tracking of the distribution of phagosomal pH within a population of single bead-containing cells. Finally, this method enables the measurement of the acidification of phagosomes formed in vivo after the administration of indicator beads.

## 2. Materials and methods

### 2.1. Materials

#### 2.1.1. Animals

All mice used were C57BL6/J wild-type (8–12 weeks old). Mice were bred and maintained under sterile conditions in the Biologic Services Unit of King's College London. Mouse handling and experimental procedures were conducted in accordance with national and institutional guidelines for animal care and use.

#### 2.1.2. Cell culture

RAW264.7 (Raschke et al., 1978) cells were obtained from ATCC. DC2.4 (Shen et al., 1997) were obtained from K. Rock (University of Massachusetts Medical School, Worcester). Cells were cultured in Dulbecco's modified Eagle's medium with 10% of heat-inactivated foetal bovine serum, penicillin (100 IU/mL), streptomycin (100 μg/mL) and glutamine (5 mM) (Life Technologies) (termed as complete medium) and maintained at 37 °C in a humidified atmosphere of 5% $CO_2$. S2 cells (Schneider, 1972) were cultured in complete Schneider medium complemented with 10% FBS, penicillin (50 IU/mL), and streptomycin (50 μg/mL).

### 2.2. Method

#### 2.2.1. Beads preparation

Fluoresbrite® BB Carboxylate Microspheres 1.75 μm (excitation 330 nm/emission 407 nm) were obtained from Polysciences Inc. (Warrington, PA, USA). pHrodo™ Red succinimydyl ester (pHrodo™ Red SE, cat. P36600) was obtained from Invitrogen, resuspended at 4 mg/ml in DMSO and conserved at −80 °C. For conjugation, beads were washed with PBS to remove preservative and resuspended in 1 mg/ml of ovalbumin in PBS overnight at 4 °C under constant agitation to coat the beads with amine-reactive residues. Next, beads were resuspended in pHrodo™ Red, SE (Invitrogen) at 2 μg/ml in PBS (pHrodo UV beads) or PBS alone (control UV beads) and incubated for 20 min at 37 °C. After washing, beads were resuspended in the same DMEM at ~8. 49 x $10^9$ particles/ml.

#### 2.2.2. In vitro phagocytosis assay

1 μl of coated beads was added to 100 μl of cell culture medium with $5.10^5$ cells/well in a 96 U-bottom wells plate and incubated 20 min at optimal growth temperature protected from light to let the beads adhere to the surface of the cells (pulse). Then cells were washed three times with PBS-BSA 1%-EDTA 2 mM, resuspended in complete medium alone, incubated for indicated time intervals at 37 °C and protected from light to let the beads accumulate in phago-lysosomes (chase). At the end of the chase, cells are washed with PBS-1% BSA 2mMEDTA and stained with fluorescent antibodies where necessary, for 25 min on ice and protected from light. After staining, cells were washed twice with FACS buffer and