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Phagocytosis of immunoglobulin-coated emulsion droplets

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ABSTRACT

Phagocytosis by macrophages represents a fundamental process essential for both immunity and tissue homeostasis. The size of targets to be eliminated ranges from small particles as bacteria to large objects as cancerous or senescent cells. Most of our current quantitative knowledge on phagocytosis is based on the use of solid polymer microparticles as model targets that are well adapted to the study of phagocytosis mechanisms that do not involve any lateral mobility of the ligands, despite the relevance of this parameter in the immunological context. Herein we designed monodisperse, IgG-coated emulsion droplets that are efficiently and specifically internalized by macrophages through *in-vitro* FcγR-mediated phagocytosis. We show that, contrary to solid polymeric beads, droplet uptake is efficient even for low IgG densities, and is accompagnied by the clustering of the opsonins in the zone of contact with the macrophage during the adhesion step. Beyond the sole interest in the design of the material, our results suggest that lateral mobility of proteins at the interface of a target greatly enhances the phagocytic uptake.

1. Introduction

Phagocytosis is a process that consists in the ability of a cell to internalize objects larger than 0.5 microns. Whereas unicellular organisms use phagocytosis to capture and eat preys, in multicellular organisms it represents a fundamental part of innate immunity, organ homeostasis and tissue remodeling. Innate immunity relies on a specialized subset of cells, the professional phagocytes, which patrol the organism, identify, ingest and eliminate pathogens. Among them, macrophages are versatile cells residing in tissues that are able to scavenge worn-out cells and participate to the activation of the adaptive immune response [1]. Phagocytosis by macrophages is triggered by the binding of the target to specific receptors present at the surface of the phagocyte. Several receptors have been identified so far, each involving different signaling pathways and ingestion mechanisms [2]. In the case of Fcγ receptors-mediated phagocytosis [3], antigens present at the surface of the target are bound by specific soluble immunoglobulins (IgGs). Fc regions of those IgGs are actively recognized [4] by the Fcγ receptors (Fcγ) from the phagocyte surface, which form clusters and trigger the internalization. Engulfment then occurs by an actin-driven membrane extension and closure [5] of a phagocytic cup around the foreign body to create a specific degradative compartment: the phagosome [6].

Most of our current quantitative knowledge on phagocytosis is based on the use of various model particles such as heat- or chemically inactivated bacteria or yeast [7] and polymer microparticles [8]. The versatility and reproducibility of the latter has allowed monitoring the influence of parameters such as size and surface chemistry [8–13], shape [14,15] and mechanical properties [16] of the target on the mechanism of phagocytosis. It was observed that the uptake of polymer particles is the most efficient for 2–3 microns-large targets, increases with the density of IgGs attached to the surface [8–12,14,17,18] and depends on the local curvature of the target in contact with the cell [14,15].

Antigens present on the surface of endogenous targets, such as erythrocytes [19], cancerous and apoptotic cells [20,21] exhibit a

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lateral mobility [22,23] that can't be mimicked by adhesive proteins adsorbed on the solid surface of common polymeric targets [24]. However, in the immunological context, phenomena as for example the formation of the immune synapse [25,26], or the fabrication of artificial antigen presenting cells [27], require the ability of antigens or receptors to be laterally mobile at the interface of the target. There is hence a need of novel particulate materials allowing the free diffusion of the adhesive molecules bound to their surface.

Oil-in-water emulsions have been already used since half a century as colloidal drug carriers for various therapeutic applications [28]. Versatile in terms of volume and surface composition, they can be fabricated with a narrow size distribution ranging from a few tenth of nanometers to several hundreds of microns [29]. Emulsions have also been used in a biophysical context as deformable objects to measure forces existing in living embryonic tissues [30] and those generated by growing actin networks *in-vitro* [31]. Carefully functionalized with biologically-relevant adhesive molecules, they are able to interact with cells in a specific manner [32] and can be used as model particles for cell adhesion modeling [33–35]. In addition to giving access to a controlled range of biomolecules densities at the surface, emulsion droplets have a liquid interface allowing proteins bound to it to be laterally mobile [34–36], as in our case the IgGs. However, to our knowledge there is no report of any observations at the scale of a single droplet interacting with a phagocyte, nor obvious elements about the possible influence of the nature of the interface (liquid *vs*. solid) on the uptake, despite its biophysical [37] and biological relevance [4,22,23].

We thus propose to use IgG-functionalized oil-in-water emulsion droplets for phagocytosis studies as probes able to mimick the lateral mobility of antigens present on the surface of cellular targets. Herein we describe a fabrication route of monodisperse, IgG-coated emulsion droplets made from soybean oil and biotinylated phospholipids. We show that IgGs-coated liquid emulsion droplets are efficiently and specifically internalized through FcγR-mediated phagocytosis *in-vitro*. During the recognition by macrophages, we show that IgGs are driven in the contact zone and colocalize with an increase of the local concentration of FcγRs, while polymerized F-actin is visible during the extension and closure of the phagocytic cup. By comparing the phagocytosis efficiency of droplets and polystyrene particles in similar conditions of IgG coating, we suggest that the lateral mobility of the IgGs at the interface of a target enhances its ability to be internalized.

2. Materials and methods

2.1. Biotinylation and opsonization of the emulsion droplets

The lipid-containing oil was obtained by dilution of DSPE-PEG(2000)-Biotin phospholipids (Avanti Lipids, Alabama, USA) in soybean oil at concentrations ranging from 0.015 to 0.15 mg mL^{-1} (30 min sonication followed by evaporation of the chloroform from the oil). This oil, cooled to room temperature, was dispersed and emulsified by hand in an aqueous continuous phase containing 15% w/w of Poloxamer 188 block polymer surfactant (CRODA, East Yorkshire, UK) and 1% w/w sodium alginate (Sigma–Aldrich, St. Louis, MO, USA) at a final oil fraction equal to 75%. The rough emulsion was sheared in a Couette cell apparatus at a controlled shear rate of 5000 s^{-1} following the method developed by Mason et al. [38] to narrow the droplet size distribution to 7 ± 2 μm. For storage and handling purposes the emulsion were diluted to an oil fraction of 60% w/w with 1% w/w of poloxamer 188 in the continuous phase and stored at 12 °C in a Peltier-cooled cabinet for several weeks. Size distribution of the emulsion droplets was measured by microscopy and image analysis. Coupling of IgGs to biotins present on the surface of the droplets was obtained after a 30 min incubation of the droplets in 0.003–0.3 mg mL^{-1} (2.10^{-8}–2.10^{-6} mol L^{-1} with a molecular weight of 150 kDa) fluorescent antibiotin IgGs solutions (Alexa Fluor 488-conjugated IgG fraction) monoclonal mouse anti-biotin (Jackson Immunoresearch, West Grove, PA, USA) at room temperature in phosphate buffer (PB, pH = 7.2, 20 mM, 0.2% w/w Tween 20). The droplets were rinsed twice in the same buffer and finally suspended in (DMEM, Life Technologies, Carlsbad, CA, USA) containing high glucose, no glutamine and no phenol red directly prior to use in cell assays.

2.2. Opsonization of the polystyrene beads by direct adsorption of IgGs

Polystyrene beads (6 μm diameter, Polysciences, Warrington, PA, USA) were functionalized by direct adsorption of rabbit anti-goat IgGs FITC conjugate (Sigma–Aldrich) at concentrations ranging from 0.4 to 1 mg mL^{-1} for one hour at room temperature.

2.3. Opsonization of the polystyrene beads using biotinylated BSA and anti-biotin IgGs

Polystyrene beads (6 μm diameter, Polysciences, Warrington, PA, USA) were incubated with biotin-conjugated BSA (Sigma–Aldrich, St. Louis, MO, USA) at a concentration of 5 mg mL^{-1} for 1 h in a PBS buffer. Beads were then washed and incubated for 30 min at 37 °C in a fluorescent anti-biotin IgGs solution (Alexa Fluor 488-conjugated mouse anti-biotin, Jackson Immunoresearch, West Grove, PA, USA) at concentration of 2.10^{-1} mg mL^{-1} in PBS.

2.4. Characterization of opsonization degree of the particles

Liquid and solid particles were characterized with a BD Accuri C6 cytometer (BD Biosciences, New Jersey, USA) according to a method we developed in the past [36] for quasi-monodisperse emulsions. In brief, the fluorescence intensity of the particles is proportional to the amount of fluorescent proteins on their surface and can be converted in a total number of fluorophores per particle using a commercial quantification kit (Quantum™ Alexa Fluor® 488 MESF beads and Quantum™ FITC-5 MESF Premix, Bangs Laboratories, Fishers, IN, USA). The number of IgGs per droplet is then estimated by dividing the number of fluorophores per droplet by the average number of dyes per IgG, which ranges from 5 to 8 for Alexa 488 and 3–4 for FITC according to the manufacturer. Using a value of 5 Alexa 488 dyes per IgG (or 3 FITC), the number of IgGs ranges from 10^3 to more than 10^5 per droplet.

2.5. Cell culture

Lifeact-mCherry RAW 264.7 murine macrophages [39] were obtained from Pierre Jurdic (IGFL, ENS Lyon) and used as model macrophages. The cells were cultured at 37 °C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (Life Technologies), 1.0 g L^{-1} D-glucose, 1.1 g L^{-1} sodium pyruvate (Life Technologies) and 1% penicillin-streptomycin (Life Technologies). The expression of the Lifeact-mCherry was maintained through the intermittent addition of 4 μg mL^{-1} puromycine (Life Technologies) to the culture medium. Confluent monolayers of cells were resuspended after trypsinization and plated in 6 wells cell culture plates lined with glass coverslips (20 × 20 mm, VWR, Radnor, PA, USA) 24 h prior to phagocytosis studies. Experiments were performed with cell densities close to 10^6 cells per glass coverslip that correspond to *ca.* 60% confluence.

2.6. Experimental setup of the phagocytosis assay

The custom-built chamber consisted of two glass coverslips assembled with 100 μm thick double-sided tape (3M, Maplewood, Minnesota, USA) to form a 20 × 20 mm experimental chamber. The coverslips with the adherent cells were first washed with DMEM without FBS to eliminate dead cells. Lipid droplets or polystyrene beads suspended at an initial concentration of two drops per cell in DMEM without FBS were injected in the observation chambers and incubated at 37 °C under 5% CO_2 for up to 120 min in the presence of the macrophages. For each time point, the solution in the chambers was replaced by a fixation solution for 20 min (4% w/w paraformaldehyde in DPBS-1X, Sigma–Aldrich). Once fixed, the cells were washed again with DPBS-1X, and a mixture of Atto 555-phalloidin and DAPI (Sigma–Aldrich, St. Louis, USA) was added to each chamber for 30 min to respectively make polymerized actin filaments and cell nuclei fluorescent for further observation. The chambers were rinsed 3 times with DPBS-1X and observed under the microscope. Unless stated, all the experiments were run in triplicate.

2.7. Microscopy

Brightfield and fluorescent images of cells with attached and internalized droplets were acquired on a Zeiss Axio Observer Z1 microscope (Oberkochen, Germany) equipped with a Clara E CCD camera (Andor Scientific, Belfast, UK) and controlled by the μManager software [40]. Confocal microscopy observations were performed on a Zeiss LSM 710 microscope. Observations were performed in DMEM without FBS at 37 °C for live-cell imaging.

2.8. Quantification of phagocytosis

Phagocytosis efficiency was characterized by manually measuring, over *ca.* 20 fields of observation, the percentage of cells having internalized from 0 to a maximum of 5 droplets on a subpopulation of around 200 randomly chosen macrophages per condition. The percentage of internalizing cells (%IC) after a given incubation time and the phagocytic index (PI) were calculated. PI is defined as the weighted arithmetic mean of the total number of internalized particles per cell. The data related to the phagocytic index are reported in the Supporting Materials. The evolution of %IC as a function of the IgG density on the droplets is fitted by a Hill equation [41]: