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A novel real time imaging platform to quantify macrophage phagocytosis

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ABSTRACT

Phagocytosis of pathogens, apoptotic cells and debris is a key feature of macrophage function in host defense and tissue homeostasis. Quantification of macrophage phagocytosis *in vitro* has traditionally been technically challenging. Here we report the optimization and validation of the IncuCyte ZOOM® real time imaging platform for macrophage phagocytosis based on pHrodo® pathogen bioparticles, which only fluoresce when localized in the acidic environment of the phagolysosome. Image analysis and fluorescence quantification were performed with the automated IncuCyte™ Basic Software. Titration of the bioparticle number showed that the system is more sensitive than a spectrofluorometer, as it can detect phagocytosis when using 20× less *E. coli* bioparticles. We exemplified the power of this real time imaging platform by studying phagocytosis of murine alveolar, bone marrow and peritoneal macrophages. We further demonstrate the ability of this platform to study modulation of the phagocytic process, as pharmacological inhibitors of phagocytosis suppressed bioparticle uptake in a concentration-dependent manner, whereas opsonins augmented phagocytosis. We also investigated the effects of macrophage polarization on *E. coli* phagocytosis. Bone marrow-derived macrophage (BMDM) priming with M2 stimuli, such as IL-4 and IL-10 resulted in higher engulfment of bioparticles in comparison with M1 polarization. Moreover, we demonstrated that tolerization of BMDMs with lipopolysaccharide (LPS) results in impaired *E. coli* bioparticle phagocytosis. This novel real time assay will enable researchers to quantify macrophage phagocytosis with a higher degree of accuracy and sensitivity and will allow investigation of limited populations of primary phagocytes *in vitro*.

1. Introduction

Macrophages are innate immune cells that respond to inflammation, tissue trauma or infection. They were first identified in the nineteenth century by Elie Metchnikoff in sea-star larvae for their ability to surround and engulf a rose thorn, a process he termed phagocytosis [1]. Together with monocytes and dendritic cells, macrophages make up the mononuclear phagocyte network and play an important role in tissue homeostasis [2]. Macrophages are equipped with a range of highly conserved pattern recognition receptors (PRRs) which bind to conserved pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) on Gram-negative bacteria [3,4]. Similarly, danger-associated molecular patterns (DAMPs), such as high mobility group box 1 [5], IL-1α [6] and uric acid [7] are released by necrotic cells during sterile injury and trigger the activation of macrophages.

Upon PRR engagement, receptor clustering brings together tyrosine kinases which initiate the internalization of foreign particles [8–11]. Downstream signaling induces the engulfment of the foreign object via protrusions of the cell membrane to form a membrane-bound vesicle [12–14]. The newly formed vesicle, called the phagosome, undergoes maturation whereby it fuses with several compartments of the endocytic system as it is transported within the cytosol [15]. Earlier work has shown that during

Abbreviations: BMDM, Bone Marrow-derived Macrophage; cDNA, complementary DNA; Ct, Cycle threshold; DAMP, Danger-associated Molecular Pattern; FBS, Fetal Bovine Serum; GEO, Gene Expression Omnibus; hiPS cell, human induced Pluripotent Stem cell; IFN-γ, Interferon-γ; IgG, Immunoglobulin G; IL, Interleukin; LPS, Lipopolysaccharide; MARCO, Macrophage Receptor with Collagenous structure; PAMP, Pathogen-associated Molecular Pattern; PMA, Phorbol 12-myristate-13-acetate; PRR, Pathogen Recognition Receptor; qPCR, quantitative Polymerase Chain Reaction; TLR, Toll-like Receptor.

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maturation, phagosomes acquire numerous proteins, including hydrolases, proton pump ATP subunits and endosome fusion proteins [16]. Changes in the phagosome membrane and its content are continuous, well-orchestrated and eventually lead to its acidification [17,18].

When phagosome maturation is complete, these organelles fuse with lysosomes which carry an arsenal of hydrolytic enzymes and anti-microbial peptides [19]. The acidic and highly oxidative environment of the phagolysosome is essential for the activation of cathepsins, oxidants and cationic peptides which eventually lead to pathogen lysis and clearance [20].

Researchers have employed a variety of assays to study phagocytosis, including light and confocal microscopy, flow cytometry and imaging flow cytometry and spectrofluorometry. However, technical and practical limitations are associated with all of these techniques, such as dye quenching, low throughput, single end point readouts and often subjective quantification. ESSEN BioScience developed the IncuCyte ZOOM®, a platform which utilizes microscopy to monitor phagocytosis in real time. In this study we report the optimization and validation of this live cell imaging platform to quantify primary murine and human macrophage phagocytosis. We show that this platform is 20 times more sensitive than spectrofluorometry and can detect signals from limited primary macrophage populations.

2. Materials and methods

2.1. Reagents

Fetal Bovine Serum (FBS), LPS (O111:B4), BSA, RPMI-1640 medium, phorbol 12-myristate-13-acetate (PMA), Bafilomycin A1, Nocodazole and Cytochalasin D were purchased from Sigma-Aldrich (Gillingham, Dorset, UK). PBS was from Lonza (Slough, UK). EDTA was purchased from VWR technologies (East Grinstead, UK). OptiMEM I reduced serum medium, Live Cell Imaging Solution, Penicillin/Streptomycin, green pHrodo® *E. coli* and red *S. aureus* Bioparticles® were from Life Technologies (Paisley, UK). Sterile IncuCyte™ green pHrodo® *E. coli* and Zymosan were purchased from ESSEN Bioscience (Welwyn Garden City, UK). Cytokines (Interferon-γ (IFN-γ), interleukin -4 (IL-4) and IL-10) were from Peprotech (London, UK).

2.2. Cell lines

Cell lines were originally from American Type Culture Collection. The RAW264.7 and THP-1 cell lines were a kind gift from Prof. Siamon Gordon, the BV-2 cell line was kindly provided from Prof. David Vaux. Macrophage cell lines at passage number 5 were cultured in RPMI-1640 medium containing 10% FBS and 1% Penicillin/Streptomycin at 37 °C/5% CO_2. Cells were passaged every three days. THP-1 cells were stimulated with 50 ng/ml PMA for four days before the phagocytosis assay.

2.3. Animals

All animal experiments were conducted with local ethical approval from the Dunn School of Pathology Local Ethical Review Committee and in accordance with the UK Home Office regulations (Guidance on the Operation of Animals, Scientific Procedures Act, 1986). Male 8–10 week old C57BL/6J mice (25–30 g) were purchased from Harlan Laboratories (Bicester, UK). All animals were housed in a 12 h light/dark cycle unit with free access to food and water.

2.4. Bone marrow-derived macrophages (BMDMs)

BMDMs were generated as previously described [21]. Briefly, tibiae and femurs from male C57BL/6J mice were flushed with PBS and bone marrow cells were re-suspended in Dulbecco Modified Eagle's Medium supplemented with 10% heat-inactivated FBS, 10–15% L929-conditioned medium [22] and 1% Penicillin-Streptomycin. Cells were cultured for seven days at 37 °C/5% CO_2 and were re-fed on day 3.

2.5. Human induced Pluripotent Stem (hiPS) cell-derived macrophages

The hiPS cell line AH017-13 was derived from dermal fibroblasts of healthy donors recruited by the Oxford Parkinson's Disease Centre (Ethics committee: National Health Service, Health Research Authority, NRES Committee South Central – Berkshire, UK – REC 10/H0505/71), reprogrammed using standardized protocols in the James Martin Stem Cell Facility, Sir William Dunn School of Pathology, and their SNP datasets and transcriptome array results are deposited in Gene Expression Omnibus (GEO) under accession numbers GSE 53426 [23]. For this study, hiPSCs were thawed and cultured as described in [23]. Differentiation to macrophages via embryoid body formation and directed differentiation was as previously described [24].

2.6. IncuCyte ZOOM® phagocytosis assay

Day 7 BMDMs in OptiMEM medium were plated into 96-well flat clear bottom black walled polystyrene tissue-culture treated microplates (Corning, Flintshire, UK) and allowed to adhere for 2 h. pHrodo® pathogen bioparticles were added at indicated concentrations and the plates were transferred into the IncuCyte ZOOM® platform which was housed inside a cell incubator at 37 °C/5% CO_2, until the end of the assay. Two images per well from two technical replicates were taken every 10 min for 1 h using a 20× objective lens and then analyzed using the IncuCyte™ Basic Software. Green channel acquisition time was 400 ms, whereas red channel acquisition time was 800 ms. In phase contrast, cell segmentation was achieved by applying a mask in order to exclude cells from background. An area filter was applied to exclude objects below 50 μm^2. Green and red channel background noise was subtracted with the Top-Hat method of background non-uniformity correction with a radius of 20 μm [25] and a threshold of 2 green and red corrected units. Fluorescence signal was quantified applying a mask (Fig. 1A; 1 mg/ml bioparticles, Fig. 1B; 10 μg/ml bioparticles). Moreover, as seen in Fig. 1C, in the absence of the edge split tool the software recognizes the indicated objects as one, whereas when edge split is applied, the objects are recognized as multiple closely-spaced objects. Edge split was therefore used as a more accurate quantification of fluorescent objects.

2.7. Confocal microscopy

BMDMs (2.5×10^4 cells per well) were seeded into IBIDI μ-plate 96 well plates (Munich, Germany) in OptiMEM medium and allowed to adhere for 2 h. Media were removed and replaced with either 200 μg/ml pHrodo red *S. aureus* bioparticles and 200 μg/ml FITC-conjugated Zymosan A or FITC-conjugated 2 μm latex beads (Polysciences Inc, PA, USA) diluted in Live Cell Imaging Solution. Cells were incubated for 1 h at 37 °C 5% CO_2, followed by extensive washing. NucBlue® Live ReadyProbes® Reagent in live cell imaging solution (ThermoFisher Scientific, MA, USA) was added to stain nuclei. Images were acquired with a 100× objective lens using an Olympus FV1200 confocal microscope (Olympus, PA, USA) fitted with a temperature controlled stage set to 37 °C and analyzed with ImageJ software v1.49.