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Macrophage activation differentially modulates particle binding, phagocytosis and downstream antimicrobial mechanisms

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ABSTRACT

Phagocytosis provides a critical first line of defense against invading pathogens. Engagement of particles through receptor-mediated binding precedes internalization and induction of cellular antimicrobial responses. Phagocytes have the capacity to differentially regulate binding and internalization processes through changes in their receptor profile and modulation of downstream events. This is necessary for the intricate control of phagocytic antimicrobial responses. Several methods are available for evaluation of phagocytosis. Unfortunately, none allow for accurate quantitation of both binding and internalization events. To overcome these limitations, we have developed a novel phagocytosis assay based on a multi-spectral imaging flow cytometry platform. This assay discriminates between internalized and surface-bound particles in a statistically robust manner and allows multi-parametric analysis of phagocytosis and downstream anti-microbial responses. We also devised a novel approach for examination of phagolysosome fusion, which provides an improved capacity for quantitative assessment of phagolysosome fusion in mixed populations of intact cells. Importantly, our approaches are likely amenable to a broad range of comparative model systems based on our examination of murine RAW 264.7 cells and a goldfish primary kidney macrophage (PKM) model system. The latter allowed us to examine the evolutionary conservation of phagocytic antimicrobial responses in a lower vertebrate model. While it has been previously reported that mixed populations of these macrophage cultures are phagocytic, it remained unclear if sub-populations within them contributed differentially to this activity. In accordance with higher vertebrate models, we found that differentiation along the macrophage pathway leads to an increased capacity for phagocytosis in goldfish PKM. Interestingly, cellular activation differentially regulated particle internalization in PKM monocyte and mature macrophage subsets. We also found differential regulation of phagolysosome fusion and downstream production of reactive oxygen intermediates (ROI). The temporal activation of specific phagocytic antimicrobial responses at distinct stages of PKM differentiation suggests specialization within the macrophage compartment early in evolution, geared to meet specific host immunity requirements within specialized niches.

1. Introduction

Phagocytosis is an important, evolutionarily conserved mechanism that is integral to host defenses against invading microorganisms. Phagocytosis is initiated by the interaction of receptors on the surface of the phagocyte with ligands on large particles, generally over 1 μm. Receptor ligation results in actin polymerization, which subsequently leads to particle internalization (Zigmond and Hirsch, 1972; Axline and Reaven, 1974; Greenberg et al., 1991; Aderem and Underhill, 1999). Once internalized, the phagosome that encloses the particle undergoes a series of maturation steps that culminates in phagolysosome fusion. In order for phagocytosis to be an effective immune defense mechanism, these two components – internalization and phagosomal maturation – must occur (Flannagan et al., 2009).

There are a number of methods available to study phagocytosis. Of these, three common ones are based on light microscopy, fluorescent microscopy, and flow cytometry techniques. Each of these assays allow for detection phagocytosis with varying levels of specificity. These techniques, however, also have caveats that limit the accurate quantification of phagocytosis and their widespread application (e.g. time, reproducibility of quenching steps to remove fluorescence from surface-bound particles, and others) (Drevets and Campbell, 1991; Hampton and Winterbourn,

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1999). Microscopy-based assays are largely limited by time requirements and low populations counts, which prevent statistically robust evaluation of phagocytosis. Flow cytometry-based assays have become increasingly popular due to the ability to analyze much higher cell counts in a short period of time. These assays, however, lack the spatial resolution of microscopy-based assays, which limit accurate quantification of internalized particles.

Using an ImageStream multi-spectral imaging flow cytometer, we developed a novel assay that incorporates the strengths of the previous assays, while overcoming the weaknesses associated with both the microscopy and flow cytometry assays. Analysis of phagocytosis responses can be further enhanced by incorporation of specific cellular markers, characterization of cellular morphology, and an increased capacity for multivariate analysis. Images can be acquired at over 100 images/second, allowing analysis of a large population of cells. We applied this novel assay to the study of phagocytosis in a teleost primary kidney macrophage (PKM) model. While it has been previously reported that mixed populations of these cultures are phagocytic, it is unclear which of the sub-populations are responsible for this activity (Neumann et al., 1998; Grayfer et al., 2008). Further, it remains unclear if these myeloid cells may display a differential capacity for phagocytosis and downstream killing mechanisms during key steps along their differentiation pathways. We sought to examine these questions among mixed populations of cells to allow for cellular crosstalk and to minimize the need for isolation of individual subpopulations, which is often associated with higher levels of basal activation.

We report here that, under basal activation conditions, mature macrophages and monocytes are the predominant phagocytes in our teleost primary kidney macrophage cultures. Upon activation with macrophage activating factor (MAF) and LPS, monocytes and mature macrophages differentially up-regulate phagocytic responses. Monocyte phagocytosis was enhanced following 6-h stimulation with MAF/LPS and was further increased with prolonged activation (48-h). Unlike the monocyte population, short activation periods (6-h) had little effect on phagocytosis in the mature macrophage population. Forty-eight hour MAF/LPS activation, however, induced a pronounced increase in mature macrophage phagocytosis. Interestingly, the kinetics of phagocytosis parallels those of respiratory burst induction in the mature macrophage, but not in the monocyte population. In our mixed cultures, we found that mature macrophages had the highest production of reactive oxygen intermediates at 48 h, while monocytes had maximal responses at 6 h. These kinetics are similar to those previously reported using sorted cell populations (Neumann et al., 2000), suggesting that production of ROI by PKM is intrinsically regulated, as cells respond similarly whether they are characterized as part of a mixed culture or following sorting of individual subpopulations. Finally, we provide a unique approach to quantify phagolysosome fusion in these mixed populations, which provides an improved capacity for quantitative assessment of phagolysosome fusion in mixed populations of intact cells. We found that, while the rate of phagolysosome fusion paralleled the rate of phagocytosis, monocytes and mature macrophages have a differential capacity to form phagolysosomes following particle internalization. This data suggests that these two cell populations may have differential capacity for degradation of internalized particles and/or rely on different mechanisms to degrade these particles.

2. Materials and methods

2.1. Animals

Goldfish (*Carassius auratus* L.) 10–15 cm in length were purchased from Mount Parnell (Mercersburg, PA) and maintained in the Aquatic Facility of the Department of Biological Sciences, University of Alberta. The fish were held at 20 °C in a flow-through water system on a simulated natural photoperiod. Unlike its smaller cyprinid relative *D. rerio*, *C. auratus* provided sufficient numbers of hematopoietic progenitors necessary for the establishment of in vitro primary macrophage cultures.

2.2. Goldfish primary kidney macrophage cultures

Primary kidney macrophages were generated by seeding isolated leukocytes and culturing in 15 mL complete MGFL-15 media (MGFL-15 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 100 μg/mL gentamicin, 10% newborn calf serum (Gibco) and 5% carp serum) with 5 mL cell-conditioned media from previous experiments and incubated for 6–9 days at 20 °C (Neumann et al., 1998, 2000). For these experiments we used PKM cultures from Day 7 cultures which were in the proliferative phase of growth (Barreda and Belosevic, 2001). Under these conditions PKM cultures consist of cells in three distinct stages of macrophage development, and are largely devoid of non-macrophage derived cells. Where indicated, cells were activated with media containing 50% self cell-conditioned media, 25% fresh complete media, 25% MAF (macrophage activating factor)-containing media and 1 μg/mL *E. coli* 0111:B4 (Sigma). MAF conditioned media was prepared as previously described (Neumann et al., 1998).

2.3. Cell lines

Jurkat T cells were cultured in RPMI 1640 supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, 1 mM sodium pyruvate, and 1× non-essential amino acids (all from Gibco). RAW 264.7 macrophage cells were cultured in complete DMEM media. Cell lines were cultured at 37 °C/5% CO_2 and passaged every 3–4 days. Where indicated, cells were activated with 100 ng/mL ultrapure *E. coli* LPS (InVivogen) for 24 h.

2.4. Phagocytosis assays

2.4.1. Light microscopy

RAW 264.7 macrophages were plated in 6-well plates at a concentration of 5×10^5 cells/mL. Cells were allowed to adhere overnight. 3 μm latex beads (Polysciences) were added at a ratio of 5:1 (zymosan: cells) and incubated for the indicated times at 37 °C/5% CO_2. Cells were then stained by HEMA 3 stain set according to the manufacturer's protocol (Fisher Scientific) and counted by light microscopy. At least 200 cells were counted.

2.4.2. Confocal fluorescent microscopy

RAW 264.7 macrophages were seeded onto sterilized glass coverslips in a 6-well plate at a concentration of 5×10^5 cells/mL. Cells were allowed to adhere overnight. Zymosan-FITC particles (Molecular Probes) were added at a ratio of 5:1 (zymosan: cells) and incubated for the indicated times at 37 °C/5% CO_2. Cells were then fixed with 1% formaldehyde for 10 min on ice and washed 2 times with 1×$PBS^{-/-}$. 100 ng/mL 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) was added to the mounting media and coverslips were mounted onto slides. Cells were imaged using a Zeiss LSM 510 laser scanning confocal microscope. Images were acquired at 40×/1.3. At least 100 cells were counted.

2.4.3. Flow cytometry

In order to evaluate our capacity to differentiate between phagocytic and non-phagocytic cells in mixed populations, we took advantage of two well-characterized cellular models: RAW 264.7 cells and Jurkat cells. RAW 264.7 cells were mixed with Jurkat T cells at a ratio of 1:1. Zymosan-FITC was added to cells at a ratio