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The macrophage marches on its phagosome: dynamic assays of phagosome function

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Preface

Professional phagocytes ingest particulate material to fulfill a diverse array of functions within a multi-cellular organism. The ancestral function of phagosomes is digestion, however, through evolution, this degradative capacity has become pivotal to the adaptive immune response by processing antigens to be presented to lymphocytes. Moreover, phagocytes have also acquired an active role in microbial killing. This Innovation article describes new assays that probe the biological activities in phagosomes. These assays provide functional insights into how the phagosome fulfills its diverse roles in homeostasis, and innate and adaptive immune responses.

Introduction

Among protists such as amoebae, phagocytosis is almost exclusively devoted to the acquisition of nutrients; however, following the adoption of a multicellular existence this behavior has evolved to fulfill more diverse and sophisticated roles. Even in social amoebae such as *Dictyostelium* spp. a subset of cells known as sentinel cells have evolved to perform anti-microbial activities, which protect the cellular community from bacterial invaders^{1,2}. In the late 1800s, Metchnikov observed the phagocytic activity of highly motile cells in dissected starfish and proposed that these cells fulfilled a key function in protecting multicellular organisms from pathogenic microorganisms. It was these observations by Metchnikov that provided the foundations of our current appreciation of the host protective functions of professional phagocytes.

We now know that phagocytic cells such as macrophages function throughout both innate and adaptive immune responses. They can, through expression of pattern recognition receptors such as toll-like receptors (TLRs), detect certain conserved molecular patterns in microorganisms that initiate an innate immune reaction, which leads to an up-regulation of anti-microbial behavior by these cells^{3–5}. Pathogen recognition also enhances the ability of macrophages to present antigens to lymphocytes thus accelerating the adaptive immune response^{5,6}. In turn, cytokines generated by activated lymphocytes feed back on macrophages changing their function to that of immune effector cells that have improved capacity to both

stimulate T cells and kill pathogens^{7–10}. Each of these events in pathogen recognition and induction of immunity revolve around the maturation, and therefore function, of the phagosomal compartment. Although many studies have been published in this area, alterations in phagosome maturation and function are predominantly inferred through the differential acquisition of cellular markers, such as lysosome-associated membrane protein 1 (LAMP1) and RAB7, rather than direct measurements of the changing milieu of the phagosomal lumen. In this article we detail observations resulting from newly developed assays that assess the changing physiology within the phagosome. These real-time readouts are contributing to a more complete appreciation of how the macrophage modifies its behavior to fulfill its many functions.

Phagosome maturation

Phagosome maturation used to be thought of as an extremely simple concept that described the degree to which phagosomes had fused with lysosomes. This notion is unfortunately no longer sufficient because phagosomes are now known to interact with many intracellular organelles during their maturation process (figure 1). Even before they are fully formed proteins, such as the NADPH oxidase complex that generates the superoxide burst, can be observed being assembled on the phagocytic cup. Once closed and the maturation process is underway, phagosomes become increasingly more acidic and hydrolytically-active and transiently fuse with the recycling endosomal system, the secretory system, including secretory lysosomes, multi-vesicular bodies such as the MHC class II (MIIC) compartment and even the endoplasmic reticulum^{11,12}. All of these sources of membrane and proteins could impact the function of phagosomes, yet these interactions are routinely assessed simply by the acquisition of “marker” or indicator proteins, and not as alterations in functional activities. Therefore, any alteration in functionality tends to be implied rather than demonstrated directly. Adopting this philosophy makes the intracellular source of a protein almost secondary to the contribution that it makes to the functionality of the phagosome. For example, enhanced acquisition of cathepsins B and L would be interpreted as increasing hydrolytic capacity, but if the cathepsins are accompanied by inhibitors of cysteine proteinase activity, such as stefin A¹³, such an interpretation would be wrong. So judging phagosome maturation by its interaction with these organelles through the presence of “marker” proteins may be seriously misleading. Finally, when discussing phagosome maturation, it seems reasonable to ask; when is a phagosome mature? In truth, this is a subjective judgment that depends on which parameter you are measuring.

Grinstein and colleagues have developed some extremely elegant methods for real-time visualization of the lipid-remodeling that accompanies phagosome biogenesis^{14–16}. These assays provide unique insight into the membrane dynamics that interface with the cytosolic machinery responsible for the fusion and fission events required for phagosome maturation. However, we are infection biologists and our interest focuses on the world of the microbial pathogen, which has to deal with the luminal environment within the phagosome, and that is the focus of this article.

Assaying pH or phagosome–lysosome fusion

By exploiting the pH-sensitive emission of the fluorescent dye carboxyfluorescein, we examined the rate of acidification of phagosomes containing IgG-coated, carboxyfluorescein-labelled beads that were taken up by mouse bone marrow-derived macrophages¹⁷. These bead-containing phagosomes reached a pH of around pH 5.0, the pH of mature lysosomes, within 12–15 minutes. This pH value represents a steady-state because it can only be maintained through the activity of proton-pumping V-ATPase complexes. So, if pH were the parameter of interest, this would indicate full maturation of the phagosome was reached 12–15 minutes post-internalization.

More recently, we developed a quantitative, fluorescence resonance energy transfer (FRET)-based assay to measure phagosome–lysosome fusion¹⁷. The assay is dependent on labeling the macrophages with a hydrophilic, acceptor fluorochrome that is endocytosed and chased into the lysosomes of the cells. The macrophages are then fed with IgG-coated beads labeled with a donor fluorochrome. The degree to which the phagosomal and lysosomal compartments fuse is measured by the magnitude of the signal generated by the transfer of the fluorescence energy of the donor fluorochrome on the bead to the acceptor fluorochrome in the phagolysosome. In essence, this assay measures the concentration of soluble, lysosomal cargo to which the donor fluorochrome-carrying particle is exposed. In macrophages, we have shown that these phagosomes containing IgG-coated beads reach a steady-state at approximately 90 minutes after internalization. This steady-state is likely maintained by the vesicular fusion and fission events that determine the concentration of lysosomal cargo in the particle-containing phagolysosomes

So, while both methods measure phagosome maturation, they indicate markedly different time-scales for the process; maximal acidification is reached in 12–15 minutes but peak concentration of lysosomal cargo is not reached until 90 minutes post-internalization. Given the divergent results generated by these functional assays, it is difficult to see how the rate of acquisition of individual lysosomal proteins, most routinely LAMP1, which is usually reported in terms of the percentage of LAMP1⁺ vesicles, can adequately describe the physiological alterations that occur in phagosomes during maturation.

Assaying hydrolytic activity

The function of the phagosome is the degradation of biological material, regardless of whether the ultimate goal is to rid the body of dead cell debris without inducing an immune response or to generate epitopes from internalized pathogens for recognition by T cells. To explore the rate of acquisition and activation of degradative hydrolases by phagosomes, another parameter of phagosome maturation, we developed assays that provide real-time readouts for a range of hydrolytic activities, including proteinase activities, lipolysis and β -galactosidase activity^{17, 18}. These assays measure the processing by specific hydrolases of a fluorogenic substrate attached to an IgG-coated bead. The increasing fluorescence is expressed as a ratio against the fluorescence of a control (calibration) fluorochrome that is also present on the beads. The readouts can then be quantified by spectrofluorometry, confocal microscopy or flow cytometry (figure 2). Unlike the pH and phagosome–lysosome fusion assays that reach a steady-state, these assays reach an endpoint determined by exhaustion of available substrate. Drugs that reduce phagosome maturation, such as the V-ATPase inhibitor Concanamycin A, in general, diminish the hydrolytic processing of the bead-bound substrate.

The number of non-internalized beads, a potential source of background, is reduced to undetectable levels through the use of fully-confluent monolayers of macrophages, and the addition of a ligand, such as IgG or mannosylated BSA, which engage extremely efficient phagocytic receptors^{18,19}. Finally, the fluorescent readouts from the substrates were not impacted by reactive oxygen or nitrogen intermediates because identical profiles were observed in macrophages from both iNOS- and NADPH oxidase-deficient mice²⁰.

The kill zone: the superoxide burst

In addition to hydrolases, one of the other enzyme activities that is acquired by the phagosome during its formation is the superoxide burst. Reactive oxygen intermediates are generated by an enzyme complex with NADPH oxidase activity that is assembled into the plasmalemma during the formation of the phagocytic cup²¹. This complex leads to generation of superoxide, which can be converted into hydrogen peroxide by superoxide dismutase. In addition, superoxide can combine with nitric oxide intermediates and iron to generate peroxynitrite and

hypervalent iron²², all of which are noxious, reactive intermediates that have potent antimicrobial activity.

We recently developed two independent assays to measure the levels reactive oxygen intermediates within phagosomes²³. The fluorescence emission of the fluorogenic probe dihydrohexafluorofluorescein increases following oxidation and, when complexed to the surface of IgG-coated beads, this probe revealed a burst of oxidative activity that terminated 20–25 minutes following internalization of the IgG-coated particle. Activation of the macrophage by pre-treatment with the TLR4 agonist lipopolysaccharide (LPS), interferon- γ (IFN γ) or both increased the intensity of the oxidative response but did not affect its duration. These data indicate that the active NADPH oxidase complex is only present in the phagosome for the first 20 minutes following its formation. Varying the concentration of substrate on the particle had no effect on the kinetics demonstrating that this was not the effect of substrate limitation²³. These kinetics were confirmed by a second assay for intraphagosomal oxidation using C18 silica particles (Nucleosil) that were coated with the oxidation sensitive lipid C11-Bodipy as the probe. C11-Bodipy undergoes a red to green spectral shift upon oxidation, which can be measured by spectrofluorometry. The actual oxidants that are detected by these two assays is unclear but preliminary data indicate that it is more likely that the probes are oxidized by secondary metabolites such as hydrogen peroxide, rather than the primary product, superoxide (D.G. Russell, B.C. Vandervan. unpublished observations).

Clearly, immune-mediated activation of the macrophage increases the superoxide burst, but stimulation of the phagocyte with LPS or IFN γ has broader effects that impact other properties of the phagosome.

Immune modulation of the intraphagosomal milieu

Effects of acute, immediate immune activation

TLRs recognize microorganism-derived molecules and are known to be recruited to the phagosome following internalization of microbial cargo²⁴. This observation gave rise to the idea that phagosomes sense, and can be specifically modulated by, the identity of their cargo. It has been reported that the presence of a TLR agonist on a phagocytosed particle can accelerate the maturation of individual phagosomes^{25,26}, as determined by LAMP1 acquisition and changes in phagosome pH, using the acidophilic probe LysoTracker. Acceleration of phagosome maturation by its cargo would have major implications for both antimicrobial activities and antigen processing and presentation. So, to further explore the effects of TLR ligation on phagosome maturation, we used LPS- or Pam3Cys-bearing beads that were coated with IgG or mannosylated BSA (ManBSA) and examined both the rate of phagosome acidification and phagosome–lysosome fusion following their uptake by macrophages using the assays described earlier²⁷. However, in all cases, we failed to detect any TLR-dependent affect on the kinetics of phagosome acidification. We have also examined the effects of TLR activation on the rate of phagosome–lysosome fusion of *Staphylococcus aureus*-containing phagosomes and on the rate of acidification of phosphatidylserine-coated particle-containing phagosomes, which mimics the uptake of apoptotic cells^{28,29}. Once again, the presence of TLR ligands had no effect on either of these phagosome maturation profiles²⁷.

Clearly, the impact of TLR stimulation on phagosome maturation and function is an issue that remains to be fully resolved. Despite the contrary observations, it is clear that activation of the innate immune system through ligation of TLRs does have a major impact on antigenicity and the robustness of subsequent immune responses, even if the underlying mechanism(s) remain unclear. TLR stimulation activates macrophages and accelerates dendritic cell (DC) maturation, and therefore it is feasible that particle-associated TLR agonists may work through

global upregulation of cell function³⁰, rather than localized modulation of phagosomal behavior.

Effects of sustained immune activation

In contrast to the contradictory data regarding the short-term effects of TLR stimulation on macrophage function, it is generally accepted that prolonged stimulation of macrophages by cytokines such as IFN γ has a major effect on macrophage function. Clues to possible functional changes between the phagosomal milieu of activated and resting macrophages come from the finding that acquisition of lysosomal hydrolases (detected by immunofluorescence studies) was delayed in activated macrophages³¹. To further address this issue we activated macrophages overnight with LPS, IFN γ or LPS plus IFN- γ and measured the rates of acidification, phagosome-lysosome fusion and acquisition of lipolytic, proteolytic and β -galactosidase activities²⁰. In contrast to previous results from short-term TLR stimulation, we observed marked differences in phagosomal behavior, which varied with the different activation stimuli used. There were subtle changes in the kinetics of phagosomal acidification, and LPS-stimulated cells showed a greater degree of acidification (0.2 pH unit lower than control). Stimulation with LPS also induced an altered profile of phagosome-lysosome fusion kinetics, such that fusion was initially (up to 90 minutes) reduced but then (after 90 minutes) increased (as indicated by protracted accumulation of lysosomal cargo) compared with the steady-state reached in the phagosomes of unstimulated macrophages at this timepoint.

However, the most notable differences between the activation states, induced by LPS, IFN γ and both LPS and IFN γ , were observed in the acquisition and activities of the various lysosomal hydrolases. These enzymes showed differing profiles according to the activation signal given to the macrophages implying that the functional constraints placed on the phagosome vary dependent upon its activation status. Most significantly, overnight stimulation with IFN γ induced a marked reduction (40–50%) in the proteolytic activity within the phagosome²⁰. To pursue the basis of this reduction in the proteolytic activity we isolated lysosomes from differentially activated macrophages and assayed the activities of their lysosomal hydrolases. However, we did not detect any disparity in total hydrolytic activities specific to the activation stimulus²⁰. These data indicate that there is differential acquisition and/or retention of individual hydrolytic activities, which cannot be explained through differences in abundance of the enzymes in the lysosomes. Alternative endosomal trafficking models that could explain such disparities have previously been proposed^{11,32,33}. These models propose that variations in phagolysosomal function results from either heterogeneity in the prelysosomal vesicles or gradients of lysosomal hydrolases within a highly tubular lysosomal network.

These data have to be placed in the context of the changing roles of the macrophage following activation with IFN γ . Under this stimulation the cell is now required to fulfill functions specific to the adaptive immune response.

Lysosomal proteolysis and antigen presentation

Antigen presentation by macrophages treated with LPS, IFN γ or LPS and IFN- γ is markedly upregulated, leading to improved efficiency of T cell stimulation³⁴. The mechanisms underlying this improvement are multiple, ranging from upregulated expression of MHC class II and co-stimulatory molecules to the more efficient processing of antigenic proteins. The studies by Blander and colleagues suggested that increased antigen processing correlates with the accelerated maturation of phagosomes, (as measured by increased accumulation of LAMP1 and LysoTracker)^{25,26}. However, in our studies, IFN γ -activated macrophage, showed a reduction in proteolytic processing within the phagosome²⁰. These data are consistent with previous reports showing that the phagosomes of macrophages are more proteolytically-active than those of DCs^{20,33}, and that alkalinization of DC phagosomes reduced proteolysis, which

led to enhanced antigen presentation³⁵. These data argue against the suggestion that increased or accelerated proteolytic processing promotes antigen presentation. To examine this question directly we incubated resting DCs and resting bone marrow-derived macrophages with IgG-coated, beads derivatized with the fluorogenic protease substrate DQ Green BSA and found that the basal proteolytic activity in DC phagosomes was approximately 50% of that observed in resting macrophage phagosomes (D.G.Russell., R.M.Yates, B.C. Vandervan. unpublished data). As dendritic cells are specialized, antigen-presenting cells this infers that reduced, not enhanced, proteolysis favors epitope generation or half-life, thus enhancing presentation.

Housekeeping versus immune function

These results question the current paradigm of how macrophages fulfill their diverse roles in tissue homeostasis and as immune effector cells during infection or injury (figure 3). The function of a tissue macrophage in a non-inflamed environment is to recognize, internalize and digest cellular debris and apoptotic bodies that are generated by normal developmental and tissue repair activities without inducing an immune response³⁶. For such activities, the cell would ideally be highly degradative, yet retain a non-inflammatory phenotype. The limited anti-microbial functions performed by such cells could likely be handled mainly through the degradative nature of their phagosomal compartment.

By contrast, once a macrophage has been activated, its behavior is changed drastically (figure 3). Its degradative capacity needs to be re-tuned to maximize proteolysis for epitope production and not for total degradation. Consistent with this rationale is the finding that two isoforms of two different protein antigens, RNase and horseradish peroxidase, which, by virtue of intra-chain cleavages, differ markedly in their susceptibility to degradation following endocytosis³⁷. Following inoculation of mice with the different isoforms of each protein absorbed onto an adjuvant (alum), the isoforms of each protein that was less susceptible to degradation, and therefore more persistent, were found to be more immunogenic. These observations, together with previously discussed findings, suggest that a reduction in proteolysis in phagosomes enhances the efficiency of antigen processing.

Although activated macrophages gain optimal activity in antigen processing and presentation, these cells must still be able to efficiently kill invading microorganisms. However, these microbicidal activities may reflect a change from the reliance on the degradative properties of the phagosome to the use of oxidative and nitrosative killing pathways. Indeed, analysis of the magnitude of the intraphagosomal superoxide burst shows that it is enhanced following activation of macrophages by TLR agonists or IFN γ ²³ (See Box 1 figure).

The functional studies that we have detailed here are augmented by more recent proteomic analysis of the phagosomes from resting versus IFN γ -activated macrophages^{38,39}. While these data do not provide any direct insights into the functional alterations within the phagosomal lumen, this information promises to be extremely insightful in understanding the underlying regulation of signaling and fusion events responsible for the reprogramming of the role of the phagosome in activated macrophages.

Taking the assays to the patient's bedside

Dynamic assays of phagosome maturation and function continue to provide new insights into the biological significance of phagosomes during health and disease. In particular, the assays can be used to provide unbiased, real-time analyses of phagosomal processes. Moreover the assays are now been used to assess phagocyte function in humans suffering from infections that may be associated with defective macrophage function. For example, alveolar macrophages are the primary barrier against respiratory infections and any defect in this cell population could have a major impact on the establishment of the infection (Box 1). We feel

that these new methods will help to identify phagocyte deficiencies that either predispose individuals to infectious disease or portend the progression of a disease process, such as the onset of AIDS.

Concluding remarks

Approaches that provide insight into phagosomal physiology are critical for understanding the contribution of macrophages and other professional phagocytes to host defense. Although immunofluorescence approaches have been invaluable in the identification of the intracellular organelles that are recruited to the phagosome during biogenesis, it is critical that we develop an understanding of the functional consequences of this process. The development of real-time assays that sample the environmental conditions within the maturing phagosome are central to raising our appreciation of how a phagosome fulfills the multiple functions demanded of it by its multicellular “host”.

Box 1. Clinical applications for assays for phagosome function

Functional assays of phagosomes may be used in a clinical setting both as diagnostic and investigative tools. For example, superoxide burst in macrophages is impaired in patients with chronic granulomatous disease⁴⁰ and is thought to be suppressed in individuals infected with HIV⁴¹. (A) Illustrates the assay that we have developed for measuring the superoxide burst within the phagosome. Murine macrophages were fed IgG-coated particles labeled with the oxidation sensitive fluorochrome dihydrohexafluorofluorescein, and the calibration dye Alexa-594. Both wild-type and macrophages deficient in the p91 subunit of the NADPH oxidase were examined in their resting and LPS-activated states, and the increase in fluorescence measured by spectrofluorometer. Activation of the macrophage with LPS enhanced the intensity but not the duration of the burst.

For clinical studies, peripheral blood monocytes, or alveolar macrophages from high-risk patients can be isolated and typed for the phagosomal competence using this assay, and the other assays detailed in this article. We are currently analyzing the intra-phagosomal superoxide burst in alveolar macrophages from HIV-infected individuals to determine the microbicidal capacity of these cells (figure B). This figure shows the flow cytometric profiles from alveolar macrophages from a patient with no beads, the beads alone, and the macrophages with beads at 10 and 60 minutes post-internalization. As the beads also carry the calibration fluorochrome Alexa-594, the result can be expressed as an Activity Index to facilitate comparison between patients, shown in figure C. This general approach can be applied with all of the phagosomal assays that we have developed, providing a panel of readouts addressing phagocyte competence. Further analysis using antibodies against cell surface activation markers or HIV proteins can be done to associate functional phenotype with the infection or activation status of the macrophages. As an array of different intra-phagosomal activities can be measured, these assays could form an important new approach for identifying phenotypes associated with susceptibility to infectious diseases, impaired immune responses, and any macrophage-associated pathologies.

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Figure 1. During maturation, the phagosome forms transient interactions with a wide range of intracellular organelles

Phagosome maturation refers to the process of phagosome remodeling through a series of independent events, following its formation at the surface of the phagocyte, and culminating in the complete fusion of the phagosome with the lysosome. Following engagement of phagocytic receptors, an area of the surface is remodeled around the particle, forming the phagocytic cup. Some protein complexes such as the NADPH oxidase complex, can be recruited and activated prior to phagosomal closure, facilitating a rapid antimicrobial response at the cell surface. The phagosome, once closed, becomes increasingly more acidic, through the accumulation of V ATPases that pump protons into the compartment, and hydrolytically-competent, through the acquisition of lysosomal enzymes. This process is marked by transient fusion events with multiple intracellular organelles including the recycling endosomal machinery, the synthetic–secretory apparatus including the endoplasmic reticulum, secretory lysosomes, and multi-vesicular bodies, which may include the MIIC compartment and/or the

autophagosome. Finally, the phagosome fuses with pre-existing, dense lysosomal bodies and equilibrates to a pH of 4.5–5.0.

Figure 2. Real-time measurement of changing phagosomal hydrolase activity in the phagosome during the maturation process

Assays have been developed that measure bulk proteinase activity, cysteine proteinase activity, lipolysis and β -galactosidase activity. The assays involve fluorogenic substrates that are linked to silica beads (A). These beads are also coupled with an opsonizing molecule, such as IgG or mannosylated BSA to facilitate their uptake by macrophages and a calibration fluorochrome. Results are usually expressed as a ratio of substrate fluorescence: calibration fluorescence. The examples illustrated show experiments with beads coupled with the proteinase substrate DQ Green BSA, which consists of albumin derivatized with a self-quenching fluorochrome. The ensuing change in fluorescence generated through the release of fluorescent peptides can be measured by several different instrument, each of which provides its own unique insight. A spectrofluorometer (B) provides a kinetic readout that represents an average value across a population of cells mounted on glass coverslips in cuvettes. A confocal microscope (C) allows visualization and quantitation of the increase in green fluorescence from the hydrolyzed substrate compared with the red fluorescence of the calibration fluorochrome at the level of the individual phagosome. These frames are from a 45 minute movie. Flow cytometry (D) enables examination of the heterogeneity of the activity across the cell population at the level of each individual cell. Analysis by flow cytometry can be combined with immunofluorescence with antibodies against pathogens, or cell surface markers. Figure 2C Images courtesy of J. Enninga [Institut Pasteur, Paris].

FIG 3. The phenotypic differences between resting and activated macrophages are reflected in the physiology of their phagosomal compartments

The functions fulfilled by macrophages are modulated by their degree of stimulation, whether this is by exogenous mediators such as microbe-derived TLR agonists or endogenous activators such as cytokines and chemokines. Resting macrophages function in the absence of any inflammatory stimuli and their primary role is cleansing the body of cellular debris such as apoptotic cells. The phagosomal compartment of the resting macrophage is highly degradative for efficient processing of this material.

In contrast, macrophages activated by exposure to TLR agonists or cytokines such as IFN γ , demonstrate alterations in several aspects of phagosome physiology. The superoxide burst is enhanced in its intensity (see also the Box 1 figure). Microbial killing in activated macrophages relies more heavily on the production of reactive oxygen and nitrogen intermediates. In addition, the phagolysosomal milieu of activated macrophages is actually less proteolytically active than that of a resting macrophage. This level of proteolysis is similar to that observed in dendritic cells, therefore, the implication is that this switch may enhance the half-life of epitopes and thereby maximize the antigen sampling and presenting capacity of activated macrophages.

Box Figure.

$$\text{Activity Index} = \frac{\text{Mean Substrate Fluorescence}}{\text{Mean Calibration Fluorescence}}$$