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Intraphagosomal measurement of the magnitude and duration of the oxidative burst

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Abstract

Generation of an oxidative burst within the phagosomes of neutrophils, dendritic cells, and macrophages is an essential component of the innate immune system. To examine the kinetics of the oxidative burst in the macrophage phagosome, we developed two new assays using beads coated with oxidation sensitive fluorochromes. These assays permitted quantification and temporal resolution of the oxidative burst within the phagosome. The macrophage phagosomal oxidative burst is short-lived, with oxidation of bead-associated substrates reaching maximal activity within 30 min following phagocytosis. Additionally, the extent and rate of macrophage phagosomal substrate oxidation was subject to immuno-modulation by activation with LPS and/or IFN- γ .

Keywords

phagocytosis; NADPH oxidase; oxidative burst

Introduction

Professional phagocytes, e.g. neutrophils, dendritic cells, and macrophages function within the innate immune system by recognizing, internalizing, and destroying invading microorganisms inside their phagosomes. Once formed, the intracellular phagosome vacuole undergoes a series of fission and fusion events, modifying both membrane and lumen contents, a process termed maturation (1). During maturation the phagosome vacuole develops into a cytotoxic organelle by acidifying, activating or acquiring hydrolytic enzymes, and by producing toxic radical compounds (2-4). Additionally, phagosome maturation is required to mount a successful adaptive immune response by destroying microorganisms and presenting antigens to lymphoid cells (5,6).

Phagocytosis prompts rapid assembly of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the phagosomal membrane. Following assembly, the NADPH oxidase reduces molecular oxygen through a one-electron transfer producing superoxide (O_2^-) within the phagosome lumen (7). Presumably, phagosomal O_2^- undergoes conversion via radical coupling to more strongly oxidizing secondary radicals such as peroxynitrite which is also considered an important phagosomal cytotoxin (8). Humans lacking component(s) of the NADPH oxidase suffer from chronic granulomatous disease, an inherited disorder characterized by recurrent pyogenic infections (9). Conversely, deregulated or inappropriate NADPH oxidase activation is thought to be involved in certain types of inflammatory tissue injury (10). Therefore, the NADPH oxidase is highly regulated to prevent inadvertent generation of damaging oxidants in surrounding tissues and to control microbial infection.

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The assembled NADPH oxidase complex is comprised of the integral membrane flavocytochrome b₅₅₈ (gp91^{phox} and p22^{phox}), and the soluble subunits p47^{phox}, p67^{phox}, and p40^{phox} (7). In unstimulated phagocytes, individual components of the NADPH oxidase system are held inactive by isolation in separate membrane and/or cytosolic locations. Upon stimulation, the soluble p47^{phox}, p67^{phox}, and p40^{phox} subunits assemble on the flavocytochrome b₅₅₈ while the GTPase Rac2 is recruited, allowing full activation of the NADPH oxidase (11). Presumably, assembly of the NADPH oxidase complex regulates the onset of the oxidative burst to coordinate maximal O₂⁻ production into a fully formed phagosome compartment. NADPH oxidase activity is transient and is maintained by continuous turnover of oxidase components (12). Thus, termination of a phagosomal oxidative burst is dependent on both the rate of assembly and the rate of disassembly, and is subject to overlapping regulatory mechanisms.

Despite the importance of oxidative phagosomal cytotoxic processes, assays designed to quantify the oxidizing potential found within the macrophage phagosome remain poorly developed. In this study, we describe two assays that specifically examine macrophage NADPH oxidase activation kinetics in real-time. These assays exploit ratio fluorometry to monitor oxidation of bead-associated substrates within the lumen of macrophage phagosomes. With these assays we have characterized phagosomal NADPH oxidase activation in live macrophages and have observed oxidase termination within 30 min following phagocytosis and clear enhancement of NADPH oxidase activity following lipopolysaccharide (LPS) and/or interferon- γ (IFN- γ) stimulation.

Results

Activation of the NADPH oxidase has been extensively studied in phagocytic cells following stimulation with soluble agonists such as fMet-Leu-Phe or phorbol 12-myristate-13-acetate (13-15). To study macrophage phagosomal NADPH oxidase activation in live cells we developed two assays using physiologically relevant model particles. These assays expand on our recent work developing fluorometric techniques to study phagosomal maturation in macrophages by quantifying functionally important processes of the phagosome lumen (3,16). To monitor phagosomal NADPH oxidase activation we employed BSA pre-labeled with dihydro-2',4,5,6,7,7'-hexafluoro fluorescein (H2HFF-OxyBURST). Oxidation of H2HFF-OxyBURST produces a fluorescein-based product which emits an intense signal at 520 nm when excited at 480 nm. Formation of the fluorescent H2HFF-OxyBURST product principally occurs in the presence of H₂O₂ and a catalyst such as peroxidase enzymes and/or heme containing proteins (17,18). To obtain phagosomal oxidation measurements carboxylated silica beads were covalently coupled via cyanamide crosslinker to BSA pre-labeled with H2HFF-OxyBURST and IgG. Lastly, these H2HFF-OxyBURST beads were covalently modified with Alexa-594 succidiny ester as a calibration fluorochrome, allowing ratiometric normalization. Since the lumen of phagosomes acidify to a final pH of ~4.8 during maturation (3) we first tested the pH sensitivity of H2HFF-OxyBURST fluorescence. H2HFF-OxyBURST beads were pre-oxidized with 0.2 U/ml horseradish peroxidase and 10 mM H₂O₂ for 30 min before washing the beads twice in PBS. Oxidized H2HFF-OxyBURST bead fluorescence was measured in acidic and neutral buffers and reported as the ratio of H2HFF-OxyBURST to Alexa-594 signal (Figure 1). Pre-oxidized H2HFF-OxyBURST fluorescence was relatively insensitive in acidic environments, displaying a near linear signal across a range of physiologically relevant pH conditions as previously reported (19).

To acquire the kinetics of phagosomal NADPH oxidase activation in macrophages the H2HFF-OxyBURST beads were bound to cell monolayers pre-established on coverslips. Unbound beads were removed after 2 minutes by vertically dipping the coverslips in assay

buffer and then placing the coverslips vertically in a cuvette containing reaction buffer at 37 °C loaded into the spectrofluorometer. Fluorescence emissions at 520 nm (H2HFF-Oxyburst signal) and 620 nm (Alexa-594 signal) were measured every 2 seconds by alternating excitation wavelengths between 480 and 594 nm, respectively. Initially, we used the IgG H2HFF-OxyBURST particles to monitor phagosomal oxidative burst from wild type cells and cells lacking gp91^{phox}. We also tested the effect of LPS pre-activation on phagosomal oxidation of H2HFF-OxyBURST particles. In these assays the calibration emission (Alexa-594) was constant throughout the assay, allowing us to normalize the readout. The kinetics of substrate oxidation was calculated as the ratio of the two fluorescent intensities, H2HFF-OxyBURST and Alexa Fluor 594, as a function of time. The kinetic data obtained from these analyses display a rapid and continued oxidation of H2HFF-OxyBURST within resting macrophage phagosomes, reaching a maximum within 25-30 min (Figure 2A). Termination of the phagosomal substrate oxidation was abrupt, concluding within approximately 30 min following phagocytosis, reflected by the plateau of H2HFF-OxyBURST signal. Pre-activating macrophages with LPS increased both the rate and the extent of phagosomal H2HFF-OxyBURST oxidation, but had little effect on its duration. To determine the specificity of the assay we performed this assay in macrophages lacking the gp91^{phox} subunit of the NADPH oxidase. As expected, gp91^{phox} deficient cells were unable to oxidize the phagosomal H2HFF-OxyBURST substrate and LPS pre-activating these cells had no effect on phagosomal H2HFF-OxyBURST oxidation (Figure 2A). To confirm that the plateau of H2HFF-OxyBURST fluorescence signal was not due to substrate limitation, beads were coated with different concentrations of H2HFF-OxyBURST and assayed in LPS activated macrophages. The maximal fluorescence signals obtained from beads coated in either 1.0 mg or 0.5 mg H2HFF-OxyBURST were comparable, indicating that the standard 1.0 mg H2HFF-OxyBURST bead coating is in excess (Figure 2B). Therefore, substrate limits can not account for the plateau of H2HFF-OxyBURST fluorescence observed in activated macrophage phagosomes.

Modulation of phagosomal cytotoxic activity is an important property inherent to macrophages and is subject to immuno-regulation (20). It is well established that LPS and IFN- γ stimulated macrophages will display enhanced intracellular killing of numerous pathogens (21,22). To determine if activation alters phagosomal H2HFF-OxyBURST oxidation, macrophages were exposed to IFN- γ (100 U/ml) and/or LPS (10ng/ml) prior to phagocytosis. Pre-activating macrophages with LPS and/or IFN- γ resulted in a dramatic increase in the rate and the extent of phagosomal H2HFF-OxyBURST oxidation compared to resting cells (Figure 3). Interestingly, the enhanced oxidation H2HFF-OxyBURST within the phagosomes of activated cells was roughly equivalent in all states of activation. Likewise, irrespective of activation status phagosomal H2HFF-OxyBURST oxidation displayed an abrupt termination 25-30 min following phagocytosis. Collectively, these data indicate that phagosomal NADPH oxidase activity terminates ~30 min following phagocytosis, this assay is specific to NADPH oxidase derived metabolites, and pre-activation enhances phagosomal oxidation of H2HFF-OxyBURST.

Next, we verified our H2HFF-OxyBURST assay observations with the oxidation sensitive fluorochrome 4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY^{581/591}). C11-BODIPY^{581/591} is a derivatized 11 carbon fatty acid in which the boron dipyrromethene difluoride (BODIPY) core is substituted with a phenyl group via a conjugated diene. Phenyl conjugation shifts the BODIPY emission spectra to a maximum at 595 nm. This conjugated diene interconnection is oxidation-sensitive and when oxidized, frees the phenyl group shifting the C11-BODIPY^{581/591} emission maxima from 595 nm (red) to 520 nm (green) (23,24). To probe macrophage phagosomes, the C11-BODIPY^{581/591} substrate was incorporated into mixed lipid monolayer-IgG particles. These particles are 3 μ m in diameter, silica-C18 modified reverse

phase HPLC matrix coated with a mixed lipid monolayer, as described previously (3). The lipid mixture on these particles also includes biotinylated phosphatidylethanolamine, allowing opsonization with biotin specific IgG antibody.

Using these lipid coated beads we compared the phagosomal C11-BODIPY^{581/591} lipid oxidation in resting and LPS pre-activated cells. To measure oxidation, the IgG opsonized C11-BODIPY^{581/591} beads were bound to macrophage monolayers established on glass bottom petri dishes in assay buffer and were analyzed with a Leica SP5 inverted confocal microscope for live imaging. The bead-associated phagosomal C11-BODIPY^{581/591} green signal increased upon oxidation when excited at 458 nm, while the red signal decreased throughout the assay when excited at 561 nm. This coordinated shift from red to green emission signal allowed us to standardize the readout and accurately control for substrate loading. To quantify bead-associated C11-BODIPY^{581/591} fluorescence, individual phagosomes containing single beads were selected and dual channel emission signals (595 nm, 520 nm) from these selections were obtained. The kinetics of C11-BODIPY^{581/591} oxidation were calculated as the ratio of the two fluorescent emission intensities, 520nm and 595 nm, as a function of time. The kinetic data obtained from these analyses display a rapid oxidation of C11-BODIPY^{581/591} substrate within the phagosome (Figure 4). As observed with the H2HFF-OxyBURST assay, termination of the phagosomal C11-BODIPY^{581/591} oxidation is abrupt, concluding approximately 25-30 min following phagocytosis. Moreover, pre-activating the cells with LPS enhanced the rate and extent of phagosomal C11-BODIPY^{581/591} oxidation which increased to a maximal activity within 15-20 min, before the assay was substrate limited. Collectively, the phagosomal oxidation of the C11-BODIPY^{581/591} and H2HFF-OxyBURST fluorochromes indicate that the oxidative burst is short-lived and is subject to enhancement by immunologically relevant agonists.

Discussion

We developed two assays to examine the kinetics of NADPH oxidase activation within macrophage phagosomes. These assays make use of normalized bead-associated substrate fluorescence, providing improved sensitivity that enables oxidation events to be quantified at either the population or the individual phagosome level. The first method relies on oxidative conversion of bead-associated H2HFF-OxyBURST to a fluorescent product within macrophage phagosomes. H2HFF-OxyBURST and the closely related compound DCFH₂ are the most commonly used fluorescent probes used for the evaluation of oxidant production in live cells (25). The kinetics of phagosomal H2HFF-OxyBURST oxidation reveals rapid and continued oxidation before an end-point equilibrium is reached when the fluorochrome is no longer oxidized. Pre-activating macrophages with LPS and/or IFN- γ enhanced both the rate and the extent of phagosomal H2HFF-OxyBURST oxidation while the timing of the end-point equilibrium remained largely unchanged. This end-point equilibrium occurs 25-30 min following phagocytosis and likely reflects termination of the phagosomal NADPH oxidase. These phagosomal H2HFF-OxyBURST oxidation kinetics are in agreement with recent reports that monitored oxidation of DCFH₂ in resting macrophage phagosomes (26,27). The second assay measures phagosomal lipid oxidation with the C11-BODIPY^{581/591} fluorochrome incorporated onto lipid-coated particles. The kinetics of phagosomal C11-BODIPY^{581/591} oxidation reveals rapid oxidation before reaching end-point equilibrium where the fluorochrome is no longer oxidized in resting macrophage phagosomes. Pre-activating macrophages with LPS enhanced both the rate and extent of phagosomal C11-BODIPY^{581/591} oxidation before substrate limitation terminates the assay.

The actual phagosomal oxidant(s) reacting with the H2HFF-OxyBURST or C11-BODIPY^{581/591} reagent is unknown. H2HFF-OxyBURST and C11-BODIPY^{581/591} are non-

discriminant probes capable of reacting with a variety of oxidants in the presence of catalyst. *In vitro* oxidation of H2HFF-OxyBURST occurs in the presence of H₂O₂ and a catalyst (peroxidases, heme containing proteins, or Fe²⁺). Similarly, C11-BODIPY^{581/591} is efficiently oxidized in the presence of hemin and organic hydroperoxide *in vitro*. Based on these *in vitro* properties, the substrates in our assays probably measure secondary radicals (not O₂⁻ directly) and iron-redox chemistries and/or peroxidase catalysts likely exist in the macrophage phagosome. As expected, macrophages lacking the gp91^{phox} subunit of the NADPH oxidase were unable to phagosomally oxidize H2HFF-OxyBURST. The gp91^{phox} subunit has a clear role in O₂⁻ production and perhaps the gp91^{phox} bound heme may also function as the catalyst in these phagosomal assays. Regardless of the oxidant being measured our assays require a functional phagosomal NADPH oxidase for oxidation of H2HFF-OxyBURST.

It is well established that the phagocyte oxidative burst can be primed by pre-exposure to certain agents that induce either a weak or no oxidative burst response themselves, but strongly enhance the oxidative burst upon exposure to a secondary stimulus. LPS and IFN- γ among others are well known agonists capable of priming the macrophage oxidative burst. With our assays we have found that the macrophage phagosomal oxidative burst is subject to priming with LPS and/or IFN- γ as indicated by the enhanced phagosomal substrate oxidation in pre-activated macrophages. These observations are consistent with previous studies that quantified extracellular O₂⁻ release from IFN- γ primed macrophages following secondary exposure to PMA (28). Although the molecular basis of NADPH oxidase priming in macrophages is not completely understood the process likely involves increased expression of the gp91^{phox}, gp22^{phox}, p47^{phox}, p67^{phox} (29-31) as well as alterations in tyrosine phosphorylation and protein kinase C signal transduction pathways (32-34). The assays detailed here provide a quantitative readout of primed phagosomal oxidative burst and make use of relevant phagocytosed particles.

Phagosome maturation is directly regulated by membrane phosphoinositide content which is continually modified by phosphoinositide kinases and phosphatases (35). The p40^{phox} and p47^{phox} subunits synchronize NADPH oxidase activity to phagosome maturation via their PX domains which bind to the membrane phosphoinositides PI(3)P, PI(3,4)P₂, PI(3,4,5)P₃ (36). Specifically, p40^{phox} dependent activation of the NADPH oxidase during Fc receptor-mediated phagocytosis requires PI(3)P on phagosome membranes (37,38). During maturation PI(3)P transiently resides on phagosomes, appearing approximately 2 minutes following phagocytosis and then disappears within 10 min following phagocytosis (39). A recent study confirmed that indeed maximal phagosomal PI(3)P levels coincided with the termination of phagosomal OxyBURST oxidation (26).

Lastly, it has been proposed for neutrophils and dendritic cells, the NADPH oxidase produces sufficient quantities of reactive oxygen species to consume protons in the phagosome and restricts phagosomal acidification and thus, protease and other hydrolase activity (40,41). These assays along with our previously developed techniques to quantify phagosomal pH and hydrolase activities (3) will now allow comprehensive analysis of the cytotoxic and degradative environment found in the phagosomes of phagocytic cells.

Materials and methods

Cells and materials

Macrophages were harvested from the bone marrow of C57BL/6 and or GP91^{phox}^{-/-} mice (Jackson Laboratories, Bar Harbor, ME, USA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate and 20% L-cell conditioned media maintained at 37°C and 7.0% CO₂ (42). For

spectrofluorometric studies, fully differentiated macrophages were transferred to petri dishes containing sterile 12.5×25 mm glass cover slips and left for 18 hr to establish a confluent monolayer. For microscopy studies cells were transferred to glass bottom petri dishes for 15 h to establish a monolayer. High purity IFN- γ (Preprotech, Rocky Hill, NJ, USA), horseradish peroxidase (Sigma), concentrated H_2O_2 (Sigma), and LPS (List Biological Laboratories, Campbell, CA, USA) were used.

Preparation of H2HFF-OxyBURST beads

Carboxylated, 3.0 μ m silica beads (Kisker Biotech, Steinfurt, Germany) were washed three times in 1 ml PBS by vortexing and centrifugation in a table top microfuge at $2000 \times g$ for 1 min. Beads were resuspended in PBS with 25 mg/ml cyanamide and agitated for 15 min. Excess cyanamide crosslinker was removed by washing the beads three times in 1 ml coupling buffer (0.1 M sodium borate pH 8.0). Beads were resuspended in 500 μ l of coupling buffer with 1.0 mg H2HFF-OxyBURST-BSA (Molecular Probes, Eugene, OR, USA) and 0.1 mg human IgG (Sigma) for 12 hours as described under argon (42). The coated beads were washed three times in quench buffer (PBS pH 7.2, 250 mM glycine) to quench unreacted cyanamide. The beads were resuspended in 1 ml coupling buffer with 10 μ l (5 mg/ml) Alexa Fluor 594 succinimidyl ester (Molecular Probes) in DMSO and agitated for 1 hour under argon. The beads were washed three times in 1 ml quench buffer and three times in 1 ml PBS.

Phagosomal H2HFF-OxyBURST bead assays

Beads coated with IgG, H2HFF-OxyBURST, and Alexa Fluor 594-SE were washed three times in PBS and resuspended in assay buffer (PBS pH 7.2, 1 mM CaCl₂, 2.7 mM KCl, 0.5 mM MgCl₂, 5 mM Dextrose and 5% FCS). Washed beads in assay buffer were bound to macrophage monolayers on cover slips at an MOI of 5 beads per macrophage, synchronized phagocytosis of labeled beads was accomplished by incubation of the cell monolayers with bead suspension in assay buffer at room temperature for 1 min. Following binding, the cover slips were washed, removing unbound beads, and placed into a cuvette containing 3 ml cuvette buffer (PBS pH 7.2, 1 mM CaCl₂, 2.7 mM KCl, 0.5 mM MgCl₂, 5 mM Dextrose) at 37 °C in a thermostat-regulated QMSE4 spectrofluorometer (Photon Technologies International, Lawrenceville, NJ, USA). Fluorescent emission was acquired from approximately 20 mm² of the macrophage monolayer or approximately 3.5×10^4 cells. Following fluorescence measurements, each cover slip was examined under a standard light microscope to ensure macrophage viability and internalization of the beads. Oxidation of the substrate yielded an increase in the H2HFF-OxyBURST fluorescence, whereas the calibration Alexa Fluor 594 fluorescence remained unchanged. The degree of substrate oxidation was determined by the ratio of the two fluorescent intensities.

Preparation and handling of Bodipy^{581/591} coated beads

3 μ m silica Nucleosil C18 reverse phase HPLC beads (Macherey-Nagel, Easton, PA, USA) were coated with a mixture of amphipathic lipids containing Bodipy^{581/591} substrate as described (43). A solution of 9.0 mg/mL of C18 particles, 2.5 mg/mL of 1,2-Dipalmitoyl-sn-Glycero-3-[Phospho-rac-(1-glycerol)] (Avanti Polar Lipids, Alabaster, AL, USA), 250 μ g/mL of 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(Cap Biotinyl) (Avanti Polar Lipids), 450 μ g/mL cholesterol, and 5 μ g/mL Bodipy^{581/591} C11 (Molecular Probes) in chloroform was evaporated under nitrogen. The lipid and bead mixture was resuspended in PBS during sonication at 40 °C. The lipid coated particles were placed on ice and washed with ice-cold PBS before incubation with mouse monoclonal anti-biotin IgG (Sigma) in PBS for 60 min. Following washing in ice-cold PBS, the IgG-opsonized lipid coated particles were resuspended in assay buffer.

Phagosomal IgG-Bodipy^{581/591} coated bead assays

For live imaging, cell monolayers were established on glass bottom petri dishes (MatTek, Ashland, MA, USA) 18 hr before use. Images were acquired with a Leica SP5 confocal laser-scanning system with an inverted microscope (Leica Microsystems GmbH, Heidelberg, Germany). For analysis the dish was maintained at 37°C with a stage heating system and imaging was performed with an HCX PL APO CS 63.0×1.20 water UV objective at zoom factor of 1.2. Green and red fluorescence signals sequentially acquired using the 458 nm and 561 nm excitation laserlines, and emission was detected in the ranges 500–530 nm and 585–620 nm, respectively. Images were acquired with a 1.0 Airy unit pinhole and scanning speed of 400 Hz, 512-by-512 optical slices at 1.0-μm Z-axis intervals were collected at each time point, providing a 30 second time interval per frame. Post acquisition, a single 3D maximum projection was performed for each time point. Oxidation of the C11-BODIPY^{581/591} substrate yielded a simultaneous decrease in the 595 nm emission, whereas the 520 nm emission increased. The degree of substrate oxidation was determined by the ratio of the two fluorescent intensities.

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Figure 1. H2HFF-OxyBURST fluorescence is pH insensitive

Fluorescence ratio of pre-oxidized H2HFF-OxyBURST beads were acquired in buffers of various pH and plotted as calibrated fluorescence using the equation $CF = OB/AF$, where CF is calibrated fluorescence, OB is H2HFF-OxyBURST fluorescence, and AF is Alexa594 fluorescence. The trace represents the averaged endpoint values over three repetitions, error bars represent SD.

Figure 2. Phagosomal H2HFF-OxyBURST oxidation profiles

H2HFF-OxyBURST oxidation kinetics were plotted as calibrated fluorescence using the equation $CF = OB/AF$. Measurements were taken every 2 seconds for 60 minutes. Activation was accomplished by LPS (10ng/ml) addition 15 hr prior to experimentation; the trace represents the averaged values over three repetitions, error bars represent SD. **B.** Beads were coated in 1.0 mg or 0.5 mg H2HFF-OxyBURST and evaluated in LPS activated macrophage phagosomes. Maximal oxidation is plotted as calibrated fluorescence using the equation $CF = OB/AF$. Activation was accomplished by pretreatment with LPS (10 ng/ml) 15 hr prior to phagocytosis. The trace represents the averaged values over two repetitions, error bars represent SD.

Figure 3. Activation enhances phagosomal H2HFF-OxyBURST oxidation

A. H2HFF-OxyBURST oxidation kinetics were plotted as calibrated fluorescence using the equation $CF = OB/AF$. Activation was accomplished by pretreatment with LPS (10ng/ml) or IFN- γ (100 U/ml) 15 hr prior to phagocytosis. For synergistic activation cells were treated with IFN- γ (100 U/ml) for 13 hr and then LPS (10ng/ml) 2 hr prior to phagocytosis. The trace represents the averaged values over three repetitions, error bars represent SD.

Figure 4. Phagosomal Bodipy^{581/591} bead oxidation profiles

A. Bodipy^{581/591} bead oxidation kinetics was plotted with the equation $CF = GF/RF$, where CF is calibrated fluorescence, GF is the green Bodipy^{581/591} signal, and RF is the red Bodipy^{581/591} signal. Measurements were taken every 20 seconds for 60 minutes. Activation was accomplished by treatment with LPS (10ng/ml) 15 hr prior to phagocytosis. The kinetic trace is from a representative experiment and is the average of 10 phagosomal bead values from each condition, error bars represent SD. **B.** Serial confocal images of phagosomal Bodipy^{581/591} beads in resting macrophages. Scale bar; 5 μ m.