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Bacterial Phagosome Acidification Within IFN-γ-Activated Macrophages: Role of Host p47 Immunity-Related GTPases (IRGs)

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Summary

Interferon-gamma (IFN-γ)–induced remodeling of the bacterial phagosome for pathogen clearance elicits the aid of a new family of GTPases termed the p47 IRGs. Members of this group reside primarily on ER-Golgi membranes before translocating to the nascent phagosome within minutes of bacterial uptake. Recruitment of p47 IRGs coincides with the acquisition of phagosome maturation and autophagy markers as well as enhanced acidification of this organelle. Here we describe a simple spectrofluorometric assay to measure luminal acidification of the bacterial phagosome within intact cells such as macrophages. This method can be applied to study the phagosomal pH (pH_{pg}) of activated cells infected with a variety of infectious microorganisms and the roles played by members of the p47 IRG family in (auto)phagolysosome biogenesis.

Key Words: Bacterial phagosome; macrophage; IFN-γ; p47 immunity-related GTPase; phagosome acidification.

1. Introduction

Interferon-gamma (IFN-γ) remains one of the most powerful natural stimuli for activating mammalian macrophages to inhibit a variety of intracellular infections ***(1)***. Its equimolar potency often greatly exceeds that of other macrophage-activating cytokines such as tumor necrosis factor (TNF-α) or type I interferons (e.g., IFN-α, IFN-β), in some cases by as much as 100,000-fold, depending on the host defense parameter being measured ***(2)***. This heightened ability of IFN-γ to endow mononuclear phagocytes with the capacity to kill ingested

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microorganisms stems largely from the complex transcriptional programs it elicits within these cells ***(3)***. Here, over 1000 genes may be engaged. Within this group are mRNA transcripts that encode host proteins with long-recognized antimicrobial activity, notably inducible nitric oxide synthase (iNOS/NOS2), phagocyte oxidase, and natural resistance associated macrophage protein-1 (NRAMP1) ***(4–6)***.

In addition to these more established pathways, a new family of IFN-γ-induced or regulated GTPases (termed immunity-related p47 GTPases or IRGs) has recently emerged that participates in host control of both intracellular bacteria and protozoa (reviewed in **refs. *7–10***). Much of their antimicrobial activity appears directed towards the pathogen's replicative niche—the phagosome—as first shown for LRG-47/Irgm1 against virulent *Mycobacterium tuberculosis* (*Mtb*), the causative agent of human TB ***(11)***. Subsequent studies have reinforced the importance of IRGs for early remodeling or disruption of the pathogen-containing vacuole prior to fusion with several target vesicles ***(12–15)***. Chief among these are secondary lysosomes and LC3- or monodansylcadavarine (MDC)-positive autophagolysosomes ***(11–15)***, both of which accumulate acidotropic dyes such as LysoTracker Red ***(11,13,15)***.

While the use of such dyes can serve as a surrogate marker for bacterial phagosome acidification, total fluorescence output is often low, making changes in luminal pH difficult to quantify ***(16)***. Moreover, protonation of a weak base to which the LysoTracker fluor is normally coupled can lead to membrane retention within endolysosomal organelles besides the phagosome ***(17)***. For this reason, direct covalent attachment of amine-reactive fluorophores to the bacterial cell envelope circumvents the problem of signals arising from nonphagosomal compartments. They also possess other advantages: photostability, sensitivity, and high quantum yield, enabling small changes in pH_{pg} to be detected for long periods ***(17)***. Carboxyfluorescein (CF; ε^1= 72,000/cm/M) serves as a prototype for following alterations in pH_{pg} since its emission intensity, like that of fluorescein itself, varies with pH ***(17)***. Extinction coefficients and fluorescence yields for CF are markedly reduced, for example, at pH <7.0. Its apparent pK_a ~6.1 also makes it well suited to monitoring the endolysosomal and autophagic pathways.

When CF is used together with a long-wavelength pH-insensitive fluorophore such as carboxytetramethylrhodamine (TAMRA; $\varepsilon^1 = 92{,}000\ cm^{-1}M^{-1}$), differences in probe concentration between samples are negated due to internal normalization at pH 4–10 ***(17)***. This approach, termed ratiometric fluorometry (R_F), relies on the invariant reference signal arising from TAMRA to be compared with that of the pH-sensitive spectrum of CF. CF spectra exhibits both pH-sensitive and -insensitive regions, but the latter emission is weak and CF lacks a consistent isobestic wavelength ***(16)***. The addition of a spectrally

distinct reference such as TAMRA thus helps separate pH-sensitive versus pH-insensitive fluorescence and, when appropriately excited by the 540-nm spectal discharge from a Xenon lamp (~1 J/flash) found in many plate analyzers, gives fluorescent quantum yields approaching that for CF ***(17)***.

Application of R_F to the study of pH_{pg} offers a simple and sensitive population-based assay for phagosome acidification within wild-type or genetically modified IFN-γ–activated macrophages ***(11,18)***. It also enables rapid screening of the p47 IRGs and other GTPases likely to be involved in this process. An important limitation of microplate R_F, however, is that information regarding the fate of individual phagosomes is not available. For assays that provide such information, the reader is directed to microscopy-assisted methods described elsewhere in this volume.

2. Materials

2.1. Primary Macrophage Isolation and Cell Culture

1. Dulbecco's modified Eagle's medium (DMEM) (Gibco/RL, Bethesda, MD)supplemented with 5% heat-inactivated goat serum, sodium pyruvate (1 m*M*), 2 m*M* L-glutamine, 100 U/mL penicillin G + 100 μg/mL streptomycin (all from Gibco/BRL), heat-inactivated 10% fetal bovine serum (FBS, Hyclone, Ogden, UT) and 20% L929 cell (ATCC, Manassas, VA) conditioned media.
2. Sodium chloride (0.2% v/v in distilled H_2O) for red blood cell (RBC) lysis.
3. Teflon cell scraper and sterile 100 mm^2 non–tissue culture agar plates (Fisherbrand, Atlanta, GA).
4. Scissors (curved and long-nosed), forceps (blunt and fine), 5-mL syringe with $23^{3/4}$-gauge needle and 70% ethanol.
5. Ca^{2+}/Mg^{2+}-free phosphate-buffered saline and ethylenediamine tetraacetic acid (EDTA) (0.5 m*M*) from GIBCO/BRL.
6. Endotoxin-free recombinant mouse IFN-γ (rMuIFN-γ) from R & D Systems (Minneapolis, MN).

2.2. Ratiometric Spectrofluorometry

1. Potassium isotonic medium: 140 m*M* KCl [10.36g/L], 15 m*M* hydroxyethyl piperazine sulfonate (HEPES; Gibco/BRL), 5 m*M* glucose [900 mg/L]. Prepare 50-mL aliquots over the following pH range for generating a standard curve: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0. Store at 4°C.
2. Nigericin (98% TLC; Sigma, St. Louis, MO [724.5g/mol; final concentration 5 μ*M*]) resuspended as 5 μ*M* aliquots in methanol/phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+}. Store dessicated at –4°C (stable for up to 3 yr).
3. Bafilomycin A_1 (Sigma) (622g/mol; final concentration 200 μ*M*) resuspend 2 μg/1.6 mL (20 μ l DMSO/PBS without Ca^{2+}/Mg^{2+}) to generate 2m*M* aliquots. Store at –20°C.

4. 5-Methoxy-2-[(4-methoxy-3,5-dimethyl-2-pyridinyl)methyl]sulfinyl]-1- benzimidazole (omeprazole). Resuspend in a small volume (50 μL) of ethanol before diluting in K_2PO_4 buffer, pH 9.0 at 10 mg/mL (final concentration 0.2 mg/mL). Store aliquots in a vacuum-purged bell jar with Drierite crystals at –20°C.
5. 5-(and -6)-Carboxyfluorescein, *N*-hydroxysuccinimidyl ester (5(6)FAM, SE; 473.4 g/mol; mixed isomers) (Molecular Probes, Invitrogen). Resuspend in a small volume (50 μL) of anhydrous DMSO before diluting in K_2HPO_4 buffer, pH 9.0 at 10 mg/mL (final concentration 0.2 mg/mL). Store aliquots in a vacuum-purged bell jar with Drierite crystals at –20°C.
6. 5-(and -6)-Carboxytetramethylrhodamine, *N*-hydroxysuccinimidyl ester(5(6)-TAMRA, SE; 527.5g/mol; mixed isomers) (Molecular Probes). Resuspend in a small volume (50 μL) of anhydrous DMSO before diluting in K_2HPO_4 buffer, pH 9.0 at 10 mg/mL (final concentration 0.2 mg/mL). Store aliquots in a vacuum-purged bell jar with Drierite crystals at –20°C.

3. Methods

Dual spectrofluorometry is highly sensitive yet dependent on establishing clear extant standards for derivation of pH_{pg}. It also requires an ionophore (nigericin; $K^+ > Rb^+ \geq Na^+ \ll Li^+$) and conductance pump inhibitors (bafilomycin A_1, omeprezole) to validate the source of fluorescent signal ***(11,16,19,20)***. Employment of a $V_{1,0}$-ATPase inhibitor such as bafilomycin A is critical since active H^+ translocation across the phagosomal membrane rather than passive H^+ leak or counterion conductance accounts for most of the luminal acidification seen in mammalian macrophages ***(20,21)***.

Choice of fluorophores for R_F is very much dependent on instrumentation and filters available. CF and TAMRA as used here are a well-established combination ***(16)*** that fits most common excitation/emission filters found on plate microfluorometers. Other pH-sensitive dyes such as cyanine CypHer5E™ (pK_a ~7.3) have recently been employed for high-throughput phagocytosis assays but require filters in the far-red range (Ex_{650}/Em_{670}) ***(22)***. Similarly, fluorescent resonance energy transfer (FRET)–based phagolysosomal fusion often enlists acceptor probes such as Alexa Fluor 594 hydrazide that excite and emit at more distant wavelengths (Ex_{594}/Em_{620}) ***(23)***. FRET at these wavelengths is highly sensitive and can be used for near-simultaneous recording from both acceptor and donor fluor PMTs. A limitation, however, is that real-time measurements are generally better suited to monitoring individual cover slips rather than large sample analysis ***(23)***. This is because maximal scan speeds for most current fluorometers or multimode analyzers typically range from ~20 to 60 s across 96- or 384-well plates, respectively ***(24)***. Such kinetics gives rise to an inherent delay in data-collection times, which make "continuous"

recording (e.g., at 4- to 6-s intervals) difficult when many samples are involved.

A simplified assay that collects data points intermittently as described below thus allows measurement of bulk samples. Spectrofluorometric instruments with a dynamic spectral range (e.g., 10^{-6} to 10^{-14} mol fluorescein) and isothermal microplate chamber for recording at incubating temperatures between 4° and 45°C are preferred ***(24)***. Dual monochromators allowing 1-nm increment scanning and narrow bandwidths (e.g., 9 nm) also lessens nonspecific fluorescence overlap while top and bottom read capabilities enable adherent cell assays to be undertaken ***(24)***. Murine macrophages lacking p47 GTPases at the chromosomal level ***(11,14)*** or human and mouse cells treated with IRG siRNAs ***(15)*** can be tested via this assay to examine their direct impact on bacterial phagosome trafficking and acidification.

3.1. Isolation and Culture of Primary Bone Marrow Macrophages

1. Affix euthanized mice to a dissection board covered with sterile laboratory diapers. Spray with 70% ethanol and remove fur from the heel (plantar flexi) to inguinal region as well as above the ilium at the hip using sterile scissors. Try to remove as much of the gastrocnemii muscle on the lower leg and quadriceps muscle on the upper leg as possible. Cut below the tibia at the ankle and above the femur near the acetabulum before wiping down the bone with sterile ethanol pads to remove residual muscle and tendon. Place the unsplintered bones into a sterile Petri dish and spray with 70% ethanol. Repeat with contra-lateral leg.
2. Shift lower limb bones to sterile biosafety hood. Pipet 3–4 mL of cold RPMI into 2 Petri dishes. Cut bone at the knee joint to separate tibia from femur. Cut off ends of bone containing the epiphyseal regions and transfer these smaller fragments into one of the two RPMI-containing Petri dishes. Place the shaft of the long bones (tibia, femur) into the other Petri dish.
3. Mince the tibia/femur heads and flush with RPMI until the bone marrow cells are dispensed into the media. Do the same with the long bones by placing the $23^{3/4}$-gauge needle into the central trabeculae until the marrow is flushed out into the RPMI. Using the tip of the needle, disperse this marrow into smaller portions, and pipet up and down 8–10 times to dispense clumps and create a single cell suspension. This is important for optimum yield.
4. Filter cell suspension through a Falcon 2350 strainer (70 μm) atop a sterile 50-mL conical tube to remove large debris. Spin at 1200*g* for 7 min. Aspirate supernatant and resuspend in 0.2% NaCl for 30 s to remove RBCs (*see* **Note 1**). Add 25 mL RPMI and spin at 1200*g* for 7 min. Resuspend in a small 5- to 10-mL volume of RPMI.
5. Count Trypan blue-negative cells using a hemocytometer and dilute to the appropriate concentration. At 4 x 10^6/mL, bone-marrow macrophages (BMMϕ) will be confluent at 5–6 d; refeed with fresh medium at day 3–4. At 2 x 10^6/mL, BMMϕ will be confluent at 6–8 d; refeed with fresh medium at day 4.

6. Add 1 mL of cells to 10 mL of prewarmed BMMϕ media in bacterial Petri dishes (not polystyrene tissue culture dishes since they will adhere too strongly) and incubate at 37°C, 5% CO_2. When refeeding just add 10 mL warm BMMϕ media to each dish. Do not remove any of the original media. These cells can normally be split two or three more times before they stop dividing.
7. Once cells are confluent, remove the original medium and replace with 5 mL cold PBS plus 20 m*M* EDTA. Place cells at 4°C for 15–20 min before gently scraping into the PBS solution. Centrifuge at 1200*g* for 7 min. Wash and resuspend in appropriate medium below for experimental use (*see* **Note 2**).

3.2. Ratiometric Spectrofluorometry

1. Fluorescent labeling of bacteria. Grow bacteria to mid-log phase (e.g., A_{600} = 0.5 corresponds to ~5 x 10^8 CFU). Wash three to four times with PBS without Ca^{2+}, Mg^{2+} before resuspending at 1–5 x 10^8 CFU/mL in PBS.
2. Label bacteria with equimolar (~150 pmol) amounts of 5(and 6)-carboxyfluorescein, succinimidyl ester (5(6)-FAM, SE), and 5(and 6)-carboxytetramethylrhodamine, succinimidyl ester (5(6)-TAMRA, SE). This requires adding 100 µL of each fluorophore (0.2 µg/mL) to 1 mL of bacterial suspension. Vortex for 5 s and incubate at 4°C for 30–60 min in the dark with tumbling.
3. Remove unreacted fluorophore by excessive washing (10–15 times in fivefold volumes of PBS) or via Sephadex G-75 columns using 2.5% sample volume/bed volume. Columns can be generated after overnight swelling of 12–15 mL/g of Sephadex G-75.
4. Following dye conjugation, check bacterial viability by plate CFU count (*see* **Note 3**).
5. Add fluorescently labeled bacteria at an MOI ~50:1 to 1 x 10^5BMMϕ/well in 96-well plates that are either untreated or pretreated for 24–48 h with 100 U/mL of rMuIFN-γ. Where V-ATPase inhibitors are being used as controls, these may be added 30–60 min prior to incubation with bacteria. Such inhibitors include Bafilomycin A_1 (200µM) or omeprazole (5µM). An uninfected blank is also required for background correction.
6. Allow bacteria to bind to cells for 60 min at 4°C. Remove nonadherent bacteria by extensive washing (five to six times) in PBS. This can be done with an automated discharge pipette and PBS removed by vacuum trap syringe or by flicking the inverted plate.
7. Add prewarmed complete media (DMEM plus 5% heat-inactivated goat serum, sodium pyruvate (1 m*M*), 2 m*M* L-glutamine, 100 U/mL penicillin G + 100 µg/mL streptomycin (all from Gibco/BRL), heat-inactivated 10% fetal bovine serum (FBS, Hyclone, Ogden, UT) and shift cells to 37°C to begin bacterial uptake.
8. At the indicated time points, wash cells with PBS and maintain in this solution for immediate recording of pH_{pg}. Depending on the bacteria used, time points may vary from 5 to 30 min or as long 6 h ***(11,23)***.

9. Record in situ pH_{pg} in triplicate using a spectrofluorometer (e.g., Molecular Probes SpectraMax Gemini EM) at $\lambda_{Ex485}/_{Em520}$ for (5,6)-FAM,SE and $\lambda_{Ex540}/_{Em590}$ for (5,6)-TAMRA,SE. Upon collection of the final time point, add nigericin (5 μ*M*) in K^+ isotonic media for 10 min to collapse the membrane pH gradient. Record fluorescence to confirm the fluorogenic signal originates from internalized bacteria (*see* **Note 4**).
10. Calculate pH_{pg} from FAM/TAMRA ratios using a calibration curve established in the same way as outlined in **steps 1–9** except that cells are fed dye-labeled bacteria in nigericin-containing K^+ isotonic media of defined pH (4.0–8.0 in 0.5 pH increments) to clamp intracellular with extracellular pH. Plot R_F versus pH for regression analysis and use this equation to ascertain pH_{pg} values.

4. Notes

1. Removal of RBCs is optional since after 6–8 d differentiation most erythrocytes have either lysed or been degraded by macrophages.
2. Primary mouse macrophages generated via this protocol are >95% $F4/80^+$ (Rat IgG_{2b}) ***(11)***.
3. Fluorescent bacteria can be stored for short periods at 4°C dependent on which pathogen is being used. In the case of slow growing mycobacteria such as *M. tuberculosis* ***(11)*** or *M. bovis BCG*, storage times can be ~3–4 wk if samples are kept in the dark.
4. After spectrofluorometry is finished, cells may be thoroughly washed in PBS and lysed for determination of total adherent cell protein via either Lowry or Bradford assays to confirm well-to-well variation.

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