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Dear Editors,

Please consider our revised manuscript, "Assaying *P.aeruginosa* based Phagocytosis in Mice" for publication in *JoVE.*

We appreciate the interest that the editors have taken in our manuscript. We have addressed the all concerns of the editors. We have also included a point-by-point response to the editor's comments in addition to making the changes in the manuscript.

Thank you again for consideration of our revised manuscript.

With regards,

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TITLE:

Alveolar Macrophage Phagocytosis and Bacteria Clearance in Mice

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KEYWORDS:

Alveolar macrophages; phagocytosis; in vitro; in vivo; bacterial clearance; pneumonia

SUMMARY:

Here we report common methods to analyze the phagocytic function of murine alveolar macrophages and bacterial clearance from the lung. These methods study in vitro phagocytosis of fluorescein isothiocyanate beads and in vivo phagocytosis of *Pseudomonas aeruginosa* Green Fluorescent Protein. We also describe a method for clearing *P. aeruginosa* in mice.

ABSTRACT:

Alveolar macrophages (AMs) guard the alveolar space of the lung. Phagocytosis by AMs plays a critical role in the defense against invading pathogens, the removal of dead cells or foreign particles, and in the resolution of inflammatory responses and tissue remodeling, processes that are mediated by various surface receptors of the AMs. Here, we report methods for the analysis of the phagocytic function of AMs using in vitro and in vivo assays and experimental strategies to differentiate between the pattern recognition receptor-, complement receptor-, and Fc gamma receptor-mediated phagocytosis. Finally, we discuss a method to establish and characterize a *P. aeruginosa* pneumonia model in mice to assess bacterial clearance in vivo. These assays represent the most common methods to evaluate AM functions and can also be used to study macrophage function and bacterial clearance in other organs.

INTRODUCTION:

AMs are the major resident phagocytes in the alveoli at the resting stage and one of the major players of innate immune responses through the recognition and internalization of inhaled pathogens and foreign particles[1,2]. It has been reported that AMs are essential for the rapid clearance of many pulmonary pathogens such as *P. aeruginosa* and *Klebsiella pneumonia*[3,4], so a deficiency in AM phagocytosis often results in respiratory infections, such as acute pneumonia, which cause higher mortality and morbidity rates.

AMs also initiate innate inflammatory responses in the lung by producing cytokines and chemokines such as TNF-α and IL-1β, which crosstalk with other cells of the alveolar environment to produce chemokines and recruit inflammatory neutrophils, monocytes, and adaptive immune cells in the lung[5]. For example, IL-1β produced by AMs helps to prime the release of the neutrophil chemokine CXCL8 from epithelial cells[6]. Moreover, AMs contribute to the phagocytosis of apoptotic polymorphonuclear leukocytes (PMNs), failure of which leads to the sustained leakage of intracellular enzymes from PMNs to the surrounding tissue, resulting in tissue damage and prolonged inflammation[7–9].

Phagocytosis by the AMs is mediated by a direct recognition of pathogen-associated molecular patterns at the pathogen surface by the pattern recognition receptors (PRRs) of the AMs or by the binding of opsonized pathogens with immune effector receptors of the AMs[10]. For the latter, AMs can recognize the targets opsonized with immunoglobulin (IgG) through their Fcγ receptors (FcγR) or the pathogens coated with complement fragments, C3b and C3bi, through their complement receptors (CR)[11]. Among complement receptors, the CR of the immunoglobulin superfamily (CRIg) is selectively expressed in tissue macrophages[12], and a recent finding highlighted the role of the CRIg in AM phagocytosis in the context of *P. aeruginosa* pneumonia[13].

Many original studies use methods to evaluate macrophage phagocytosis to describe the molecular mechanisms of macrophage function[14–15]. However, methods like in vivo phagocytosis require a precise quantification of phagocytosis. Here, we summarize a detailed methodology for both in vitro and in vivo phagocytosis using fluorescein isothiocyanate (FITC)-glass beads and *P. aeruginosa* green fluorescent protein (GFP), respectively. Further, we explain the method of differentiating among PRR-, CR-, and FcγR-mediated phagocytosis. Finally, we report a method to characterize bacterial clearance in mouse with respect to P. *aeruginosa* pneumonia.

PROTOCOL:

This protocol follows the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Eastern Virginia Medical School.

1. Fluorescent Beads Phagocytosis

1.1. Euthanize the mouse (C57BL/6J, 6 weeks old, female) by CO_2 asphyxiation as per IACUC protocols for the ethical euthanasia of animals.

1.2. Lay the mouse belly-up on a dissection board covered with paper towels. Pin its paws down with its limbs spread-eagle and hook a string under its front teeth to pull its head back so that the trachea is positioned straight and level.

1.3. Wet the mouse's throat, chest, and belly with 70% ethanol to disinfect and prevent the fur from sticking to the tools.

1.4. Using regular forceps, pull up the skin at the centerline of the body, and cut with surgical scissors up the centerline to the top of the throat.

1.5. Using the blunt end of standard surgical scissors, carefully move away the muscle and connective tissues on the throat and use spring scissors (microscissors) to expose the trachea.

1.6. Gripping a cartilage ring with the forceps, carefully make a small incision (~1.5 mm), using microscissors, on the ventricle face of the trachea and insert an 18 G needle into the trachea.

1.7. Gently lavage 3 mL of phosphate-buffered saline (PBS), 1 mL at a time. Each time gently withdraw the fluid into the syringe and reinfuse it back into the lung, 3x in succession. Transfer the collected PBS (~2.8 mL), which is bronchoalveolar lavage fluid (BALF), to a tube, centrifuge at 1,000 x *g* for 10 min, and collect the pellet. Add 1 mL of fresh PBS to the tube and centrifuge at 1,000 x *g* for 10 min to wash the debris and collect the pelleted alveolar macrophages.

1.8. Resuspend the pellet in 2 mL of Dulbecco's modified Eagle's medium (DMEM) with 10% nonheat-inactivated fetal bovine serum (FBS) and culture primary alveolar macrophages in the same media for 2 days on a glass-bottom dish at 37 °C in a humidified atmosphere.

1.9. Aspirate the old media, wash it with 1 mL of PBS, and add 2 mL of fresh media. Add FITC beads (carboxylated latex beads, 2 µm in diameter, 50 beads/cell) and incubate for 1 h at 37 °C in a humidified atmosphere.

1.10. Wash extensively with PBS (1 mL at a time, for a total of five washes) to remove extracellular beads. Image 100 cells randomly and count the cells with intracellular beads (488 nm).

NOTE: Phagocytic indexes are the number of ingested beads divided by the total number of macrophages; the percentage of phagocytic cells is the number of macrophages that ingest at least one bead divided by the total number of macrophages[16].

1.10.1. Alternatively, after a 1 h incubation with beads, wash the cells with 3 mL of PBS and process them for flow cytometry for the quantification of phagocytosis. Similarly, process AMs without beads as unstained cells or control cells. Calculate the percentage positivity and mean fluorescence intensity, using flow cytometry software, by selecting those options in the software.

2. FcγR- and CR-mediated Phagocytosis

2.1. For the opsonization, incubate 2 x 10^8 sheep red blood cells (SRBCs) with 50 µL of rabbit anti-SRBC-IgM or 50 µL of rabbit anti-SRBC-IgG for 30 min at room temperature[11].

2.2. Incubate IgM-opsonized SRBCs with 50 µL of C5-deficient (C5D) human serum for 30 min at 37 °C to fix the complement fragments C3b and C3bi on IgM-coated SRBCs.

2.3. Seed murine macrophage cells (MH-S cells) (10,000 cells/well) in a 96-well plate and incubate overnight to get a ~70% confluence. Add 100 µL of 1 x 10^7/mL opsonized SRBCs to each well of MH-S cells and incubate for 1 h at 37 °C. Wash unbound SRBCs very quickly (~1 min) with 100 µL of ammonium chloride-potassium (ACK) lysis buffer.

2.4. Lyse the cells with 0.1% SDS and add 50 µL of 2,7-diaminofluorene (DAF) containing 3% hydrogen peroxide and 6 M urea. Measure the absorbance of the hemoglobin-catalyzed fluorene blue formation at 620 nm.

2.5. Determine the number of SRBCs by using a standard curve at 620 nm absorbance values with a known number of SRBCs. Similarly, process MH-S cells incubated with nonopsonized SRBCs to use as negative controls.

3. PRR-mediated Phagocytosis

3.1. Follow steps 1.1–1.9 for the isolation and culturing of mouse primary alveolar macrophages.

3.2. After 2 days, remove the media, wash the cells with 1 mL of PBS, and add 500 µL of fresh media containing Alexa Fluor-488-conjugated zymosan-A bioparticles (100 particles/dish).

3.3. Incubate for 1 h at 37 °C. Stop the phagocytosis by adding 500 µL of ice-cold PBS.

3.4. Wash the cells extensively with PBS (1 mL at a time, for a total of five washes). Fix the cells with 4% paraformaldehyde for 10 min at room temperature.

3.5. Wash the cells extensively with PBS (1 mL at a time, for a total of five washes) and keep the cells in 500 µL of PBS.

3.6. Image the cells under differential interference contrast and a fluorescent channel at 488 nm. Count AMs containing zymosan-A bioparticles and determine the percentage of phagocytosis.

4. *In Vivo* Phagocytosis by Alveolar Macrophages

NOTE: Inoculate *P. aeruginosa* GFP on a nutrient agar plate and incubate the plate at 37 °C overnight. On the next day, inoculate the single colony to 2 mL of nutrient broth and grow the bacteria at 37 °C overnight.

4.1. The next day, anesthetize mice with an intraperitoneal administration of 10 mg/mL ketamine and 1 mg/mL xylazine mixture. Confirm proper anesthetization via a lack of response to the toe pinch.

4.2. Lay the mouse on a flat board with a rubber band across the upper incisors and place it in a semirecumbent (45°) position with the ventral surface and rostrum facing upward. Using curved

forceps, partially retract the tongue. Using a microsprayer, intratracheally administer 50 µL (5 x 10^6 colony-forming units [CFU])[17] of *P. aeruginosa* GFP into the lungs of the anesthetized mice.

4.3. After 1 h of infection, follow steps 1.1–1.7.

4.4. Resuspend the cells in PBS and cytocentrifuge them (1,000 x *g*, 1 min at room temperature) onto a glass slide.

4.5. Differentially stain the cytospin slides for alveolar macrophages, neutrophils, and lymphocytes, according to the manufacturer's instructions.

4.6. Randomly select 100 AMs, count the AMs containing intracellular bacteria, and determine the percentage of phagocytosis.

5. *In Vivo* Bacteria Clearance Using *P. aeruginosa*

5.1. Inoculate *P. aeruginosa* on a *P. aeruginosa* isolation agar plate and incubate the plate at 37 °C overnight. Inoculate the single colony to 2 mL of lysogeny broth (LB) and grow the bacteria at 37 °C overnight. Calculate the CFU, using the following formula.

CFU/mL = (number of colonies x dilution factor)/volume of the culture plate.

Dilute the culture with PBS to get the desired CFU/mL.

5.2. In trial 1, intratracheally inject a sublethal dose of ~2.5 x 10^5 CFU/mL *P. aeruginosa* into anesthetized wild-type (WT) and TRIM72^{KO} mice, as stated in step 4.2. Measure the body weight daily for 6 days.

5.3. In trial 2, inject a second dose of *P. aeruginosa* (5 x 10^5 CFU/mL) into the mice that survived in trial 1 and measure the body weight for 4 days.

5.4. In trial 3, using a different set of mice, inject WT and TRIM72^{KO} mice with a lethal dose (3 x 10^7 CFU/mL) of *P. aeruginosa* and record the mortality within 2 days of injection.

5.5. Either at death or after euthanasia at day 2 after the injection, collect the whole-lung tissue for the quantification of the lung bacterial burden at peak infection.

5.6. To test the lung bacterial burden, add 200 µL of normal saline to the lung tissues and homogenize them, using a previously tested setting on an electronic homogenizer that completely disrupts the lung tissue without breaking bacteria. Adjust the total volume of the lung homogenate to 1 mL and plate 100 µL of lung lysate on *Pseudomonas* isolation agar plates at 10-fold serial dilutions.

5.7. Incubate the plates at 37 °C for 24 h and count the bacterial colonies to determine the CFU per whole lung.

6. Statistical Analysis

6.1. Use Student's *t*-test to determine the statistical significance of the difference between the two groups. Consider a difference statistically significant when $p < 0.05$. All data are presented as means ± standard error of the mean (SEM).

REPRESENTATIVE RESULTS:

We first performed the experiment to analyze phagocytosis by mouse primary AMs. Throughout all analyses, we compared AMs isolated from WT and TRIM72^{KO} mice. As shown in **Figure 1A**, fluorescence microscopy revealed that phagocytosis of FITC-glass beads by mouse primary AMs occurs after 1 h of incubation. **Figure 1B** shows the analysis of phagocytosis by flow cytometry. The quantification of phagocytosis measured by microscopy and flow cytometry is represented in **Figure 1C** and **Figure 1D**, respectively. FcγR- and CR-mediated phagocytosis by MH-S cells is represented in **Figure 2A**, and **Figure 2B** shows the quantification. These results show that the expression of TRIM72 in MH-S cells resulted in a more than fivefold decrease in complement phagocytosis. Representative images of Alexa Fluor-488-conjugated zymosan-A particle ingestion by primary AMs isolated from WT or TRIM72^{KO} mice are shown in **Figure 2C**, and the quantification is presented in **Figure 2D**. In vivo phagocytosis results are represented in **Figure 3**. **Figure 3A** shows differential staining identifying the presence of AMs, neutrophils, and lymphocytes, and GFP^{+} phagocytic cells. The percentage of BALF cells and the quantification of phagocytosis is represented in **Figure 3B** and **Figure 3C**, respectively. The percentage of body weight loss in mice after the intratracheal administration of a sublethal dose of *P. aeruginosa* is shown in **Figure 4A**, and the percentage of survival of mice at day 2 after a lethal dose of *P. aeruginosa* is indicated in **Figure 4B**. **Figure 4C** shows a scatter plot for the whole-lung bacterial burden at death or at day 2 after the *P. aeruginosa* infection in mice.

FIGURE LEGENDS:

Figure 1: Phagocytosis in mouse primary alveolar macrophages. (**A**) Representative images of low versus high phagocytic indexes showing primary AMs containing green fluorescent beads (left); representative images showing low and high percentages of phagocytic AMs. Arrows: low phagocytic index AMs; arrowheads: high phagocytic index AMs. The scale bar = 25 µm for the left two images and 50 µm for the right two images. (**B**) Representative flow cytometry detection of phagocytizing cells in no-beads control, WT + beads, and TRIM72^{KO} + beads AMs. The bars define the bead-containing cell population (in percentage) and the mean fluorescence intensity (MFI). (**C**) Statistics of the average phagocytic index and the percentage of phagocytic AMs in WT and TRIM72^{KO} AMs; $n = 5$ for both groups, $^{*}p < 0.05$. (**D**) Statistics of flow cytometry MFI and the percentage of FITC^{+} cells in WT and TRIM72^{KO} AMs; $n = 3$ for both groups, $^{*}p < 0.05$. This figure is reprinted with permission of the American Thoracic Society[13].

Figure 2: FcγR- and CR-mediated phagocytosis. (**A**) Representative images of opsonized sheep red blood cells' (SRBCs) phagocytosis by MH-S cells. The arrows point to some SRBCs. The scale bar = 25 µm. (**B**) Quantification of SRBCs phagocytosis by MH-S cells overexpressing TRIM72 ($TRIM72^{OE}$) in the presence of IgG (FcγR-mediated phagocytosis) or IgM (CR-mediated phagocytosis); $n = 6$ for each group, $*p < 0.05$ and $**p < 0.005$ compared with WT control. (**C**) Representative images of Alexa Fluor-488-conjugated zymosan particle ingestion by primary AMs isolated from WT or $TRIM72^{KO}$ mice. The arrows in the panel A and B indicate zymosan + cells. The scale bar = 50 µm. (**D**) Statistical results of the percentage of zymosan-containing AMs; $n = 3$ for each group, $p > 0.05$. This figure is reprinted with permission of the American Thoracic Society[13].

Figure 3: In vivo phagocytosis of *P. aeruginosa* GFP. (**A**) Representative images of bronchoalveolar lavage fluid (BALF) cell cytospin slides from WT and $TRIM72^{KO}$ mice 1 h after the injection of *P. aeruginosa* GFP. Kwik-Diff staining identifies AMs (large, round cells) and neutrophils; GFP identifies phagocytic cells (white arrows) and GFP^{+} differential interference contrast (DIC) identifies internalized GFP bacteria (black arrows) in AMs. The scale bars = 50 µm. (**B**) Quantification of the percentage of AMs and neutrophils in BALF of WT and $TRIM72^{KO}$ mice 1 h after the *P. aeruginosa* injection. (**C**) Quantification of the percentage of phagocytic AMs in WT and $TRIM72^{KO}$ mice; $n = 3$ for each group, $*p < 0.05$. The data are presented as mean (± SEM). This figure is reprinted with permission of the American Thoracic Society[13].

Figure 4: In vivo bacteria clearance using *P. aeruginosa* (P.A.). (**A**) The percentage of body weight (B.W.) loss of naive WT and $TRIM72^{KO}$ mice after the first intraperitoneal injection of 2.5×10^5 CFU/mL PAO1 (a clinical isolate of *P. aeruginosa*); $n = 13$ for WT (black squares), $n = 8$ for $TRIM72^{KO}$ (red circles), $*p < 0.05$, $**p < 0.005$ compared with WT. (**B**) The percentage of survival at day 2 after the 3×10^7 CFU/mL *P. aeruginosa* intraperitoneal injection; $n = 10$ for WT (solid black circles) and $TRIM72^{KO}$ (solid red squares), $*p < 0.05$ for WT versus $TRIM72^{KO}$ groups. (**C**) A scatter plot of the whole-lung bacterial burden at day 2 of the *P. aeruginosa* injection in WT and $TRIM72^{KO}$. The gray dashed line designates the injected bacterial dose; ^ designates mice who have died. For WT versus $TRIM72^{KO}$ groups, $p < 0.05$. This figure is reprinted with permission of the American Thoracic Society[13].

DISCUSSION:

While performing a gas exchange function, the lung persistently confronts foreign particles, pathogens, and allergens. AMs provide the first line of defense by virtue of their main function, namely phagocytosis. AMs also coordinate with other immune cells in destroying the pathogens and in the resolution of inflammation. Here, we described methods for specifically assessing phagocytosis by AMs isolated from the mouse lung. The protocol presented in this manuscript explains a detailed study of phagocytosis both in vivo and in vitro, which can also be used to study the macrophage function and bacterial clearance in other organs.

The described in vitro phagocytosis relies on the simple idea of incubating serum-treated FITC beads or opsonized SRBCs with cultured AMs to initiate phagocytosis. This method includes steps of extensive washing and the lysis of nonphagocytized RBCs to reduce the background. Care must

be taken not to detach the adhered AMs. The wash step involving ACK lysis solution should be done within 1 min, as a longer washing time leads to the lysis of the macrophages.

In addition, in the imaging analysis, we characterized both the percentage of phagocytizing cells and the phagocytic index to gain a comprehensive view of the AM phagocytosis function. We have also explained the method to differentiate among PRR-, FcγR-, and CR-mediated phagocytosis in the murine macrophage MH-S cell line. In conjunction with genetic modulation, this step is useful when trying to gain mechanistic insights on the specific phagocytosis pathway that was affected by the target gene manipulation.

Previous reports documented methods to evaluate the bacterial uptake; the most notable method is the gentamicin protection assay[14]. In the protocol presented here, we have also shown an imaging method to measure in vivo phagocytosis. There are a few key factors that make this method better in comparison to the previously published methods. The method described here involves an intratracheal injection of *P. aeruginosa* GFP followed by differential staining of BALF cells. This method highlights the use of fluorescent bacteria, which helps to identify ingested bacteria within the phagocytes. In addition, the differential staining of BALF cells helps to specifically differentiate the phagocytic capacity of AMs from other immune cells in the in vivo environment and to evaluate the relative contribution of different phagocytes to clear the bacterial loads from the lung. A minor limitation of this method is that it requires a careful intratracheal administration to avoid variability between mice.

Further, we explained the method to establish a *P. aeruginosa* bacteria clearance in mice in the context of pneumonia. To characterize this method, we determined the body weight loss and mortality after the intratracheal administration of *P. aeruginosa* and used a quantitative assay to determine the lung bacteria burden. The success of the assay will be determined by a standardization of the timing of the injection, the quality of the pathogen and the dose of injection, and the optimization of a homogenization method that completely disrupts the lung without breaking the bacterial cell membrane.

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DISCLOSURES:
The authors have nothing to disclose.

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recruitment but decreases bacterial clearance and survival. *Infection and Immunity*. **65**, 1139-1146 (1997).
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Figure 1

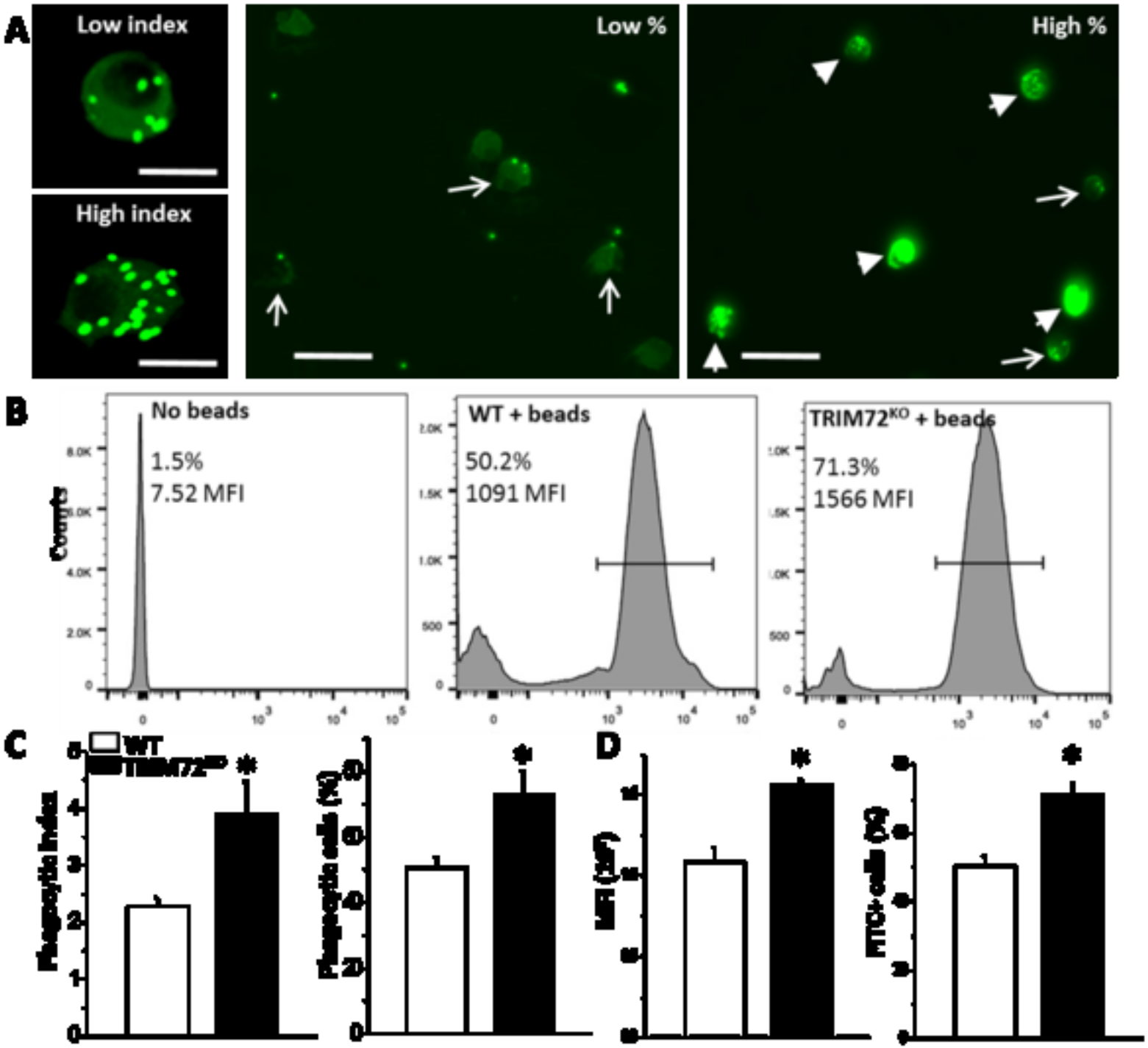

Figure 4

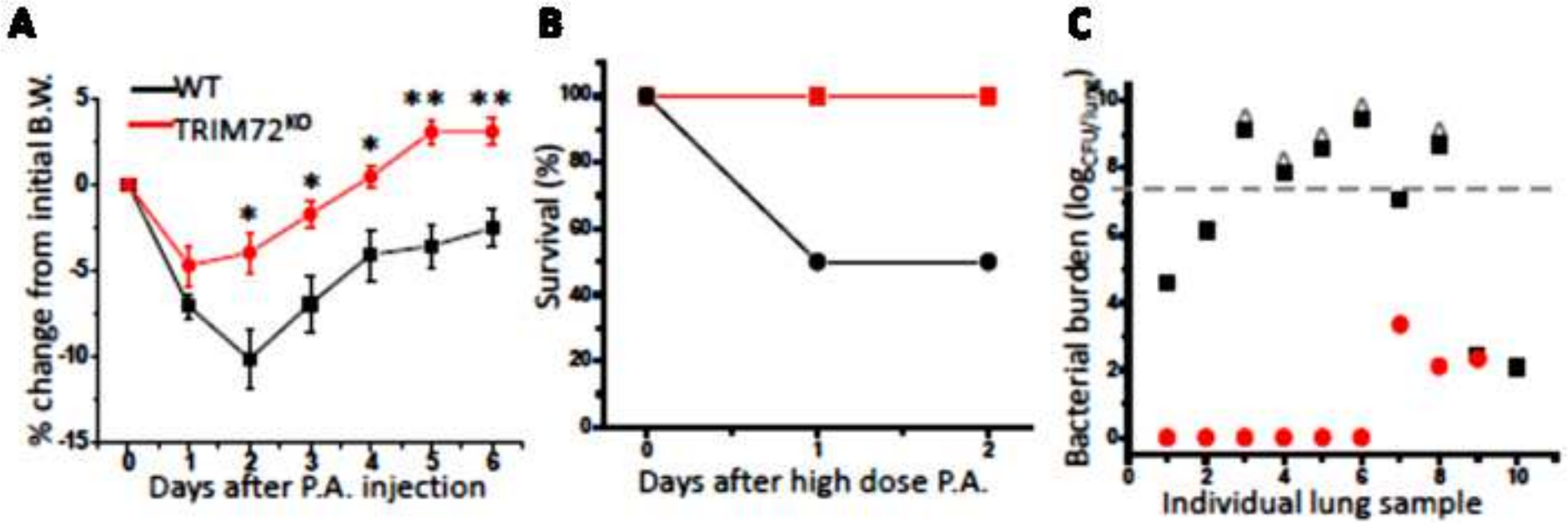

Figure 2 revised

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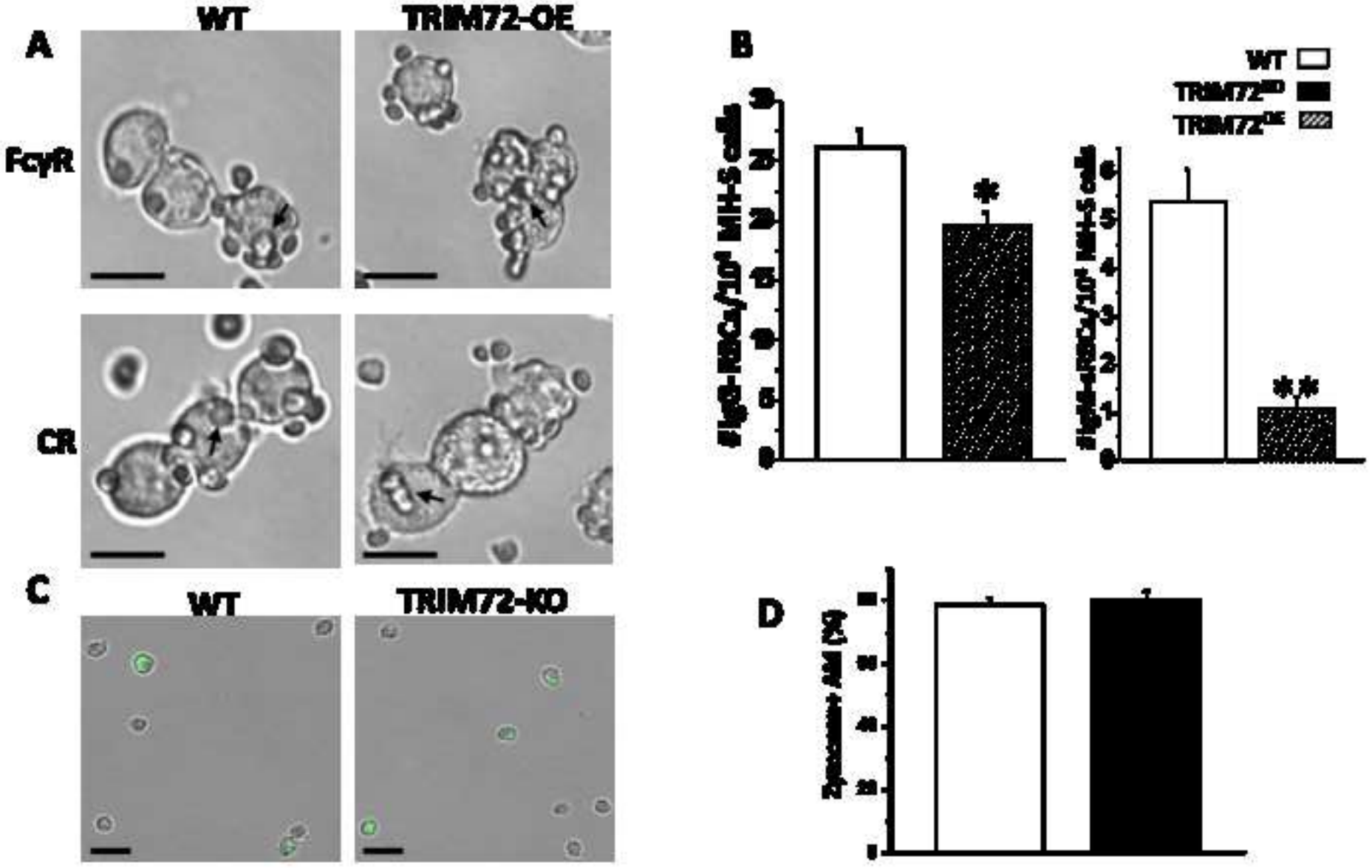

Figure 3 revised

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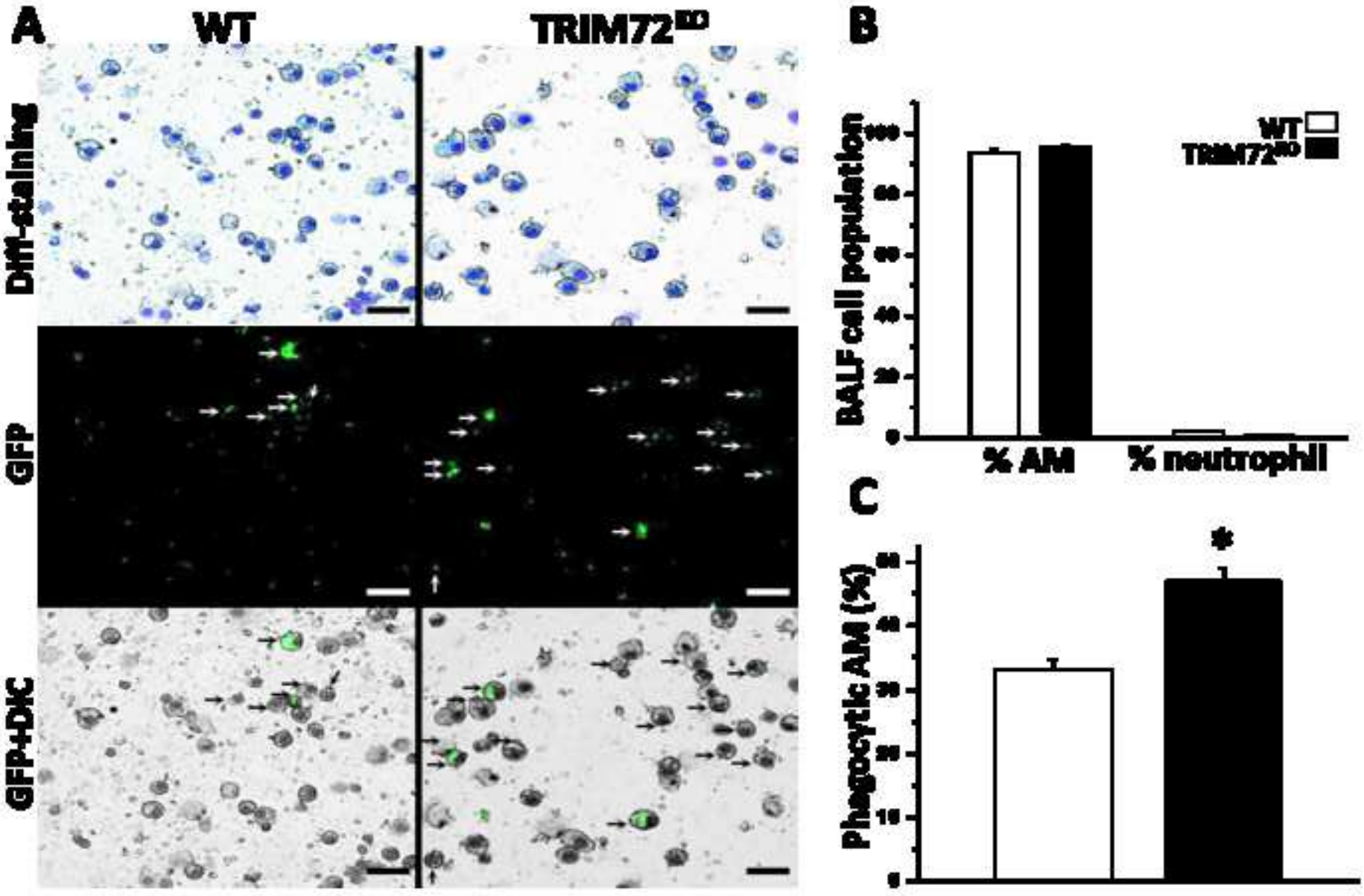

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
18-G Needle	Nipro Medical	CI+1832-2C	Molecular Biology grade
2,7-diaminofluorene (DAF)	Sigma-Aldrich	D17106	Molecular Biology grade
70% Ethanol	Decon Labs Inc.	18C27B	Analytical grade
96-well plate	Corning	3603	Cell Biology grade
ACK lysis buffer	Life Technologies	A10492	Molecular Biology grade
Alexa fluor-488 Zymosan-A-bioparticle	Thermofisher Scientific	Z23373	Molecular Biology grade
C5 deficient serum	Sigma-Aldrich	C1163	Biochemical reagent
Centrifuge	Labnet International	C0160-R	
Cytospin 4 Cytocentrifuge	Thermofisher Scientific	A78300101 Issue 11	
DMEM Cell Culture Media	Gibco	11995-065	Cell Biology grade
FBS	Atlanta Biologicals	S11550	Cell Biology grade
Flow Cytometer	BD Biosciences	FACSCalibur	
Flow Jo Software	FlowJo, LLC		
Forceps	Dumont	0508-SS/45-PS-1	Suitable for laboratory animal dissection
FITC-carboxylated latex beads	Sigma-Aldrich	L4530	Cell Biology grade
GFP-*P. aeruginosa*	ATCC	101045GFP	Suitable for cell infection assays
Glass bottom dish	MatTek Corp.	P35G-0.170-14-C	Cell Biology grade
High-Pressure Syringe	Penn-Century	FMJ-250	Suitable for laboratory animal use
Homogenizer	Omni International	TH-01	
Hydrogen peroxide	Sigma-Aldrich	H1009	Analytical grade
Inverted Fluorescence Microscope	Olympus	IX73	
Ketamine Hydrochloride	Hospira	CA-2904	Pharmaceutical grade
Shandon Kwik-Diff Stains	Thermofisher Scientific	9990700	Cell Biology grade
LB Agar	Fisher Scientific	BP1425	Molecular Biology grade
LB Broth	Fisher Scientific	BP1427	Molecular Biology grade
MicroSprayer Aerosolizer	Penn-Century	IA-1C	Suitable for laboratory animal use
Paraformaldehyde	Sigma-Aldrich	P6148	Reagent grade
PBS	Gibco	20012-027	Cell Biology grade
rabbit anti-SRBC-IgG	MP Biomedicals	55806	Suitable for immuno-assays

rabbit anti-SRBC-IgM	Cedarline Laboratories	CL9000-M	Suitable for immuno-assays
Scissors	Miltex	5-2	Suitable for laboratory animal dissection
Small Animal Laryngoscope	Penn-Century	LS-2	Suitable for laboratory animal use
Sodium Dodecyl Sulfate (SDS)	BioRad	1610301	Analytical grade
Spring Scissors (Med)	Fine Science Tools	15012-12	Suitable for laboratory animal dissection
Spring Scissors (Small)	Fine Science Tools	91500-09	Suitable for laboratory animal dissection
sheep red blood cells (SRBCs)	MP Biomedicals	55876	Washed, preserved SRBCs
Urea	Sigma-Aldrich	U5378	Molecular Biology grade
Xylazine	Akorn Animal Health	59399-110-20	Pharmaceutical grade

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Title of Article: Assessing the alveolar macrophage phagocytosis: a comprehensive methodology

Author(s): Nagaraja Nagre, Xiaofei Long, Andrew Pearson, Xiaoli Zhao

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Dear Dr. Steindel and reviewers,

I would like to thank each of you for your insightful comments and concerns. Detailed below are the changes made in response to each comment individually. Your comments will be copied verbatim in black, and our response will be in blue.

Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been carefully proofread for spelling and grammar mistakes, and all identified issues have been corrected.

2. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Copyright permission email is attached and Figures are cited in the Figure Legend

3. Figures: Please include a scale bar for images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend. Please also define error bars in the figure legend.

Scale bars have been added to each microscope image, and scale has been defined within the figure legends

4. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

Figures are uploaded individually.

5. Table of Equipment and Materials: Please revise to include the name, company, and catalog number of all relevant materials. Please provide lot numbers and RRIDs of antibodies, if available. Please sort the items in alphabetical order according to the Name of Material/ Equipment.

The Table of Equipment and Materials has been updated to include all requested information.

6. Please revise the title to avoid punctuation.

The title has been revised to exclude punctuation.

7. Please provide an email address for each author.

Email addresses for each author have been included in the manuscript.

8. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis).

Reference numbers have been changed to superscript following proper placement as per editorial suggestion.

9. Line 30: The references should be numbered in order of appearance; 11 and 12 should be 3 and 4.

References were re-numbered in order of appearance.

10. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Centrifuge speeds were converted to centrifugal force (x g) rather than rpm.

11. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Sigma-Aldrich, MP Biomedicals, Cedarline Laboratories, Life Technologies, Thermo Fisher, etc.

All commercial language was removed from the manuscript.

12. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Ethics statement was added to manuscript before numbered protocol steps.

13. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Language throughout the protocol was revised to include only imperative tense instructions.

14. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Protocol steps were divided to reduce the number of actions per step.

15. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Detail within protocol steps has been increased to avoid any potential confusion of those wishing to replicate these procedures.

16. 1.1: Please specify the euthanasia method and the surgical instruments used to expose the trachea.

Requested details have been included.

17. 1.2: What volume of DMEM with 10 % non-heat inactivated FBS is used? Please specify the temperature for culturing primary alveolar macrophages. Please specify the volume/weight of FITC-beads added.

Requested details have been included.

18. 1.3: What volume of PBS is used to wash?

Requested details have been included.

19. 1.4: Please describe how to quantify phagocytosis and how to calculate percentage positivity and mean fluorescence intensity.

Requested details have been included.

20. 2.1: Please specify incubation temperature.

Requested details have been included.

21. 4.1: Please specify growth conditions.

Requested details have been included.

22. 4.2: Please specify the dose of ketamine-xylazine. Please mention how proper anesthetization is confirmed.

Requested details have been included.

23. 4.3: Please describe how to collect the bronchoalveolar lavage fluid (BALF). Please specify centrifugation conditions.

Protocol has been edited to refer the reader to steps 1.1-1.6, which detail exact procedure for BALF collection and macrophage pelleting.

24. 5.1: Please mention how the mice are anesthetized.

Protocol has been edited to refer the reader to step 4.2 for anesthetization procedure.

25. Please include single-line spaces between all paragraphs, headings, steps, etc.

Manuscript has been edited to use single spacing throughout.

26. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

The steps that should be visualized are highlighted.

27. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

These instructions were considered when highlighting the protocol for the video.

28. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

These instructions were considered when highlighting the protocol for the video.

29. Discussion: Please also discuss the significance with respect to existing methods and any future applications of the technique.

30. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:

Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

References were edited to use this format.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The focus of this manuscript is supposed to be a 'comprehensive methodology' (per the title) to determine phagocytic uptake by alveolar macrophages. Not only does the manuscript not reflect a comprehensive methodology, it describes methodology that does not rigorously measure phagocytosis.

We would like to thank the reviewer for careful and thorough reading of this manuscript and for the thoughtful comments and constructive suggestions, which help to improve the quality of this

manuscript.

Major Concerns:

1) The use of 'comprehensive' in the title and in the manuscript is misleading since there are other, and better, methods to quantitatively measure phagocytic uptake, particularly of bacteria. For example, the gentamicin protection assay and even microscopic assays that distinguish the plasma membranes of the cells.

The title has been edited to remove the term ‘comprehensive’. Relevant references are cited.

2) Important: the described assays do NOT distinguish between internalized bacteria or beads and those that are cell-surface associated. The assays described assess total cell-associated particulates, not internalized. No rigorous methodology is described to distinguish between associated and internalized beads (Section 1) or fluorescent particles or bacteria (Sections 3, 4).

Where it is sufficient to remove all extracellular fluorescent material, the protocol outlines the use of multiple PBS washes (Sections 1, 3). Where PBS washes would be ineffective, the protocol details the use of ACK lysis buffer to remove extracellular SRBCs (Section 2).

3) The description of alveolar macrophage isolation is deficient, as is a description of why MH-S cell may (or might not) be used in place of alveolar macrophages.

The macrophage isolation procedure has been edited to include more detail.

Use of MH-S cells is beneficial due to potential for genetic modulation, as is mentioned in the third discussion paragraph.

4) Section 5 does not belong in the protocol, it is not specific to alveolar macrophages nor does it rigorously describe phagocytosis. It more likely describes PMN-mediated killing by a variety of mechanisms.

Although a previous report suggests that PMNs are major innate immune cells fighting *P. aeruginosa* infection, we believe that TRIM72, which is used for a comparison purpose in this protocol, does not regulate PMN function, because it is not expressed in neutrophils. As AM phagocytosis of invading pathogens occurred earlier than any significant amount of neutrophil recruitment to the airspace, we believe the observed bacterial clearance is due to AMs and regulated by TRIM72.

5) The figures are poor. The representative figure of zymosan (Fig.2) is hard to see and not informative, the same is true of Fig. 3A, and the circles/squares of Fig. 4 are not accurately described in the legend with respect to the figure.

Quality of specified figures has been improved. Figure 4’s circles and squares are now accurately described in the legend.

6) TRIM72 was not introduced in the text but is featured in the results. The role and inclusion of TRIM72 is unnecessary to this methods proposal.

The inclusion of TRIM72 was for demonstration purposes. Our lab has used this protocol to study the effects of TRIM72 on macrophage phagocytosis, so this data was available to use as a representation of the functionality of these methods.

7) The statistical tests in this manuscript are not identified.

Statistical analysis section was added to the manuscript.

Minor Concerns:

1) P. aeruginosa should be italicized throughout the text.

Each mention of *P. aeruginosa* has been italicized.

Reviewer #2:

Manuscript Summary

This is an interesting protocol.

We appreciate the positive feedback from the reviewer.

Minor Concerns:

please change the title in assessing murine alveolar macrophages phagocytosis...

This is more informative and human alveolar macrophages are different from the same murine cells.

The title has been edited to specify murine alveolar macrophages.

I would also underline the importance of using the appropriate controls for FITC-beads considering that the autofluorescence of the alveolar macrophages is in the same spectrum of FITC.

We have used non-treated AMs as unstained cells or controls in flow cytometry.

Reviewer #3:

Manuscript Summary:

In this manuscript the authors present a method for analyzing alveolar macrophage phagocytic function and bacterial clearance from the lung. This method involves isolation of macrophages and treatment with fluorescent beads to determine phagocytic capacity. They also provide methods to differentiate among PRR, CR and FcγR mediated phagocytosis.

We greatly appreciate the positive feedback from the reviewer. We thank the reviewer for thorough reading of this manuscript.

Major Concerns:

None

Minor Concerns:

While this is a comprehensive method to evaluate phagocytic function of alveolar macrophages, there are other methods that are not discussed here (e.g., pHrodo).

The relevant methods are cited.

Reviewer #4:

Manuscript Summary:

Manuscript be Nagre et al claims to provide comprehensive methodology of assessing alveolar macrophage phagocytosis, however I have not found anything in that manuscript that was not published previously, moreover techniques described are quite standard and commonly used in the laboratories across the world.

In addition although authors aim to 'report methods for comprehensive analysis of the phagocytic function of AMs using in vitro and in vivo assays, and experimental strategies to differentiate pattern recognition receptor-, complement receptor- and Fc gamma receptor-mediated phagocytosis', they somewhat interchangeably use primary murine alveolar macrophages and MH-S cell line.

Use of MH-S cells is beneficial due to potential for genetic modulation, as is mentioned in the third discussion paragraph.

Also there is no mention of the controls which should be done in each of the protocols.

In Flow cytometry experiment, unstained AMs were used as controls. We have used TRIM72 genetic modification for a comparison purpose, and we used comparison between WT, $TRIM72^{KO}$ and $TRIM72^{OE}$.

Not clear why 'Characterization of *P. aeruginosa* pneumonia model' was included in the manuscript.

P. aeruginosa model is included to bring more insight into *in vivo* protocol. Although *P. aeruginosa* GFP clearance highlights the methodology for *in vivo* phagocytosis, we believe, including an assay to explain *P. aeruginosa* bacterial clearance mechanism in pneumonia mouse model will be an added advantage.

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Dear Editor,

I would like to thank you for your insightful comments and concerns. Detailed below are the changes made in response to each comment individually. Your comments will be copied verbatim in black, and our response will be in blue

So invitro, invivo and the P.aeruginosa pneumonia methods are different? Needs more clarity on this part.

Edited as suggested.

What is the significance of this with respect to phagocytosis?

We believe, explaining an additional method of bacterial clearance will have more advantage.

Please revise the Introduction to include all of the following:

Edited as suggested.

Citation.

Relevant citations are added.

Need to bring out the clarity in the protocol with respect to in vivo and in vitro

Relevant editions are made.

Age, sex, strain of mice used?

Required information is added.

How small of an incision? What was used for the incision?

Required information is added.

So everytime you are withdrawing and re-infusing it back. Or you are just infusing 3ml of PBS. How do you maintain the sterility in this case?

In our hand, this method gives higher cell number. Every time we withdraw and re-infuse back 1 mL PBS, and transfer the collected BALF to a sterile tube.

Please mention that the PBS above is called BALF here. Volume collected?

Required details are added.

How do you call it as alveolar macrophages at this stage? How about cell debris.

Required changes are made

Do you wash before culturing? Do you filter?

Required details are added.

What kindo of beads are these.

Bead details are already mentioned.

Do you check for any macrophage-based marker at this stage? How do you get rid of non-specific bindings.

In our experience, under resting conditions, more than 95 % of cells are macrophages and they look very different from other cells. We have also tried using cell sorting by flow cytometry. But, under resting condition, differential staining and microscopy is good enough to identify AMs.

Citation

New references are included.

Wouldn't this lyse the cells with RBC as well? Why can't just use PBS instead? Volume of the buffer used?

Care should be taken not to exceed washing time more than 1 minute. This will only wash outside RBCs. same is discussed in discussion and relevant reference is added.

What are the culture conditions? How many cells per dish?

Required details are added.

Grow how? Also do you grow on agar or broth?

Required details are added.

How is this done?

Required details are added.

Conditions speed temp time etc ?

Required details are added.

You cannot call this Model since more specific studies are needed. Please reword.

Corrections are made.

What is the desired CFU and how do you calculate this. Please include a one liner note for this.

A note is added.

This is on a different set of mice?

Yes.

Please include a one-liner title for the figures representing all panels together.

A one-liner title is added.

As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

Changes are made.

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

2. Please address all the specific comments marked in the manuscript.

Specific comments are addressed.

3. Once done please ensure that the protocol is no more than 10 pages and the highlights are no more than 2.75 pages including heading and spacings.

4. Figure 3A would benefit from borders on the microscopic images. It is difficult to separate the different images visually.

Changes are made.

5. Please provide copyright permissions for the Figures if they are to be re-used in publication.

The copy of copyright permissions we submitted before covers both text and figures to be reused.

Dear Editor,

I would like to thank you for your comments. Detailed below are the changes made in response to each comment individually. Your comments will be copied verbatim in black, and our response will be in blue.

Where is the explanation on TRIM2^{OE} as seen in panel B.?

The explanation is included in the figure legend. TRIM72 over expression (TRIM2^{OE}) is used for a comparison purpose.

Not shown in the figure.

SRBCs are indicated by an arrow.

Missing scale bar.

Scale bar is added

Please check the citation number – this is reference 13

Reference number is corrected.

Included this abbreviation here. Please check

Thank you. Adding an abbreviation here is fine.

Please upload the reprint permission for the figures as it was never uploaded before or there might have been some problem in uploading. Please upload this as a .doc file in the editorial manager account.

A PDF version of the reprint version is uploaded.

Figure 3: The new figure 3A is missing scale bar and 3C is missing the x-axis label.

Scale bar is added. A symbolic labeling of the bar diagram representing x-axis is already shown on the top right corner of the figure 3.

For the protocol section, the highlight is around one page only. Since the upper limit is 2.75 pages, please highlight section 1 and 2 of the protocol as well for making the video. This will make the most cohesive story.

Section 1 and 2 are also highlighted.

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