

Video Article

Measuring Granulocyte and Monocyte Phagocytosis and Oxidative Burst Activity in Human Blood

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Abstract

The granulocyte and monocyte phagocytosis and oxidative burst (OB) activity assay can be used to study the innate immune system. This manuscript provides the necessary methodology to add this assay to an exercise immunology arsenal. The first step in this assay is to prepare two aliquots ("H" and "F") of whole blood (heparin). Then, dihydroethidium is added to the H aliquot, and both aliquots are incubated in a warm water bath followed by a cold water bath. Next, *Staphylococcus aureus* (*S. aureus*) is added to the H aliquot and fluorescein isothiocyanate-labeled *S. aureus* is added to the F aliquot (bacteria:phagocyte = 8:1), and both aliquots are incubated in a warm water bath followed by a cold water bath. Then, trypan blue is added to each aliquot to quench extracellular fluorescence, and the cells are washed with phosphate-buffered saline. Next, the red blood cells are lysed, and the white blood cells are fixed. Finally, a flow cytometer and appropriate analysis software are used to measure granulocyte and monocyte phagocytosis and OB activity. This assay has been used for over 20 years. After heavy and prolonged exertion, athletes experience a significant but transient increase in phagocytosis and an extended decrease in OB activity. The post-exercise increase in phagocytosis is correlated with inflammation. In contrast to normal weight individuals, granulocyte and monocyte phagocytosis is chronically elevated in overweight and obese participants, and is modestly correlated with C-reactive protein. In summary, this flow cytometry-based assay measures the phagocytosis and OB activity of phagocytes and can be used as an additional measure of exercise- and obesity-induced inflammation.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54264/>

Introduction

The granulocyte and monocyte phagocytosis/oxidative burst (OB) activity assay is a simple, straight-forward technique that is frequently used to gather information about innate immune function following prolonged and intensive exercise.¹⁻⁴ When studying the response of the immune system to a stimulus, granulocytes and monocytes are of particular interest because they play a central role in host defense and are the first immune cells to accumulate at the site of infection.⁵ Neutrophils, a type of granulocyte, are the first cells to translocate into damaged muscle tissue following intensive exercise.⁶ Phagocytosis and OB activity are the most common measures of granulocyte and monocyte function,⁷ and are preferred as indicators of innate immune cell function because of their central role in both the infection process and the repair process.

The assay described in this manuscript utilizes a basic flow cytometer to provide a quantitative determination of granulocyte and monocyte phagocytosis and OB activity in whole blood. The use of whole blood in this technique is advantageous because it allows the assay to be conducted without a time-consuming purification procedure. This assay can easily be modified to accommodate a variety of research designs and lab capabilities. For example, Liao *et al.* utilized a similar technique to show that neutrophil OB activity is increased and neutrophil phagocytosis is decreased following traumatic brain injury.⁸ In another example, McFarlin *et al.* described an elegant modification of this technique that uses allophycocyanin (APC)-conjugated CD66b and high-performance tandem APC-conjugated CD45 labeling with image-based flow cytometry to classify granulocytes in terms of their phagocytosis and OB activities.^{7,9}

Our research group has used various adaptations of this technique as an indicator of innate immune function in overweight, obese, and athletic populations for almost 20 years.^{10,11} The text below will provide the reader with step-by-step instructions for performing this assay and highlight areas that the reader may adapt to meet the needs of their research.

Protocol

NOTE: All blood collection procedures were conducted in accordance with the guidelines set forth by the Appalachian State University (ASU) Institutional Review Board (IRB).

1. Assay Preparation

1. Gather, prepare, and organize the materials specified for the procedure (please refer to Table of Specific Materials/Equipment).
 1. Label 12 x 75 mm tubes.
 1. Label two tubes for each participant: one tube for the phagocytosis assay (label this tube with black ink and an "F" for fluorescein isothiocyanate [FITC]) and one tube for the OB activity assay (label this tube with red ink and an "H" for dihydroethidium [HE]).
NOTE: The use of sterile 12 x 75 mm tubes is recommended to minimize unintended cellular activation and associated increase in background noise.
 2. Label three tubes for the assay controls: one tube for the phagocytosis assay (FITC control: label this tube with black ink and an "F"), one tube for the OB activity assay (HE control: label this tube with red ink and an "H"), and one tube for the negative control (Blood only: label this tube with green ink and "blood only").
 2. Prepare stock solutions at least one day before the day of the assay.
 1. Prepare the sterile phosphate-buffered saline (PBS). Dilute 100 ml of 10x PBS with 900 ml of 18.2 MΩ H₂O. Filter sterilize with a 0.2 μm filter. Store at 4 °C until use.
 2. Prepare the sterile PBS-glucose. Dissolve 1.0 g of glucose in 100 ml of PBS. Filter sterilize with a 0.2 μm filter. Store at 4 °C until use.
 3. Prepare the sterile 0.1 M citrate buffer. Dissolve 1.2 g of citric acid and 1.1 g of sodium citrate in 100 ml of 18.2 MΩ H₂O. Adjust the pH to 4.0. Filter sterilize with a 0.2 μm filter. Store at 4 °C until use.
 4. Prepare the HE stock solution. Resuspend 1.0 mg of HE in 1.0 ml of dimethyl sulfoxide (DMSO). Mix gently, aliquot, and store at -20 °C until use.
 5. Prepare the FITC-labeled *Staphylococcus aureus* (*S. aureus*) stock solution (2 x 10⁹ particles/ml). Resuspend 10 mg of FITC-labeled *S. aureus* in 1.5 ml (or appropriate amount) of PBS. Divide into three 500 μl aliquots and sonicate according to the manufacturer's recommendation. Aliquot and store at -20 °C until use.
 6. Prepare the unlabeled *S. aureus* stock solution (2 x 10⁹ particles/ml). Resuspend 100 mg of unlabeled *S. aureus* in 15 ml (or appropriate amount) of PBS. Divide into thirty 500 μl aliquots and sonicate according to the manufacturer's recommendation. Aliquot and store at -20 °C until use.
 3. Prepare fresh working solutions on the day of the assay.
 1. Prepare the HE working solution. Transfer 10 μl of thawed HE stock solution to 990 μl of PBS-glucose. Protect from light and keep on ice until use.
 2. Prepare the FITC-labeled *S. aureus* working solution (133,333 particles/μl). Transfer 70 μl of thawed FITC-labeled *S. aureus* stock solution to 980 μl of PBS. Protect from light and keep on ice until use.
 3. Prepare the unlabeled *S. aureus* working solution (133,333 particles/μl). Transfer 70 μl of thawed unlabeled *S. aureus* stock solution to 980 μl of PBS. Protect from light and keep on ice until use.
 4. Prepare the quench solution. Add 750 μl of 0.4% trypan blue solution to 11.25 ml of sterile 0.1 M citrate buffer. Keep in an ice-water bath until use.
 4. Have a certified phlebotomist draw blood according to the World Health Organization's guidelines for drawing blood.
 1. Draw blood into one 4 ml blood collection tube containing K₂EDTA. Invert blood collection tube according to the manufacturer's instructions. Keep blood collection tube at room temperature on a bench-top rocker until use. Use this blood for the complete blood count (CBC) with white blood cell (WBC) differential analysis described in Step 1.2.1.
 2. Draw blood into one 4 ml blood collection tube containing lithium heparin. Invert blood collection tube according to the manufacturer's instructions. Keep blood collection tube at RT on a bench-top rocker until use. Use this blood for the monocyte and granulocyte phagocytosis and OB activity assay described in Step 2.
2. Determine the amount of bacteria (*i.e.*, *S. aureus*) that will be needed to complete the assay for each sample.
 1. Use a hematology analyzer to perform a CBC with WBC differential analysis on the blood as described in the manufacturer's instructions. Make a note of the WBC count (in cells/ml), %Neutrophil, and %Monocyte values.
 2. Use the equations below to determine the neutrophil and monocyte counts for each sample.

$$\text{Neutrophil count (in cells/ml)} = \% \text{Neutrophil} \times \text{WBC (in cells/ml)}$$

$$\text{Monocyte count (in cells/ml)} = \% \text{Monocyte} \times \text{WBC (in cells/ml)}$$
 3. Use the equations below to determine the phagocyte count for each sample and the number of phagocytes present in 100 μl of sample.

$$\text{Phagocyte count (in cells/ml)} = \text{Neutrophil count (in cells/ml)} + \text{Monocyte count (in cells/ml)}$$

$$\text{Number of phagocytes in 100 } \mu\text{l} = (\text{Phagocyte count (in cells/ml)})/10$$
 4. Use the equation below to determine the number of bacteria (*i.e.*, *S. aureus*) needed for each sample and the amount of FITC-labeled *S. aureus* or unlabeled *S. aureus* working solution (133,333 particles/ml) that should be added to each tube.
 NOTE: The target bacteria:phagocyte ratio is 8:1.

$$\text{Number of bacteria needed} = \text{Number of phagocytes in 100 } \mu\text{l} \times 8$$

$$\text{Volume of FITC or unlabeled working solution needed (in } \mu\text{l)} = (\text{number of bacteria needed})/(133,333 \text{ particles}/\mu\text{l})$$

2. Perform Assay

1. Prepare whole blood for the assay.
 1. For each participant and control tube, use an extended-length pipette tip to transfer 100 μ l of whole blood from the lithium heparin blood collection tube to the bottom of an appropriately labeled 12 x 75 mm tube. Replace caps.
NOTE: Be careful to avoid getting blood on the sides of the 12 x 75 mm tubes.
 2. Use a micropipette to add 10 μ l of HE working solution to the tubes labeled with red ink and an "H", including the HE control tube. Replace caps and vortex briefly.
 3. Incubate all tubes in a 37 °C water bath for 15 min. Then, immediately transfer all tubes to an ice-water bath for 12 min.
NOTE: Use an open metal rack and gently shake the rack every 5 min to ensure that the tubes are quickly and adequately warmed or chilled.
2. Add bacteria (*i.e.*, *S. aureus*) to the sample and control tubes.
 1. Use a micropipette to add the appropriate amount (calculated in Step 1.2.4) of unlabeled *S. aureus* working solution to the tubes labeled with red ink and an "H", including the HE control tube. Replace caps and vortex briefly.
 2. Use a micropipette to add the appropriate amount (calculated in Step 1.2.4) of FITC-labeled *S. aureus* working solution to the tubes labeled with black ink and an "F", including the FITC control tube. Replace caps and vortex briefly.
 3. Vortex the "blood only" control tube, but do not add either type of bacteria (*i.e.*, *S. aureus*) to it.
 4. Incubate all tubes in a 37 °C water bath for 20 min. Use an open metal rack and gently shake the rack every 5 min to ensure that the tubes are quickly and adequately warmed. After the incubation period, immediately transfer all tubes to an ice-water bath for 1 min.
3. Wash the cells.
 1. Use a repeater pipette to add 100 μ l of ice-cold quench solution to all tubes. Replace caps, vortex briefly, and then incubate all tubes on ice for 1 min.
 2. Use a repeater pipette to add 1 ml of ice-cold PBS to all tubes. Vortex briefly, and then add an additional 2 ml of ice-cold PBS to all tubes and replace caps. Centrifuge all tubes for 5 min at 4 °C and 270 x g, and then aspirate the supernatant.
 3. Repeat Step 2.3.2 one time (total of 2 washes).
4. Prepare samples for flow cytometry.
 1. Use a repeater pipette to add 50 μ l of ice-cold fetal bovine serum to all tubes. Briefly vortex all tubes.
 2. Transfer all tubes to an appropriate carousel and use an automated cell lyse preparation workstation with red blood cell (RBC) lysing and WBC fixing reagents to lyse the RBCs and fix the WBCs as described in the manufacturer's instructions.
 3. After the automated cell lyse preparation workstation run has completed, wrap tubes in aluminum foil and store in a 4 °C refrigerator until the flow cytometry analysis can be performed.

3. Flow Cytometry Acquisition

1. Setup the flow cytometry system.
 1. Warm up the flow cytometry system as directed in the user manual provided by the manufacturer. Then, check the reagent and waste tank levels. If needed, fill the reagent tanks and/or empty the waste tank.
 2. Use the "Clean Panel" feature to run the system clean cycle. Then, run the alignment and fluidics verification fluorospheres, and ensure that the half-peak coefficient of variation (HPCV) for each detector is within the predefined range described in the quality control (QC) protocols provided by the manufacturer.
2. Create assay-specific acquisition and instrument setting protocols.
 1. Create assay-specific acquisition protocols for the phagocytosis and OB activity assays.
NOTE: The acquisition protocols described here can be adapted for use on any flow cytometer with a blue laser (488 nm), a filter (530/30) for FITC, and a filter (575/26) for HE.
 1. Open the flow cytometry system acquisition software and create a "New Protocol".
 2. Choose the "FL1 Parameter" for the phagocytosis assay (FITC) and the "FL2 Parameter" for the OB activity assay (HE).
 3. Create the plots (*i.e.*, forward scatter [FSC] vs. side scatter [SSC] dot plot, FL1 histogram, FL2 histogram) required for acquisition.
 4. Create polygonal regions for the WBC (white blood cells), granulocyte and monocyte populations on the FSC vs. SSC plot and create linear regions on the FL1 and FL2 histograms. Set a "Stop Count" of 50,000 events for the WBC gate.
 5. Save the protocols with assay-specific names.
 2. Create assay-specific instrument setting protocols for the phagocytosis and OB activity assays.
 1. Drag and drop the "assay-specific acquisition protocols" (defined in Step 3.2.1) from the "Resource Explorer" to the "Acquisition Manager" on the flow cytometry system acquisition software.
NOTE: Information added in this way will always appear at the end of the worklist.
 2. Enter the sample information into the worklist, and save the worklist.
 3. Remove the control sample tubes from the 4 °C refrigerator and allow them to equilibrate to room temperature for 15 min. Then, briefly vortex each control sample tube.
 4. Use the "assay-specific acquisition protocols" defined in Step 3.2.1 to run the negative control sample (*i.e.*, blood only) and the positive control samples (*i.e.*, HE control, FITC control) through the flow cytometry system. Review the histograms and adjust the photomultiplier tube (PMT) voltages of the FITC and phycoerythrin (PE) channels as needed.

5. Vortex the control samples and transfer them to the flow cytometry system carousel according to the order on the worklist. Then, place the carousel in the sampling chamber and click on the Cytometer Toolbar "Start" button to acquire data for the control samples. Save the "xxx Setting Pro" setting protocol.
3. Acquire study samples on the flow cytometry system.
 1. Remove the study sample tubes from the 4 °C refrigerator and allow to equilibrate to room temperature for 15 min.
 2. While the samples are equilibrating, drag the predefined study-specific acquisition protocols to the worklist space to make a project worklist.
 3. Confirm that the "setting protocol" is linked to the "assay-specific setting protocols" defined in Step 3.2.2. Then, enter the identifying information (e.g., study name, draw number, visit number, subject ID) for each tube into the work list.
 4. After the 15-min equilibration period, vortex study samples and transfer them to the flow cytometry system carousel according to the order on the worklist. Then, place the carousel in the sampling chamber and click on the Cytometer Toolbar "Start" button to acquire data for the study samples.
 5. Review the quality of the data by inspecting the RBC lysis efficiency and the WBC cell subgroup distribution, proportions, absolute counts, and fluorescence intensities on the report panel of each sample.
NOTE: The data quality is considered to be acceptable if all of these criteria are within their predefined limits. If all criteria are not met, sample should be reprocessed.
 6. Clean and shut down the flow cytometry system. Store the flow cytometry data on two different computer drives to prevent accidental data loss.

4. Data Analysis

1. Perform the phagocytosis analysis.
 1. Consolidate all of the phagocytosis (i.e., F-labeled) sample and control list mode data (LMD) data files into a single folder. Then, open the flow cytometry analysis software and drag the files into the sample space.
 2. Set the WBC, Granulocyte, Monocyte, and Lymphocyte gates.
 1. Double-click on the file name to open any of the "FITC control samples". Use the drop-down menus to set the x-axis to "FSC" and "linear" and the y-axis to "SSC" and "linear".
 2. Use the "polygon gate selector" to prepare a gate of the total WBC population.
NOTE: Be sure to include all of the cells at the edges of the chart. Label this gate "WBC".
 3. Double-click on the "new WBC gate" and use the drop-down menus to set the x-axis to "FSC" and "Linear" and the y-axis to "SSC" and "Linear".
 4. Use the "polygon gate selector" to set the "Granulocyte gate" and the Monocyte gate", and then use the "elliptical gate selector" to set the "Lymphocyte gate". Label each gate accordingly. Drag the "gate data (%)" off to the side so that the cells are easily viewed.
 3. Set the "Phagocytosis Positive gate" and the "Phagocytosis Negative gate" for the granulocyte and monocyte populations.
 1. Double-click on the "FITC control sample" used in Step 4.1.2.1. Then, click on the "Granulocyte gate" and use the drop-down menus to set the x-axis to "FL1" and "Log" and the y-axis to "SSC" and "Linear".
 2. Use the "square gate selector" to set a "Phagocytosis Negative gate" and a "Phagocytosis Positive gate".
NOTE: Since the FITC control samples did not receive bacterial (i.e., *S. aureus*) stimuli, all of the cells should theoretically be phagocytosis negative. Use discretion when establishing the boundaries for these populations.
 3. Repeat Step 4.1.3.1 and Step 4.1.3.2 for the monocyte population.
 4. Add statistics for "% of WBC", "fluorescence intensity", and "% phagocytosis positive cells" for the granulocyte and monocyte populations.
 1. Click on the "FITC control sample" used in Step 4.1.2.1. Highlight the "Granulocyte gate", and then click on "Workspace" and "Add Statistic". Highlight "Geometric Mean" and "FL1 Log" and click "Add". Then chose the "Frequency of Parent" statistic and click "Add".
NOTE: This will give the fluorescence intensity (geometric mean) of the entire granulocyte population and the percentage of WBC (parent) that are granulocytes.
 2. Click on the "FITC control sample" used in Step 4.1.2.1. Highlight the "Phagocytosis Positive gate" of the granulocyte population, and then click on "Workspace" and "Add Statistic". Highlight "Geometric Mean" and "FL1 Log" and click "Add". Then choose the "Frequency of Parent" statistic and click "Add".
NOTE: This will give the fluorescence intensity (geometric mean) of the phagocytosis positive granulocytes and the percentage of the granulocytes (parent) that are phagocytosis positive.
 3. Repeat Step 4.1.4.1 and Step 4.1.4.2 for the monocyte population.
 4. Click on the "FITC control sample" used in Step 4.1.2.1. Highlight the "Lymphocyte gate", and then click on "Workspace" and "Add Statistic". Highlight the "Frequency of Parent" statistic and click "Add".
NOTE: This will give the percentage of WBC (parent) that are lymphocytes.
 5. Highlight all of the gates and statistics created in the FITC control sample used in Step 4.1.2.1. Drag them into "All Samples" in the "Group" panel at the top of the screen.
NOTE: This will apply these settings to all of the study samples.
 6. Save and name work as a .wsp file in the same folder that contains the LMD folder.
5. Adjust individual sample gates.

1. Click on the "first sample" in the data set and confirm that the WBC gate appropriately fits the cell distribution on the screen. If it does not, click and drag the edges of the gate adjust the gate for that sample. Click on the "right arrow" (*i.e.*, ">") to advance to the next sample. Repeat this step until all samples have been reviewed.
2. Click on the "WBC gate" for the first sample in the data set and confirm that the "Granulocyte gate", "Monocyte gate", and "Lymphocyte gate" for each sample appropriately fit the cell distribution on the screen. Adjust as needed. Click on the "right arrow" (*i.e.*, ">") to advance to the next sample.
3. Repeat Step 4.1.5.2 until all samples have been reviewed. Then, click "Save".
6. Display the data in an electronic spreadsheet program.
 1. Click on the "Table Editor" icon at the top of the screen. Click "Edit", "Keywords", and "\$DATE" to include the sampling date on the final spreadsheet. Then, click "Edit", "Keywords", and "@SAMPLEID1" to include the sampling identification on the final spreadsheet. Repeat for "@SAMPLEID2", "@SAMPLEID3", and "@SAMPLEID4".
 2. Adjust the "Table Editor" screen and the "sample" screen so they can both be viewed on the computer screen. Select any "sample" on the sample sheet. Then, drag each statistic of interest to the "Table Editor".
 1. Drag the "Freq. of Parent" statistic listed under the "Granulocyte gate" to the "Table Editor". Type "% WBC as Granulocytes" under the "Name" column of the "Table Editor".
 2. Drag the "Geometric Mean" statistic listed under the "Granulocyte gate" to the "Table Editor". Type "Geometric Mean Fluorescence, Granulocytes" under the "Name" column of the "Table Editor".
 3. Drag the "Freq. of Parent" statistic listed under the "Granulocyte/Phagocytosis Positive gate" to the "Table Editor". Type "% Phagocytosis Positive Granulocytes" under the "Name" column of the "Table Editor".
 4. Drag the "Geometric Mean" statistic listed under the "Granulocyte/Phagocytosis Positive gate" to the "Table Editor". Type "Geometric Mean Fluorescence, Phagocytosis Positive Granulocytes" under the "Name" column of the "Table Editor".
 5. Drag the "Freq. of Parent" statistic listed under the "Monocyte gate" to the "Table Editor". Type "% WBC as Monocytes" under the "Name" column of the "Table Editor".
 6. Drag the "Geometric Mean" statistic listed under the "Monocyte gate" to the "Table Editor". Type "Geometric Mean Fluorescence, Monocytes" under the "Name" column of the "Table Editor".
 7. Drag the "Freq. of Parent" statistic listed under the "Monocyte/Phagocytosis Positive gate" to the "Table Editor". Type "% Phagocytosis Positive Monocytes" under the "Name" column of the "Table Editor".
 8. Drag the "Geometric Mean" statistic listed under the "Monocyte/Phagocytosis Positive gate" to the "Table Editor". Type "Geometric Mean Fluorescence, Phagocytosis Positive Monocytes" under the "Name" column of the "Table Editor".
 9. Drag the "Freq. of Parent" statistic listed under the "Lymphocyte gate" to the "Table Editor". Type "% WBC as Lymphocytes" under the "Name" column of the "Table Editor".
 3. Drag and drop the parameters visible in the "Table Editor" until they are arranged in the preferred order.
 4. Click "Save". Then, click "Create Table" to display the data in a spreadsheet format. Click on "save as" to save and name work as .xlsx file.
2. Repeat Step 4.1 for the OB activity assay (*i.e.*, H-labeled) samples and control LMD data files.
NOTE: Be sure to substitute all references to phagocytosis with references to OB activity.

Representative Results

Prolonged and intensive exercise has profound effects on innate immune function, including natural killer cell function, macrophage cytokine-mediated response to viral infection, and granulocyte and monocyte phagocytosis and OB activity. Multiple studies indicate that granulocyte and monocyte phagocytosis increases significantly post-exercise, reflecting the inflammation induced by muscle damage and metabolic demands. In contrast, granulocyte and monocyte OB activity decreases post-exercise and may be one immune indicator of increased susceptibility to infection. For example, **Figure 1** shows that phagocytosis of *S. aureus* by granulocytes is increased immediately and 1.5-hr after a 75-km cycling bout (70% $\text{VO}_{2\text{MAX}}$), returning to normal by the next morning. Monocyte phagocytosis is similarly affected by this level of exercise. **Figure 2** shows that granulocyte OB activity is decreased for at least 21-hr after 75-km cycling. Monocyte OB activity is also decreased after 75-km cycling.

Granulocyte and monocyte phagocytosis of *S. aureus*, but not granulocyte and monocyte OB activity, are correlated to systemic inflammation biomarkers. **Figure 3** shows the modest correlation between granulocyte phagocytosis and serum C-reactive protein (CRP) levels (Pearson product-moment correlation; $r = 0.44$; $P < 0.01$) in 106 overweight/obese women. Serum CRP is also correlated to monocyte phagocytosis of *S. aureus* (Pearson product-moment correlation; $r = 0.30$; $P < 0.01$). In addition, blood neutrophil/leukocyte counts (Pearson product-moment correlation; $r = 0.62$ and $r = 0.61$, respectively; $P < 0.001$), body mass index (Pearson product-moment correlation; $r = 0.50$ and $r = 0.40$, respectively; $P < 0.001$), serum insulin levels ($r = 0.30$ and $r = 0.23$; $P < 0.03$), and blood $\text{A}_{1\text{C}}$ levels (Pearson product-moment correlation; $r = 0.28$ and $r = 0.21$, respectively; $P < 0.04$) are correlated to granulocyte and monocyte phagocytosis of *S. aureus*. In general, these data indicate that high granulocyte and monocyte phagocytosis in resting subjects is indicative of systemic inflammation.

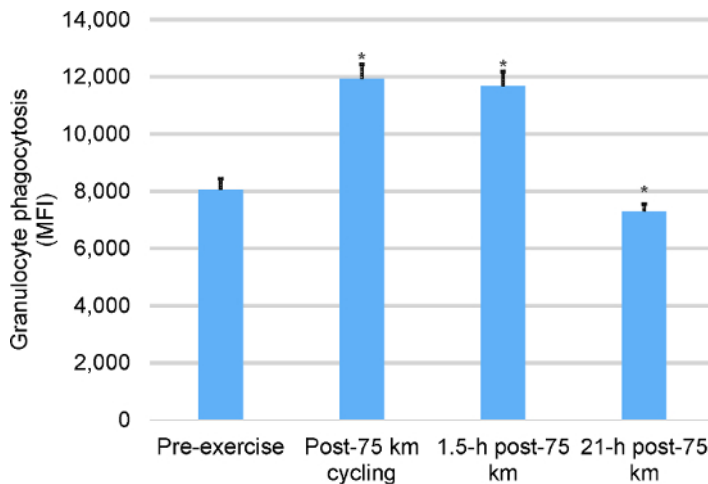


Figure 1: Granulocyte Phagocytosis is Affected by Intense Endurance Exercise. Granulocyte phagocytosis increased immediately and 1.5-hr after a 75-km cycling bout at 70% of VO_{2MAX} . By 21-hr post-exercise, granulocyte phagocytosis returned to slightly below pre-exercise levels. *indicates different from pre-exercise (Repeated measures ANOVA with paired t-test post-hoc analysis, when appropriate; $N = 19$; $P < 0.05$). Values are mean $\pm$ standard error. [Please click here to view a larger version of this figure.](#)

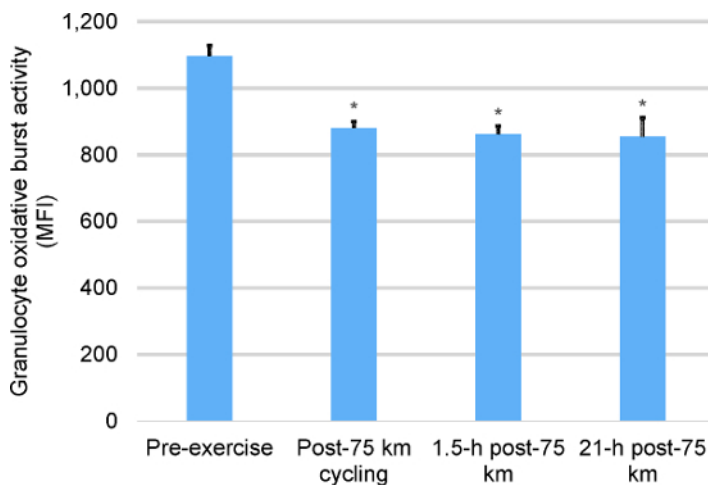


Figure 2: Granulocyte Oxidative Burst Activity is Affected by Intense Endurance Exercise. Granulocyte oxidative burst activity decreased immediately, 1.5-hr, and 21-hr after a 75-km cycling bout at 70% of VO_{2MAX} . *indicates different from pre-exercise (Repeated measures ANOVA with paired t-test post-hoc analysis, when appropriate; $N = 19$; $P < 0.05$). Values are mean $\pm$ standard error. [Please click here to view a larger version of this figure.](#)

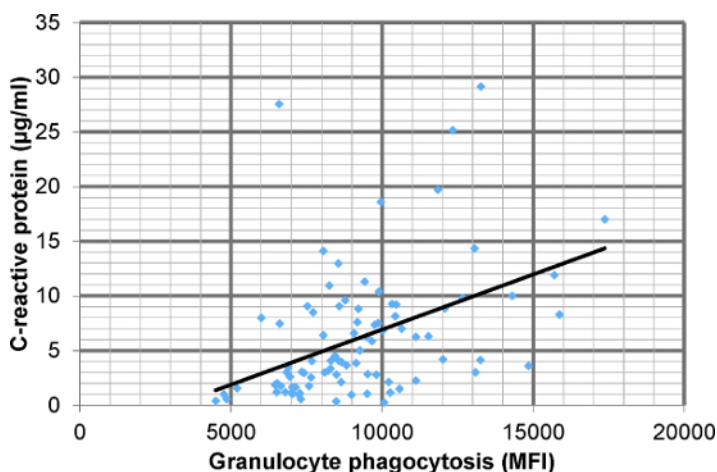


Figure 3: Granulocyte Phagocytosis is Correlated with Systemic Inflammation in Overweight/Obese Women. Granulocyte phagocytosis was correlated to serum C-reactive protein, a widely accepted biomarker of systemic inflammation (Pearson product-moment correlation; $N = 87$; $r = 0.44$; $P < 0.01$). [Please click here to view a larger version of this figure.](#)

Discussion

This manuscript provides a step-by-step protocol for the determination of two indicators of granulocyte and monocyte function. We have identified a few steps that are critical to the success of this assay. One such step is the ice-bath incubation that occurs immediately prior to the addition of bacteria. Thorough cooling of all samples will minimize the effect of temperature on phagocytosis and OB activity. Another critical step is the addition of bacteria to each sample. A 8:1 bacteria:phagocyte ratio produces optimal results; however other researchers may find that this ratio will need to be modified to produce suitable results. Another critical step is the use of RBC lysing and WBC fixing reagents to lyse RBCs and fix WBCs. The use of these reagents ensures complete lysis of the RBCs and enables researchers to safely store processed samples at 4 °C overnight, and then run them through the flow cytometer the next day. Previous work (unpublished) indicates that RBC lysing and WBC fixing can be used to delay the flow cytometry of fixed samples by as many as 12 hr without compromising data integrity.

This procedure can easily be modified to meet the capabilities and interests of the researcher, but it should be emphasized that optimization is necessary when adaptations are made. A few of the most common adaptations that have been reported are to the target bacteria, the label/probe, and the incubation time. Researchers have produced exceptional results when *Escherichia coli*^{7,8} is used in place of *S. aureus* as the target pathogen (both at the 8:1 ratio). A fungus, such as *Saccharomyces cerevisiae*, may also be suitable as a substitute for *E. coli*. In addition, researchers frequently use a pH-sensitive molecular probe,^{7,12} instead of FITC, as the probe for phagocytosis. Likewise, probes other than HE are commonly used for measuring OB activity, including dichlorofluorescein diacetate (DCF-DA)¹⁰ and dihydrorhodamine 123 (DHR).¹³ Reported incubation times vary from 20 min to 120 min,^{12,14,15} with McFarlin reporting that the maximal response time may vary by bacterial target.⁷ It should also be noted that the optimal incubation times may vary between the phagocytosis and OB aspects of the assay.⁷

As with all laboratory assays, troubleshooting may be necessary when establishing this technique in a new research setting. The authors advise that optimization of the bacteria:phagocyte ratio is the key to success for this assay. The authors also note that the data from a sample should be discarded from analysis if fewer than 80% of the cells in the granulocyte population from a non-diseased participant are FITC positive; in this situation, the sample can be re-collected and re-analyzed, if possible.

There are a few limitations to the granulocyte and monocyte phagocytosis and OB activity assay described in this manuscript. One such limitation is the exclusive use of FSC and SSC to identify the cell populations of interest. The use of cell surface markers would allow researchers to positively identify cell populations.¹⁰ However, the addition of this feature would require more than a basic flow cytometer, and is thus beyond the scope of this manuscript. Another limitation of this assay is that it relies on the efficacy of trypan blue to quench the fluorescence of probes that have not been internalized by the immune cells of interest. A pH-sensitive molecular probe^{7,12} can be used as an alternative to FITC in situations where internalization is a concern. Since such a probe is only fluorescent in acidic environments such as those in endocytic vesicles, the acquired fluorescence represents internalized particles only. In addition, several researchers have criticized the current assay because it does not include a parallel set of samples that is incubated in ice water instead of at 37 °C to control for baseline immune cell activity. On several occasions, we included this "ice condition" when performing the granulocyte and monocyte phagocytosis and OB activity assay, and found that it does not significantly improve the quality of the data (unpublished).

In summary, this manuscript describes a technique for measuring phagocytosis and OB activity in granulocytes and monocytes. This is a simple, straightforward assay that can be easily modified to account for laboratory capabilities and research interests. In exercise science- and obesity-related research, this measure of innate immune function will allow the investigator to explore the effects of many potential countermeasures, including weight loss, supplement usage, dietary interventions, and other lifestyle changes. Upon mastering this technique, researchers will be able to monitor acute and chronic changes in immune cell function in response to diet, exercise, and many other stimuli.

Disclosures

The authors have nothing to disclose.

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References

1. Nieman, D. C. *et al.* Influence of pistachios on performance and exercise-induced inflammation, oxidative stress, immune dysfunction, and metabolite shifts in cyclists: a randomized, crossover trial. *PLoS One*. **9**, e113725 (2014).
2. Knab, A. M. *et al.* Effects of a freeze-dried juice blend powder on exercise-induced inflammation, oxidative stress, and immune function in cyclists. *Appl Physiol Nutr Metab*. **39**, 381-385 (2014).
3. Konrad, M. *et al.* The acute effect of ingesting a quercetin-based supplement on exercise-induced inflammation and immune changes in runners. *Int J Sport Nutr Exerc Metab*. **21**, 338-346 (2011).
4. Nieman, D. C. *et al.* Immune and inflammation responses to a 3-day period of intensified running versus cycling. *Brain Behav Immun*. **39**, 180-185 (2014).
5. Nieman, D. C. *et al.* Carbohydrate supplementation affects blood granulocyte and monocyte trafficking but not function after 2.5 h of running. *Am J Clin Nutr*. **66**, 153-159 (1997).
6. Tidball, J. G. Inflammatory cell response to acute muscle injury. *Med Sci Sports Exerc*. **27**, 1022-1032 (1995).
7. McFarlin, B. K., Williams, R. R., Venable, A. S., Dwyer, K. C., & Haviland, D. L. Image-based cytometry reveals three distinct subsets of activated granulocytes based on phagocytosis and oxidative burst. *Cytometry A*. **83**, 745-751 (2013).

8. Liao, Y., Liu, P., Guo, F., Zhang, Z. Y., & Zhang, Z. Oxidative burst of circulating neutrophils following traumatic brain injury in human. *PLoS One*. **8**, e68963 (2013).
9. McFarlin, B. K., Venable, A. S., Prado, E. A., Henning, A. L., & Williams, R. R. Image-based flow cytometry technique to evaluate changes in granulocyte function in vitro. *J Vis Exp*. (2014).
10. Nieman, D. C. *et al.* Immune response to obesity and moderate weight loss. *Int J Obes Relat Metab Disord*. **20**, 353-360 (1996).
11. Nieman, D. C. *et al.* Immune function in female elite rowers and non-athletes. *Br J Sports Med*. **34**, 181-187 (2000).
12. Ichii, H. *et al.* Iron sucrose impairs phagocytic function and promotes apoptosis in polymorphonuclear leukocytes. *Am J Nephrol*. **36**, 50-57 (2012).
13. Gomes, A., Fernandes, E., & Lima, J. L. Fluorescence probes used for detection of reactive oxygen species. *J Biochem Biophys Methods*. **65**, 45-80 (2005).
14. Gruger, T. *et al.* Negative impact of linezolid on human neutrophil functions in vitro. *Chemotherapy*. **58**, 206-211 (2012).
15. Dementhon, K., El-Kirat-Chatel, S., & Noel, T. Development of an in vitro model for the multi-parametric quantification of the cellular interactions between *Candida* yeasts and phagocytes. *PLoS One*. **7**, e32621 (2012).