BECKMAN COULTER
Life Sciences

Phagocytosis of Bacteria in a Whole Blood Assay: The Influence of Opsonization on Monocyte and Granulocyte Phagocytosis

Data kindly provided by Andreas Spittler, MD, of the Center of Translational Research, Medical University of Vienna.

Introduction

Human health relies on several inborn protections from illness-inducing organisms. These defense mechanisms take several forms – including physical barriers, such as the skin, mucous membranes, and their cilia, the inflammatory cascade, as well as a cadre of cellular host-defense systems – collectively known as the immune system. Cellular immunity is divided into two classes, the innate immune system and the adaptive immune system. The innate immune system is tuned to providing a quick, knee-jerk, nonspecific response to invading organisms, and it relies heavily on neutrophil-, granulocyte-, and monocyte-mediated recognition and clearance of the threat. Diminished phagocytic activity in these cells can render the immune system less efficient at squelching infection. Understanding how key immune cell populations respond to and protect against non-self cells is essential to better understanding how we may exploit those cells for human health.

In the innate immune system, phagocytosis is the primary process by which cells identify, isolate, and destroy invaders by engulfing and digesting them. Among the phagocytic cells of the immune system, neutrophilic granulocytes (neutrophils) are the most abundant type of leukocyte (white blood cells) in mammalian circulation and the first line of defense against invading microorganisms. Of the leukocytes, monocytes are the largest and are capable of differentiating into macrophages

Objectives

- Discover how the various cells of the adaptive immune system respond to opsonization
- Learn to evaluate phagocytosis in single cells using flow cytometry
- Determine optimal *E. coli*-to-cell ratios and assay conditions for optimal data interpretability
- Follow along with step-by-step gating instructions for acquiring multiparameter data across the cell types analyzed

Opsonization adorns invading cells, like bacteria, with molecular pro-phagocytosis signals, enhancing their uptake and clearance. This process specifically tags foreign cells with opsonin molecules, such as circulating antibodies or complement proteins. Analogous to molecular "handles," opsonins strengthen binding between immune cells and their target cells to increase their visibility to the immune system, increasing the likelihood of phagocytosis (Figure 1). Opsonization is, indeed, essential to the efficient functioning of the innate immune system, and this application note demonstrates the important role that opsonisation plays in phagocytosis.

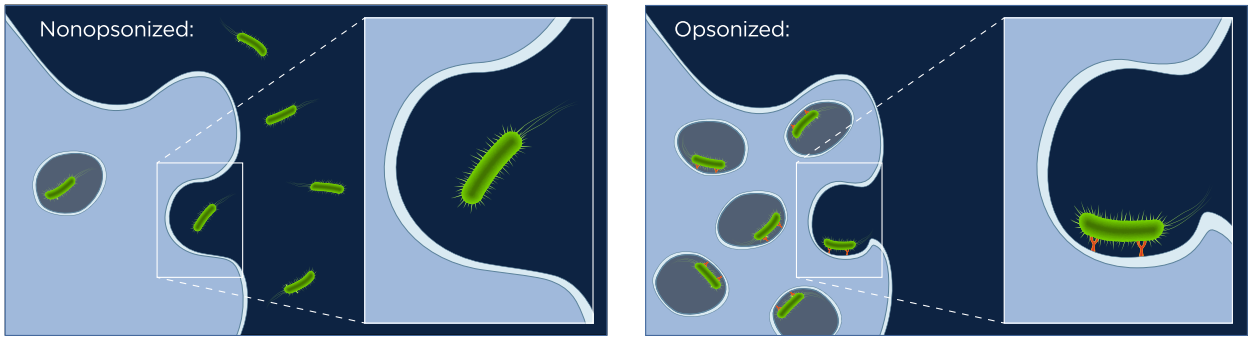

Figure 1. How opsonins increase the efficiency of phagocytosis. In the absence of opsonization (left panel), phagocytic cells are slower to respond to foreign cells (green), although they do – eventually – engulf and destroy the invaders. Nonopsonized phagocytic events (left inset) are somewhat stochastic, relying on the speed of the phagocyte and the mobility of the prey. When the invading cells are opsonized (right panel), the rate and efficiency of phagocytosis is markedly improved. The difference is largely due to the anchoring effect of opsonins (red) on foreign cells, bringing them closer to the immune cell's membrane and facilitating phagocytosis (right inset).

There are many effective methods to evaluate phagocytosis, such as enzyme-linked immunosorbent assay (ELISAs) and Western blotting, however, these assays look at cell populations as a whole and require substantial hands-on time. Flow cytometry enables a detailed analysis of the progression of phagocytosis in a population of cells, bringing the advantage of high-throughput, single-cell analysis. Multicolor flow cytometry offers an even higher throughput, while increasing the number of targets analyzed in a single run.[1] Fluorescent labeling of cell-surface markers enables the quantification of specific immune cell types (Table 1).

Table 1. Cell-surface markers for the detection of immune cells

Cell Marker	Target Cell
CD3*	Mature T cells
CD10	Immature B cells
CD13, CD33, CD117	Myeloid cells
CD14*	Monocytes
CD16, CD56*	Natural killer (NK) cells
CD45*	All leukocytes

*Demonstrated in this application note.

As demonstrated in Table 1, above, a variety markers either expressed on the cell surface or bound at the cell surface can promote the phagocytic capability of specific cells of the immune system. For example, neutrophils and monocytes express surface receptors that increase the efficiency of phagocytosis when the bait is coated with immunoglobulin or complement. This is, perhaps, most relevant to human health when, due to defects in the expression of complement receptors on granulocytes and/or macrophages, patients have a diminished immune response and increased or recurrent infections.[2] In patients with multiple or long-lasting infections, a patient's population of monocytes might become exhausted or depleted, setting up an increased likelihood of future infections.[3] In neonates, the phagocytic capacity of monocytes is inversely correlated with gestational age, and the occurrence of sepsis correlates with massively depleted monocyte phagocytic capacity.[4, 5] This correlation, however, is not a hard and fast rule, as infections can also cause an upregulation of phagocytosis.[6] Circulating monocytes and neutrophils provide insight into the function of the innate immune system via their measured phagocytic capacity.

This application note demonstrates the use of multicolor flow cytometry for the analysis of phagocytosis. Analyzing both opsonized and nonopsonized *E. coli*, the influence of opsonization and temperature on phagocytic activity of whole blood immune cells is assessed. Opsonized *E. coli* are precoated with immunoglobulin and complement, thus providing optimal conditions for phagocytosis in vitro. Both opsonized and nonopsonized *E. coli* are tagged with fluorescein isothiocyanate (FITC), a green fluorescent compound. These analyses will determine whether or not opsonisation has an impact on whole blood-derived immune cell phagocytosis across immune-cell types.

Materials

- Heparinized blood collection tube
- Anti-CD45 Krome Orange (Beckman Coulter, Brea, CA) Cat. # A96416 (100 µg/mL)
- Anti-CD14 PC7 (Beckman Coulter, Bream CA) Cat. # A22331 (12.5 µg/mL)
- Anti-CD3 APC (Beckman Coulter, Brea, CA) Cat. # IM2467U (12.5 µg/mL)
- Anti-CD56 PE (Beckman Coulter, Brea, CA) Cat. # IM2073U (3.0 µg/mL)
- VersaLyse™ Lysing Solution (Beckman Coulter, Brea, CA) Cat. # A09777
- PHAGOTEST reagent kit (Glycotope™ Biotechnology, Heidelberg Germany)
- Phosphate-buffered saline (PBS) without Ca^{2+}/Mg^{2+}
- CytoFLEX™ Flow Cytometer (Beckman Coulter, Brea, CA)
- Vortex mixer
- 37 °C water bath (with digital thermometer)
- Laboratory standard cell counting method

Protocol

1. Using a heparinized blood-collection tube, draw a whole-blood sample.
2. Immediately place 500 µL of the whole-blood sample on ice for 30 min to attenuate the metabolism of the cells.
3. Place the opsonized and nonopsonized *E. coli* from the PHAGOTEST Kit on ice, in the dark, for at least 30 min.
4. Label four 5 mL (12 x 75 mm) as follows: '37-O' (positive, opsonized), '4-O' (negative, opsonized), '37-NO' (positive, nonopsonized), and '4-NO' (negative, nonopsonized).
5. Enumerate leukocytes using the Vi-CELL® Cell Counter or your standard laboratory method.
6. Aliquot 100 µL of precooled whole blood into each flow tube, and place on ice.
7. Vortex the opsonized and nonopsonized *E. coli* vigorously to ensure dispersion.
8. Pipet 20 µL of opsonized FITC-labeled *E. coli* into each of the flow tubes labeled '37-O' and '4-O'.
9. Pipet 20 µL of nonopsonized FITC-labeled *E. coli* into each of the flow tubes labeled '37-NO' and '4-NO'.
10. Incubate the flow tubes labeled '37-O' and '37-NO' at 37 °C (positive samples), and incubate the flow tubes labeled '4-O' and '4-NO' on ice (4 °C, negative controls). Incubate both sets of tubes for precisely 20 min, in the dark.
11. At the end of the 20-minute incubation, place the positive samples on ice.
12. Add anti-CD45 KrO, anti-CD14 PC7, anti-CD3 APC, and anti-CD56 PE to each blood sample as an antibody cocktail, and incubate for 20 minutes on ice, in the dark.
13. Add 1 mL of VersaLyse Lysing Solution to all four samples, and immediately vortex for 1 second.
14. Incubate for 10 minutes at room temperature (18 to 25 °C), in the dark.
15. Wash the cells three times with PBS without Ca^{2+}/Mg^{2+} at 350 g for 5 minutes in a benchtop centrifuge like the Allegra® X-12R from Beckman Coulter Life Sciences.
16. Completely resuspend the pelleted cells in 250 µL of 1X PBS, and then place on ice.
17. Analyze the cells immediately on the CytoFLEX Flow Cytometer using the standard instrument setup and filter configuration. Refer to the gating strategy detailed in Figures 2 and 3.

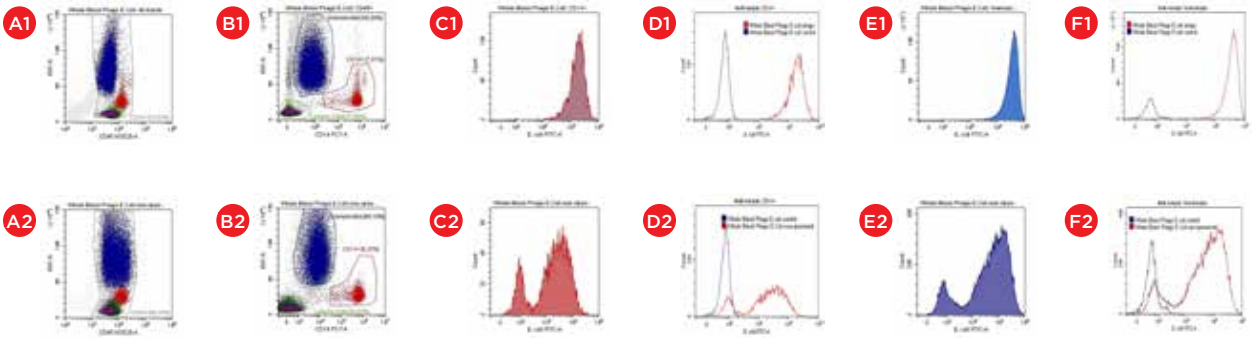

Figure 2. Phagocyte gating strategy. The gating strategy for the analysis of immune-cell phagocytosis of opsonized (top row) and nonopsonized (bottom row) *E. coli* was as follows: The initial gate applied was on CD45-positive events (A1, A2), which identified all leukocytes. Based on the CD45+ population, cells were then gated to identify populations of monocytes (CD14+), granulocytes (Granulocytes), and lymphocytes (Lympho Gate), in green (B1, B2). A histogram compared the relative counts of FITC-A+ monocytes, indicating the total amount of active phagocytosis in the opsonized (C1) versus the nonopsonized cocultures (C2). Subsequently, *E. coli* fluorescence in the FITC-A channel was used to identify the relative proportion of monocytes that had phagocytosed bacteria (D1, D2). The negative control was incubated at 4 ˚C (blue histogram) and the positive control was incubated at 37 ˚C (red histogram). Likewise, the phagocytic activity of granulocytes was assessed (E1, E2), and demonstrated a phagocytic response similar to the monocytes (F1, F2).

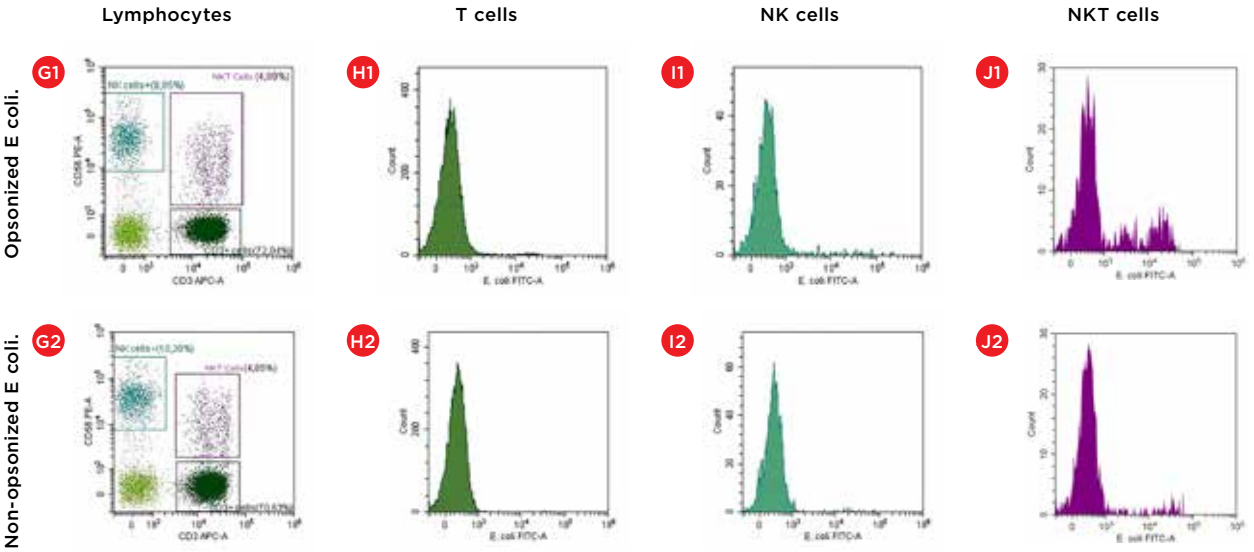

Figure 3. Lymphocyte gating strategy. The gating strategy for identifying phagocytosis of opsonized (top row) and non-opsonized (bottom row) *E. coli* by non-phagocytic immune cells was as follows: Gated lymphocytes (Lympho Gate) were applied to a CD3 X CD56 dot-plot (G1, G2). Gates were set corresponding to CD3+ cells (T-lymphocytes), CD56+ cells (NK cells), and CD3+/CD56+ cells (NKT cells). Data for the T lymphocytes (H1, H2), NK cells (I1, I2), and NKT cells (J1, J2) were gated against *E. coli* fluorescence in the FITC-A channel.

Results and Discussion

Initial gating determined that both the opsonized and nonopsonized whole-blood samples were composed of ~80% (64.93% and 96.41%, respectively) leukocytes, indicating that lysis and removal of red blood cells from the samples had been effective (Figure 2, panels A1 and A2). The relative abundance of granulocytes, lymphocytes, and monocytes was similar across opsonized and nonopsonized samples (Figure 2, panels B1 and B2), consistent with expectation since the same blood sample was used for both analyses.

Analysis of *E. coli* fluorescence identified a single peak for monocytes that had been fed opsonized *E. coli* (Figure 2, panel C1), and two distinct peaks in the nonopsonized samples (Figure 2, panel C2). The opsonized *E. coli* was greater in count and intensity than was the nonopsonized sample (Figure 2, panels D1 and D2). This was consistent with expectations, as opsonization increases the binding strength between phagocytes and their targets. Opsonization not only enhanced phagocytosis in monocytes, but led to the apparent disappearance of the *E. coli* population (Figure 2, panel D1). In the nonopsonized sample (Figure 2, panel D2), the smaller peak indicates the proportion of *E. coli* that was not phagocytosed, which can be seen when compared with the negative control. However, the larger, opsonized peak reveals that, even without opsonization, a baseline level of phagocytosis occurs in the monocyte population.

The temperature dependence of phagocytosis in monocytes is depicted in Figure 2, panels D1 and D2, as neither opsonized nor nonopsonized *E. coli* were phagocytosed by the monocytes at 4˚C (blue line); these data suggest that all analyses of phagocytosis must be conducted under strictly controlled conditions. Analysis of the granulocyte population similarly showed both a temperature dependence and an increase in phagocytosis of opsonized vs. nonopsonized *E. coli* (Figure 2, panels E and F).

Across the nonphagocytic cell types (Figure 3, panels G1 and G2), quantification of T lymphocytes (H1, H2), NK cells (I1, I2), and NKT cells (K1, K2) identified that populations were consistent across opsonized (top panels) and nonopsonized (bottom panels) samples, and only trace amounts of phagocytosis were seen in these populations. This is to be expected, as none of the cells are phagocytes.

The breakdown of cell types found in each whole-blood sample are listed in Table 2, confirming that the proportion of cell types was maintained across samples following treatment with VersaLyse to preferentially remove erythrocytes. The difference between total non-erythrocyte cells in the opsonized vs. nonopsonized samples underscores the importance of normalizing assays by removing nonessential cells before analysis.

Table 2. Relative proportions of non-erythrocyte blood cells in whole blood in the presence of opsonized and nonopsonized *E. coli*

Whole-blood Cell-type Breakdown	Nonopsonized *E. coli*	Opsonized *E. coli*
Total non-erythrocyte	96.41%	64.93%
Monocytes	8.26%	7.01%
Granulocytes	60.16%	60.59%
Lymphocytes	28.89%	27.99%
T lymphocytes	70.63%	72.04%
NK cells	10.20%	8.05%
NKT cells	4.85%	4.89%

Tips for success

- Prepare the heparinized whole-blood sample for analysis immediately after collection. This protocol is optimized for use with heparin and may not perform as expected with other anticoagulants.
- Keep all samples containing the FITC-labeled *E. coli* in the dark at all times to prevent photobleaching.
- The concentration of *E. coli* is calculated for a normal leukocyte count of 4,000 – 10,000 leukocytes/µL. Adjust the concentration of *E. coli* and/or whole blood when the leukocyte count is outside of this range.
- VersaLyse only lyses the red blood cells. It must be used at room temperature (18 to 25 °C). Allow to equilibrate to room temperature and let stand for 1 hour prior to use.
- Phagocytes incubated at 37 °C vs. 4 °C will have different size and granularity, and this difference must be accounted for during gating.

Conclusions

Opsonization effectively amplifies the innate immune system's response to bacterial challenge, increasing the phagocytic efficacy of whole-blood derived immune cells. The effect of opsonization is robust, as demonstrated by a complete clearance of opsonized bacteria from the 'positive/opsonized' cocultures. Flow cytometry enables precise analysis of the influence of opsonization on the efficiency of phagocytosis on a single-cell level. This application note successfully demonstrates the use of FITC-labeled *E. coli* to monitor the progression of phagocytosis in opsonized and nonopsonized cells with the CytoFLEX™ Flow Cytometer, enabling the generation of valuable results within 90 minutes.

Notes

The results demonstrated in this application sheet represent those generated on the Beckman Coulter CytoFLEX™ Flow Cytometer. As differences exist in the performance between analyzers, the author cannot guarantee a similar appearance with the use of other flow Cytometers.

References

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