NovoCyte® Flow Cytometer

Application Note No. 18

Examining the Kinetics of Neutrophil Phagocytosis by Flow Cytometry

The immune system has evolved specialized cells which engulf and kill invading pathogens in a process known as phagocytosis. These cells, known as phagocytes, are essential for elimination of dangerous microorganisms as well as apoptotic bodies from the host. Phagocytosis can be divided into two key stages: the uptake of particles by ingestion, followed by clearance of the particle by degradation. Initiation of phagocytosis begins with the interaction of receptors on the phagocyte with ligands on the target particle. These ligands can be components of the target particle, such as lipopolysaccharide (LPS), or host proteins that coat a target particle and are recognized by phagocytes, also known as opsonization. Proteins used for opsonization include immunoglobulins, components of the complement cascade, and other serum proteins. There are several types of phagocytes including neutrophils, macrophages, dendritic cells, and others. Neutrophils account for 50-70% of circulating leukocytes and are the first line of defense against many pathogens. During an inflammatory response neutrophils are rapidly recruited to the site of infection and fight pathogens by both phagocytosis and release of antimicrobial molecules. Neutrophils are extremely efficient phagocytes that can internalize IgG opsonized targets in less than 20 seconds. Therefore, studying neutrophil phagocytosis is essential to understand the initial host response to pathogens.

Two methods for measuring phagocytosis are routinely used, microscopy and flow cytometry. Both techniques measure the capability of phagocytic cell to ingest a fluorescent particle (labeled microorganisms or beads). There are several advantages to using flow cytometry to measure phagocytosis. Microscopy requires time consuming manual counting of ingested particles, which limits the total number of cells analyzed. Conversely, flow cytometry can analyze thousands of cells per second facilitating statistical analysis while offering multi-parametric measurements for simultaneous evaluation of neutrophil features. Finally, neutrophils are easy to prime and activate by manipulation in the lab, which is a necessary process for microscopic analysis of phagocytosis. Flow cytometry allows the measurement of neutrophil phagocytosis in white blood cells without creating artifacts and therefore may be more representative of phagocytosis *in vivo*. In this application note, we measured neutrophil phagocytosis on the NovoCyte flow cytometer under various conditions and quantified the effect of a potent actin remodeling inhibitor, cytochalasin D.

Neutrophil phagocytosis increases both with incubation time and particle concentration

Cell phagocytosis can be measured using flow cytometry by quantifying the engulfment of fluorescent particles. These studies often use cultured bacteria, yeast polysaccharides, or latex/polystyrene beads as the target particle for phagocytosis. Particles opsonized by immunoglobulins are recognized by Fcγ receptors that specifically interact with the Fc portion of IgG resulting in receptor activation and the initiation of phagocytosis. The kinetics of neutrophil phagocytosis were measured by adding IgG coated FITC-labeled latex beads to leukocytes and quantifying the percentage of cells that had engulfed the beads **(Figure 1)**. Under these conditions, phagocytosis of opsonized beads steadily increases over time, reaching ~70% at 160 minutes. Increased phagocytosis also results in an increase in cell side scatter (SSC) of neutrophils due to the fact that the cells have ingested solid beads **(Figure 1A)**. The phagocytosis of control beads did not change over time indicating these results are due to specific IgG mediated phagocytosis.

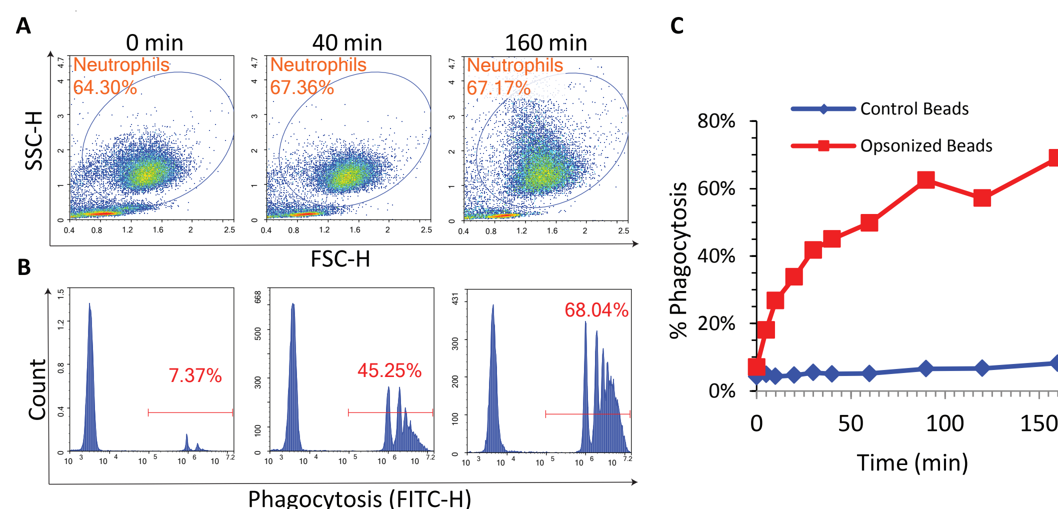

Figure 1: Increased Neutrophil Phagocytosis with incubation time 2µm FITC latex beads coated with IgG were mixed with isolated white blood cells (WBCs) at a ratio of 10:1. Cells were incubated for the indicated times (0-160 minutes) then washed with cold PBS + 0.02% EDTA followed by addition of Trypan blue to quench extracellular fluorescent signal. Monocytes adhered to plate leaving only granulocytes and lymphocytes. Lymphocytes are eliminated by FSC (A). Representative plots of phagocytosis of opsonized beads at 0, 40, and 160 minutes are shown for FSC/SSC (A) and FITC (B). Kinetic data for frequency of phagocytic cells is shown for both opsonized (Red) and control FITC Beads (Blue) (C).

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Next, we examined how an increased ratio of target beads to neutrophils affects the rate of phagocytosis **(Figure 2)**. Phagocytosis increases linearly with an increased ratio of opsonized beads to neutrophils. Almost 90% of cells had undergone phagocytosis at a ratio of 50:1 where an apparent increase in SSC is observed **(Figure 2A)**. However, when the ratio of beads to neutrophils is greater than 10:1 a noticeable increase in phagocytosis occurs in the control beads, reaching 20% phagocytic cells at 50:1. This data indicates that the ideal ratio of beads to neutrophils that should be used for these assay conditions is 10:1, to avoid an increase in nonspecific phagocytosis of target beads.

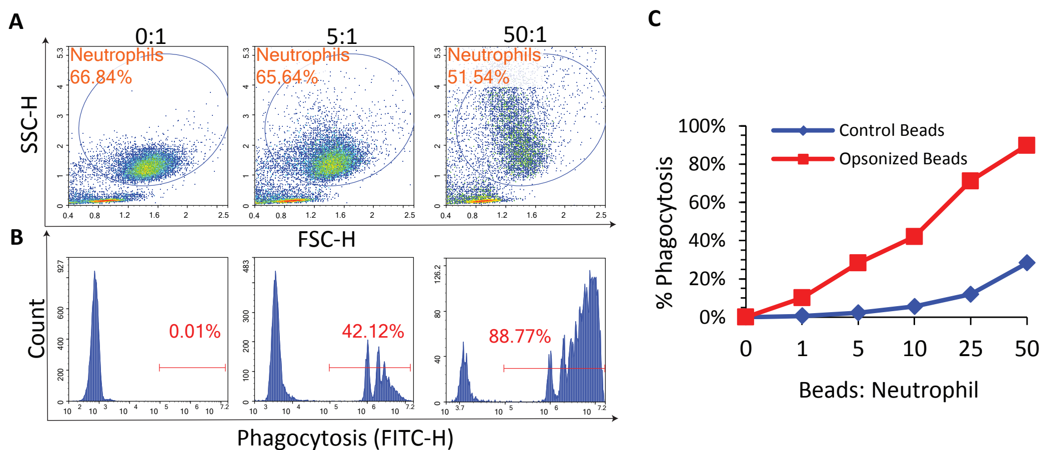

Figure 2: Neutrophil phagocytosis increases with increased bead to cell ratio. 2um FITC latex beads coated with IgG were mixed with isolated WBCs at the indicated ratios beads to neutrophils for 40 minutes, then washed with cold PBS + 0.02% EDTA followed by addition of Trypan blue to quench extracellular fluorescent signal. Representative plots for ratios at 0:1 (no beads), 5:1, and 50:1 are shown for FSC/SSC (A) and FITC (B). Frequency of phagocytic cells are quantified for both opsonized (Red) and control FITC Beads (Blue) (C).

Inhibition of phagocytosis with cytochalasin D

A variety of conditions and agents have been reported to impair phagocytosis, including inhibition of actin rearrangement, which is necessary during the uptake of large particles. Cytochalasins are antagonists of actin remodeling and commonly used to inhibit phagocytosis, therefore, the effect of cytochalasin D on neutrophil phagocytosis was examined. Increasing concentrations of cytochalasin D dramatically inhibited neutrophil phagocytosis, which was nearly halved at 370nM as the IC50 was calculated to be 0.439µM **(Figure 3)**. Complete ablation of phagocytosis was achieved with 10uM cytochalasin D, and a dose dependent effect was observed. This data demonstrates that cytochalasin D has a strong concentration-dependent inhibitory effect on phagocytosis and measurements by flow cytometry are specific and sensitive enough for these types of assays.

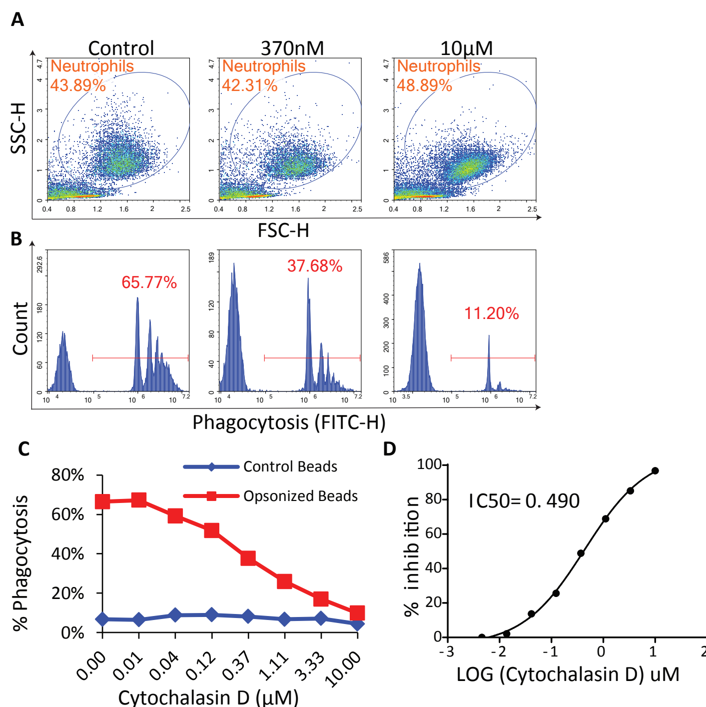

Figure 3: Neutrophil phagocytosis decreases with the addition the actin remodeling inhibitor, cytochalasin D. 2µm FITC latex beads coated with IgG were mixed with isolated WBCs at a ratio of 20:1 with indicated concentrations of cytochalasin D. Cells were incubated for 30 minutes, then washed with cold PBS + 0.02% EDTA followed by addition of Trypan blue to quench extracellular fluorescent signal. Representative plots of control (no inhibitor), 370nM, and 10µM cytochalasin D are shown for FSC/SSC (A) and FITC (B). The frequency of phagocytic cells are shown for both opsonized (Red) and control FITC Beads (Blue) (C). cytochalasin D concentration-dependent inhibitory effect on phagocytosis; IC50 is 0.439µM (D).

Conclusion

In this application note, we investigated the effect of time and bead to cell ratio on neutrophil phagocytosis. Increasing both the time and bead concentrations enhanced IgG induced neutrophil phagocytosis. It was also seen that cytochalasin D is a potent inhibitor, capable of complete abrogation of neutrophil phagocytosis. Measuring phagocytosis with the NovoCyte flow cytometer allows rapid analysis of millions of cells, making results easier to achieve.

References

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