Phagocytosis of FITC labelled opsonized and non-opsonized *E. coli* bacteria by monocytes and granulocytes in a whole blood assay

APPLICATION NOTE

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IN THIS PAPER YOU WILL

- Learn how to use flow cytometry to evaluate phagocytosis in monocytes and granulocytes.
- Learn optimal E. coli to cell ratios and assay conditions to make data interpretable.
- Learn how easy it is to use the compensation library feature enabled by CytoFLEX flow cytometer fiber array diodes
- Step-by-step instructions for instrument set up for acquiring multiparameter data

Principal of the Technique

Background

Phagocytosis is a process by which cells ingest or engulf particles or other cells. It is an essential part of the innate immune response and therefore necessary for defensive reactions against invasion of the body by foreign antigens. Neutrophilic granulocytes are the first defense line against invading microorganisms. They have a high capacity to engulf bacteria and to destroy these particles by intracellular mechanisms such as oxygen radical production. Circulating monocytes are also capable to ingest bacteria and other particles. In addition to neutrophils monocytes have the possibility to present small peptides of the digested particles on their surface in combination with products of the major histocompatibility complex (MHC).

Neutrophils and monocytes express on their surfaces receptors which make the recognition and the ingestion of with immunoglobulin and/or complement coated particles more effective. However changes of the phagocytic activity can lead to reduced phagocytic capacities and therefore to dramatic clinical situations. For example defects in the expression of complement receptors on granulocytes and/or macrophages can lead to a reduced phagocytic activity and subsequently to recurrent infections in these patients. [1] In patients with long lasting infections the monocyte fraction might be exhausted which also leads to a diminished phagocytic activity.[2] In neonatal children it has been reported that the phagocytic capacity of monocytes is reversely dependent on gestational aging and that the appearance of sepsis correlates with a massively diminished monocyte phagocytic capacity.[3,4] On the other hand at the beginning of infections from time to time an enhancement of phagocytosis can be observed.[5] Therefore the determination of the phagocytic capacities of both, circulating monocytes and neutrophils give a profound insight in the function of the innate immune system.

BECKMAN COULTER Life Sciences

Research Applications

Introduction

Flow cytometry is an ideal method to determine the phagocytic activity of neutrophils and monocytes in whole blood. The method is easy to perform and provides results within 90 minutes. The test kit used here has been previously developed without the possibility to additionally stain surface antigens. In the original test kit quenching solution is provided to eliminate the fluorescence signal which is set free from the FITC dye of *E. coli* bacteria which are bound to the cell surface but not ingested into the cell. The staining with antibodies has the big advantage that populations can be much better discriminated instead of gating via the scattergram. In the following protocol non-opsonized and opsonized *E. coli* bacteria were used and compared. *E. coli bacteria* which are opsonized are coated with immunoglobulin and complement. This allows better phagocytic properties than using non-opsonized *E. coli* bacteria. However using non-opsonized particles give the plainest results since these particles have to be first opsonized by the immunoglobulins from the circulating blood before particles can effectively be ingested. When opsonized particles are used the best phagocytic starting conditions are provided to the cells which possibly might not be relevant in the overall context.

Protocol

Standard Procedure

1. Draw heparinized whole blood and process the blood immediately.
2. Precool 500 µL of whole blood on ice for 30 minutes to delimitate the metabolism of the cells.
3. Prepare two flow tubes (75 mm x 12 mm) and put them on ice. Label one with '+' (positive sample) and the other one with '-' (negative control on ice).
4. Enumerate leukocytes per lab method: ViCell, Hemacytometer, or automated instrumentation.
5. Add 100 µL of precooled whole blood to each tube and incubate with 20 µL of opsonized or non-opsonized FITC labeled E. coli bacteria for precise 20 minutes at 37°C (positive sample) and on ice (4°C, negative control).
   Important: The E. coli flask has to be rigorously vortexed before usage. The concentration of E. coli bacteria is calculated for the normal leukocyte count (4,000 – 10,000 leukocytes/µL). Please adjust *E.coli* concentration and/or whole blood when leukocyte count is out of range.
6. After 20 minutes incubation time put the positive sample immediately back on ice.
7. Blood is then stained for 20 minutes on ice with anti-CD45 KrO ,anti-CD14 PC7, anti-CD3 APC and anti-CD56 PE antibodies (all antibodies from Beckman Coulter, Table 1).
8. Red cells are lysed (VersaLyse Lysing Solution, Beckman Coulter)
9. Cells are washed three times with PBS without Ca^{2+}/Mg^{2+} at 350g for 5 minutes (Allegra X-12R Centrifuge, Beckman Coulter) .
10. Cells are immediately analyzed on a CytoFLEX with standard instrument setup and with standard filter configuration.

Table 1

Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	FITC	PE	ECD	PC5	PC7	APC	APC-A700	APC-A750
Marker	CD45						CD56			CD14	CD3		
Clone	J.33						N901 (HLDA6)			RMO52	UCHT1		

Results

Whole blood was incubated and stained as described above. Cells were immediately analyzed on a CytoFLEX. For opsonized and non-opsonized E. coli phagocytosis the gating strategy was the same which is described as follows. In a first step CD45 positive leukocytes were gated as shown in Figures 1 and 2a; Figures 1 and 2b are then gated on CD45+ events. Subsequent gates were set around the CD14+ monocyte population (CD14+), around the granulocytes (Granulocytes) and around the lymphocytes (Lympho Gate). CD14+ monocytes are then the parent populations for the histograms in Figures 1 and 2c. To demonstrate the phagocytic capacity of these cells, an overlay was created which includes the negative population incubated at 4°C (Fig. 1 and 2d). The same was done with the granulocytes as depicted in Figures 1 and 2e and 1 and 2f, respectively. Subsequently the gated lymphocytes were transferred to a new dot plot (Fig. 1.2g) and gates were set according CD3+ and/or CD56+ cells. CD3+/CD56- are T-lymphocytes, CD3-/CD56+ cells are Natural Killer cells (NK cells) and CD3+/CD56+ cells are cytotoxic T-cells. In Figure 1,2h (CD3+ cells), Figure 1/2i (NK cells) and Figure 1,2k (cytotoxic T-cells) only few positive FITC signals are seen which are either due to phagocytosis and/or due to E. coli bacteria bound on the cell surface. As expected the phagocytosis of non-opsonized particles was much lower than of opsonized particles. This effect displays the necessity of the whole blood to opsonize first the bacteria with immunoglobulins and/or complement before they can effectively ingested.

Conclusion

Flow cytometry is a valuable tool to evaluate the innate immune system; however, there are several critical points to consider. Whole blood phagocytosis only works well when heparin is used as anti-coagulant during blood collection. Precooling of the blood is necessary to get a negative control. Also the exact E. coli incubation time (20 minutes), the exact temperature condition (37°C) and the E. coli to leukocyte ratio are indispensable otherwise data obtained are difficult to interpret or repeat. If all critical points are carefully taken into account the E. coli phagocytosis is a functional assay which delivers excellent results and provides profound insight into the innate immune system.

References

1. Varin A, Gordon S. Immunobiology. 2009, 214(7):630-41
2. Xiu F, Jeschke MG. Shock. 2013, 40(2):81-8.
3. Hallwirth U, Pomberger G, Zaknun D, Szepfalusi Z, Horcher E, Pollak A, Roth E, Spittler A. Early Hum Dev. 2002, 67(1-2):1-9
4. Hallwirth U, Pomberger G, Pollak A, Roth E, Spittler A. Pediatr Allergy Immunol. 2004, 15(6):513-6
5. Spittler A, Razenberger M, Kupper H, Kaul M, Hackl W, Boltz-Nitulescu G, Függer R, Roth E. Clin Infect Dis. 2000, 31(6):1338-42

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.

Reagent Details

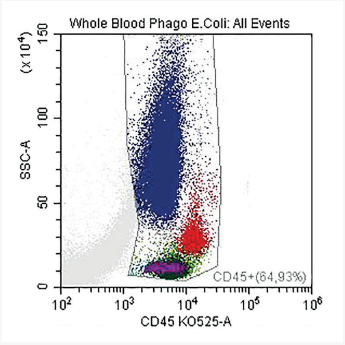
Reagent	Supplier	Order Details
Phagotest	GLYCOTOPE Biotechnology	GLYCOTOPE BIOTECHNOLOGY GmbH Czernyring 22 69115 Heidelberg Germany

Vortex mixer
Water bath for 37°C positive sample incubation
Digital thermometer to control the temperature of the water bath

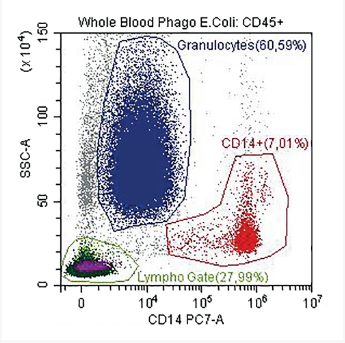
Figure 1:

Phagocytosis of FITC labelled and **opsonized** *E. coli* bacteria in whole blood

1a



1b



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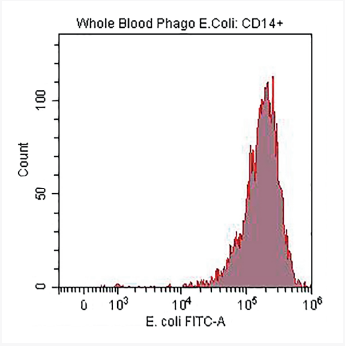

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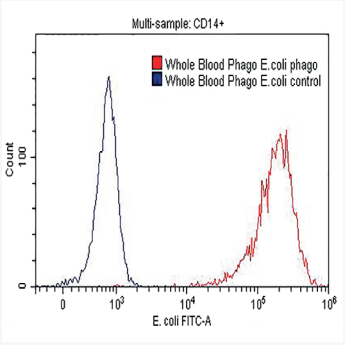
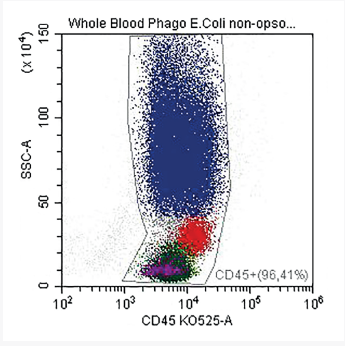


Figure 2:

Phagocytosis of FITC labelled and **non-opsonized** *E. coli* bacteria in whole blood

2a



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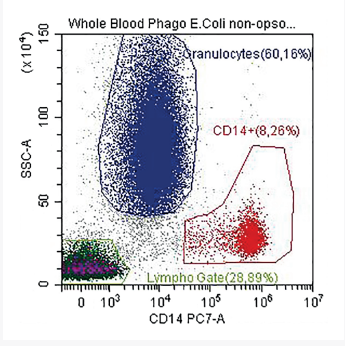

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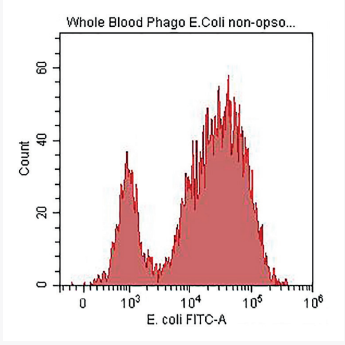

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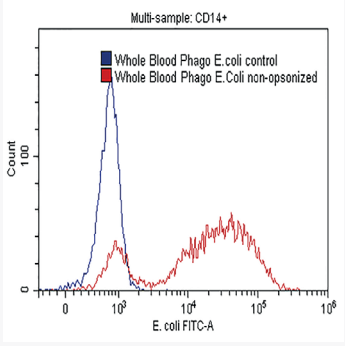
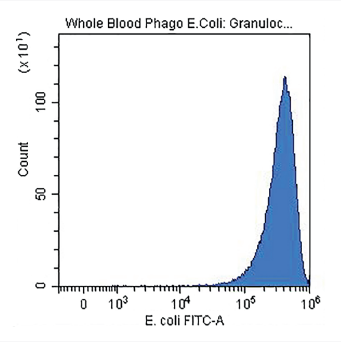


Figure 1:

Phagocytosis of FITC labelled and **opsonized** *E. coli* bacteria in whole blood

1e



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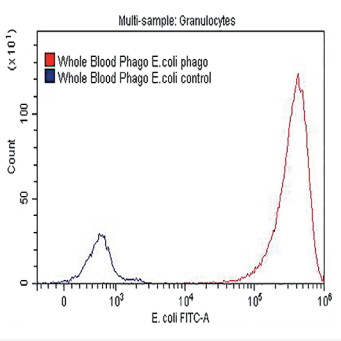

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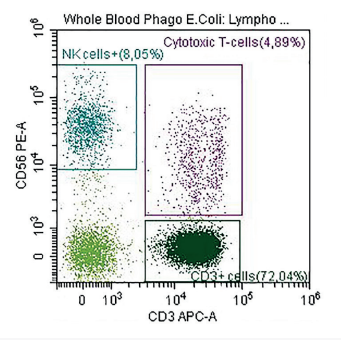

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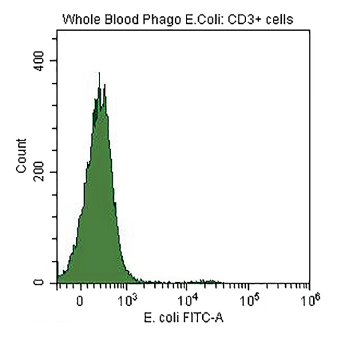
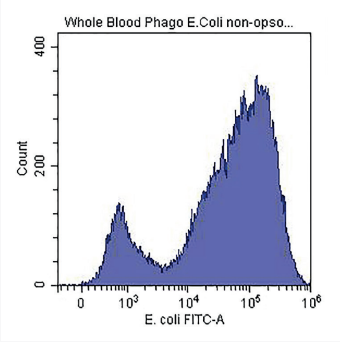


Figure 2:

Phagocytosis of FITC labelled and **non-opsonized** *E. coli* bacteria in whole blood

2e



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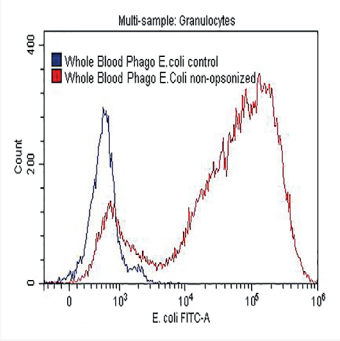

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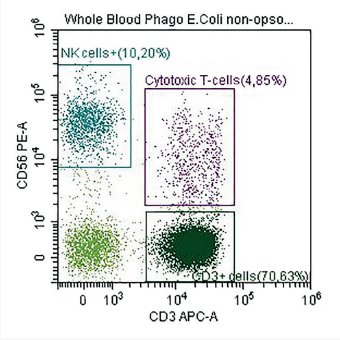

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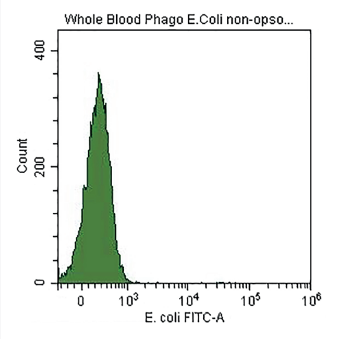
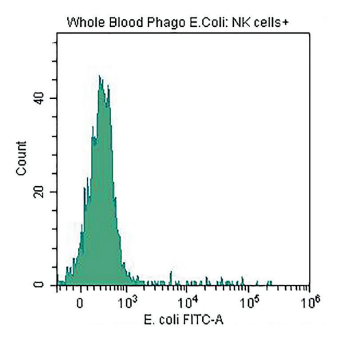


Figure 1:

Phagocytosis of FITC labelled and **opsonized** *E. coli* bacteria in whole blood

1i



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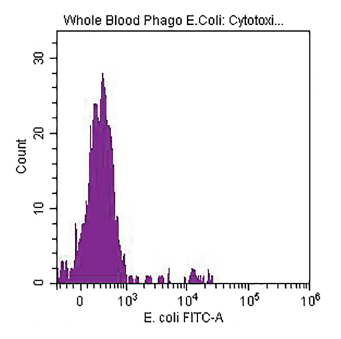
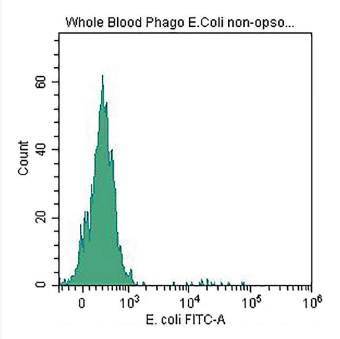


Figure 2:

Phagocytosis of FITC labelled and **non-opsonized** *E. coli* bacteria in whole blood

2i



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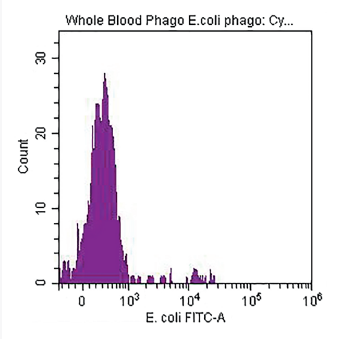

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