Method for analysis of surface molecule alteration upon phagocytosis by flow cytometry

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Summary

In this study, we introduce an application of flow cytometry for the concurrent detection of phagocytotic cells and surface molecules involved in the phagocytic process. *E. coli* expressing green fluorescent protein (GFP) were applied as the phagocytosable particles. Blood samples were incubated with *E. coli* expressing GFP, followed by indirect immunofluorescence using four candidate monoclonal antibodies (mAbs). Granulocytes that had phagocytosed *E. coli* exhibited high levels of GFP intensity, in contrast to the non-phagocytosed cells. By comparing the level of expression of molecules expressed on phagocytosed granulocytes with that of non-phagocytosed cells by flow cytometry, it enabled the determination of the expression and alteration of the cell surface molecules upon phogocytosis. Of the four mAbs used in this study, upon phagocytosis, molecules recognized by mAbs WK13, COSA5A and COSA33NL were up-regulated. However, CD15 recognized by mAb VIMD5 was down-regulated. The proposed method will benefit the study of phagocytic mechanisms in the future. *(Asian Pac J Allergy Immunol 2010;28:170-6)*

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Introduction

Phagocytosis, first observed and defined by Elie Metchnikoff,[1,2] is the process by which phagocytic leukocytes use in taking up large particles such as pathogenic microorganisms into the cells. This process has long been known to play important roles in clearing up pathogenic microorganisms and apoptotic cells, as well as linking innate to acquired immune responses. Simplistically, the phagocytic process involves the recognition of the pathogens, internalization, directing the pathogens towards the phagolysosome for digestion, and exocytosis of the digested particles.[3] During this past decade, the knowledge regarding the molecular mechanism underlying each process has been broadened. The recognition mechanisms are brought about by the interaction between the membrane receptors on phagocytic cells and the ligands on the microorganisms. Several of these receptors have been characterized and their respective ligands have also been identified. Substantially characterized to date are Fc receptors (FcRs) and complement receptors (CRs) that initiate the internalization of the pathogens after binding of the Fc regions of the antibody molecules bound to the antigens in case of FcRs, and the complements that are directly bound to the pathogens or the antibodies specifically bound to the antigens for the CRs.[4-8] Additional receptors involved in the phagocytic process include scavenger receptors (SR), lectins such as the mannose receptor and dectin-1, Toll-Like Receptors (TLRs), as well as other integrins.[9-11] These receptors recognize diverse sets of ligands[3,12,13] encompassing both self and non-self antigens, hence entailing phagocytosis as an integral part of several vital biological systems. Multiple types of phagocytic receptors can be engaged simultaneously during an encounter of a single pathogen, leading to the recruitment and subsequent activation of a wide variety of

signaling molecules.[5-7,14,15] As a result, different signaling pathways are activated and work collectively to ensure an effective elimination of the pathogen by phagocytic cells. Though a large number of receptors and molecules involved in the phagocytic process have been described, many more are expected to be discovered and investigated in order to unravel the complexity of this process. Finding new phagocytic receptors or proteins involved in the regulation of phagocytosis and investigating their roles in phagocytic process, are essential for the understanding of the pathogenesis of infectious diseases, which in turn may provide a basis for the development of improved therapeutic approaches to microbial infections.

An early method used for determining whether the phagocytic process of cells had occurred was the nitroblue tetrazolium reductase test (NBT test).[16] This method utilized the ability of the phagocytic cells to take up nitroblue tetrazolium dye and reduce it to yield a blue crystal product. A more recent technique is flow cytometry[17,18] that determines phagocytosis based on the presence of the fluorochrome-labeled phagocytosable particles that have been taken up inside phagocytic cells. In this study, we introduce the concept of applying flow cytometry in simultaneously detecting the phagocytic cells and the alteration of surface molecules as a consequence of the engulfing process.

Methods

Phagocytosable particles

To construct the GFP expressing bacteria, the gene coding for GFP (GenBank accession number X83959) was amplified from cDNA by PCR using the following primer sequences: forward primer GLICF (5' gac gac gac aag atg agt aaa gga gaa gaa ct 3') and reverse primer GLICR (5' gag gag aag ccc ggt taa ttt gta tag ttc at 3'). The amplified product was cloned into pTriEx-4-EK/LIC vector according to the manufacturer's protocol (Novagen, Darmstadt, Germany), and the resulting vector was transformed into *E. coli* strain Origami™ B(DE3) competent cells (Novagen). The bacteria were grown overnight at 37°C using SB broth (Bio Basic, Ontario, Canada) supplemented with 70 μg/ml kanamycin, and 10 μg/ml tetracycline (Sigma, St. Louis, MO, USA). The bacteria were subsequently heat killed at 60°C for 30 minutes. The fluorescent intensity of the heat-killed bacteria was checked by flow cytometry prior to use. The same strain of *E. coli*, those without a vector or with the pTriEx-4 Ek/LIC vector containing the non-fluorescent protein (F2 domain of P-glycoprotein), were used as negative controls. The bacterial concentration was determined by measuring the optical density of the bacterial suspension at 600 nm.

Antibodies

Monoclonal antibodies (mAb), clones WK13 (IgG), COSA5A (IgG) and COSA33NL (IgM) were generated in our laboratory. The anti-CD15 mAb clone VIMD5 (IgM) was provided by Dr. Stephen Gadd, University of Vienna, Austria. The phycoerythrin (PE)-conjugated goat anti-mouse IgG antibody (stock concentration 0.5 mg/ml) and the PE- conjugated goat anti-mouse IgM antibody (stock concentration 0.5 mg/ml) were purchased from eBioscience. (San Diago, CA, USA). All mAbs used were undiluted culture supernatants. Prior to use, the binding reactivity of each mAb was validated using flow cytometric methods.

Detection of phagocytosis by flow cytometry

One hundred microliters of blood using EDTA as an anticoagulant were mixed with 100 μl of $5x10^8$ cells/ml *E. coli* expressing GFP and incubated at 37°C or on-ice for negative controls for one hour. After incubation, the tubes were immediately placed on-ice. Then, 50 μl of RBC lysing solution [4.5% formaldehyde (Sigma)] and 10% diethylene glycol (Fluka, Buchs, Switzerland) in PBS pH7.2] were added and incubated on-ice for an additional 10 minutes. Subsequently, 1 ml of distilled water was added to complete the lysis. The tubes were centrifuged at 14,000 x g in a microcentrifuge for 10-15 seconds to collect the white blood cells (WBCs). The cells were washed twice with 0.1% NaN_3 in PBS pH 7.2 prior to being resuspended with 1% paraformaldehyde and analyzed by a flow cytometer (Cytomics™ FC500, Beckman Coulter, Miami, FL, USA).

Indirect immunofluorescent staining of cell surface molecules

To study the cellular distribution of the cell surface molecules recognized by the mAbs, 100 μl of EDTA blood were added to 50 μl of the culture supernatant of each mAb and incubated on ice for 30 minutes. All cells were washed twice with 0.1% NaN_3 in PBS pH 7.2, then resuspended with 30 μl of 1% BSA in PBS, pH 7.2 and 25 μl

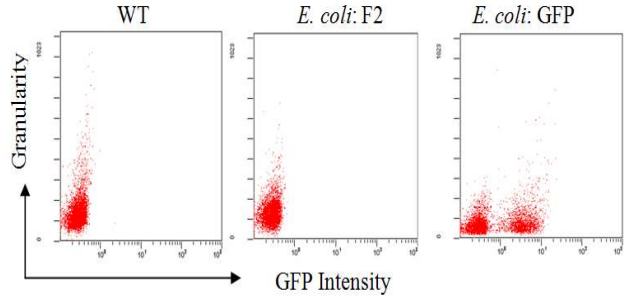

Figure 1. Fluorescence intensity of *E. coli* expressing GFP. The fluorescence activity of the GFP expressed by wild-type *E. coli* (WT), *E.coli* expressing a non-fluorescent protein (F2) (*E. coli*: F2) and *E. coli* expressing GFP (*E. coli*: GFP) was assessed by flow cytometry.

of PE labeled anti-mouse IgG (dilution 1:200) plus anti- mouse IgM (dilution 1:50) antibodies, followed by incubation on ice for 30 minutes in the dark. Then the RBC lysing solution was added and incubated further for 10 minutes on ice, followed by the addition of distilled water. The cells then were washed twice and resuspended with 500 µl of 1% paraformaldehyde and analyzed by a flow cytometer. To study the alteration of cell surface molecules upon phagocytosis, 100 µl of EDTA blood were incubated with 100 µl of $5x10^8$ cells/ml *E. coli* expressing GFP at 37°C for one hour prior to staining with mAbs by the indirect immunofluorescent staining method described above.

Results

Fluorescence intensity of E. coli expressing GFP

The *E. coli* expressing GFP were genetically engineered in our laboratory. Prior to using this *E. coli* as phagocytosable particles, the fluorescent intensity of the GFP expressed by the *E. coli* was assessed by means of flow cytometry. When compared to the wild-type *E. coli* (WT), or the *E. coli* genetically engineered to express non-fluorescent protein (*E. coli*: F2), the *E. coli* expressing GFP (*E. coli:*GFP) exhibited two groups of bacteria, one with non-fluorescence as was observed in WT and *E.* coli:F2, and another with a high fluorescent level (Figure 1.). These results indicated that the GFP reserves its fluorescent activity inside the bacteria and the *E. coli* expressing GFP could be detected by flow cytometry. This GFP expressed *E. coli* were then used in the phagocytosis assay.

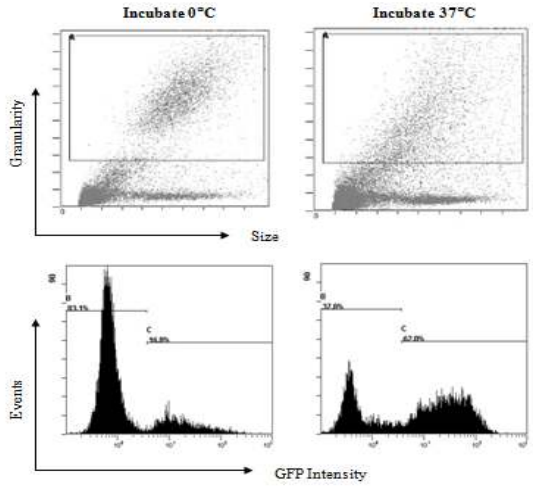

Figure 2. Phagocytosis of the granulocytes determined by flow cytometry. Blood samples from healthy individual were mixed and incubated with *E. coli* expressing GFP at 37°C or on-ice for 60 minutes. Red blood cells were lysed and the remained white blood cells were analyzed by flow cytometry. Granulocytes were gated according to their size and granularity (Top panel). Granulocytes that have ingested bacteria expressing GFP were determined by flow cytometry according to the presence of GFP (Lower panel). This figure represents one of the three experiments using different donors.

Detection of phagocytosis by flow cytometry

We next tested the utilization of *E. coli* expressing GFP as phagocytosable particles to determine the phagocytic activity of the granulocytes via flow cytometry. Blood was incubated with the GFP-expressing *E. coli* either on-ice or at 37°C for one hour and analyzed by flow cytometry. The granulocytic population was gated according to their size and granularity (Figure 2., top panel). Then, the gated cells were evaluated to determine whether they had ingested GFP-*E. coli* by measuring the percentage of cells containing GFP activity. When incubated on-ice, during which time, the cells were presumed to have no phagocytic activity, it was found that the percentages of the granulocytes containing GFP activity in three different subjects were much lower than those of cells incubated at 37°C in which the phagocytic process had occurred (16.8%, 16.4 and 16.8% at on-ice *vs.* 62.0%, 58.4% and 63.3% at 37°C , respectively [Figure 2., bottom panel]). At 37°C incubation, the phagocytosed and non-phagocytosed granulocytes

could be differentiated by our flow cytometric system using *E. coli* expressing GFP as a phagocytosable particle.

Cellular distribution of cell surface proteins recognized by mAbs WK13, COSA5A, COSA33NL and VIMD5

Four mAbs, WK13, COSA5A, COSA33NL, and VIMD5 were used to study the alteration of cell surface proteins on granulocytes upon phagocytosis. To determine whether the granulocytes expressed molecules recognized by the mAbs, indirect immunofluorescent staining was employed. The results of immunofluorescent staining indicated that the granulocytes expressed molecules recognized by all tested mAbs albeit at different levels. The cell surface molecules recognized by mAbs WK13 and COSA5A were expressed at low levels whereas those recognized by mAbs COSA33NL and VIMD5 were expressed at moderate and high levels respectively (Figure 3.).

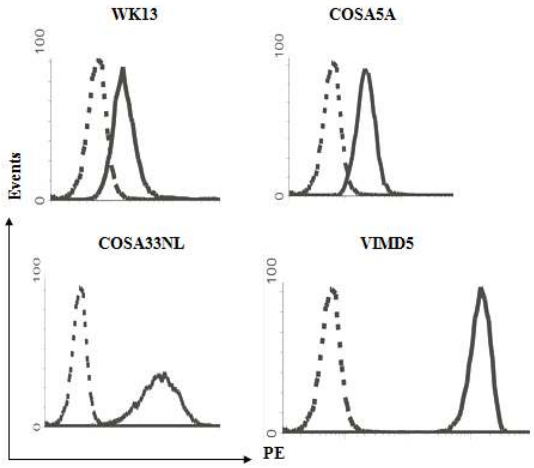

Figure 3. Expression of granulocyte surface molecules recognized by mAbs WK13, COSA5A, COSA33NL and VIMD5. Peripheral blood cells were stained with the indicated mAbs by indirect immunofluorescence using PE-conjugates and analyzed by flow cytometry. Granulocytes were gated according to their size and granularity. Conjugate controls are shown as dashed lines, while granulocytes stained with the indicated monoclonal antibodies are represented by solid lines. A representative result from one of three donors is shown.

Detection of the alteration of cell surface molecules upon phagocytosis

Lastly, we investigated whether the phagocytic activity of the granulocytes caused any alteration of their cell surface molecules. We used the two-color flow cytometric method to detect these two parameters simultaneously. Cells were incubated with *E. coli* expressing GFP at 37°C to initiate the phagocytic process, followed by the immunofluorescent staining with the monoclonal antibody of interest using PE-conjugates. Then the occurrence of phagocytosis and the alteration of cell surface molecules were detected and analyzed by flow cytometry. The granulocytes were first gated based on their size and granularity. Then the gated granulocytes were divided into two groups; the one that has presumably ingested *E. coli*:GFP which exhibited high level of GFP activity (gates G2 and G4 of Figure 4.), and the one that had not ingested *E. coli*:GFP which exhibited low level of GFP activity (gates G1 and G3 of Figure 4.). Subsequently, the changes of the expression of cell surface molecules on the granulocytes that had ingested *E. coli* expressing GFP were analyzed using the expression levels of those molecules on the surface of the granulocytes that had not ingested the *E. coli* expressing GFP as the baseline. The granulocytes that had ingested bacteria and showed the higher level of expression of the cell surface molecules over the base line, indicated an up-regulation of the expression of cell surface molecules upon phagocytosis. On the contrary, those that showed the lower level indicated a down-regulation of the expression upon phagocytosis.

As shown in Figure 4., the occurrence of phagocytic process resulted in an increased expression of cell surface molecules recognized by mAbs WK13, COSA5A and COSA33NL. On the other hand, the level of expression of the CD15 molecule recognized by mAb VIMD5 was found to be decreased upon phagocytosis. These data, therefore, clearly demonstrated that the two-color flow cytometric method can be used to simultaneously measure the phagocytosis of phagocytes and the alteration of their cell surface molecules on the phagocytic process.

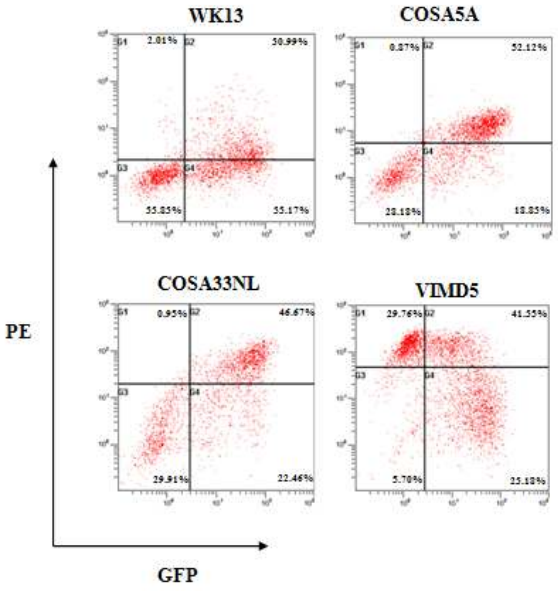

Figure 4. Alteration of cell surface molecules upon phagocytosis. Blood was incubated with *E. coli* expressing GFP at 37°C for one hour prior to staining with the indicated monoclonal antibodies using PEconjugates and the alteration of cell surface molecules on the granulocytes that have phagocytosed bacteria (GPF positive; G2 and 4) and have not phagocytosed bacteria (GFP negative; G1 and 3) was determine by flow cytometry. A representative result from one of three donors is shown.

Discussion

Phagocytosis is an important defense mechanism of the innate immune response. It is a process characterized by phagocytes, mainly macrophages, monocytes and granulocytes, extending their cell membrane to wrap around foreign particles, ingest and destroy them. This process involves several cell surface receptors**,** that upon binding to their respective ligands on the pathogens**,** trigger various signaling pathways that ultimately lead to the clearance of the ingested microorganisms.[3,10] In this study, we have developed a simple flow cytometric method for the determination of the occurrence of phagocytosis and for identifying cell surface molecules involved in the phagocytic process. In our system, we genetically engineered *E. coli* expressing GFP. After proper excitation, these *E. coli* fluoresce green. As expected, by flow cytometric analysis, the generated *E. coli* were detected under the fluorescent detector of flow cytometer (Figure 1.). However, some transformed *E.coli* did not express GFP and showed non-fluorescence. After transformation with the GFP harboring plasmid, GFP expression was induced by IPTG. In our experiments, it is possible that the GFP induction was not done under theoretically optimal conditions. Some of the bacteria, therefore, expressed GFP but some did not. However, this did not affect the phagocytosis experiment. The proportion of GFP expressing and not expressing bacteria was in the ratio of approximately 1:1. In our phagocytosis assay, all bacteria were incubated with phagocytes. As a single phagocyte can phagocytose several bacterial cells, both GFP and non-GFP expressing bacteria were simultaneously affected. As a result, all phagocytosed phagocytes show fluorescence upon flow cytometric determination.

The *E. coli* expressing GFP was, then, used to develop a method for measuring phagocytosis. When the *E. coli* expressing GFP were used as the phagocytosable particles to detect phagocytosis, it was possible to separate granulocytes into two distinct populations; the non-phagocytosed cells and the phagocytosed cells (Figure 2.). The obtained result was a resemblance to what has been seen in other reports using fluorochrome-labeled particles as phagocytosable particles.[18-20] Furthermore, in this study, we introduced a new means of determining the phagocytic activity of cells and identifying cell surface molecule alterations upon phagocytosis, in a single experiment. In illustration, we selected four mAbs against human granulocytes (WK13, COSA5A, COSA33NL, and VIMD5) to test whether the molecules recognized by these mAbs are involved with the phagocytic process by following their expression levels on phagocytosed and non-phagocytosed cells. WK13, COSA5A and COSA33NL were mAbs generated in our laboratory. These monoclonal antibodies react to unknown molecules expressed on granulocytes (Figure 3.). VIMD5 is a CD15 mAb.[21] CD15 molecule is a carbohydrate epitope (3-fucosyl-N-acetyl lactosamine) attached to lipid or protein on the cell membrane and used as a marker for mature granulocytes.[21, 22] Additionally, it was shown that CD15 combined with CD62P and played an important role in cell adhesion.[21, 22]

Furthermore, we studied the alteration of the molecules recognized by the four mentioned mAbs upon phagocytosis. We found that the molecules recognized by mAbs WK13 and

COSA5A were expressed at relatively low levels on the granulocytes before the initiation of phagocytosis, whereas the molecules recognized by mAbs COSA33NL and VIMD5 were expressed at moderate and high levels, respectively (Figure 3.). Phagocytosis was then initiated by incubating blood cells with *E.* coli expressing GFP at 37°C, and the alteration of the expression of these molecules after phagocytosis was determined. To analyze the alteration, by flow cytometric analysis, granulocytes were first divided into two populations based on the presence of GFP. The first population contained low GFP activity**,** which is equivalent to granulocytic cells that had not phagocytosed the *E.* coli. The second population containing high GFP activity were cells that have already phagocytosed *E.* coli. The alteration of the cell surface molecules, as a result of the occurrence of phagocytosis, was then assessed by comparing its expression on the GFP positive and negative populations. We found that molecules recognized by mAbs WK13, COSA5A and COSA33NL expressed at low to moderate levels before phagocytosis were increased on *E. coli* phagocytosed cells. In contrast, the CD15 molecule recognized by mAb VIMD5 that were expressed at a high level before the phagocytosis showed a decreased expression (Figure 4.). The results indicated that these cell surface molecules were phagocytic activation-associated molecules and may be involved in the phagocytic process. CD15 is a carbohydrate epitope strongly expressed on granulocytes.[21,22] This molecule is involved in granulocytic function, including chemotaxis, phagocytosis and respiratory bursts.[21-24] The CD15 monoclonal antibodies could possibly suppress granulocyte phagocytosis of both Fc and Fc+C3b opsonized particles indicating the involvement of CD15 on granulocytic phagocytosis.[23,24] The down-regulation of CD15 expressed on granulocytes upon phagocytosis, observed in this study, confirms the involvement of CD15 in the phagocytic process of granulocytes reported in previous studies. With mAbs WK13, COSA5A and COSA33NL, the up-regulation of these molecules upon phagocytosis observed in the present study is of interest. As these molecules are still un-defined, they collectively may play an important role in the functioning of phagocytes. Further study is needed for a detailed characterization.

In summary, in this study, we introduced a two-color flow cytometric method for simultaneously determining phagocytosis and the detection and alteration of cell surface molecules during phagocytosis. The introduced method is valuable for the identification of molecules involving the regulation of phagocytosis. Hopefully, this will lead to a better understanding of phagocytic mechanisms and the pathogenesis of infectious diseases. Additionally, it may provide the basis for the development of preventive and therapeutic approaches for microbial infections.

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