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# Biochemical and functional analysis of COS3A, a novel CD63-specific monoclonal antibody

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## Abstract

**Background:** Monoclonal antibodies (mAbs) have become essential tools in life science research and in medicine, because of their extreme specificity. Several mAbs against leukocyte surface molecules have been generated in our laboratory. From these, mAb COS3A was selected for biochemical and functional analysis.

**Objective:** To analyze the properties and function of the mAb COS3A.

**Method:** Cellular distribution was analyzed by immunofluorescence staining and flow cytometry. Biochemical characterization of the molecular target of COS3A was approached by immunoprecipitation, Western blotting and amino acid sequencing using LC-MS. N-glycosidase F treatment of COS3A-precipitated protein and culture of U937 cells in the presence of tunicamycin before cell lysate preparation were used to study the glycosylation state of proteins. Phagocytosis was examined by flow cytometry.

**Results:** MAb COS3A bound specifically to a molecule expressed on the surface of various human hematopoietic cells and cell lines but not on erythrocytes. The antigen had a molecular weight of 30-70 kDa, which was reduced to 25 kDa by elimination of N-linked glycan. LC-MS data and immunoprecipitation indicated that mAb COS3A bound specifically to the CD63 molecule. Remarkably, functional analysis demonstrated that mAb COS3A dramatically reduced granulocyte phagocytosis.

**Conclusions:** The mAb COS3A recognized the CD63 molecule and strongly diminished granulocyte phagocytosis of *E. coli*, suggesting that CD63 may play a crucial role in the initial step of phagocytosis. MAb COS3A is, therefore, suitable for both biochemical and functional studies of CD63, and may be used for further study of the mechanism of phagocytosis and also in therapeutic approaches.

**Keywords:** monoclonal antibody, COS3A, leukocyte surface molecule, CD63, phagocytosis

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## Introduction

CD63 is a member of the tetraspanin family of four-transmembrane domain proteins. This molecule was originally identified as a surface protein of activated platelets, and was previously known as platelet glycoprotein 40 (Pltgp40).[1] It is also expressed in the early developmental stage of human melanoma, and termed melanoma antigen 491 (ME491).[2] This molecule is also known as lysosome-associated membrane protein 3 or LAMP-3, due to its location in late endosomal and lysosomal membranes. CD63 interacts either directly or indirectly with many different molecules such as some types of integrins, other tetraspanins, cell surface receptors (MHCII, CD3, FcεRI, CXCR4), kinases, adapter proteins (AP-2, AP-3, AP-4) and many other proteins including L6 antigen, syntenin-1, TIMP-1, $H^+$,$K^+$-ATPase and membrane-type 1 matrix metalloproteinase (MT1-MMP).[3,4]

Although the function of CD63 is not fully understood, several reports have suggested that it is involved in protein trafficking,[4,5] leukocyte recruitment,[6] T cell activation,[7] HIV replication[8-10] and metastasis of tumor cells.[11] Moreover, CD63 is known as a biomarker for activation of several cell types including granulocytes and platelets. Because of its significantly increased expression upon their activation,[12] analysis of CD63 expression in human basophils, the so-called basophil activation test, has been used for allergy diagnosis.[13,14] It has also been reported that CD63 is involved in phagocytosis of yeast.[15,16] Several pathogens express surface molecules that can be sensed by phagocytic receptors, leading to opsonin-independent phagocytosis. These receptors have been shown to contribute to innate resistance to a number of bacterial pathogens. However, knowledge of the involvement of CD63 in the phagocytosis of bacterial cells is limited.

In this study, the mAb clone COS3A was characterized and was found to bind specifically to a member of the human tetraspanin protein family, CD63. The mAb was further used as a tool to track the involvement of surface CD63 in granulocyte phagocytosis of *Escherichia coli (E. coli)*. Remarkably, the mAb COS3A reduced the phagocytic activity of granulocytes, suggesting that the mAb prevented binding of CD63 to its natural ligands on pathogens, and implying that CD63 plays an essential role in the recognition step of *E. coli* phagocytosis.

## Declaration

### *Human Ethics*

The study was conducted according to the guidelines for human handlings based on the declaration of Helsinki. The documentation required for human studies was approved by the Ethics Committee for Researchers Involving Human Subjects, Suranaree University of Technology, Nakhon Ratchasima, Thailand on October 3rd, 2012.

## Methods

### *Cells*

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood of healthy donors by Ficoll–Hypaque density gradient centrifugation. Human T cell lines (Molt4, SupT1 and Jurkat), B cell lines (Daudi, Ramos and Raji), myeloid cell lines (KG-1a, U937 and THP-1) and an erythroid cell line (K562) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS (Gibco, Grand Island, NY), 40 mg/ml gentamicin and 2.5 mg/ml amphotericin B in a humidified atmosphere of 5% $CO_2$ at 37°C. Monkey kidney cell line COS7 was cultured in DMEM (Gibco, Grand Island, NY) supplemented with 10% FCS and antibiotics.

### *Antibodies*

The monoclonal antibodies COS3A, anti-13M and the anti-CD99 mAb MT99/3 were produced in our laboratory. These mAbs all belong to the IgG2a isotype. An anti-CD63 mAb, MEM259 was provided by Prof. Dr. Vaclav Horejsi, Institute of Molecular Genetics, Prague, Czech Republic. FITC-conjugated sheep $F(ab')_2$ anti-mouse immunoglobulin antibodies (FITC-conjugates) were purchased from Chemicon (Victoria, Australia). Horseradish peroxidase conjugated rabbit $F(ab')_2$ anti-mouse immunoglobulins (HRP-Igs) was obtained from Dako (Glostrup, Denmark).

### *Intracellular staining*

COS7 cells ($5x10^4$ cell/ml) were grown on coverslips in DMEM supplemented with 10% FCS, 40 µg/ml gentamicin and 2.5 µg/ml amphotericin B in a humidified atmosphere of 5% $CO_2$ at 37°C. After one day of cultivation the adhering cells were washed, fixed with 4% formaldehyde in PBS pH 7.4 for 15 min at room temperature and treated with permeabilizing buffer (0.1% TritonX-100 in PBS pH 7.4) for 5 min on ice. The cells were stained by adding 100 µl of permeabilizing buffer containing either mAb COS3A or isotype-matched control mAb anti-13M at a final concentration of 20 µg/ml. After 30 min on ice, the cells were washed and then incubated on ice for another 30 min with Alexa fluor (AF) 488-conjugated goat anti-mouse IgG (Molecular Probes, CA). DAPI (Molecular Probes) was used for nuclear staining according to manufacturer's instruction. After washing, the stained cells on coverslips were mounted with 50% glycerol in PBS pH 7.2 on glass slides, and sealed with nail polish. The cells were examined under an A1Rsi laser scanning microscope system from Nikon (Japan).

### *Immunofluorescence assay*

Cells ($1\times10^7$ cells/ml) or 0.3% RBC suspension in PBS pH 7.2 were pre-incubated with 2% human IgG at 4°C for 30 min. Fifty microliters of either mAb COS3A or isotype-matched control mAb anti-13M at a final concentration of 20 µg/ml was added into an equal volume of the cell suspension. After incubation at 4°C for 30 min, the cells were washed twice with washing buffer (1% BSA in PBS containing 0.02% azide) and incubated at 4°C with FITC-conjugates at a dilution of 1:100 for another 30 min. The stained cells were washed, fixed with 1% paraformaldehyde in PBS pH 7.2, and analyzed by a flow cytometer (BD Biosciences). Individual populations of blood cells were gated according to their forward- and side-scatter characteristics. COS7 cells were analyzed using an A1Rsi laser scanning microscope system from Nikon (Japan).

### *Western blotting*

U937 cells ($1\times10^7$ cells) were harvested, washed and solubilized for 30 min on ice in 1 ml of lysis buffer (1% Triton X-100, 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µM pepstatin A, 10 µg/ml aprotinin and 5 mM iodoacetamide). Clear cell lysates were obtained by centrifugation at 14,000 rpm at 4°C for 10 min. Cell lysates were resolved on 10% SDS-PAGE, and then electrophoretically transferred onto a polyvinylidenefluoride (PVDF) membrane. The membrane was blocked for 1 h in 5% skimmed milk in PBS pH 7.2 at room temperature, then incubated for an additional 1 h with 2 µg/ml of mAb COS3A or control mAb at room temperature. The membranes were washed 5 times with 0.1% Tween-20 in PBS pH7.2 and then incubated with HRP-Igs for 1 h at 25°C. After thorough washing, the signals were detected by the

enhanced chemiluminescence method.

***Immunoprecipitation***

U937 cell lysates were prepared as previously described for Western blotting. The cell lysates were pre-cleared with CNBr-activated Sepharose beads (GE Healthcare Life Sciences, Uppsala, Sweden) coated with human immunoglobulins. The pre-cleared cell lysates were then incubated with CNBr-activated Sepharose beads coated with mAb COS3A or isotype-matched control mAb anti-13M and rotated for 3 h at 4°C. After five times washing, the proteins were eluted from the beads by addition of 1x non-reducing Laemmli sample buffer, boiled for 5 min, and then resolved by 10% SDS-PAGE. The protein bands were examined either by blue-silver Coomassie staining[17] or Western blotting.

***Phagocytosis analysis***

Phagocytosis was determined using flow cytometry as previously described.[18] Briefly, 100 µl of EDTA-treated whole blood was mixed with 100 µl of *E. coli* strain DH5α expressing GFP (dilution 1:300 of cell suspension with $A_{600}$ = 0.6 in PBS pH 7.2) in the absence or presence of mAb COS3A or isotype-matched control mAb anti-13M at a final concentration of 20 µg/ml. The cells were incubated at 37°C or on ice for 1 h and then immediately placed on ice. Fifty microliters of RBC lysing solution containing 4.5% formaldehyde and 10% diethylene glycol in PBS pH7.2 was added and incubated on ice for an additional 10 min, then 1 ml of distilled water was added to complete the lysis. White blood cells were collected by centrifugation at 14,000x g for 30 seconds. The cells were washed twice with PBS pH 7.2 containing 0.1% $NaN_3$. The cells were resuspended in PBS pH 7.2 containing 1% paraformaldehyde and phagocytosis was analyzed by a flow cytometer. Fractional phagocytosis was calculated from the following equation:

$$\% \text{ Phagocytosis} = \frac{(\text{number of GFP positive cells at 37°C-number of GFP positive cells at 0°C})_{\text{with mAb}}}{(\text{number of GFP positive cells at 37°C-number of GFP positive cells at 0°C})_{\text{without mAb}}} \times 100$$

***N-glycosylation analysis***

For N-glycosidase F treatment, the immunoprecipitated proteins were dissociated from the beads by adding 40 µl of 20 mM phosphate buffer containing 1% SDS, boiled for 5 min, and 160 µl of 20 mM phosphate buffer containing 1% Triton X-100 then added. To remove N-linked sugars, five units of N-glycosidase F (Roche applied science, Mannheim, Germany) were added to the eluates, followed by incubation for 16 h at 37°C. The deglycosylated proteins were analyzed by Western blotting.

For tunicamycin treatment, U937 cells ($1x10^6$ cells/ml) were cultured in RPMI-1640 medium with or without tunicamycin (R&D Systems) at final concentration 5 µg/ml for 48 h. The treated cells ($1x10^7$ cells) were solubilized for 30 min on ice in 1 ml lysis buffer. The cell lysates were then subjected to Western blotting.

***Preparation of phagocytosable particles***

The gene encoding green fluorescent protein (GFP) was subcloned from pEGFP-N1 vector (Clontech Laboratories, Inc., Mountain View, CA) by restriction enzyme digestion and inserted into the pQE-Tri expression vector (QIAGEN) at the *BamHI* and *NotI* sites using enzyme quick ligase (New England Biolabs, Beverly, MA). The recombinant vectors were transformed into *E. coli* strain DH5α competent cells. The bacteria were grown for 18 h at 37°C in Luria-Bertani broth supplemented with 0.1 mM penicillin and subsequently heat-killed at 60°C for 30 min. The fluorescence intensity of the heat-killed bacteria was checked by flow cytometry before use. The same strain of *E. coli*, transformed with pQE-Tri empty vector, was used as a negative control. The bacterial cell suspension used in this study had an optical density at 600 nm ($A_{600}$) of 0.6.

## Results

***Cellular expression of the molecule recognized by mAb COS3A***

Monoclonal antibody COS3A was originally generated during the study of three different immunogen preparation strategies for production of anti-CD4 mAbs.[19] The mAb COS3A, however, was not specific for CD4. As the mAb was produced using the COS7 cell transfection system, we first determined whether mAb COS3A reacted with COS7 cell proteins. We found that mAb COS3A bound strongly both to cell surface proteins (**Figure 1A**) and to intracellular proteins in the monkey kidney cell line, COS7 (**Figure 1B**).

Human cell surface analysis by immunofluorescence staining and flow cytometry indicated that the mAb COS3A bound strongly to antigens on various human hematopoietic cell lines such as monocytes and granulocytes and weakly to lymphocytes but did not bind to erythrocytes (**Figure 2A,2B**). These results suggested a close homology between monkey and human forms of the target molecule. Additionally, cellular expression of this molecule was strongly increased upon phytohemaglutinin (PHA) activation for up to 72 hours (**Figure 2C**) indicating that it is an activation-associated molecule.

***MAb COS3A recognizes a highly N-glycosylated protein, CD63***

We further investigated the biochemical properties of the mAb COS3A target by immunoprecipitation and Western blotting, using U937 cell lysates as a protein source. A broad band of immunoreactive proteins, of molecular weight 30-70 kDa, was obtained by immunoprecipitation and Western blotting (**Figure 3A,3B**). However, this was converted to a sharp band of 25 kDa either by N-glycosidase F treatment (**Figure 3B**) or by inclusion of tunicamycin, an inhibitor of N-glycosylation in the endoplasmic reticulum, in the cell culture medium (**Figure 3C**). A band found at molecular weight 50 kDa is presumed to be an incompletely deglycosylated form of the target molecule of the mAb COS3A. These results indicate

that the target molecule is an extensively N-glycosylated protein. Moreover, mAb COS3A recognized the protein after glycan elimination, indicating that it binds specifically to the core protein of its target.

To identify the target of mAb COS3A, U937 cells were first treated with tunicamycin to remove the glycan part of the

**Table 1. Amino acid sequencing of the molecule recognized by mAb COS3A, using LC/MS and analysis by Mascot database.**

| Protein band (kDa) | Access number | Matched score* | Sequence coverage (%) | Matched peptides** | Identified Protein |
|---|---|---|---|---|---|
| 25 | F8VV56 | 47 | 15 | 1 MLVEVAAAIA **GYVFRDKVMS EFNNNFR**QQM **ENYPKNNHTA SILDR**MQADF<br>51 KCCGAANYTD WEKIPSMSKN RVPDSCCINV TVGCGINFNE KAIHKEGCVE<br>101 KIGGWLRKNV LVVAAAALGI AFVEVLGIVF ACCLVKSIRS GYEVM | CD63 (Homo sapiens) |
| 50 | F8VNT9 | 51 | 7 | 1 MAVEGGMKCV KFLLYVLLLA FCACAVGLIA VGVGAQLVLS QTIIQGATPG<br>51 SLLPVVIIAV GVFLFLVAFV GCCGACKENY CLMITFAIFL SLIMLVEVAA<br>101 AIAGYVFRDK **VMSEFNNNFR** QQMENYPKNN HTA | CD63 (Homo sapiens) |

* Individual scores > 42 indicate identity or extensive homology ($p<0.05$)
** Matched peptides shown in Bold Red

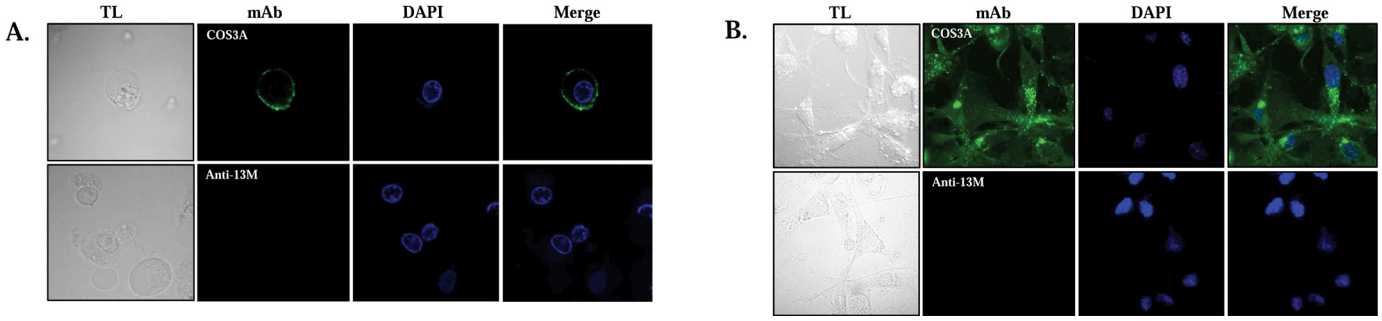


**Figure1. Expression of the molecule recognized by mAb COS3A on COS7 cells** (A) Suspensions of COS7 cells were surface-stained by indirect immunofluorescence with either mAb COS3A or an isotype-matched control mAb, anti-13M. (B) COS7 cells were fixed, permeabilized and stained on coverslips with either mAb COS3A or an isotype-matched control mAb, anti-13M, by indirect immunofluorescence. AF488-conjugated goat anti-mouse IgG was used as the secondary antibody. Cell nuclei were shown by DAPI staining. The analysis was performed using an A1Rsi laser scanning microscope system from Nikon with 100x magnification. In addition to the fluorescence a transmitted light (TL) image was recorded. A representative result from one of three independent experiments is shown.

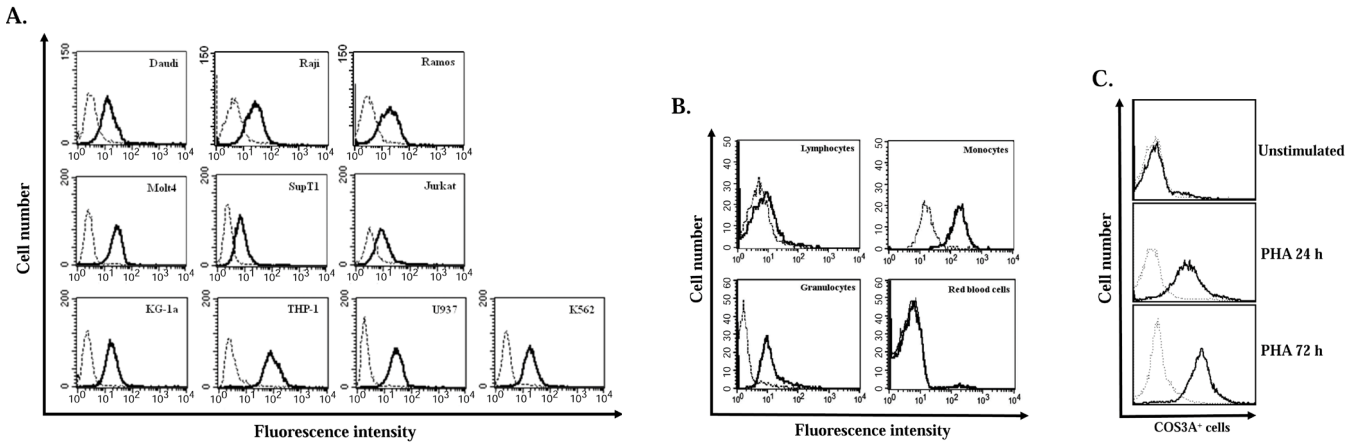


**Figure 2. Cell surface expression of the molecule recognized by mAb COS3A.** (A) Various human hematopoietic cell lines or (B) peripheral blood lymphocytes, monocytes, granulocytes and erythrocytes or (C) PBMCs ($1x10^6$ cell/ml) were stimulated with PHA at a final concentration of 5 μg/ml at various time points, were stained by indirect immunofluorescence with 20 μg/ml of mAb COS3A as the primary antibody, FITC-conjugated goat anti-mouse IgG as the secondary antibody, and analyzed by flow cytometry. Results with conjugate controls are shown as dashed lines, while cells stained with the mAb are represented by solid lines. A representative result from one of three independent experiments is shown.

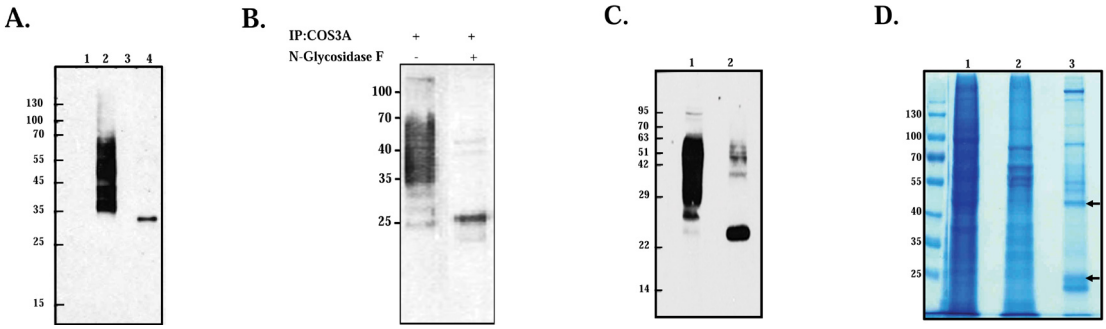


**Figure 3. Biochemical characterization of the cell surface antigen recognized by COS3A.** (A) U937 cell lysates were resolved under non-reducing conditions by 10% SDS-PAGE and subjected to Western blotting (lane1: conjugate control; lane2: mAb COS3A; lane3: isotype-matched control mAb; lane4: mAb MT99/3, a positive control). (B) U937 cell lysates were subjected to immunoprecipitation using immobilized mAb COS3A. The precipitated proteins were eluted and N-linked glycan chains were removed by incubation without (lane 1) or with five units of N-glycosidase F (lane 2). The proteins were then resolved by 10% SDS-PAGE under non-reducing conditions and subjected to Western blotting using 2 μg/ml of mAb COS3A as primary antibody. (C) U937 cells were cultured in the absence (lane1) or presence (lane 2) of 10 μg/ml tunicamycin for 48 h at 37°C under 5% $CO_2$. The cells were lysed and proteins were resolved by 10% SDS-PAGE under non-reducing conditions and subjected to Western blotting using 2 μg/ml of mAb COS3A as primary antibody. (D) Lysates from tunicamycin-treated U937 cells were subjected to immunoprecipitation. Cell lysates (lane 1), unbound proteins (lane 2), and the precipitated proteins from COS3A-coated beads (lane 3), were resolved under non-reducing conditions by 10% SDS-PAGE. The protein bands were visualized by blue-silver Coomassie staining. A set of representative results from one of four independent experiments is shown

protein before cell lysate preparation and the lysate were subjected to immunoprecipitation. The precipitated proteins from mAb COS3A-coated beads were resolved by 10% SDS-PAGE under non-reducing condition and visualized by blue-silver Coomassie staining (**Figure 3D**). Two protein bands of molecular weight about 50 kDa, an incomplete deglycosylated form, and a 25 kDa non-glycosylated form were cut out and sent for amino acid sequencing by LC/MS analysis at 1st BASE Services Company.

The amino acid sequence of each peptide fragment was analyzed using the Mascot database, which showed that the sequences of both the 50 kDa and the 25 kDa protein bands matched that of human CD63. The sequence coverage, match score and sequence of the identified sequences are shown in **Table 1**. Thus, the mAb COS3A target appears to be human CD63. To confirm the LC/MS data, immunoprecipitation and Western blotting were performed using a known anti-CD63 mAb, MEM-259, for detection of the CD63 molecule. We first used MEM-259, COS3A or a control mAb, anti-13M, for immunoprecipitation from U937 cell lysates. The precipitated proteins were then subjected to Western blotting, using either MEM-259 or COS3A for detection. As expected, both of the anti-CD63 mAbs, MEM-259 and COS3A, recognized the proteins that were precipitated with both MEM-295- and COS3A-coated beads, but not the protein precipitated with the control mAb anti-13M (**Figure 4A**). Moreover, the band of CD63 that was observed when the mAb COS3A was used for both immunoprecipitation and Western blotting was stronger than those obtained using MEM-259 at the same concentration in all experiments. This suggests that the binding affinity of the mAb COS3A is higher than that of mAb MEM-259.

Taken together, these results confirm that the mAb COS3A recognizes a highly N-glycosylated protein, CD63.

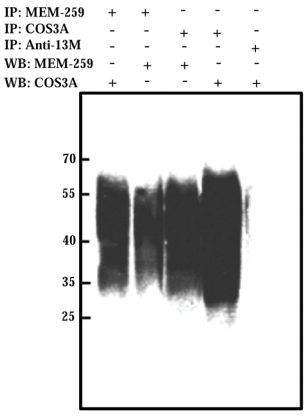


**Figure 4. MAb COS3A binds specifically to human CD63.** U937 cell lysates were subjected to immunoprecipitation using mAb COS3A, MEM-259 (CD63 mAb) or an isotype-matched control mAb (anti-13M). The precipitated proteins were then subjected to Western blotting using either mAb COS3A or MEM-259 as a primary antibody. Goat anti-mouse HRP-IgG was used as the secondary antibody, and the protein bands were visualized using a chemiluminescence detection system. The result shown here is from one of three independent experiments.

### *MAb COS3A inhibits phagocytosis by granulocytes*

Phagocytosis contributes to the first line of defense against infectious pathogens, binding of the ligands on pathogens to membrane receptors of the phagocytes being an essential first step in successful phagocytosis. It has been reported that CD63 is translocated from the cell surface to intracellular organelles containing phagocytosed yeast;[15] furthermore, CD63 was selectively recruited to phagosomes containing a pathogenic yeast, *Cryptococcus neoformans*, in an acidification-dependent

manner.[16] However, since both reports related to yeast, we investigated whether surface CD63 also participates in bacterial phagocytosis. To answer this question, mAb COS3A was used as a tool to follow the phagocytosis of bacterial cells by granulocytes. To track the phagocytosis, *E. coli* cells expressing GFP were prepared and used as phagocytosable particles. The transformed *E. coli* showed strong fluorescence intensity compared to wild type, which can be identified by flow

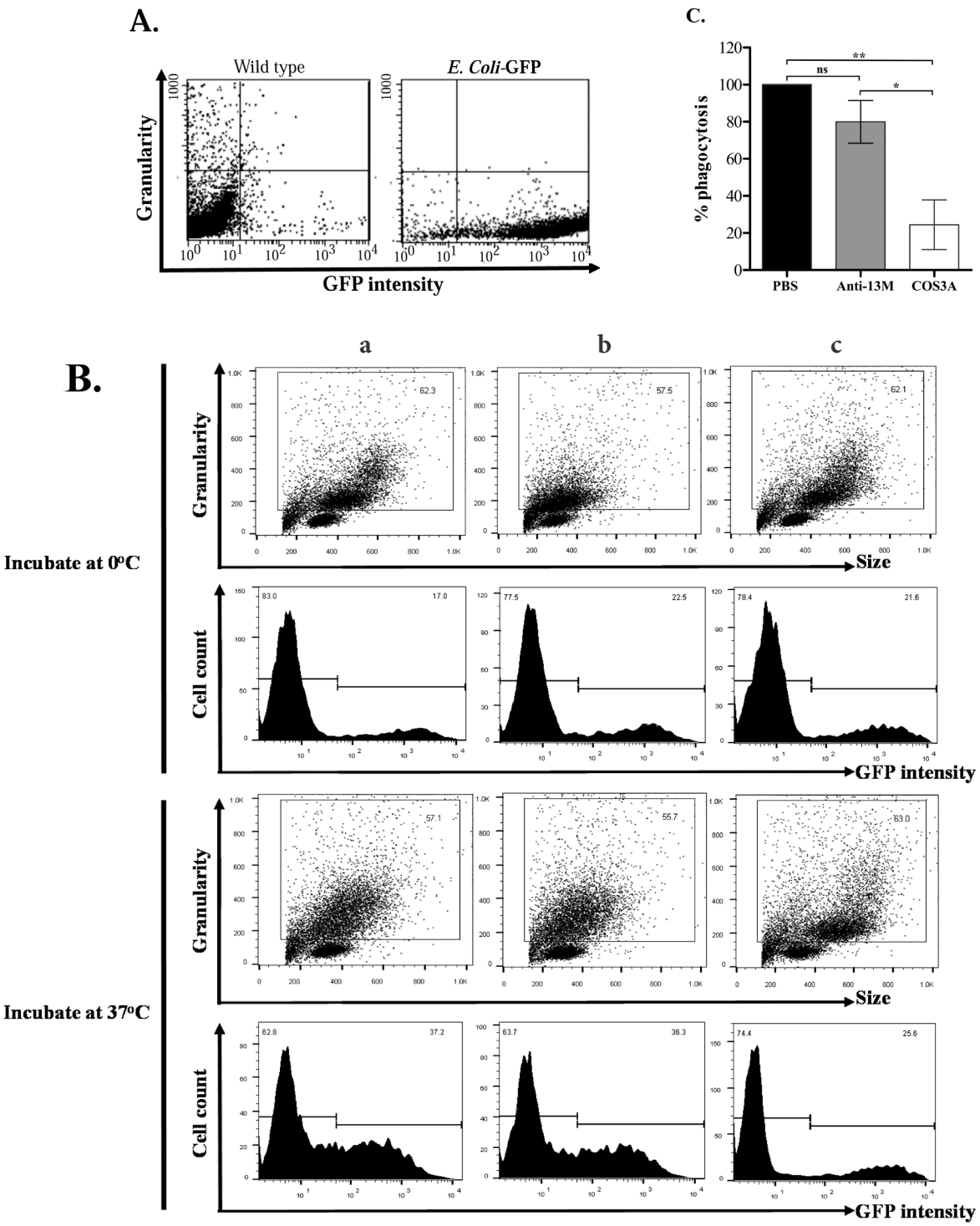


**Figure 5. MAb COS3A inhibited granulocyte phagocytosis.** (A) FACS profiles represent the fluorescence intensity of wild-type *E. coli* and *E. coli* expressing GFP (*E. coli*-GFP). (B) Whole blood was incubated on ice or at 37°C for 1 h with *E. coli* expressing GFP in the absence of antibody (a) or in the presence of 20 μg/ml of either isotype matched control mAb, anti-13M (b) or mAb COS3A (c). RBCs were lysed and the granulocyte phagocytosis of *E. coli* expressing GFP was examined by flow cytometry recording the presence of GFP. A representative result from one of four independent experiments is shown. (C) Bar graph showing the percentage of granulocyte phagocytosis calculated from results of four independent flow cytometry experiments, according to the equation stated in the methods (ns not significant, *$p<0.05$, **$p<0.01$)

cytometry (**Figure 5A**) and was further used in phagocytosis assays. Interestingly, granulocyte phagocytosis of *E. coli* was significantly reduced when the mAb COS3A was added into the system, while the control mAb anti-13M had no effect (**Figure 5B**). At 0°C, the granulocytes were expected to have no phagocytic activity and the measured fluorescence originated from binding of bacteria to the surface of the granulocytes. The fractional phagocytosis is shown in a bar graph (**Figure 5C**), which shows that mAb COS3A decreased phagocytosis to 20% of control values. The mAb anti-13M did not inhibit the phagocytosis, indicating that the inhibitory effect did not occur through FcγR or opsonin-dependent pathways but through an opsonin-independent pathway. These results indicate that engagement of cell surface CD63 with the mAb COS3A blocks the interaction between ligands on *E. coli* and their specific receptor on phagocytes. Consequently ingestion of the bacterial cells was inhibited, suggesting that CD63 plays an essential role in the recognition step of phagocytosis.

## Discussion

Monoclonal antibodies have become vital tools in numerous areas of research and also in medicine. The classical method for generating monoclonal antibodies is the so-called hybridoma technique, which was established in 1975 by Georges J.F. Köhler and César Milstein.[20] Using this technique, the mAb COS3A was produced using CD4-expressing COS7 cells as the immunogen for antibody induction in a Balb/c mouse.[19] The resultant mAb reacted strongly with a molecule expressed by COS7 cells, both on the surface and intracellularly, but not with the CD4 molecule. With the exception of erythrocytes, the antibody also bound to a molecule expressed on the surface of several human hemaopoietic cells and cell lines. Additionally, up-regulation of this molecule upon PHA activation was observed, suggesting that the COS3A target is an activation-associated molecule. The FACS profiles obtained using the generated mAb were the same as those described in previous reports for CD63.[7,21,22] Biochemical analysis and amino acid sequencing by LC/MS specified that the molecule recognized by COS3A is a highly N-glycosylated protein, human CD63.

The structure of CD63 includes four putative transmembrane domains, a small (EC1) and a large (EC2) extracellular domain, short cytoplasmic N- and C-termini, and three N-linked glycosyl chains.[23,24] The biochemical properties found in this study correlate well with those of the CD63 molecule, and as the mAb COS3A was able to bind to cell surface CD63, and to both glycosylated and non-glycosylated forms of CD63, it is suggested that the antigenic determinant is located on the extracellular domain of the core protein.

The tetraspanin CD63 is ubiquitously expressed and is localized both within the endosomal system and on the cell surface.[4] The molecular function of CD63 is not yet clear, but it has been reported to play an important role in several fundamental cell processes including intracellular protein trafficking and migration[4,5,15] under normal as well as pathological conditions. Furthermore, CD63 is known as a marker of activation of numerous cell types such as mast cell and basophils.[25] A significant increase in CD63 expression was found on activation of basophils and this is now used in allergy testing.[13,14] However, information about the involvement of CD63 in phagocytosis is limited.

Phagocytosis has been acknowledged as a critical component of the innate and adaptive immune response. The whole process of phagocytosis can be divided into three fundamental stages, namely (i) recognition (ii) internalization and (iii) degradation.[26] The cells involved in this mechanism are called phagocytes and include monocytes, macrophages and granulocytes. Unlike macropinocytosis, phagocytosis involves the recognition and binding of ligands on pathogens by cell surface receptors, a so-called a receptor-mediated event. Two types of phagocytosis have been described; the first is opsonin-dependent phagocytosis, in which antibody or complement opsonizes the pathogen and promotes its phagocytosis via Fcγ or complement receptors on phagocytes. The second type is opsonin-independent phagocytosis, in which phagocytes recognize the microorganism via pattern-recognition receptors, such as scavenger and mannose receptors. CD63 was reported to be specifically recruited to phagosomes internalizing *S cerevisiae*[15] and *Cryptococcus neoformans*, a pathogenic yeast.[16] Since phagosomes are plasma membrane-derived, the primary composition of phagosomes varies, depending on the selective engagement of surface molecules. Many pathogens exhibit surface molecules that do not exist in higher organisms. These molecules can be sensed by several phagocytic receptors, such as mannose receptors, CD14 and CD36, which respectively bind to mannose units in polysaccharides present on the surface of some yeast cells, lipopolysaccharides of bacterial cell walls and plasmodium falciparum-infected erythrocytes.[26] Although a large number of phagocytic receptors and the molecules involved in phagocytosis have been defined, this does not cover the complete spectrum of infectious agents confronted by phagocytes. CD63 is abundant in late endosomes, lysosomes and mature phagosomes that are essential for the microbicidal function of phagosomes. Although it has been reported that cells lacking LAMP proteins are incapable of destroying *Neisseria gonorrheae*, a pathogenic bacterium,[27] there is no information about the involvement of CD63, known also as LAMP-3, in the recognition step of bacterial phagocytosis. In this study, we observed the effect of the mAb COS3A on granulocyte phagocytosis of *E. coli* and found significant obstruction of the induction of phagocytosis, compared to controls. There are two possible reasons for this. First, CD63 may act as a phagocytic receptor for a ligand that is expressed on *E. coli*, so that binding of the mAb COS3A to CD63 blocks the natural ligand-receptor interaction, leading to failure of phagocytosis. Second, CD63 has been reported to interact with several molecules, so it may not act as a phagocytic receptor at all, but binding of this molecule by the mAb COS3A may trigger negative signals that indirectly inhibit the internalization of *E.coli* by granulocytes.

In conclusion, we have identified a novel clone of mAb specific for CD63, named COS3A. The antibody is suitable for both biochemical and functional studies of the CD63 molecule. In addition, mAb COS3A significantly inhibits phagocytosis of *E.coli* by granulocytes, and we show here for the first time

that CD63 may play an important role in the recognition step of bacterial phagocytosis through an opsonin-independent pathway. This observation opens the question of whether CD63 acts as a phagocytic receptor or is involved in the regulation of the phagocytic process, especially for the gram-negative bacterium *E. coli*, and further study is required to elucidate the actual mechanism.

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## Competing interests

The authors declare that they have no competing interests.

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