Human anti-peptidoglycan-IgG-mediated opsonophagocytosis is controlled by calcium mobilization in phorbol myristate acetate-treated U937 cells

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Recently, we demonstrated that human serum amyloid P component (SAP) specifically recognizes exposed bacterial peptidoglycan (PGN) of wall teichoic acid (WTA)-deficient Staphylococcus aureus ΔtagO mutant cells and then induces complement-independent phagocytosis. In our preliminary experiments, we found the existence of human serum immunoglobulins that recognize S. aureus PGN (anti-PGN-IgGs), which may be involved in complement-dependent opsonophagocytosis against infected S. aureus cells. We assumed that purified serum anti-PGN-IgGs and S. aureus ΔtagO mutant cells are good tools to study the molecular mechanism of anti-PGN-IgG-mediated phagocytosis. Therefore, we tried to identify the intracellular molecule(s) that is involved in the anti-PGN-IgG-mediated phagocytosis using purified human serum anti-PGN-IgGs and different S. aureus mutant cells. Here, we show that anti-PGN-IgG-mediated phagocytosis in phorbol myristate acetate-treated U937 cells is mediated by Ca²⁺ release from intracellular Ca²⁺ stores and anti-PGN-IgG-dependent Ca²⁺ mobilization is controlled via a phospholipase Cγ-2-mediated pathway. [BMB Reports 2015; 48(1): 36-41]

INTRODUCTION

Host innate immunity utilizes various pattern recognition receptors (PRRs) for recognition of pathogen-associated molecular patterns (PAMPs) (1). Upon binding of PRRs to PAMPs, activation of host innate immune responses induces secretion of cytokines to promote inflammation and attracts immune cells, such as macrophages, neutrophils, and natural killer cells, on-
ocytosis may also use intracellular calcium signaling to achieve phagocytosis.

Here, we provide the molecular evidence that specific inhibitors involved in intracellular calcium signaling also inhibit human anti-PGN-IgG-mediated calcium ion increase, suggesting that intracellular calcium signaling may be involved in regulation of anti-PGN-IgG-mediated phagocytosis and cytokine production.

RESULTS

Purified anti-PGN-IgGs specifically bind to S. aureus PGN
First, to confirm binding specificity of purified anti-PGN-IgGs, we examined the binding abilities of purified anti-PGN-IgGs against fluorescein isothiocyanate (FITC)-labeled purified S. aureus WTA-coupled PGN and WTA-depleted PGN using flow cytometry (Fig. 1A, 1B). As expected, anti-PGN-IgGs bind to WTA-depleted PGN (Fig. 1A-i), but not WTA-coupled PGN (Fig. 1B-i), indicating that purified anti-PGN-IgGs have binding specificity against S. aureus PGN. To further confirm the binding specificity by competition assay, anti-PGN-IgGs were incubated with insoluble purified WTA-coupled PGN or WTA-depleted PGN in the presence or absence of soluble PGN. The binding ability of anti-PGN-IgGs to S. aureus insoluble WTA-depleted PGN was decreased with the addition of soluble PGN (Fig. 1A-ii), but no difference was observed in the WTA-coupled PGN (Fig. 1B-ii). These results suggest that the purified anti-PGN-IgGs specifically recognize S. aureus PGN.

Anti-PGN-IgGs induce engulfment of WTA-depleted ΔtagO S. aureus mutant cells into macrophages
Recent studies have shown that anti-PGN-IgGs induce phagocytosis by association with FcyRs (7) and that human SAP induces phagocytosis of WTA-depleted S. aureus ΔtagO mutant cells onto human neutrophils (8). To examine whether anti-PGN-IgGs induce phagocytosis of WTA-coupled S. aureus Δspa mutant or WTA-depleted ΔtagO/Δspa double mutant cells, FITC-labeled ethanol-killed S. aureus Δspa mutant or ΔtagO/Δspa double mutant cells were incubated with purified anti-PGN-IgGs in the presence of phorbol myristate acetate (PMA)-treated U937 macrophage cells and S. aureus Δspa mutant treated human serum (Fig. 2). When S. aureus cells engulfed in the 100 macrophages were counted after 30 min incubation, anti-PGN-IgGs induced the phagocytosis of ΔtagO/Δspa S. aureus double mutant cells (370 ± 15, column 8), but not S. aureus Δspa mutant cells (50 ± 14, column 4). The moderate phagocytosis was observed by incubation with S. aureus ΔtagO/Δspa double mutant cells and Δspa-mutant treated human serum (120 ± 18, column 7), sug-

![Fig. 1](image1.png)

![Fig. 2](image2.png)
gesting a possibility that unidentified antibodies in the serum can induce phagocytosis. These results demonstrate that anti-PGN-IgGs can bind to PGN of ΔtagO S. aureus mutant cells and induce phagocytosis in a PGN-dependent manner.

**PMA-treated U937 macrophage cells control intracellular Ca^{2+} release via phospholipase Cγ2 (PLCγ2) pathway**

To investigate which intracellular signaling pathway and what kinds of molecules are involved in the anti-PGN-IgG-mediated phagocytosis, we firstly examined the possible involvement of the calcium signaling pathway using calcium-sensitive fluorescent dye and several calcium signaling inhibitors in U937 macrophages. When ΔtagO Δspa S. aureus double mutant cells pretreated with anti-PGN-IgGs were incubated with PMA-treated U937 cells, the calcium signal rapidly increased (Fig. 3A), indicating that anti-PGN-IgG-mediated phagocytosis activates intracellular calcium signaling pathway. Recently, it was suggested that extracellular cyclic adenosine diphosphate ribose (cADPR) enhances FcγR-mediated phagocytosis (10), we wondered whether cADPR may be involved in the calcium signaling pathway of anti-PGN-IgG-mediated phagocytosis. When 8-Br-cADPR, an antagonist of cADPR (11), was pretreated to confirm this possibility, as expected, 8-Br-cADPR decreased anti-PGN-IgG-mediated Ca^{2+} release (Fig. 3B). Furthermore, when Xestospongin C (XeC), an inositol triphosphate (IP₃) receptor blocker (12), was pretreated to examine whether IP₃ is also involved in this calcium signal, Ca^{2+} signal was decreased (Fig. 3C). To further verify the effect of protein kinase C (PKC), when Go6976 (13), an inhibitor of PKCα isoenzyme, was pretreated onto U937 macrophages, no changes were observed on the intracellular calcium mobilization (Fig. 3D). Also, as tyrosine phosphorylation of phospholipase Cγ2 (PLCγ2) is known to play a pivotal role in lipopolysaccharide (LPS)- and PGN-mediated activation of macrophages and dendritic cells, leading to calcium mobilization (14), U73122 (15), an inhibitor of PLCγ2, was preincubated onto U937 cells. Surprisingly, anti-PGN-IgG-mediated Ca^{2+} signal was completely disappeared (Fig. 3E). Next, it has been reported that NAADP (nicotinic acid adenine dinucleotide phosphate) is generated in lysosome-related acidic organelles after cADPR production which leads to intracellular calcium release (16). When Ned-19, a NAADP antagonist (17), was preincubated with U937 macrophages, calcium signal slightly decreased (Fig. 3F).

Additionally, we performed phagocytic assay in order to further quantify our previous data of calcium ion change by calculating the phagocytic index of U937 macrophages. When anti-PGN-IgG-mediated phagocytic efficiency index was estimated using five different calcium inhibitors (Fig. 3G), U73122 greatly decreased the phagocytic index by 57%. 8-Br-cADPR and XeC reduced the number of engulfed bacteria cells by 29 and 34%, respectively. Ned-19 and Go6976 did not affect the phagocytic index. These results are the same as the Ca^{2+} measurement data above. Taken together, these results strongly suggest that anti-PGN-IgG-mediated intracellular calcium signaling and phagocytosis are mainly mediated by PLCγ2.

**Anti-PGN-IgG-dependent phagocytosis is mediated by calcium release from intracellular calcium store**

PMA-treated U937 cells were pretreated with both 8-Br-cADPR and XeC in order to determine which intracellular calcium stores were related to calcium release within the U937 macrophages. It was reported that cADPR directly binds to ryanodine receptor (RyR) to induce Ca^{2+} release from the endoplasmic reticulum (ER) calcium store (11). When we treated 8-Br-cADPR and XeC simultaneously, anti-PGN-IgG-induced Ca^{2+} signal was decreased (Fig. 4B), indicating that the ER functions as a major internal Ca^{2+} store in U937 macrophages. When macrophages were incubated with both Ned-19 and XeC treatment (Fig. 4C), calcium release was more inhibited than with the Ned-19 alone, indicating that inhibition effect of calcium release mainly results from XeC. Therefore, acidic-like organelles as an intracellular calcium store may not be related to Ca^{2+} release in U937 macrophages. The combination of Go6976 and XeC did not reduce Ca^{2+} release (Fig. 4D), suggesting that PKC inhibitor did not affect Ca^{2+} release via the PLCγ2 pathway.

To determine whether the initial calcium peak is mediated...
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Fig. 4. Anti-PGN-IgG-induced calcium release is controlled via PLC2-mediated pathway. Shown is the effect of 200 μM 8-Br-cADPR, 2 μM Xestospongin C, 500 nM Go6976, 10 μM Ned-19, and 100 μM 8-Br-ADPR on anti-PGN-IgG-induced calcium increase in PMA-treated U937 cells. Each data represent the mean ± S.D. from three independent experiments. *P < 0.01(*) versus anti-PGN-IgG group.

by calcium influx from the extracellular environment, the U937 macrophages were treated in a Ca²⁺ free buffer. As a result, calcium release was inhibited (Fig. 4E), suggesting that initial rapid calcium increase may be mediated by calcium entry from extracellular medium. To examine whether the calcium channel is involved, we performed a Ca²⁺ measurement experiment using a calcium channel blocker. We first pretreated with 8-Br-ADPR, and calcium entry to the cells was largely reduced (Fig. 4F). 8-Br-ADPR is an agonist of ADPR; it leads to conversion of cADPR into ADPR and then leads to calcium influx by activating TRPM2 (transient potential receptor melastatin-2), a non-selective calcium permeable cation transport (18). These results showed that anti-PGN-IgG-induced Ca²⁺ signal in PMA-treated U937 cells is involved in the influx from the extracellular medium through calcium transport and the release of calcium from intracellular Ca²⁺ stores.

Finally, when the phagocytic index was estimated in a Ca²⁺ free buffer, bacterial cells were engulfed by 54% (Fig. 4G). Similar reduction effect of phagocytosis was also obtained when macrophages were pretreated with both 8-Br-cADPR and XeC (48% decrease). However, Go6976, Ned-19, and 8-Br-ADPR did not affect the phagocytic index.

We see that, IP3 and cADPR as calcium signaling messenger are closely linked to anti-PGN-IgG-induced phagocytosis in U937 macrophages, while PKC, ADPR, and NAADP seem to not have any connection with phagocytosis of anti-PGN-IgG-opsonized S. aureus ΔtagO/Δspa double mutant cells. Taken together, these results demonstrate that anti-PGN-IgG-mediated phagocytosis in PMA-treated U937 cells is mediated by Ca²⁺ release from intracellular ER Ca²⁺ store and anti-PGN-IgG-dependent Ca²⁺ mobilization is controlled via PLC2-mediated pathway.

DISCUSSION
Recently, our group reported that human serum MBL binds to S. aureus WTA, a cell wall glycopolymer (19). This work prompted further screening to identify additional serum proteins that recognize S. aureus cell wall components. When human serum was incubated with 10 different S. aureus mutants, serum amyloid P component (SAP) specifically bound to a WTA-deficient S. aureus ΔtagO mutant, but not to tagO-complemented WTA-expressing cells, indicating that human SAP functions as a host defense factor, similar to other PGN recognition proteins and NOD-like receptors (8). Furthermore, human serum SAP can function as an opsonin that is capable of enhancing human neutrophil-mediated phagocytosis. But, the molecular mechanism of human anti-PGN-IgG-mediated phagocytosis has not been fully understood. In this study, we show the importance of PLC in regulating calcium signals that is necessary for anti-PGN-IgG-mediated phagocytosis. The main achievement of this study is that calcium signal in U937 cells is composed of multiple calcium signaling messengers and requires IP3 and cADPR downstream of the PLC pathway.

As a result of this study, we propose a plausible clearance mode of infected S. aureus cells in a host. The infected cells will be engulfed into phagocytic cells, such as neutrophils or macrophages and then WTA will be removed in the acidic conditions of phagolysosome as shown in our previous works (8). Then, if engulfed WTA-depleted PGN-exposed S. aureus cells can escape from phagocytic cells, human serum SAP or anti-PGN-IgGs will induce complement-independent FcγRs-dependent phagocytosis or complement-dependent opsonophagocytosis, respectively. During this opsonophagocytosis, intracellular calcium signal will be induced and be controlled mainly via PLC-mediated pathway.

MATERIALS AND METHODS

Materials and reagents
Bovine serum albumin (BSA), 8-Br-cADPR, PMA and U73122 were purchased from Sigma-Aldrich. RPMI 1640 medium, fetal bovine serum, and antibiotic-antimycotic solution were purchased from Hyclone Laboratories, Inc. Fluo-4, AM, and Go6976 were purchased from Invitrogen. Xestospongin C was purchased from Calbiochem. Ned-19 was purchased from Santa Cruz Biotechnology, Inc. U937 cells were purchased from ATCC (American type culture collection).
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**Bacteria and sera**
Bacterial strains and functions of deleted genes in *Staphylococcus aureus* mutant strains are summarized as follows. *S. aureus* RN4220 is used as a parental strain (20). Strain M0107 is an IgG-binding protein A-deficient Δspa mutant of RN4220 (21). Strain T258 is a WTA- and protein A-deficient ΔtagO/Δspa double mutant (19). The tagO gene encodes the first-step enzyme TagO for WTA biosynthesis, and spa gene encodes protein A that binds to the Fc region of IgG. *S. aureus*-recognizing IgG-deficient serum was prepared using *S. aureus* Δspa mutant cells as described previously (23).

**Purification of *S. aureus* PGN**
Insoluble PGN and soluble PGN were purified as described previously (8, 23). Insoluble PGN was obtained from *S. aureus* RN4220 and ΔtagO/Δspa double mutant, respectively. WTA-depleted soluble PGN was obtained from RN4220 by treated 5% (w/v) trichloroacetic acid and lysostaphin (200 μg) as described previously (8).

**Purification of anti-PGN-IgGs from human intravenous immunoglobulin (IVIG)**
Anti-PGN-IgGs was purified as described previously (19, 23). Briefly, 200 μg of purified PGN in 200 μl of PBS was coated onto a nitrocellulose membrane (10 × 3 mm, Whatman, pore 0.45 μm), and baked at 100°C for 1 h. Twenty pieces of nitrocellulose membrane were prepared for each sample and were incubated with 50 mg of commercially available human intravenous immunoglobulin (IVIG, SK Chemicals, Seoul) in 10 ml of 10 mM Tris buffer (pH 7.4) containing 140 mM NaCl and 0.4% BSA (buffer A) for 30 min at 37°C with shaking and then kept on ice for 2 min. During the opsonization of *S. aureus* cells, prepared PMA-treated U937 macrophages were incubated with 5 μM Fluo-4, AM in HBSS at room temperature for 40 min and then the cells were washed twice with HBSS. After washing, the U937 cells were incubated with various Ca2+ signaling inhibitors at 37°C in 5% CO2 before adding anti-PGN-IgG-opsonized *S. aureus*. The concentration of treated calcium signaling inhibitors is summarized as follows: 200 μM 8-Br-cADPR (11), 100 μM 8-Br-ADPR (18), 5 μM U73122 (15), 2 μM Xestospongin C (12), 10 μM Neda-19 (17), 500 nM G66976 (13). Intracellular calcium concentration was measured by level of fluorescence in the U937 cells as previously described (9).

**Phagocytic assay**
This experiment was performed as previously described with some modification (24). In brief, *S. aureus* T258 and M0107 mutant strains grown in LB 10 at 37°C overnight were harvested, killed with 70% ethanol, labeled with 0.1 mM FITC in 0.1 M Na2CO3 buffer (pH 8.5) for 30 min at room temperature, and suspended in HBSS. FITC-labeled bacteria (equivalent to 1.5 × 107 CFU) were opsonized with 10% prepared sera with anti-PGN-IgGs in 20 μl buffer A for 30 min at 37°C with shaking. The PMA-treated U937 cells were incubated with various calcium signaling inhibitors at 37°C in 5% CO2 as described above. Then, the PMA-treated U937 cell suspension (1.5 × 107 cells, 35 μl) prepared earlier was added to 5 μl of the opsonized bacteria (corresponding to 3.8 × 106 CFU; multiplicity of infection ~25) and incubated at 37°C for 60 min with shaking. Finally, phagocytosed FITC-labeled *S. aureus* cells in the U937 macrophages were counted under fluorescent phase-contrast microscopy. More than 100 cells of U937 macrophage were counted. Extracellular FITC-labeled *S. aureus* was quenched by 0.2% trypan blue. The phagocytic index (%) of calcium signaling inhibitors was calculated as follows: percentage of number of *S. aureus* cells engulfed by 100 macrophages, which means inhibition effect about anti-PGN-IgG-induced phagocytosis alone without calcium signaling inhibitor as a control.

**Statistical analysis**
Data were expressed as the mean ± S. D. of the data from at least three independent experiments. Statistical analyses were performed using a student t-test and P values of < 0.01 were considered significant and indicated in the figures.
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