

BMB Reports – Manuscript Submission

Manuscript Draft

**Manuscript Number:** BMB-19-018

**Title:** CpG-DNA induces bacteria-reactive IgM enhancing phagocytic activity against *Staphylococcus aureus* infection

**Article Type:** Article

**Keywords:** CpG-DNA; IgM; phagocytosis; *Staphylococcus aureus*; TLR9

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**CpG-DNA induces bacteria-reactive IgM enhancing phagocytic activity against  
*Staphylococcus aureus* infection**

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**Running title:** Bacteria-reactive IgM enhances phagocytic activity

**Keywords:** CpG-DNA, IgM, phagocytosis, *Staphylococcus aureus*, TLR9

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**ABSTRACT**

CpG-DNA triggers the proliferation and differentiation of B cells which results in the increased production of antibodies. The presence of bacteria-reactive IgM in normal serum was reported; however, the relevance of CpG-DNA with the production of bacteria-reactive IgM has not been investigated. Here, we proved the function of CpG-DNA for the production of bacteria-reactive IgM. CpG-DNA administration led to increased production of bacteria-reactive IgM both in the peritoneal fluid and serum through TLR9 signaling pathway. When we stimulated B cells with CpG-DNA, production of bacteria-reactive IgM was reproduced *in vitro*. We established a bacteria-reactive monoclonal IgM antibody using CpG-DNA stimulated-peritoneal B cells. The monoclonal IgM antibody enhanced the phagocytic activity of RAW 264.7 cells against *S. aureus* MW2 infection. Therefore, we suggest that CpG-DNA enhances the antibacterial activity of the immune system by triggering the production of bacteria-reactive IgM. We also suggest the possible application of the antibodies for the treatment of antibiotics-resistant bacterial infections.

## INTRODUCTION

Immunoglobulin M (IgM) exists as two types: natural IgM and immune IgM (1). Natural antibodies, consisting mostly of the natural IgM, are the antibodies found in the sera of humans and mice without any exposure to antigens (2). In contrast to the natural IgM, immune IgM is the first antibody produced in response to antigenic stimulation (3). Both IgMs have an important role in pathogen defense, especially bacterial infection (4). Several investigators have suggested that the administration of IgM in both *in vitro* and *in vivo* models leads to protection against bacterial infection, such as *Streptococcus agalactiae* (*S. agalactiae*), *Escherichia coli* (*E. coli*), *Borrelia hermsii* (*B. hermsii*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Francisella tularensis* (*F. tularensis*) (5-9). Using IgM knock out mice, some reports have also discovered the protective role of IgM expression against bacterial infection, including *Streptococcus pneumoniae* (*S. pneumoniae*) and *B. hermsii* (10, 11).

DNA containing CpG dinucleotide motifs (CpG-DNA), which are found in bacterial DNA, stimulate Toll-like receptor 9 (TLR9) on immune cells, leading to cell proliferation and cytokine production (12). Furthermore, CpG-DNA activates B cells, producing T cell-independent antibodies (13). These roles of CpG-DNA in the immune system are closely related to the prevention of bacterial infection (14-17). Investigators have reported that CpG-DNA activated dendritic cells and the secreted type-1 cytokine, IFN- $\gamma$ , have protective roles against bacterial infection in murine models (14, 15). The antibacterial effects of reactive oxygen species (ROS) produced by the administration of CpG-DNA in osteoblast-like cell lines have also been shown in *S. aureus* infection (16). Furthermore, it was reported that CpG-DNA treatment activated macrophages to promote phagocytosis in *S. aureus*-infected conditions through the JNK/P38 signaling pathway (17).

Previously, we reported that bacteria-reactive IgG produced by the administration of CpG-

DNA enhances the antibacterial effect in murine models (18); however, the biological functions of IgM produced by CpG-DNA administration are not known. Here, we confirmed that the production of TLR9-mediated IgM occurred both in the peritoneal cavity and sera of mice and that IgM was produced in the peritoneal B cells *in vitro*. We isolated a bacteria-reactive monoclonal IgM antibody induced by CpG-DNA administration and found for the first time that the IgM enhances phagocytosis against bacterial infection.

## RESULTS

### **IgM antibodies reactive to bacteria were generated by CpG-DNA administration through the TLR9 signaling pathway**

In our previous studies (18), we confirmed that the survival of mice after *S. aureus* MW2 infection was enhanced by injection of CpG-DNA. Based on our observations, we assumed that bacteria-reactive IgM could be produced by the injection of CpG-DNA 1826 into the peritoneal cavity. To identify the production of IgM by the CpG-DNA treatment in mice, we harvested the peritoneal fluid and serum from the mice after intraperitoneal injection of CpG-DNA 1826 followed by intravenous injection with *S. aureus* MW2, and measured the levels of IgM reactive to *S. aureus* MW2 using the ELISA assay. When the mice were intraperitoneally injected with CpG-DNA 1826, the IgM levels were significantly increased both in the peritoneal fluid and serum, especially in the peritoneal fluid. The concentration of the IgM was decreased slightly in the peritoneal fluid and serum following the infection with *S. aureus* MW2; however, the pretreatment of CpG-DNA 1826 before *S. aureus* MW2 infection showed a significant increase of the IgM concentration (Fig. 1A and B).

To analyze the binding ability of IgM produced by the CpG-DNA administration to various bacterial species, we selected Gram-positive bacterial species (*Staphylococcus* and *Streptococcus*) and Gram-negative bacterial species (*Klebsiella*, *Escherichia*, *Acinetobacter*

and *Pseudomonas*) and performed ELISA assays. The results show that the levels of each bacteria-reactive IgM were increased in the peritoneal fluids and serum by intraperitoneal injection of CpG-DNA 1826, and the rate of increase in the peritoneal fluids was significantly higher than that of the serum (Fig. 1C-F).

To determine whether the IgM production induced by the CpG-DNA administration was due to the activation of the TLR9 signaling pathway, we performed the same experiments using BALB/c TLR9<sup>-/-</sup> mice. There were no significant changes in IgM production both in the peritoneal fluid and sera of the TLR9<sup>-/-</sup> mice even with the injection of CpG-DNA 1826 (Fig. 1G-J). Therefore, the results suggest that the TLR9 signaling pathway is involved in the production of bacteria-reactive IgM induced by CpG-DNA stimulation.

#### **Induction of bacterial-reactive IgM in mouse peritoneal cavity cells by CpG-DNA treatment *in vitro***

To identify where the bacteria-reactive IgM was produced by the CpG-DNA administration, the mice were intraperitoneally injected with CpG-DNA 1826 or non-CpG-DNA 2041, and then the peritoneal fluid, spleen, mesenteric lymph nodes (MLN), and cervical lymph nodes (CLN) were harvested from the mice 7 days after the injection. The cells from the tissues were cultured for 2 days, and the levels of IgM were analyzed in the cell culture supernatants (Fig. 2A). The IgM amounts were increased in the culture supernatants of cells prepared from the CpG-DNA 1826-injected peritoneal fluid, but were not increased in the other tissues by CpG-DNA 1826 injection. To determine whether IgM is produced by CpG-DNA stimulation *in vitro*, the cells from the tissues were stimulated *in vitro* with PBS, CpG-DNA 1826 or non-CpG-DNA 2041 for 2 days and the levels of IgM were analyzed in the cell culture supernatants (Fig. 2A). The results show that the amounts of IgM were significantly increased in the peritoneal fluid, but only slightly increased in the other tissues by CpG-DNA

1826 stimulation *in vitro* compared to the PBS control or non-CpG-DNA 2041. When the CpG-DNA was pre-treated prior to the isolation of the peritoneal cells, the CpG-DNA-induced production of IgM was significantly reduced. To examine the bacteria-reactivity of the IgM produced by the CpG-DNA stimulation of the peritoneal cells *in vitro*, we measured the levels of bacteria-reactive IgM with the Gram-positive bacteria species. The levels of bacteria-reactive IgM were significantly increased by stimulation with CpG-DNA compared to PBS or non-CpG-DNA 2041 (Fig. 2B). To determine which B cells are associated with bacteria-reactive IgM secretion following CpG-DNA stimulation, mice were intraperitoneally injected with the PBS control or CpG-DNA 1826. Nine days after the injection of CpG-DNA 1826, we sorted CD23<sup>-</sup>CD19<sup>+</sup> B cells (B1 cells) and CD23<sup>+</sup>CD19<sup>+</sup> B cells (B2 cells) from the peritoneal cavity lymphocytes (Fig. 2C). The sorted B cells were treated with CpG-DNA 1826 *in vitro* for 2 days, and the amounts of IgM in the supernatants of the cell cultures were measured by ELISA (Fig. 2D). The secreted amounts of IgM in response to CpG-DNA treatment were increased both in the B1 and B2 cells. However, the increase of IgM production by B cells was reduced when the mice were pre-injected with CpG-DNA before the isolation of peritoneal cells, indicating that CpG-DNA influenced the B cell activity in terms of antibody production (Fig. 2D). The amounts of bacteria-reactive IgM in the B cells were increased by CpG-DNA stimulation *in vitro*; however, the production of the bacteria-reactive IgM by the B2 cells was significantly reduced when CpG-DNA was pre-treated prior to the isolation of the peritoneal cells (Fig. 2E). The results suggest that bacteria-reactive IgM was produced by the CpG-DNA treatment both in the B1 and B2 cells of the peritoneal cavity; however, there can be some difference in the regulation between the two subpopulations.

### **Production of bacteria-reactive monoclonal IgM antibody**

To obtain the bacteria-reactive monoclonal IgM antibody from the mouse peritoneal cavity cells, we screened the hybridoma cells secreting CpG-DNA-induced IgM. Seven days after the intraperitoneal injection of CpG-DNA 1826, the peritoneal cavity cells were harvested from the mice, and they were fused with myeloma SP2/0 cells, and screened in selective medium. After screening a hybridoma clone (3F3F5) secreting the bacteria-reactive monoclonal IgM antibody, the cells were cultured serum-free medium for 5 days. The bacteria-reactive monoclonal IgM antibody was purified from the culture supernatants using ion-exchange chromatography, followed by gel filtration chromatography. The IgM protein fractions were collected, loaded and run on a SDS-PAGE, and stained with Coomassie brilliant blue R solution (Fig. 3A). The fractions including the purified IgM were analyzed by Western blotting (Fig. 3B). We confirmed the ability of the purified monoclonal IgM antibody to bind several Gram-positive bacteria by ELISA (Fig. 3C).

#### **Bacteria-reactive monoclonal IgM antibody enhances phagocytosis of *S. aureus* MW2 in RAW 264.7 cells**

To perform the phagocytosis assay *in vitro*, we selected murine macrophage cell line RAW 264.7. To investigate the phagocytosis efficacy of the bacteria-reactive monoclonal IgM antibody, we incubated fluorescein isothiocyanate (FITC)-labeled *S. aureus* MW2 with PBS, normal mouse IgM, or bacteria-reactive monoclonal IgM antibody (3F3F5 IgM). The RAW 264.7 cells were treated with the antibody-bound *S. aureus* MW2, and then, phagocytosis was observed by confocal microscopy (Fig. 4A, B). The results indicate that the efficacy of phagocytosis was increased in the presence of the bacteria-reactive monoclonal IgM antibody or normal mouse IgM. However, there was no difference in phagocytic efficacy between the two IgM suggesting that the amounts of bacteria-reactive IgM were increased in the peritoneal cavity and sera following the injection of CpG-DNA 1826 (Fig. 1) but the



phagocytic ability of the bacteria-reactive antibody was not enhanced by the administration of CpG-DNA 1826 compared to normal mouse IgM.

## DISCUSSION

*S. aureus* has been recognized as a major cause of many infectious diseases including life-threatening diseases such as pneumoniae, sepsis, and endocarditis (19). Excessive use of multiple antibiotics to treat *S. aureus* infection has led to the emergence of methicillin-resistant *staphylococcus aureus* (MRSA) (20). As the efficacy of antibiotics decreases and the emergence of MRSA increases, a new strategy to replace antibiotics is urgently required. Several reports have suggested the development of vaccination and passive immunization using protective antibodies as an alternative strategy against MRSA infections (21-23).

In our previous experiments, we confirmed that CpG-DNA treatment enhanced the host protection following *S. aureus* infection, producing bacteria-reactive IgG antibodies which also have a protective role against infection (18). Here, we hypothesized that the CpG-DNA-induced production of antibodies, especially IgM, would be involved in the anti-bacterial effect of CpG-DNA. Because the levels of the bacteria-reactive IgM antibodies were significantly increased both in the peritoneal cavity and serum of mice following the administration of CpG-DNA (Fig. 1), we predicted that CpG-DNA triggered the activation of some B cell populations, producing bacteria-reactive IgM.

Natural antibodies, which exist without any exposure to antigens, have a broad antibacterial activity, and prevent several bacterial infections (24). Here, we confirmed that the amount of IgM produced by the CpG-DNA treatment was greatly increased in the peritoneal cavity of the mice and had a broad reactivity against several bacteria (Fig. 1). We suppose that the bacteria-reactive IgM has broad reactivity because the epitopes of the antibody might be common bacterial surface components such as carbohydrate, glycoprotein,

and/or metabolites.

In addition, the secretion of IgM antibodies by the CpG-DNA treatment was significantly increased in the peritoneal cells when we isolated the peritoneal cells, spleen, MLN, and CLN and stimulated them with CpG-DNA *in vitro* (Fig. 2). It has been reported that most of the B1 cells were in the peritoneal cavity and produced broad bacterial-reactive antibodies against microbial agents (25, 26). We found that the bacteria-reactive IgM was produced both in the peritoneal B1 and B2 cells, but higher levels of IgM were produced in the B1 cells than in the B2 cells (Fig. 2).

Researchers have shown the efficacy of antibody-mediated phagocytosis against bacterial infection as a measure of the anti-bacterial effect in phagocytic cells (24, 27). When we examined the effects of the bacteria-reactive monoclonal IgM antibody (3F3F5 mIgM) against bacterial infection, the efficacy of phagocytosis against *S. aureus* MW2 infection in RAW 264.7 cells was enhanced by the monoclonal IgM antibody which was comparable to normal IgM (Fig. 4). Based on the results, we consider that 3F3F5 mIgM could be a potent therapeutic agent against bacterial infection in emergency.

In conclusion, we proved that the bacteria-reactive IgM produced by CpG-DNA has protective roles against *S. aureus* infection by enhanced antibody-mediated phagocytosis in murine macrophage cell line. Despite numerous studies on the development of therapeutic antibodies against bacterial infection, no clinical antibodies have yet been approved. Therefore, future studies on infection therapy should include the development of a humanized antibody against MRSA infection, and this development might be useful to treat infectious diseases.

## MATERIALS AND METHODS

**CpG-DNA administration**

CpG-DNA 1826 and non-CpG-DNA 2041, whose backbones of the sequences were modified with phosphorothioate, were synthesized by GenoTech Co. The following sequences were used: CpG-DNA 1826, 5'-TCCATGACCGTTCTGACCGTT-3' and non-CpG-DNA 2041 as a negative control, 5'-CTGGTCTTTCTGGTTTTTTTCTGG-3'. The oligodeoxynucleotides were dissolved in distilled water, injected intraperitoneally in BALB/c mice or TLR9<sup>-/-</sup> mice (50 µg/ mouse).

***In vitro* stimulation of mouse primary cells with CpG-DNA**

Primary cells were harvested from the mice, homogenized, and re-suspended in RPMI 1640 medium containing 5% FBS. After removing the erythrocytes, the cells were cultured in RPMI 1640 medium containing 5% FBS with 100 U/ml of penicillin and 100 µg/ml of streptomycin. Next, each cell culture plate was treated with PBS or 5 µg/ml of CpG-DNA 1826 or 5 µg/ml of non-CpG-DNA 2041. After 48 h, the cell culture supernatants were harvested and analyzed by ELISA to quantify the IgM levels.

**Sorting B cells from mouse peritoneal cells**

Peritoneal cells were stained with antibodies in sorting buffer (1 mM EDTA, 25 mM HEPES pH 7.0, 1% FBS diluted in PBS). To isolate the B cells, the peritoneal cells were incubated with anti-mouse CD19 (BD Biosciences, San Jose, CA, USA) and anti-mouse CD23 (eBioscience, San Diego, CA, USA). To sort non-B cells from the peritoneal cells, anti-mouse CD3 (BD Bioscience) was used to isolate T cells. B1 cells and B2 cells were sorted using a FACS Aria<sup>TM</sup> II system (Becton Dickinson Inc.).

**Purification of monoclonal IgM antibody**

IgM-secreting hybridoma cells (3F3F5 clone) were applied to the Hybridoma-SFM (Thermo Fisher Scientific Inc.), and grown at 125 rpm in a shaker incubator containing 8% CO<sub>2</sub> at 37°C. After 5 days, cell culture supernatants were obtained by centrifugation at 3,000 rpm for 10 min, and purified using DEAE Sepharose<sup>TM</sup> Fast Flow (GE Healthcare Co.) and HiPrep<sup>TM</sup> 16/60 Sephacryl<sup>TM</sup> S-300 HR (Ge Healthcare Co.). The samples were loaded onto a SDS-PAGE, analyzed by Coomassie blue staining and Western blotting (28).

### **Western blotting**

The purified IgM was resolved, and transferred onto nitrocellulose membranes, which were blocked with 5% Skim milk in PBS-T for 1 h at room temperature. Membranes were incubated with HRP-conjugated goat anti-mouse IgM ( $\mu$ -chain specific) antibody (Merck Millipore Co.) for 2 h at room temperature. Immuno-reactive protein band intensities were measured with chemiluminescence reagent (Thermo Fisher Scientific Co.) as previously described (29).

### ***In vitro* phagocytosis assays**

The mouse RAW 264.7 macrophage cell line was purchased from the American Type Culture Collection (ATCC). The cells were cultured overnight on poly-L-lysine-coated cover glass (Sigma-Aldrich Co.) in 12-well plates (Nunc Inc.). The FITC-labeled *S. aureus* MW2 was treated with PBS or IgM for 1 h, and then, the bacteria were added to the plate cultured with RAW 264.7 cells. The cells were incubated for 1 h and fixed with 4% paraformaldehyde (Affymetrix Inc.). The fixed cells were washed with PBS, and then, Hoechst No. 33258 (Sigma-Aldrich Co.) was added at room temperature. The mounted cells were analyzed using a LSM 710 laser scanning microscope (Carl Zeiss Co.) previously described (18). The phagocytic index was measured by counting the number of FITC-labeled *S. aureus* MW2

phagocytosed by RAW 264.7 cells as previously described (18).

Additional Material and Methods are provided in the Supplementary Material

## ACKNOWLEDGMENTS

This work was supported by grants from the National Research Foundation (2017R1A2B2007373, 2009-0093812) funded by the Ministry of Science and ICT in the Republic of Korea.

## CONFLICTS OF INTEREST

The authors declare no conflict of interest.

## FIGURE LEGENDS

**Figure 1. Production of bacteria-reactive IgM in the mouse peritoneal cavity and serum by administration of CpG-DNA 1826.** (A, B) BALB/c mice were intraperitoneally injected with CpG-DNA 1826. After 7 days, the mice were intravenously injected with *S. aureus* MW2 ( $1 \times 10^7$  CFU). Peritoneal fluid and serum were collected from the mice two days after the bacterial infection. Bacteria-reactive IgM in the peritoneal fluid (A) and serum (B) was captured using *S. aureus* MW2-coated plates (n=3/group) and the concentrations of IgM were measured by ELISA. 1826, CpG-DNA 1826. MW2, *S. aureus* MW2. (C-F) BALB/c and (G-J) TLR9<sup>-/-</sup> mice were intraperitoneally injected with CpG-DNA 1826. Seven days after the administration of CpG-DNA 1826, the peritoneal fluids (C, E, G, I) and serum (D, F, H, J) were collected. To measure the amounts of IgM reactive to bacteria, the indicated bacteria were added to poly-L-lysine-coated plates, and the amount of bound IgM was determined by ELISA (n=3/group). The results presented are representative of three independent

experiments.  $*p < 0.05$ ;  $**p < 0.005$ ;  $***p < 0.0005$ .

**Figure 2. Production of bacteria-reactive IgM in the mouse peritoneal cavity cells by CpG-DNA 1826 treatment *in vitro*.** (A) BALB/c mice were intraperitoneally injected with PBS, CpG-DNA 1826, or non-CpG-DNA 2041. After 7 days, the cells from the peritoneal cavity (PerC), spleen, mesenteric lymph nodes (MLN), and cervical lymph nodes (CLN) were harvested and then treated with PBS, CpG-DNA 1826, or non-CpG-DNA 2041. The cell culture supernatants were collected after 48 h, and the amounts of total IgM were determined by ELISA ( $n=3/\text{group}$ ). (B) The peritoneal cells were harvested from PBS-injected mice, and the cells were treated with PBS, CpG-DNA 1826, or non-CpG-DNA 2041. The amounts of IgM reactive to the indicated Gram-positive bacteria in the culture supernatants were determined by ELISA ( $n=3/\text{group}$ ). (C) B1 and B2 cells were isolated from PBS- or CpG-DNA 1826-injected peritoneal cavity using a FACS Aria<sup>TM</sup> II system and fluorescence-labeled anti-mouse CD19 and anti-mouse CD23 antibodies. (D, E) Isolated B1 and B2 cells from the peritoneal cavity were stimulated with PBS or CpG-DNA 1826 for 4h. The cell culture supernatants were collected, and the amount of total IgM (D) and IgM reactive to Gram-positive bacteria (E) were determined by ELISA ( $n=3/\text{group}$ ). 1826, CpG-DNA 1826. 2041, non-CpG-DNA 2041. The results presented are representative of three experiments.  $***p < 0.0005$ .

**Figure 3. Purification and characterization of monoclonal IgM antibody produced by CpG-DNA 1826 stimulation.** (A, B) IgM-secreting hybridoma cells (clone 3F3F5) were cultured in Hybridoma-SFM for 5 days. The supernatants of the cell cultures were collected, and subjected to DEAE-sepharose column chromatography, and then, the protein fractions containing IgM were purified by gel filtration. The fractions containing monoclonal IgM

antibody were analyzed by Coomassie brilliant blue staining (A) and Western blotting (B). S, cell culture supernatants; HC, heavy chain; LC, light chain. (C) The bacteria-reactivity of the purified IgM was measured by ELISA using plates coated with Gram-positive bacteria (n=3/group). The results presented are representative of three experiments.

**Figure 4. Enhanced phagocytosis activity in RAW 264.7 cells by the bacteria-reactive monoclonal IgM antibody.** FITC-labeled *S. aureus* MW2 cells ( $3 \times 10^8$  CFU/mL) were incubated with PBS, normal mouse IgM (normal mIgM), or bacteria-reactive monoclonal IgM antibody (3F3F5 IgM) (10 µg/mL) for 1 h at 37°C, and then added to RAW 264.7 cells *in vitro*. After 1 h, the cells were washed with PBS, fixed, and stained with Hoechst No. 33258 to visualize the nuclei (blue). (A) Confocal images revealing phagocytosis of *S. aureus* MW2. Scale bars, 10 µm. MW2, *S. aureus* MW2. (B) The phagocytic index was analyzed (n=5/group). The results presented are representative of three experiments. \*\*\* $p < 0.0005$ .

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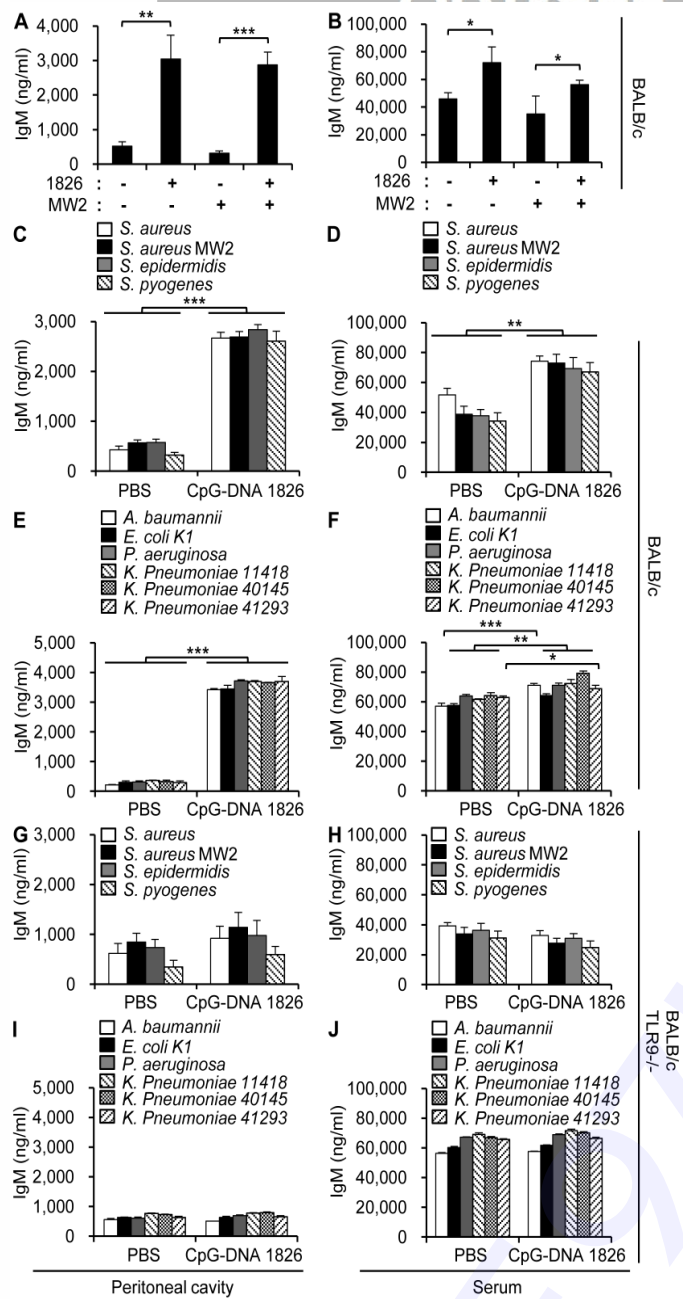


Fig. 1. Figure 1. Production of bacteria-reactive IgM in the mouse peritoneal cavity and serum by administration of CpG-DNA 1826.

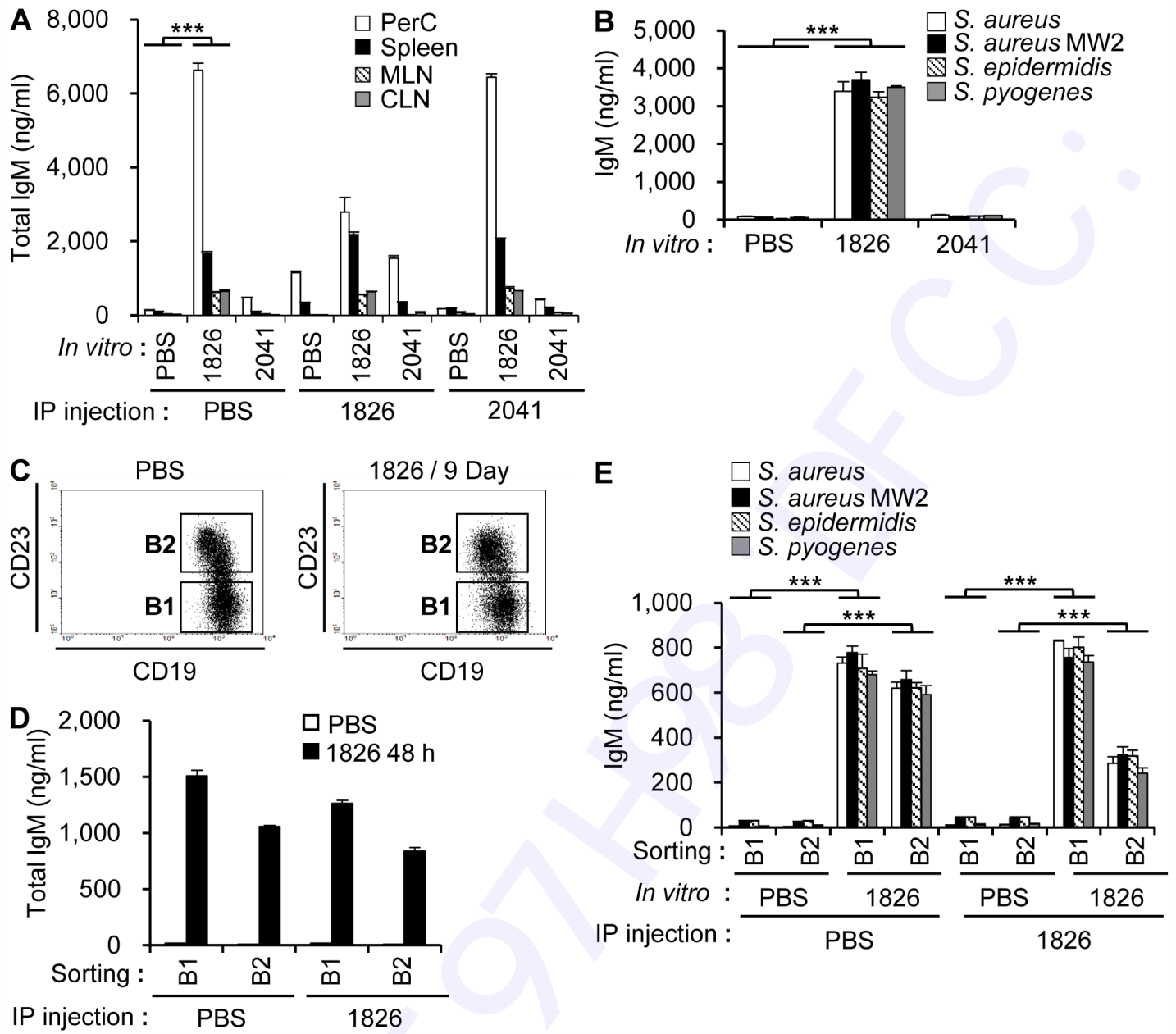


Fig. 2. Figure 2. Production of bacteria-reactive IgM in the mouse peritoneal cavity cells by CpG-DNA 1826 treatment in vitro.

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(kDa) S 33 37 41 45 49 53 57 : Fraction No.



**B** MW  
(kDa) S 33 37 41 45 49 53 57 : Fraction No.

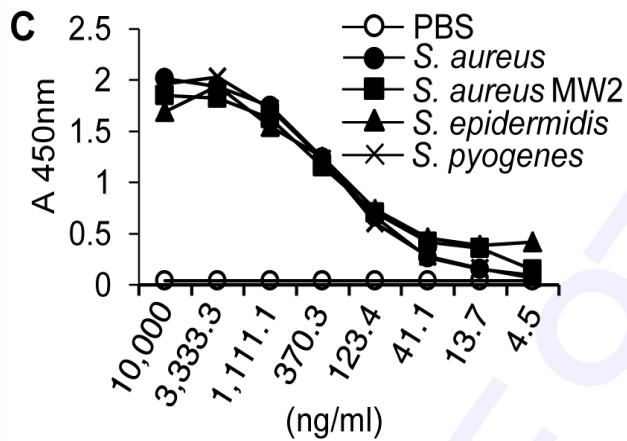
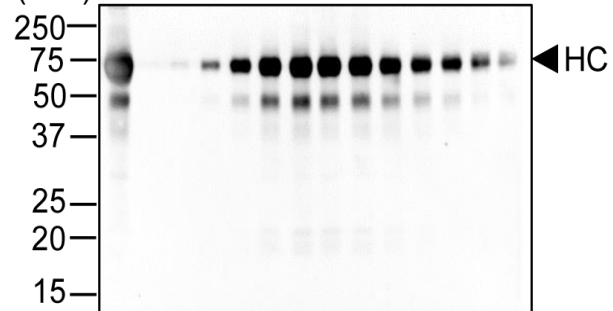


Fig. 3. Figure 3. Purification and characterization of monoclonal IgM antibody produced by CpG-DNA 1826 stimulation.

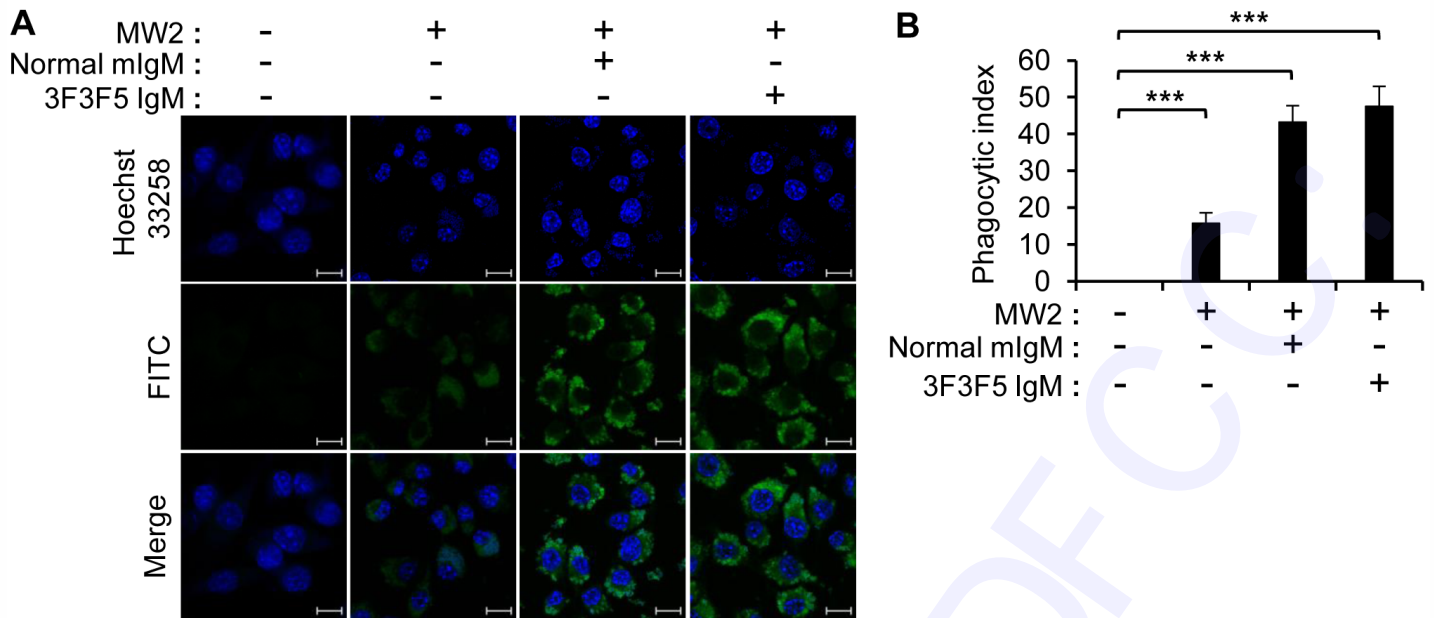


Fig. 4. Figure 4. Enhanced phagocytosis activity in RAW 264.7 cells by the bacteria-reactive monoclonal IgM antibody.

## Supplementary Methods

### Mice

Eight-week old BABL/c mice (Nara Biotech Inc.) and BALB/c TLR9 knockout mice (TLR9<sup>-/-</sup>) (Oriental BioService Inc.) were purchased for the experiments. The mice were kept under specific-pathogen-free (SPF) conditions with the optimal temperature (20-25°C) and humidity (32-37%). Sera were obtained by retro-orbital bleeding under isoflurane (2-3%, JW Pharmaceutical Inc.) inhalation anesthesia to minimize pain. After the experiments were terminated, the mice were sacrificed by CO<sub>2</sub> inhalation and all efforts were made to minimize suffering. All animal study protocols were approved by the Hallym University Institutional Animal Care and Use Committee (Permit Number: Hallym 2017-43).

### Bacteria

*Staphylococcus aureus* (*S. aureus*, KCCM 12103), *Staphylococcus epidermidis* (*S. epidermidis*, KCCM 40416), *Streptococcus pyogenes* (*S. pyogenes*, KCCM 11873), *Klebsiella pneumoniae* 11418 (*K. pneumoniae*, KCCM 11418), *Klebsiella pneumoniae* 40145 (*K. pneumoniae*, KCCM 40145), *Klebsiella pneumoniae* 41293 (*K. pneumoniae*, KCCM 41293), *Escherichia coli* (*E. coli* K1, KCCM 12119), *Acinetobacter baumannii* (*A. baumannii*, KCCM 40203), and *Pseudomonas aeruginosa* (*P. aeruginosa*, KCCM 11803) were purchased from the Korean Culture Center of Microorganisms (KCCM). All bacteria were grown at 37°C in Lysogeny broth (LB). The *S. aureus* strain MW2 (*S. aureus* MW2, MRSA) was grown at 37°C in Columbia broth supplemented with 2% NaCl (1). All bacteria were grown overnight, re-cultured in fresh media at a 1/50 dilution until the mid-log phase (OD<sub>600</sub> 0.5-0.6) and harvested, and colony forming units (CFU) were determined on agar plates.

## ELISA

To determine the production of bacteria-reactive antibodies following CpG-DNA 1826 administration in mice, we used poly-L-lysine coated plates (Corning Inc.). The bacteria were grown overnight, washed twice with PBS by centrifugation at 10,000 rpm for 15 min, and re-suspended in conventional ELISA coating buffer. Each well was coated with 100  $\mu$ l of re-suspended bacteria ( $5 \times 10^7$  CFU /well) and incubated overnight at 4°C. After incubation, the bacteria were fixed with 0.5% glutaraldehyde in PBS for 15 min at room temperature. After washing twice with PBS, each well was incubated with RPMI 1640 medium containing 100 mM glycine and 0.1% BSA for 30 min at room temperature to block the glutaraldehyde and washed twice with PBS. The bacteria-coated wells were then blocked with PBS containing 1% BSA for 1 h at room temperature. Serum, peritoneal cavity fluid, and culture supernatants of cells from primary tissues such as peritoneal cells (PerC), spleen, mesenteric lymph nodes (MLN), and cervical lymph nodes (CLN) or purified antibodies were serially diluted, added to each well, and incubated for 1 h at room temperature. The samples were washed three times with PBS-T (0.2% Tween-20 in PBS) and antibodies, including horseradish peroxidase (HRP)-labeled goat anti-mouse IgM (Southern Biotech Inc.) were added to the wells and incubated for 1 h at room temperature. After washing with PBS-T four times, the TMB Microwell Peroxidase Substrate Kit (KPL Inc.) was used to detect the peroxidase-labeled conjugates (blue-color expression), followed by the addition of the TMB Stop solution (KPL) (yellow-color), and the absorbance was measured at 450 nm using a SpectraMax 250 microplate reader (Molecular Devices Inc.). To determine the concentrations of the total IgM, the plates were coated with goat anti-mouse Ig (Southern Biotech Inc.) and analyzed using anti-mouse IgM antibody by ELISA as previously described (2).



**Production of hybridoma cells from B cells in the peritoneal cavity**

BALB/c mice were intraperitoneally injected with 50 µg of CpG-DNA 1826. After 7 days, the peritoneal cells were harvested from the mice, and fused with mouse SP2/0 myeloma cells, and then, bacteria-reactive IgM antibody-producing hybridoma clones were isolated using a standard hybridoma technique (2).

**Fluorescent labeling of bacteria**

*S. aureus* MW2 was cultured to OD<sub>600</sub> 0.5-0.6 ( $3 \times 10^8$  CFU), washed with PBS, and fixed with 70% ethanol in PBS for 1 h. The fixed bacteria were dissolved in 0.1 M Na<sub>2</sub>CO<sub>3</sub> buffer (pH 8.5) with 0.02 mM FITC (Sigma-Aldrich Co.) for 30 min at room temperature, washed with serum-free Hank's Balanced Salt Solution (HBSS), and re-suspended in HBSS containing 2 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 10 mM HEPES, 150 mM NaCl, and 0.4% BSA.

**References**

1. Brown S, Xia G, Luhachack LG et al (2012) Methicillin resistance in *Staphylococcus aureus* requires glycosylated wall teichoic acids. *Proc Natl Acad Sci U S A* 109, 18909-18914
2. Wu G, Kim D, Kim JN et al (2018) A Mucin1 C-terminal Subunit-directed Monoclonal Antibody Targets Overexpressed Mucin1 in Breast Cancer. *Theranostics* 8, 78-91