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Original article

Streptococcus pyogenes CAMP factor attenuates phagocytic activity of RAW 264.7 cells

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Abstract

Streptococcus pyogenes produces molecules that inhibit the function of human immune system, thus allowing the pathogen to grow and spread in tissues. It is known that *S. pyogenes* CAMP factor increases erythrocytosis induced by *Staphylococcus aureus* β-hemolysin. However, the effects of CAMP factor for immune cells are unclear. In this study, we investigated the effects of CAMP factor to macrophages. Western blotting analysis demonstrated that all examined strains expressed CAMP factor protein. In the presence of calcium or magnesium ion, CAMP factor was significantly released in the supernatant. In addition, both culture supernatant from *S. pyogenes* strain SSI-9 and recombinant CAMP factor dose-dependently induced vacuolation in RAW 264.7 cells, but the culture supernatant from Δ*cfa* isogenic mutant strain did not. CAMP factor formed oligomers in RAW 264.7 cells in a time-dependent manner. CAMP factor suppressed cell proliferation via G2 phase cell cycle arrest without inducing cell death. Furthermore, CAMP factor reduced the uptake of *S. pyogenes* and phagocytic activity indicator by RAW 264.7 cells. These results suggest that CAMP factor works as a macrophage dysfunction factor. Therefore, we conclude that CAMP factor allows *S. pyogenes* to escape the host immune system, and contribute to the spread of streptococcal infection.

Keywords: Innate immunity; *Streptococcus pyogenes*; Virulence factor; CAMP factor; Phagocytosis

1. Introduction

Streptococcus pyogenes is a Gram-positive bacterium that causes numerous diseases, including superficial infections, such as pharyngitis and pyoderma, invasive infections, such as necrotizing fasciitis and streptococcal toxic shock syndrome, and the post-infectious diseases such as rheumatic fever [1]. *S. pyogenes* produces multiple virulence-associated proteins, which contribute to its ability to evade the innate immune system [2]. These streptococcal proteins show cytotoxicity against immune cells and inhibit the recognition of *S. pyogenes* or subsequent phagocytosis. For example, *S. pyogenes* streptolysin O (SLO) confers resistance to macrophage killing by preventing phagolysosome acidification [3]. The C5a-binding protein of *S. pyogenes* inhibits the chemotactic function of C5a and promotes avoidance of detection by human neutrophils [4]. Furthermore, *S. pyogenes* streptococcal pyrogenic exotoxin B degrades C3b, and promotes evasion of phagocytosis by neutrophils [5]. Sda1 suppresses the innate immune response by preventing Toll-like receptor 9-dependent recognition and the bactericidal activity of macrophages [6]. By evading the innate immune system, *S. pyogenes* is able to invade tissues [7−11] and proliferate.

Abbreviations: RAW cells, RAW 264.7 cells; SLO, streptolysin O; THY broth, Todd Hewitt broth supplemented with 0.2% yeast extract; HRP, horseradish peroxidase; rCAMP factor, recombinant CAMP factor; GTBS, Tris-buffered saline containing 0.25% gelatin; LPS, lipopolysaccharide; LDH, lactate dehydrogenase; PI, propidium iodide.

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Christie Atkins Munch-Petersen (CAMP) factor is known to induce CAMP reaction, which was first described by Christie et al. as the cooperative lysis of sheep erythrocytes by *Streptococcus agalactiae* and *Staphylococcus aureus* [12]. The CAMP reaction has also been detected when *S. pyogenes* [13] or *Propionibacterium acnes* [14] is co-cultured with *S. aureus* or *Clostridium perfringens*. The sequences of genes that encode the CAMP factors of *S. pyogenes* [13], *S. agalactiae* [15], and *P. acnes* [16] are highly homologous. Lang et al. reported that *S. agalactiae* CAMP factor is a pore-forming toxin that creates pores in sheep erythrocyte membranes pre-treated with sphingomyelinase and oligomerizes on liposome membranes *in vitro* [17]. Moreover, *P. acnes* CAMP factor and the acid sphingomyelinase of host cells enhance bacterial virulence and confer cytotoxicity to host cells [18]. However, little is known about the effect of *S. pyogenes* CAMP factor on immune cells. The aim of this study was to determine whether CAMP factor is actually expressed by *S. pyogenes* and to examine its effects on macrophages.

2. Materials and methods

2.1. Bacteria

Invasive *S. pyogenes* clinical strains 466 (serotype M1), SSI-9 (serotype M1), SSI-1 (serotype M3), SSI-7 (serotype M3), and SSI-8 (serotype M3) were isolated from wound site of patients with streptococcal toxic shock syndrome. Other *S. pyogenes* clinical isolates used in this study, including strains 142 (serotype M1), 144 (serotype M1), and TW3384 (serotype M3), were used as non-invasive strains isolated from pharynx. All strains were grown in Todd Hewitt broth (Becton Dickinson, MD, USA) supplemented with 0.2% yeast extract (THY broth) at 37 °C.

2.2. Cells

RAW 264.7 cells (RAW cells; mouse monocyte-macrophages) were obtained from DS Pharma Biomedical Co. Ltd. (Osaka, Japan) and grown in DMEM (Wako, Osaka, Japan) supplemented with 10% heat-treated FBS (Japan Bio Serum Co. Ltd., Hiroshima, Japan), 100 U/ml penicillin, and 100 μg/ml streptomycin (Wako) at 37 °C in 95% air and 5% CO_2. Human myeloid THP-1 cells were grown in RPMI 1640 (Wako) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.6 mg/ml glutamine at 37 °C in 95% air and 5% CO_2. THP-1 cells were forced to differentiate by treatment with 100 nM 4-alpha-phorbol 12-myristate 13-acetate (LC Laboratories, MA, USA) for 1 day.

2.3. Animals

All animal procedures were performed according to protocols reviewed and approved by the established institutional policies and guideline of Niigata University for animal care and use. ICR mice were obtained from Clea (Tokyo, Japan). Thioglycollate-elicited macrophages were isolated from the peritoneal cavities of mice. Macrophages were cultured in RPMI at 37 °C in 95% air and 5% CO_2.

2.4. Materials

A rabbit antibody against *S. pyogenes* CAMP factor was generated by Eurofins Genomics (Tokyo, Japan). A rabbit antibody against *S. pyogenes* GAPDH/Plr was prepared as described previously [4]. Horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG was obtained from GE Healthcare (Buckinghamshire, UK). Thiazolyl blue tetrazolium bromide was purchased from Sigma (MO, USA). Western blotting protein marker was obtained from Life Technologies-Invitrogen (CA, USA). $CaCl_2$ and $MgCl_2$ were purchased from Wako.

2.5. Construction of recombinant CAMP factor expression vector

A recombinant (r) CAMP factor expression plasmid was constructed using a pGEX-6P-1 vector (GE Healthcare). The forward primer (5′-GCACTCAGTTGGATCCGATGATGCTTCAAATC-3′) and reverse primer (5′-CCCGAATTCCTATGACTTAAGAGCTGTTTCCAAAG-3′) were used to amplify the *cfa* gene of *S. pyogenes* strain SSI-9 by PCR. The resultant PCR fragment was cloned into a pGEX-6P-1 vector. The pGEX-6P-1 vector containing the *cfa* gene was transformed into *Escherichia coli* strain Able-K (Stratagene, CA, USA) by heat shock method. The Able-K transformants were grown in Luria−Bertani broth (Nacalai Tesque, Kyoto, Japan) supplemented with 100 μg/ml ampicillin (Meiji Seika, Tokyo, Japan) to select for the pGEX-6P-1 vector, allowing its expression. Then, the rCAMP factor protein was purified by glutathione-sepharose 4B (GE Healthcare), and the GST tag was cleaved by prescission protease (GE Healthcare). The purified rCAMP factor protein was dialyzed against Tris-buffered saline containing 0.25% gelatin (GTBS). The amount of lipopolysaccharide (LPS) in 1 μg of purified rCAMP factor protein was determined to be less than 2 pg using LPS detection kit (GenScript, NJ, USA).

2.6. Integration mutagenesis using targeted plasmid

PCR product of the internal portion of *cfa* gene was amplified using 5′-GGGGAATTCTTTTCAAAGAGATGCTCT-3′ and 5′-CCCGGATCCTCTGATAATGCTTCTACT-3′ as primers, and ligated into a suicide vector pSF151 [19]. The resultant plasmid pMK7 was transformed into wild type strain SSI-9 by electroporation and the inactivated mutant strain was selected on kanamycin (Meiji Seika, Tokyo, Japan)-containing agar plate.

2.7. Western blot analysis

S. pyogenes strains were incubated in THY broth at 37 °C for 20 h under various conditions. Cell extracts and culture supernatants were collected by centrifugation at 3000× *g* for

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10 min. The samples were mixed with 2% SDS sample buffer and heated at 99 °C for 3 min, and then separated by SDS-PAGE using 12.5% gels (Gellex International, Tokyo, Japan) and transferred to polyvinylidene difluoride membranes (Millipore, MA, USA). The membranes were incubated with blocking reagent (Nacalai) to block nonspecific binding and probed with the anti-CAMP factor antibody diluted in Tris-buffered saline containing 0.05% Tween 20 (TaKaRa, Shiga, Japan). The membrane was then incubated with a HRP-conjugated secondary antibody in Tris-buffered saline containing 0.05% tween 20. The membrane was treated with HRP substrates and analyzed by chemiluminescence detector (Fujifilm, Tokyo, Japan). RAW cells (3×10^4 in 300 μl) were treated with rCAMP factor (5 μg/ml) at 37 °C for various time periods (0, 1, 3, 6, 12, 24, 48, and 72 h) in a 48-well plate (BD Falcon, NJ, USA). Thereafter, the medium was removed, and the cells were washed with DMEM, then incubated in 2% SDS sample buffer in preparation for SDS-PAGE using 5–20% gradient gels (BIO CRAFT, Tokyo, Japan) and western blotting using the anti-CAMP factor antibody.

2.8. Cell vacuolation assay

Vacuolation was assayed by direct microscopy or by calcein AM (Molecular Probes, OR, USA) uptake. For the direct microscopy assay, RAW cells (1×10^4 in 200 μl) were seeded into a 96-well plate (BD Falcon) and incubated with the indicated concentrations of rCAMP factor, heat-treated rCAMP factor, or rCAMP factor treated with an anti-CAMP factor antibody for various time periods. rCAMP factor (1.0 mg/ml, 100 μl) was incubated at 100 °C for 60 min (heat-treated), or with 200 μl of anti-CAMP factor antibody stock solution at 37 °C for 60 min (antibody neutralization). Otherwise, 5 μl of *S. pyogenes* wild-type strain SSI-9 and isogenic *cfa*-deficient (Δ*cfa*) mutant strain culture supernatants were added, and the cells were incubated at 37 °C. *S. pyogenes* wild-type strain and mutant strain culture supernatants were obtained by ultrafiltration using filters (Millipore), and were sterilized with filters (Sartorius Stedim Biotech, Göttingen, Germany). Viable cells were defined as those having well-defined cytoplasmic and nuclear outlines, and vacuolated cells were defined as the presence of one or more intracytoplasmic vacuoles within viable cells. For each determination, RAW cells were counted with a fluorescence microscopy (Keyence, Osaka, Japan), and the percentage of vacuolated cells was calculated as follows: vacuolated cells/viable cells. For the calcein AM uptake assay, RAW cells (1×10^5 in 2 ml) were treated with rCAMP factor (5 μg/ml), heat-treated rCAMP factor (5 μg/ml), or rCAMP factor (5 μg/ml) treated with the anti-CAMP factor antibody at 37 °C for 24 h in a glass bottom dish (MatTek, MA, USA). The medium was removed, and the cells were washed with DMEM. And then, 2 ml of DMEM and 4 μl of 1 mg/ml calcein AM in dimethyl sulfoxide (1:500 dilution; Nacalai) were added. RAW cells were incubated at 37 °C for 5 min, washed twice with DMEM, and then observed with a confocal laser scanning microscopy (Zeiss, Jena, Germany).

2.9. Cytotoxicity assay

RAW cells (1×10^4 in 300 μl) were seeded into a 48-well plate (BD Falcon) and stimulated with 3 different concentrations of rCAMP factor (0.2, 1, and 5 μg/ml) or 0.1% Triton X-100 at 37 °C for 24 h or 30 min, respectively. Cytotoxicity was determined as the amount of lactate dehydrogenase (LDH) using a LDH measurement kit (Roche Diagnostics K.K., Tokyo, Japan) according to the manufacturer's instructions. In addition, the further cytotoxic analyses were performed by staining with propidium iodide (PI; Life Technologies-Invitrogen) and performed with an MTT assay. For PI staining, RAW cells were stained with 0.3 μl of PI (1:1000 dilution) and counted by fluorescence microscopy application (Keyence). Then, the cells were fixed with 4% glutaraldehyde (Sigma) in DMEM at room temperature for 1 h, washed with PBS, stained with 0.6 μl of DAPI (1:500 dilution; Dojindo, Kumamoto, Japan), and enumerated by fluorescence microscopy application. The percentage of PI-positive cells was calculated as follows: PI positive cells/cells with DAPI-stained nuclei. For the MTT assay, 75 μl of thiazolyl blue tetrazolium bromide (2 mg/ml in PBS) was added to each well. RAW cells were incubated at 37 °C for an additional 2 h, and then the medium was removed by suctioning. Formazan blue that formed in the cells was dissolved with 100 μl of DMSO. Absorbance at 571 nm was measured with an absorbance detector (Thermo Scientific, MA, USA).

2.10. Cell cycle assay

RAW cells were treated with rCAMP factor (5 μg/ml) at 37 °C for 24 h. Cell cycle was investigated using a cell cycle assay kit (Biocolor Life Science Assays, Carrickfergus County Antrim, UK) according to the manufacturer's instructions.

2.11. Phagocytic activity assay

S. pyogenes strain SSI-9 was cultured until the mid-log phase (optical density at 600 nm = 0.2). RAW cells were treated with rCAMP factor (5 μg/ml) at 37 °C for 24 h. The medium was discarded, and RAW cells were washed with DMEM, and then DMEM containing 10% FBS was added. RAW cells were infected with *S. pyogenes* at 37 °C for 1 h, and RAW cells were washed twice with DMEM. Merged cells were defined as cells that touched or captured *S. pyogenes*. For a quantitative phagocytic assay, the medium was suctioned, and RAW cells were washed with DMEM. Then, phagocytic activity indicator (Life Technologies-Invitrogen) in Hank's balanced salt solution (Life Technologies-Invitrogen) containing 20 mM HEPES (Nacalai) was added to each well, and the plate was incubated at 37 °C for 30 min. The cells stained with phagocytic activity indicator were counted by fluorescence microscopy application, and fluorescence intensity per cell was calculated.

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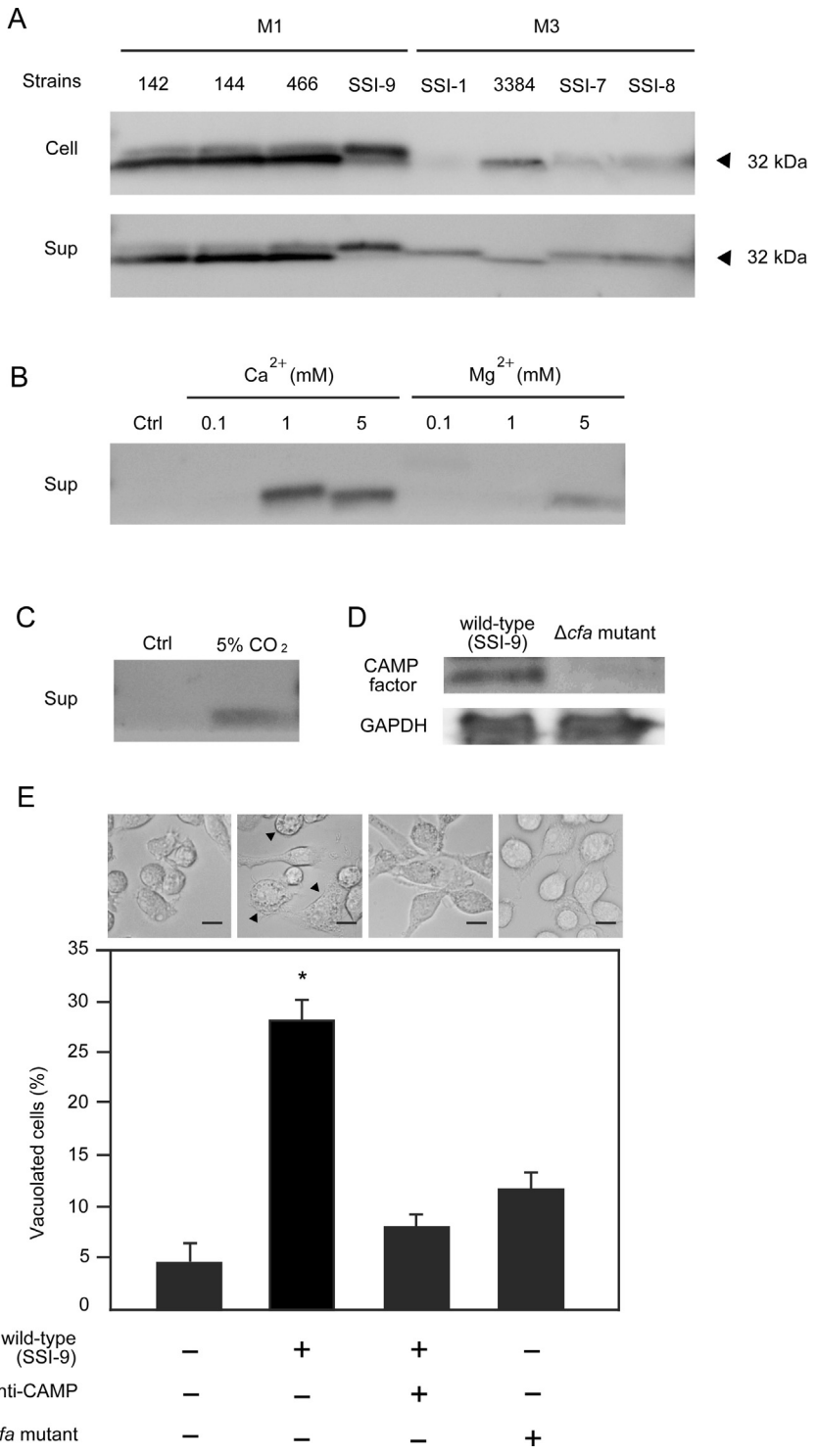

Fig. 1. Expression and localization of CAMP factor in *S. pyogenes* and vacuolation of RAW cells induced by *S. pyogenes* strain SSI-9 culture supernatant. A) Serotypes M1 and M3 *Streptococcus pyogenes* strains were incubated in THY broth at 37 °C for 20 h. Bacterial cell extracts and culture supernatants were separated by SDS-PAGE and analyzed by Western blotting using an anti-CAMP factor antibody. B) *S. pyogenes* strain SSI-9 was incubated in 50% THY broth containing 0.1, 1, and 5 mM $CaCl_2$ or $MgCl_2$ at 37 °C for 20 h. C) *S. pyogenes* strain SSI-9 was incubated at 37 °C for 20 h in 95% air and 5% CO_2. The optical density at 600 nm of each sample was adjusted to 0.5. Culture supernatants were analyzed by Western blotting using the anti-CAMP factor antibody. Cell: bacterial cell extracts, Sup: culture supernatants. D) Protein expression of CAMP factor and GAPDH/Plr from *S. pyogenes* wild-type strain SSI-9 and its Δ*cfa* isogenic mutant strain was detected by Western blotting. E) RAW cells were incubated with 5 μl of THY broth, *S. pyogenes* wild-type strain SSI-9 culture supernatant, wild-type strain culture supernatant treated with an anti-CAMP factor antibody, or Δ*cfa* mutant strain at 37 °C for 24 h. The percentage of vacuolated cells was calculated by direct microscopic visualization. The arrowheads indicate vacuolated RAW cells. Data are shown the mean ± SE; $n = 3$; $^*p < 0.01$, compared to the percentage of vacuolated control cells. Scale bar: 10 μm.

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2.12. Statistical analysis

All assays were performed in triplicate as independent experiments. Most of the presented data are expressed as the mean ± standard error (SE). Mean values among experimental groups were compared using Dunnett's *t*-test or one-way ANOVA, and *p* values less than 0.05 were considered to be statistically significant.

3. Results

3.1. S. pyogenes *secretes CAMP factor into the culture supernatant*

First, we determined whether several *S. pyogenes* strains possess the *cfa* gene that encodes CAMP factor. In silico analyses, the *cfa* genes were detected in *S. pyogenes* strains for which complete genome sequences are available from GenBank (serotype M1; 476, MGAS5005, SF370 and SSI-9, serotype M2; MGAS10270, serotype M3; MGAS315 and SSI-1, serotype M4; MGAS10750, serotype M5; Manfredo, serotype M6; MGAS10394, serotype M12; MGAS2096 and MGAS9429, serotype M18; MGAS8232, serotype M28; MGAS6180, serotype M49; 591, NZ131 and Alab49, and serotype M59; MGAS15252). And then, the all deduced mature CAMP factors were calculated 32 kDa. The expression of CAMP factor protein was examined in 8 strains of *S. pyogenes*. All the strains examined in this study expressed CAMP factor proteins, which were secreted into the culture supernatant (Fig. 1A). Next, we investigated the effects of divalent metal ions on the expression of CAMP factor from *S. pyogenes* strain SSI-9. When *S. pyogenes* was incubated at

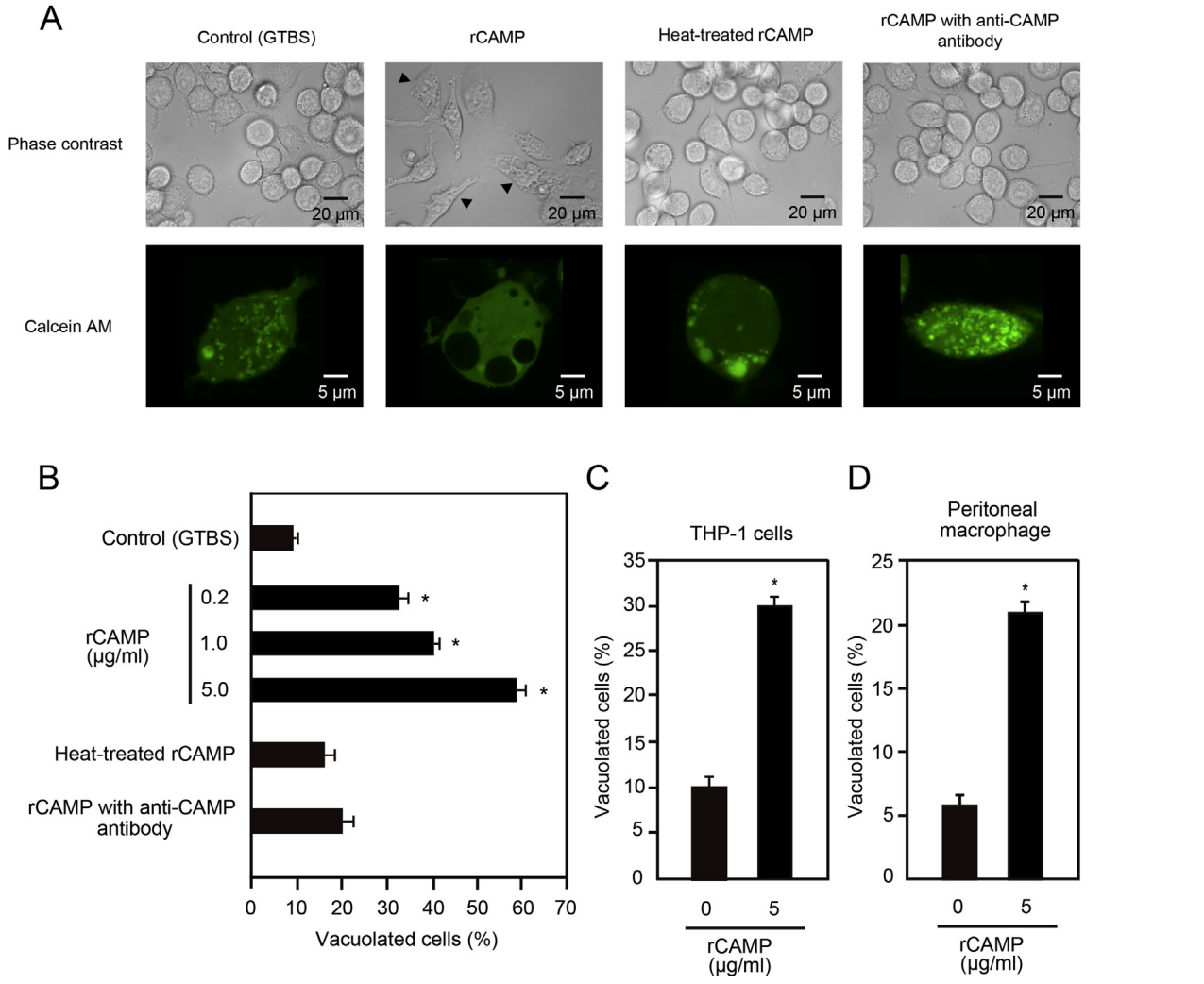

Fig. 2. Effects of recombinant CAMP factor on the vacuolation of RAW cells. RAW cells were incubated with Tris-buffered saline containing 0.25% gelatin (GTBS) as a control, rCAMP factor (5 μg/ml), heat-treated rCAMP factor (5 μg/ml), or rCAMP factor (5 μg/ml) treated with the anti-CAMP factor antibody at 37 °C for 24 h. A) RAW cells were observed under a fluorescence microscopy in DIC and a confocal laser scanning microscopy following staining with calcein AM as green images. The arrowheads indicate vacuolated RAW cells. B) RAW cells were incubated with GTBS as a control or various concentrations of CAMP factor at 37 °C for 24 h. C) THP-1 cells and D) peritoneal macrophages were incubated with GTBS or rCAMP factor (5 μg/ml) at 37 °C for 24 h. Data are shown as the mean ± SE; $n = 3$; $*p < 0.01$, compared to control cells.

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37 °C with $CaCl_2$ or $MgCl_2$, CAMP factor was strongly expressed in a $CaCl_2$ or $MgCl_2$ concentration-dependent manner (Fig. 1B). In addition, the expression of CAMP factor was significantly induced in 5% CO_2 condition than atmosphere condition (Fig. 1C).

3.2. S. pyogenes *strain SSI-9 culture supernatant induces vacuolation of RAW cells*

We next confirmed the effects of *S. pyogenes* strain SSI-9 culture supernatant on RAW cells. *S. pyogenes* wild-type strain culture supernatant induced the production of a number of large vacuoles in the cytoplasm of RAW cells. In contrast, *S. pyogenes* Δ*cfa* isogenic mutant strain did not express CAMP factor proteins (Fig. 1D), and the culture supernatant had lower rate of vacuoles than that of wild-type strain (Fig. 1E). In addition, the wild-type strain culture supernatant treated with an anti-CAMP factor antibody did not induce vacuolation (Fig. 1E).

3.3. Recombinant CAMP factor induces vacuolation of RAW cells

In Fig. 1E, *S. pyogenes* culture supernatant induced vacuolation of RAW cells. We constructed and purified recombinant (r) CAMP factor, and then we investigated the effect of this rCAMP factor on RAW cells. RAW cells treated with rCAMP factor at 37 °C for 24 h contained vacuoles that were similar to those observed in the cells treated with *S. pyogenes* culture supernatant. In contrast, heat-treated rCAMP factor, and rCAMP factor treated with an anti-CAMP factor antibody did not induce vacuolation (Fig. 2A). When RAW cells were incubated in the presence of 10 pg of LPS, the vacuoles were not observed (data not shown). Fig. 2B shows that after 24 h of treatment with rCAMP factor, the percentage of vacuolated cells increased dose-dependently as assessed by direct counting (phase-contrast microscopy). Notably, in the presence of 5 μg/ml rCAMP factor, the percentage of vacuolated cells reached almost 60%. Moreover, vacuoles were also observed in THP-1 cells and peritoneal macrophages from ICR mice treated with rCAMP factor (Fig. 2C and D).

3.4. rCAMP factor oligomerizes on RAW cells

Next, the time course of vacuolation was investigated. When RAW cells were treated with rCAMP factor, vacuoles were formed in a time-dependent manner, and the number of vacuoles reached a maximum at 24 h (Fig. 3A). It has been reported that most pore-forming toxins oligomerize on cell membranes [17,20–23]. Therefore, we examined whether rCAMP factor oligomerizes on the membranes of RAW cells. Some rCAMP factor oligomers were observed and those formed in a time-dependent manner (Fig. 3B). This result is in agreement with the findings presented in Fig. 3A, which shows that CAMP factor time dependently induces the formation of vacuoles in RAW cells.

3.5. rCAMP factor inhibits growth of RAW cells

To examine whether rCAMP factor induces cytotoxicity in RAW cells, we assessed cell viability after incubation with rCAMP factor by PI staining and LDH assays. As shown in Fig. 4A, after 24 h of treatment with rCAMP factor, the percentage of PI-positive cells was nearly equal to that of the vehicle control as assessed by fluorescence microscopy application. Similarly, there were no significant differences in intracellular and extracellular LDH release between the vehicle control and rCAMP factor-treated cells (Fig. 4B). The PI staining assay and LDH release assay revealed that rCAMP factor didn't possess cytotoxicity. Next, we investigated the activity of intracellular oxidoreductase enzymes in RAW cells using MTT reagent. Absorbance value of the cells treated with rCAMP factor was lower than that of control cells (Fig. 4C). These results suggested that CAMP factor have relation to cell proliferation. Therefore, we counted total cell number. The total cell number decreased after 24 h of treatment with

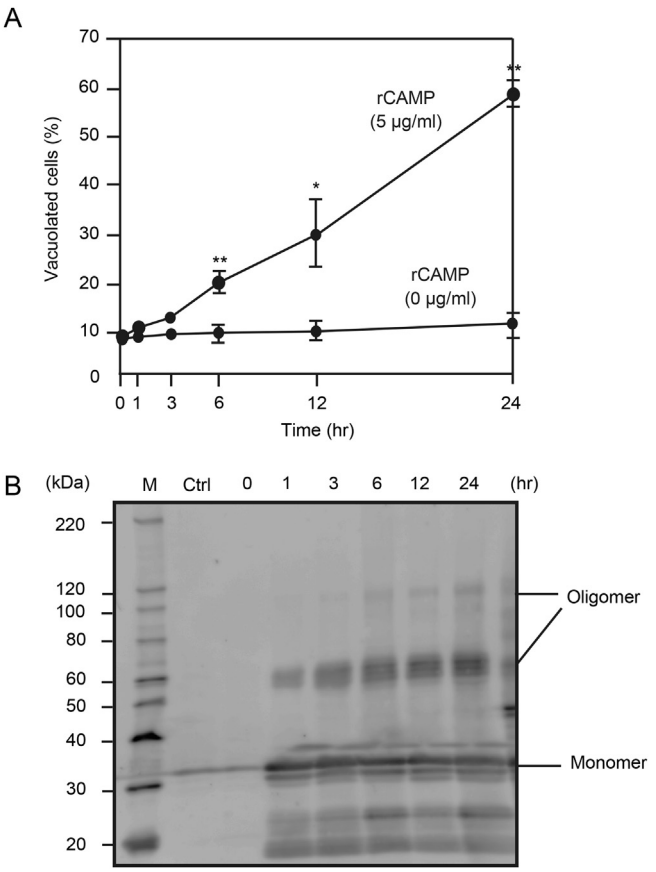

Fig. 3. Binding of rCAMP factor to RAW cells. RAW cells were incubated with GTBS as a control or CAMP factor (5 μg/ml) at 37 °C for the indicated times. A) The percentage of vacuolated cells was calculated by direct microscopic visualization (visual observation). Data are shown as the mean ± SE; $n = 3$; $^{*}p < 0.05$; $^{**}p < 0.01$, compared to the percentage of vacuolated control cells. B) RAW cells were washed and scraped using 2% SDS sample buffer with 2-mercaptoethanol, followed by heating at 37 °C for 30 min. The proteins in cell lysates were separated by 5–20% gradient SDS-PAGE and then subjected to Western blotting using the anti-CAMP factor antibody. M, protein marker.

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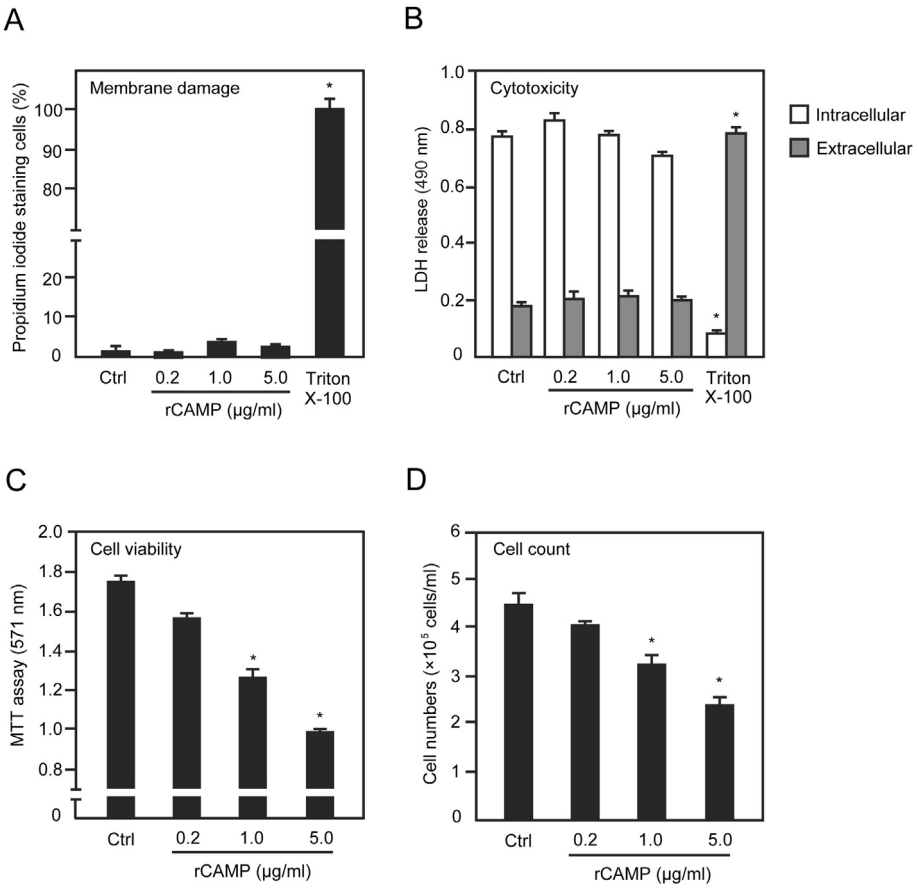

Fig. 4. Cytotoxicity of rCAMP factor in RAW cells. RAW cells were incubated with GTBS as a negative control, various concentrations of CAMP factor, or 0.1% Triton X-100 as a positive control at 37 °C for 24 h. A) Total cells were detected by staining nuclei with DAPI, and dead cells were stained with propidium iodide (PI). The percentage of PI-positive cells was calculated by fluorescence microscopy application. Data are shown as the mean ± SE; $n = 3$; *$p < 0.01$, compared to the number of PI-positive cells in the negative control. B) The amount of lactate dehydrogenase (LDH) released was determined with the LDH measurement kit, and is shown as absorbance at 490 nm. White bars and gray bars show the intracellular and extracellular LDH levels, respectively. Data are shown as the mean ± SE for 3 independent experiments. $n = 3$; *$p < 0.01$, compared to the absorbance in negative control cells. C) Cell viability was determined by MTT assay, and is shown as absorbance at 571 nm. Data are shown as the mean ± SE; $n = 3$; *$p < 0.01$, compared to absorbance in control cells. D) Total cells were counted by visual observation. Data are shown as the mean ± SE; $n = 3$; *$p < 0.01$, compared to the cell number in control samples.

rCAMP factor in a dose-dependent manner (Fig. 4D), suggesting that rCAMP factor inhibited the growth of RAW cells without inducing cell death.

3.6. rCAMP factor causes cell cycle arrest at G2 phase in RAW cells

Next, we investigated the effects of rCAMP factor on the cell cycle. The images obtained in experiments using the cell cycle assay kit are presented in Fig. 5A. The percentages of RAW cells in the G1 and M phase decreased after 24 h of treatment with rCAMP factor (Fig. 5B and D), whereas that of cells in the S/G2 phase increased (Fig. 5C). These results indicated that rCAMP factor induced G2 phase cell cycle arrest in RAW cells.

3.7. rCAMP factor reduces the phagocytic activity of RAW cells

We showed that the rCAMP factor induced the vacuolation and cell cycle arrest of RAW cells. Then, we investigated the effect of rCAMP factor on phagocytic activity, which is one of the main functions of macrophages like RAW cells. RAW cells treated with rCAMP factor were infected with *S. pyogenes* strain SSI-9 at 37 °C for 1 h. In vacuolated cells, the percentage of merged cells (harboring or contacting *S. pyogenes*) was significantly lower than that of merged intact cells (Fig. 6A). To quantitatively evaluate phagocytic activity, we measured the uptake of phagocytic activity indicator in vacuolated cells, as shown by fluorescence intensity. The fluorescence intensity in vacuolated cells treated with rCAMP factor was lower than that in intact cells after 30 min of treatment with phagocytic activity indicator (Fig. 6B). These results indicated that rCAMP factor reduced the phagocytic activity of RAW cells.

4. Discussion

Both the protein–protein BLAST and the conserved domain database analyses demonstrated that CAMP factor is mainly detected in Streptococci. In addition, previous reports show that CAMP factor is specifically expressed in pathogenic

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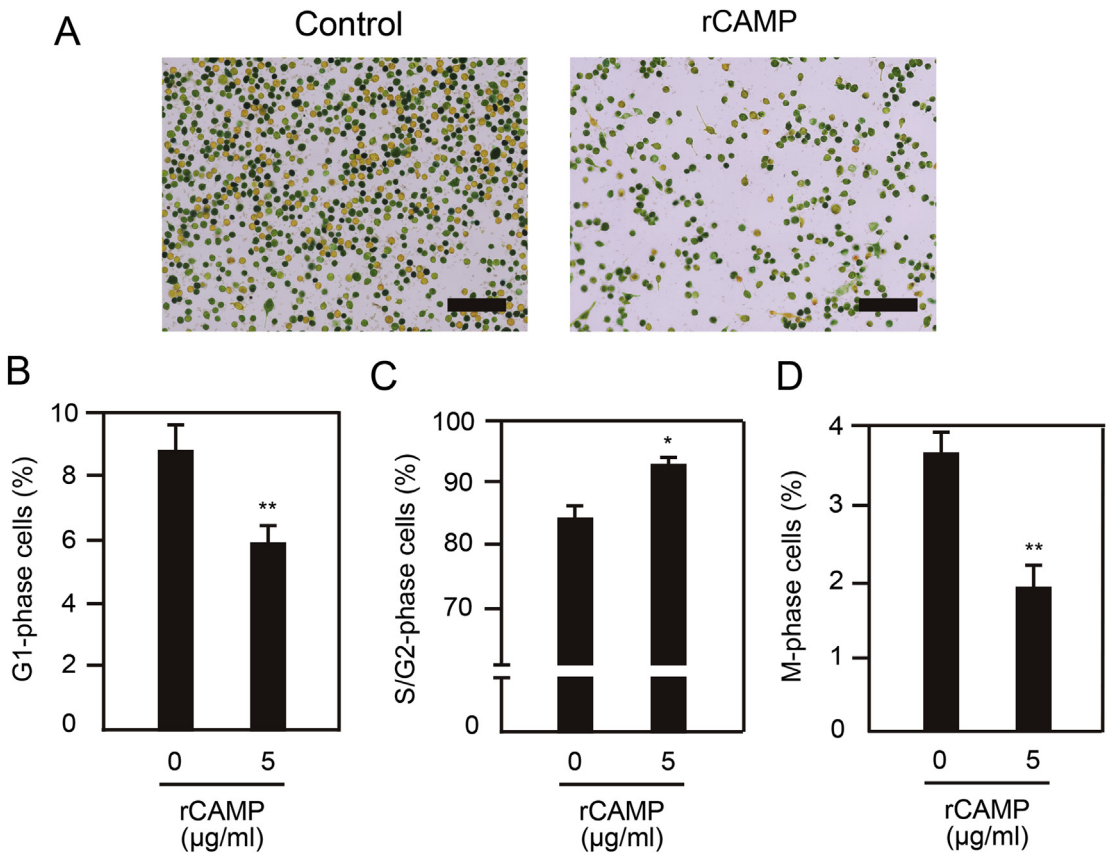

Fig. 5. Effects of rCAMP factor on the cell cycle in RAW cells. RAW cells were incubated with GTBS as a control or CAMP factor (5 μg/ml) at 37 °C for 24 h. A) Based on the images obtained with a cell cycle assay kit, the percentages of cells in each of the following phases B) G1-phase; C) S/G2-phase; and D) M-phase were calculated. Data are shown as the mean ± SE; $n = 3$; *$p < 0.05$; **$p < 0.01$, compared to the percentage of control cells in each phase. Scale bar: 200 μm.

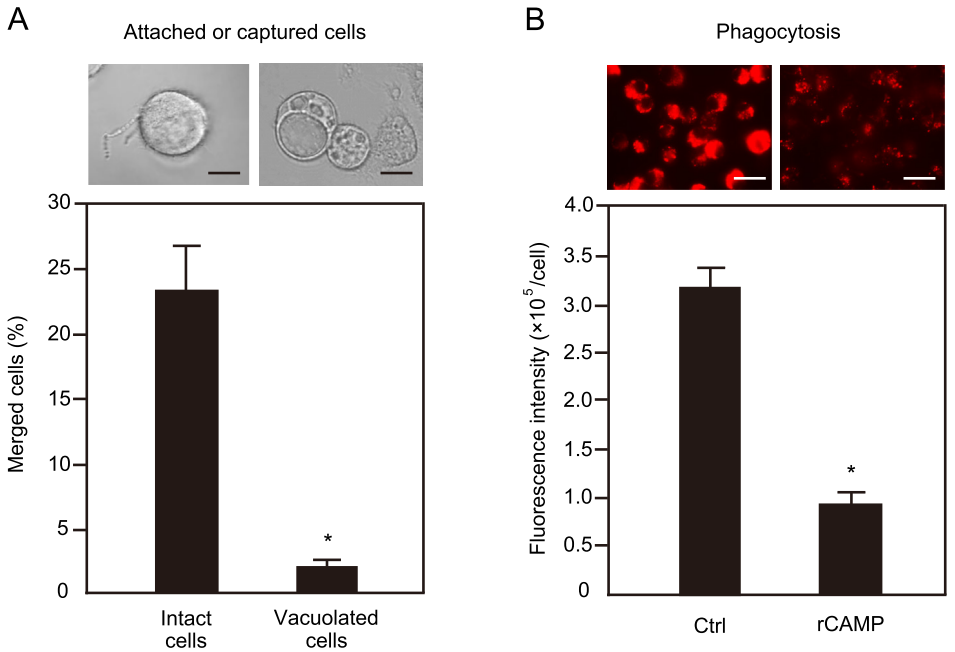

Fig. 6. Effects of rCAMP factor on the phagocytic activity of RAW cells. RAW cells were incubated with GTBS as a control or CAMP factor (5 μg/ml) at 37 °C for 24 h. A) Then, RAW cells were infected with *S. pyogenes* for 1 h. The percentage of cells merged with *S. pyogenes* was calculated by direct microscopic visualization. Data are shown as the mean ± SE; $n = 3$; *$p < 0.01$, compared to the percentage of cells merged with *S. pyogenes* in the control. Scale bar: 10 μm. B) Cells were stained with phagocytic activity indicator, and fluorescence intensity per cell was calculated by fluorescence microscopy application. Data are shown as the mean ± SE; $n = 3$; *$p < 0.01$, compared to the fluorescence intensity in intact cells. Scale bar: 20 μm.

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bacteria that cause skin infections, such as *S. agalactiae*, *Streptococcus uberis*, *P. acnes*, and *S. pyogenes*. *S. pyogenes* also causes severe invasive diseases, and the number of fatalities by the severe infections was presumed to reach at least 650,000 each year [24]. It is well-known that the first phase of *S. pyogenes* infection begins from bacterial adhesion onto human epithelial cells and internalization through its adhesin proteins [8,25,26]. Thereafter, it has been speculated that the invading bacteria could escape from human immunity and would grow in the tissues [4,5,27,28], which is the higher concentrations of CO_2 and calcium and magnesium ion in extracellular fluid than on the skin surface. Our data revealed that CAMP factor was strongly expressed in *S. pyogenes* when incubated at 37 °C with $CaCl_2$ or $MgCl_2$ (Fig. 1B) and in 5% CO_2 (Fig. 1C). It therefore suggested that the expression of CAMP factor is remarkably enhanced when *S. pyogenes* invades in host tissues. Jiang et al. reported that two-component system CsrRS in *S. agalactiae* regulated expression of CAMP factor [29]. *S. pyogenes* possesses CovRS that was the homologous molecule of *S. agalactiae* CsrRS. Moreover, it was shown that the activity of CovRS depends on the concentration of magnesium ion [30]. Our data revealed that the expression of CAMP factor in *S. pyogenes* was increased in the presence of $MgCl_2$ (Fig. 1B). These results suggested that expression of CAMP factor is controlled under CovRS in *S. pyogenes* and CAMP factor plays an important role in *S. pyogenes* pathogenesis. In addition, it was reported that *S. pyogenes* SLO and *Helicobacter pylori* VacA cause vacuolation after several hours [31,32]. In contrast, rCAMP factor gradually induced vacuolation in nearly 1 day (Fig. 3A). Consequently, it is thought that CAMP factor contributes to the persistence and exacerbation of inflammation during streptococcal infections in deeper tissues.

Several reports have revealed that some bacterial virulence factors lead to pore formation in cell membranes. These pore-forming toxins, such as SLO [33], *S. agalactiae* CAMP factor [17], VacA [22], and *C. perfringens* ε-toxin [20,21,23], oligomerize on host cell membranes. In this study, both the culture supernatant of *S. pyogenes* strain SSI-9 and rCAMP factor of *S. pyogenes* induced the formation of vacuoles in RAW cells (Figs. 1E and 2A). In addition, we detected the formation of rCAMP factor oligomers in RAW cells by Western blotting (Fig. 3B). The native conformations of VacA [34] and ε-toxin [20,21] are dodecamer and heptamer, respectively. In contrast, CAMP factor forms a tetramer, which is a small oligomer. This result suggested that the oligomer formation and toxic effects of CAMP factor in RAW cells were different from those of VacA and ε-toxin.

Previous studies suggested that some compounds induced both vacuole formation and cell cycle arrest, resulting in the death of host cells [35−37]. However, Tsai et al. reported that ethambutol induced G0/G1 phase cell cycle arrest and the formation of cytoplasmic vacuoles and reduced the phagocytic activity of RPE50 cells without causing cell death [38]. We demonstrated that rCAMP factor induced vacuolation (Fig. 2A) and inhibited cell proliferation of RAW cells without causing cell death (Fig. 4). Moreover, we showed that rCAMP factor caused G2 phase cell cycle arrest (Fig. 5) and reduced the phagocytic activity of RAW cells (Fig. 6). Our results suggested that CAMP factor induced the formation of vacuoles such as mimic phagocytic vacuoles in RAW cells.

In conclusion, CAMP factor induced the formation of vacuoles, suppressed the growth of RAW cells through G2 phase cell cycle arrest, and inhibited phagocytic activity. The activity of CAMP factor might differ from that of other known pore-forming toxins, because oligomer formation of CAMP factor differed from those of other toxins, and CAMP factor did not induce cell death. Previous studies revealed that *S. pyogenes* organisms internalize into epithelial cells [8,25,26]. Therefore, we speculate that invading *S. pyogenes* expresses CAMP factor in host tissues under high concentration of CO_2 and calcium or magnesium ion. Furthermore, our results suggested that the secreted CAMP factor attacks macrophages and works as a macrophage dysfunction factor that allows *S. pyogenes* to escape from the immune system, and then CAMP factor contributes to exacerbation of inflammation during streptococcal infections.

Future investigations are needed to clarify the mechanism of vacuolation induced by CAMP factor, the details regarding the interaction between CAMP factor and the cell membranes, and the relationship between CAMP factor oligomerization and pore formation in host cells.

Conflict of interest

The authors declare no conflict of interest.

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