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Recombinant Human REG Iα Aggregates *Staphylococcus aureus*—Exhibits a Lectin-Like Function

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Abstract

Staphylococcus aureus is pathogenic to humans with worldwide health care concern due to its ability to evade the immune system and develop resistance to multiple drugs. Reg family proteins are C-type lectins with antimicrobial properties. Bacterial aggregation through binding to microbial cell surface sugar and/or lipid moieties is key mechanism employed in the process. In the present study we have analysed the antimicrobial effect of human REG Iα on *S. aureus*. Aggregation of mid-log phase culture of *S. aureus* was observed in presence of purified recombinant REG Iα. Therefore REG Iα can be applied in eliminating *S. aureus* infections in humans.

Keywords

Reg Proteins, Lectin-Like Activity, Bacterial Aggregation

1. Introduction

Staphylococcus aureus continues to pose a challenge to human health owing to its ability to evade the immune system by acquiring novel genetic elements and developing resistance to antibiotics [1]. Therefore, along with developing new conventional drugs it is of interest and health benefit to look into alternative strategies for managing *S. aureus* infections that can be used preferably in synergy with existing antibiotics.

Role of C-type lectin superfamily in the immune system is well documented [2]. Serum mannose binding protein (MBP), a typical Ca^{2+} dependent carbohydrate binding lectin and ficolins activate the lectin complement pathway after binding carbohydrates on cell surfaces of infecting microbes, followed by opso-

nization and phagocytosis [3] [4] [5] [6]. Lectin receptors on the surface of immune cells bind pathogens via glycan recognition on microbial surfaces [7] [8] [9]. C-type lectin receptors initiate signaling pathways resulting in endocytosis, phagocytosis and production of inflammatory cytokines [10].

Reg proteins are members of *Reg* (*Regenerating gene*) multigene family [11] [12] [13], classified as C-type lectins that exhibit carbohydrate specificity via C-type lectin like domain (CTLD) [14]. Class III Reg family proteins show antibacterial activity against *Salmonella* Typhimurium, *Yersinia pseudituberculosis*, *Bacillus subtilis*, *Enterococcus faecalis*, *Listeria innocua* and *L. monocytogens* [15]-[20] owing to their ability to bind to molecules displayed on bacterial cell surfaces [15] [16] [20] [21]. Reg proteins, unlike the conventional C-type lectins, are relatively small, monomeric proteins which although do not recognize monosaccharides [22] [23] [24], they significantly bind to disaccharides [24], polysaccharides [15] [21] [24] and lipids [16] [17].

Class I Reg proteins are identified as pancreatic β-cell regeneration factors [25] amongst which human REG Iα is a 16 kDa secretory protein expressed primarily in pancreas with low expression in gastric mucosa and trace levels in kidneys [26]. REG Iα is reported to stimulate pancreatic β-cell proliferation *in vitro* and ameliorate diabetes in NOD mice [27] [28].

Amino acid sequence and structure analyses reveal a C-type lectin-like domain in human REG Iα [29] [30]. Iovanna, *et al.* (1993) have previously detected human REG Iα stimulated aggregation of bacteria from human feces including *Bacteroides*, *Eubacterium*, *Escherichia coli* (KH 802), *Peptostreptococcus* and *Bifidobacterium* [22]. However, antimicrobial activity of REG Iα towards pathogenic bacteria has not been investigated so far. In the present study we have analysed the lectin-like properties of human REG Iα against the cell surface polysaccharides of gram-positive *S. aureus.* For our purpose we expressed recombinant human REG Iα in *E. coli* and purified the gene product from inclusion bodies, on anion exchange chromatography column and used the purified protein to analyse the stimulation of *S. aureus* aggregation.

2. Materials and Methods

2.1. Bacterial Strains

Staphylococcus aureus 209P (ATCC 6538P) strain was used in this study.

2.2. Expression System

Human *REG Iα* gene encoding the mature peptide was PCR amplified using sense primer [5'-GTTGATTTGCCTCTTAAGCAAG-3'] and antisense primer [5'-AATTGCTGGATCAGTTCTAGAC-3'] and cloned into pBluscriptSK$^-$ T vector for sequence confirmation. The confirmed gene sequence was introduced into expression vector pTriEx-4 (Novagen), between SmaI and XhoI within the multiple cloning site. pTriEx-4 includes gene sequence for a 15 amino acids long S-tag that is cloned at the N-terminal of the target gene. In our study, we have deleted this sequence from the vector, by inverse PCR using sense primer

5'CAAGAGGCCCAGACAGAGTTGCCCCAG-3' and antisense primer 5'-GTGATGGTGGTGATGGTGTGCCATGGT-3', thus our finished product does not have any S-tag at the N-terminal. The S-tag deleted plasmid was transformed into *E. coli* host, Rosetta 2 (DE3) (Novagen).

2.3. Expression and Refolding of REG Iα

Two ml pre-culture of recombinant Rosetta 2 was incubated overnight in Luria Broth (LB) at 37˚C in presence of 50 µg/ml Ampicillin and 34 µg/ml Chloramphenicol. 25 ml LB with fresh antibiotics was inoculated with pre-culture the next day and further incubated at 37˚C till A_{600} reached 0.80. The culture was cooled at room temperature for 30 min, before protein expression was induced at 25˚C for 4 hours with isopropyl-1-thio-β-D-galactopyranoside at final concentration of 1 mM. The cells were harvested by centrifugation at 6,500 g for 15 min at 4˚C. Cell pellet was re-suspended in 1/5 culture volume of inclusion body (IB) wash buffer (20 mM Tris-HCl, 10 mM EDTA, and 1% Triton-X 100, pH 7.5) and ruptured by sonication in aliquots of 0.5 ml. Each aliquot was sonicated 8 times, on ice, (Power 1, Output 6), in bursts of 30 seconds. The aliquots were chilled on ice for 2 minutes between every two bursts.

Expression of REG Iα was detected in the inclusion body fraction. Insoluble inclusion bodies were collected by centrifugation at 10,000 g, for 10 min and homogenized in a dounce homoginizer in 1/5 volumes of IB wash buffer. Pellet was collected by centrifugation and homogenized once more. At the end of the second round of homogenization and centrifugation, the inclusion bodies were solubilized in 1/25 volumes of re-suspension buffer (7 M Guanidine-HCl, 0.1 M Tris-HCl, 0.15 M Reduced Glutathione, and 2 mM EDTA, pH 8.0) and incubated at room temperature, rotating end-to-end for 2 hours. Solubilized protein was collected as supernatant after centrifugation and refolded, by adding it dropwise into 2 volumes of 50 mM Tris-HCl containing 500 mM Arginine-HCl and 0.6 mM Oxidized Glutathione, pH 8.0, at room temperature, while continuous stirring. This method has been adopted from Cash, *et al.* (2006), after necessary modifications [31]. After incubation for 24 hours at 4˚C the refolded protein was dialysed against 10 volumes of 25 mM Tris-HCl, 2 mM $CaCl_2$, pH 8.0, for a total of 6 hours, changing the buffer once after 3 hours.

2.4. Anion Exchange Chromatography

One ml HiTrapQ anion exchange chromatography column (GE Healthcare) was equilibrated with 5 column volumes of 20 mM Tris-HCl, 100 mM NaCl, pH 8.5. Refolded protein was filtered through 0.45 µm syringe filter and loaded on the column using NGC™ automated chromatography system (BioRad). Unbound proteins were washed with 20 column volumes of same buffer. For elution, a linear gradient of NaCl was applied from baseline ionic strength of 100 mM, increasing up to 450 mM for 35 column volumes. Flow rate was maintained at 2 ml/min throughout the run. REG Iα was eluted as a single peak in 6 fractions of 1 ml each. Purity of elution fractions was confirmed by SDS-PAGE (15% gel)

followed by Coomassie staining. Concentration of purified protein was calculated from absorbance measured at 280 nm and 235 nm on a spectrophotometer (Biospec Nano, Shimadzu Biotech) using the method of Whitaker and Granum, (1980) [32]. Purified protein was saved at −80˚C in 50 - 100 µl aliquots and used for downward application within 1 week post purification.

2.5. Bacterial Aggregation

S. aureus was grown to mid-log phase (A_{600} = 0.75) followed by centrifugation at 5000 g for 5 minutes. Culture medium was discarded and cells were re-suspended in buffer containing 20 mM Tris-HCl, 150 mM NaCl, and 2 mM $CaCl_2$, at pH 7.5. Twelve point five folds dilution of this cell preparation was incubated with 10 µM REG in 100 µl of reaction mixture, at room temperature. Images of aggregated cells were captured under light microscope at magnification of 600 diopter.

To analyse the dose dependency of bacterial aggregation, 50 folds dilution of the cell preparation was incubated with (2.5, 5 and 10 µM) REG I*α* in a 100 µl reaction mixture at room temperature and free cells were counted using a hemocytometer. Time course of aggregation was measured in presence of 5 µM REG I*α* at 0, 30 and 60 minutes post incubation. For the control experiments, 50 folds dilution of cells was incubated in buffer without REG I*α* and free cells were counted at the indicated time points. Cell count per ml of incubation mixture was calculated using Hemocytometer Calculation Tools at http://www.currentprotocols.com/WileyCDA/CurPro3Tool/toolId-10.html. Percent free cells at indicated time points post incubation were used for statistical analysis and figure representations, with reference to the number of free cells at 0 minute incubation treated as 100%.

2.6. Data Analysis

REG I*α* addition versus control was analysed using two tailed Student's *t*-test. Average values from 3 or 5 experiments were used for calculations. $P < 0.05$ was considered statistically significant.

3. Results and Discussion

C-type lectins recognize carbohydrate moieties on invading pathogens, thereby playing a role in their elimination from the human body [33] [34]. Furthermore, role of Class III Reg family proteins in aggregation followed by killing of bacteria such as *Salmonella*, *Yersinia*, *Listeria* and *Enterococcus* is documented [16] [17] [19] [20]. Hence, in the present study we examined the antimicrobial effect of human REG I*α* against *S. aureus* which is a class I Reg family member.

Although REG I*α* has been reported as a glycosylated protein [35], we employed *E. coli* expression system for preparing recombinant protein which is unable to glycosylate the finished protein products. Previously, lectin activity has been reported in other Reg proteins such as recombinant human REG III and REG IV that were expressed in *E. coli* [24] [31]. Therefore, we assumed that

compromising glycosylation on REG Iα will not affect its lectin activity. Since eukaryotic proteins expressed in *E. coli* form inclusion bodies in the bacterial cytoplasm, for purification we adopted the previously tried method established for obtaining human HIP/PAP and mouse Reg IIIγ from inclusion bodies [31]. Inclusion bodies were solubilized under denaturing conditions, followed by refolding in presence of Arginine, as described in materials and methods. The refolded sample was purified on anion exchange column. The protein was eluted as a single peak between 220 mM and 260 mM concentrations of NaCl (**Figure 1(a)**). Elution fractions from the peak contained 16 kDa protein as confirmed by SDS-PAGE (**Figure 1(b)**), which is in agreement with the size of nonglycosylated recombinant REG Iα reported elsewhere [26] [36]. Approximately 1.5 mg of purified protein was obtained from 25 ml bacterial culture.

Purified REG Iα was added to *S. aureus* suspended in aggregation buffer and images were captured after 30 minutes of incubation. (**Figure 2(a)**). *S. aureus* in liquid culture appears in singlets, doublets, short chains or sometimes in small clusters. Cells appeared in clusters after 30 minutes post incubation with REG Iα. On the other hand clusters of bacteria were not found when incubated without REG Iα (**Figure 2(b)**). Thus, these results indicate that human REG Iα induces aggregation of *S. aureus*.

Next, we analysed the dose dependency and time course of bacterial aggregation in presence of purified REG Iα. Bacterial cells that did not aggregate were counted as free cells. The difference in amount of free cells post incubation in presence of REG Iα was used to determine the extent of bacterial aggregation in the assay. REG Iα aggregated *S. aureus* in a dose dependent manner (**Figure 3**). Incubation with 2.5 μM purified REG Iα resulted in a 4% reduction in total number of free cells after 60 minutes compared to samples incubated without REG Iα. 5 μM REG Iα was able to aggregate 32% of free cells and 10 μM protein aggregated 41% of free cells in the incubation mixture after 60 minutes. Furthermore, extent of aggregation by 5 μM REG Iα was compared after 30 and 60 minutes. Incubation for 30 minutes resulted in significant aggregation. The difference in extent of aggregation post 30 minutes and post 60 minutes did not bear statistical significance (**Figure 4**). Therefore, our results reveal that 5 μM REG Iα is sufficient for aggregation of *S. aureus* in 30 minutes. A clear cytotoxic effect of REG Iα against *S. aureus* was not observed (data not shown).

The role of lectins in innate immune mechanism have recently been highlighted, suggesting that host lectins bind to carbohydrates on microbial cell surfaces and activate the complement system via lectin-complement pathway of innate immunity [3] [4] [5] Bacterial aggregation by host lectins followed by opsonization, phagocytosis chemotaxis, activation of leukocytes or direct killing of the pathogen have been elucidated [37]. Thus our results strongly commend the role of REG Iα in elimination of *S aureus* from human body via aggregation of the bacteria.

Class III and Class IV Reg family proteins are expressed primarily in the gastrointestinal tract, with the exception of human HIP/PAP which is also found in

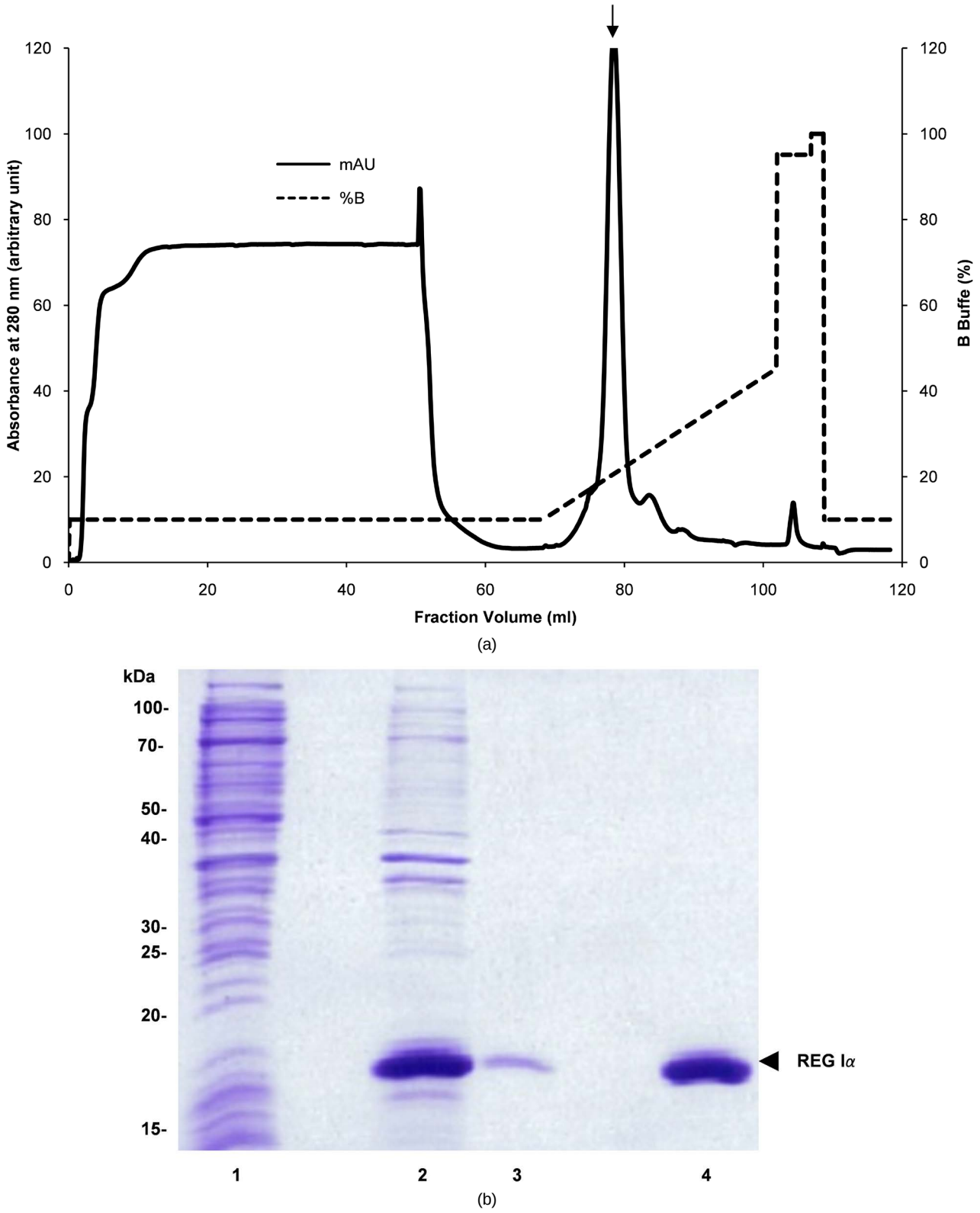

Figure 1. (a) Purification of recombinant REG I*α* on anion exchange sepharose resin. Elution profile was monitored online by measuring absorbance at 280 nm (left axis, solid line). Linear gradient of NaCl was applied between 10% and 45% of B buffer (20 mM Tris-HCl, 1 M NaCl, pH 8.5) for 35 column volumes (right axis, broken line) at the flow rate of 2 ml/min. (b) SDS-PAGE analysis of REG I*α*. Lane 1. Supernatant after sonication of whole cell fraction. Lane 2. Pellet of whole cell fraction after sonication. Lane 3. Dialysed sample after protein refolding, Lane 4. Eluate from anion exchange column.

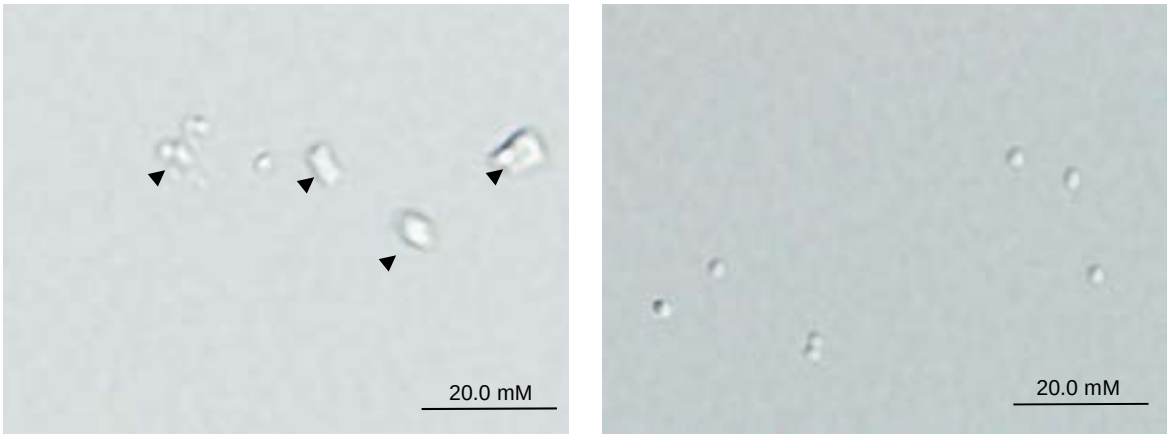

Figure 2. Aggregation of *S. aureus* by human REG I*α*. Mid-log phase culture of *S. aureus* was incubated with (a) or without (b) 10 μM purified REG I*α* in 100 μl of reaction mixture. Images of bacterial aggregates were captured 30 minutes post incubation. Cell clusters are indicated by arrows.

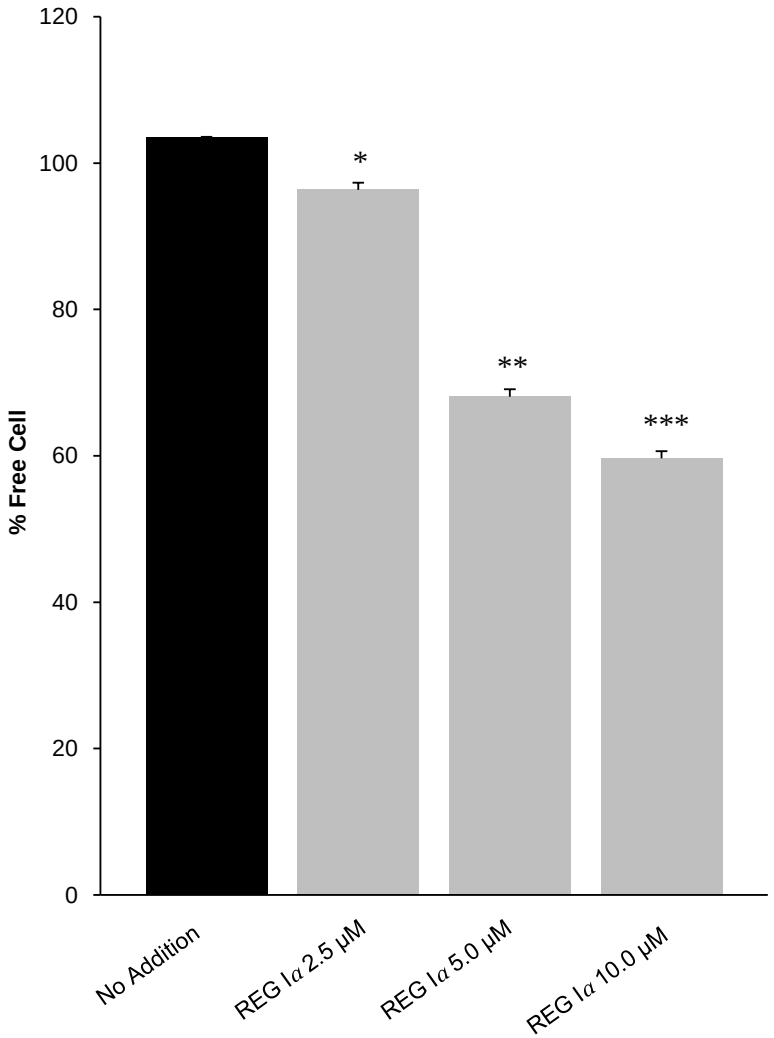

Figure 3. Dose dependent aggregation of *S. aureus* by REG I*α*. Bacterial aggregation was measured 60 minutes post incubation. Black bar represents no addition of REG I*α*. Addition of 2.5 μM, 5 μM and 10 μM REG I*α* is shown by grey bars; error bars represent standard error from 5 experiments; *P < 0.05, **P < 0.005, ***P < 0.0005 (Student *t* test).

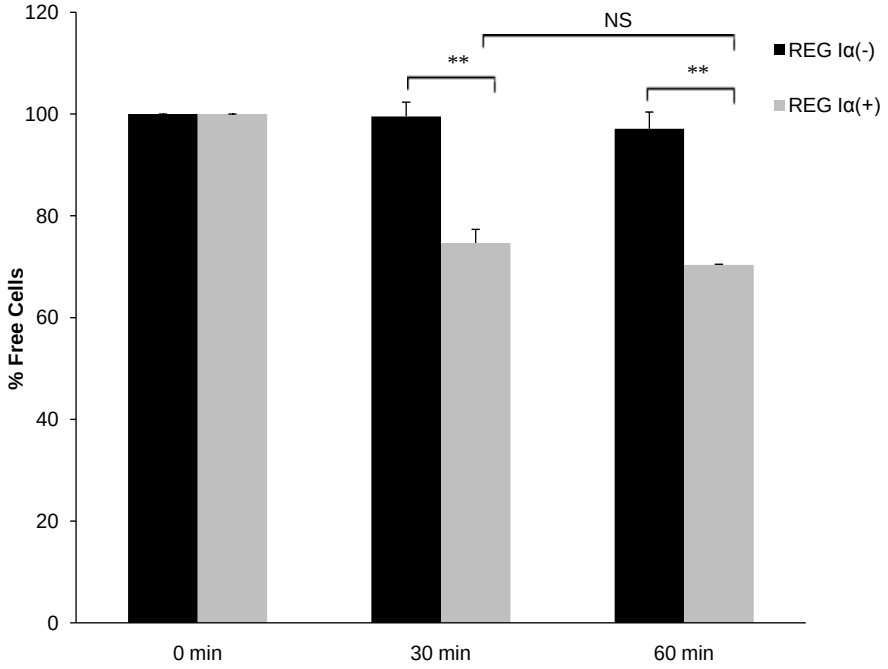

Figure 4. Time course of *S. aureus* aggregation by REG I*α*. Bacterial aggregation measured at 0, 30 and 60 minutes post incubation. Black bars represent no addition of REG I*α*, grey bars represent 5 μM REG I*α* added to bacterial cells; error bars represent standard error from 3 experiments; **P < 0.005 (Student *t* test).

pancreas. REG I*α* is however primarily expressed in pancreas. The REG I*α* mRNA levels were found to be 10 folds higher in pancreas than in the gastrointestinal tract [26], marking it characteristically different from other Reg family members. Secretion of REG I*α* from the pancreas leading to its load into the small intestine via the route of pancreatic duct, bile duct and duodenum perhaps has a role in pathogen clearance in the bile duct and the upper section of intestine—where Class III and Class IV Reg proteins secreted from the gastrointestinal tract are less likely to reach.

The *in vivo* receptor of REG I*α* on macrophages or other immune cells remains to be determined. Direct structural data of REG I*α* interaction with its ligand is also not known to date. Thus, further research is required to establish a link between bacterial aggregation and stimulation of immune system via REG I*α*. Nevertheless, aggregation of *S. aureus* by REG I*α* suggests its potential in combating infections. An elicited immune response against the human recombinant REG I*α* is not a concern, therefore it can be used in synergy with existing conventional methods of managing and treating *S. aureus* infections. Since colony formation by infectious bacteria is initiated by adhesion to the site of infection [38], ectopic application of REG I*α* at typical routes of entry into human body can prevent or slow the process of bacterial adhesion and colonization. Further, it may also stimulate the lectin-complement pathway of innate immunity.

Investigation into the potential of REG I*α* as an alternative strategy or its usage in synergy with existing treatment options in management of infection is

required.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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