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Original Article

Elucidation of cellular signaling mechanism involved in *Vibrio cholerae* chitin-binding protein GbpA mediated IL-8 secretion in the intestinal cells

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ABSTRACT

Background: *Vibrio cholerae N*-acetylglucosamine-binding protein (GbpA) is a four-domain, secretory colonization factor which is essential for chitin utilization in the environment, as well as in adherence to intestinal cells. GbpA is also involved in inducing intestinal inflammation by enhancing mucin and interleukin-8 secretion. The underlying cell signaling mechanism involved in the induction of the pro-inflammatory response and IL-8 secretion has yet to be deciphered in detail.

Methods: Herein, the process through which GbpA triggers the induction of IL-8 in intestinal cells was investigated by examining the role of GbpA in intestinal cell line HT 29.

Results: GbpA, specifically through the fourth domain, forms a binding connection with Toll-like receptor 2 (TLR2) and additionally, recruits TLR1 along with CD14 within a lipid raft micro-domain to initiate the signaling pathway. Notably, disruption of this micro-domain complex resulted in a reduction in IL-8 secretion. The lipid raft association served as the catalyst that invoked a downstream cellular inflammatory signaling pathway. This cascade involved the activation of various MAP kinases and NFκB and assembly of the AP-1 complex. This coordinated activation of signaling molecules eventually leads to enhanced IL-8 transcription via increased promoter activity. These findings suggested that GbpA is a crucial protein in *V. cholerae*, capable of inciting a pro-inflammatory response during infection by orchestrating the formation of the GbpA-TLR1/2-CD14 lipid raft complex. Activation of AP-1 and NFκB in the nucleus eventually enhanced IL-8 transcription through increased promoter activity.

Conclusion: Collectively, these findings indicated that GbpA plays a pivotal role within *V. cholerae* by triggering a pro-inflammatory response during infection. This response is instrumented by the formation of the GbpA-TLR1/2-CD14 lipid raft complex.

1. Introduction

The attachment of *Vibrio cholerae* to the intestinal epithelium is the first important step in the pathogenesis of cholera. This adherence to the intestinal epithelium initiates the colonization and micro-colony formation of *V. cholerae*. Several cell surface factors are involved in this adhesion process [1]. Cholera pathogenesis is multifactorial and depends on several factors, including toxin-coregulated pili [2], outer membrane proteins [1], and lipopolysaccharide [3]. *N*-acetylglucosamine-binding protein A (GbpA), the gene product of locus VCA0811, plays a key role in the adhesion process. GbpA is a chitin-binding protein (CBP) and is known to be a common adhesin for chitinous surfaces as well as the intestinal epithelium [4].

GbpA, a four domain protein [5], is an important part of the *V. cholerae* chitin utilization programme and is essential for bacterial attachment to chitinous surfaces for chitin utilization. This CBP has been determined to be the most important factor for the specific attachment of *V. cholerae* to chitin [6] and deletion of GbpA has resulted in reduced interaction with chitin leading to diminished chitin utilization [7]. GbpA has been identified as an adhesion factor for surfaces displaying *N*-acetylglucosamine (GlcNAc) and GlcNAc oligomers [7].

Abbreviations: AP-1, Activating protein-1; NFκB, Nuclear factor κB; TLR, Toll like receptor; CD, Cluster of differentiation; MAP kinase, Mitogen activated protein kinase; CBP, Chitin binding protein; c/EBP, CCAAT enhancer binding protein; TNF-α, Tumour necrosis factor α; ChIP, Chromatin immunoprecipitation.

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GlcNAc is a crucial component of mucin, a complex glycoprotein that is abundant in mucus [8], and is also the monomer in chitin. The mucin layer serves as a receptor for the attachment of *Vibrio* species [9] and other bacteria, which is facilitated by chitin-binding protein. In particular, the CBP of *Lactobacillus plantarum* and CBP21 of *Serratia marcescens* have been shown to interact with mucin during the colonization of intestinal epithelial cells [10,11]. GbpA has been identified as a factor that bridges *V. cholerae* and mucin in the host [12]. Structural analysis has revealed that the first domain of GbpA directly binds to intestinal mucus during colonization, while the domains 2 and 3 anchor the protein on the bacterial cell surface [5].

GbpA is a secretory colonization factor of *V. cholerae* and mainly remains in the culture supernatant of bacteria [4]. However, GbpA is also found on bacterial surfaces in a bound form [4,12]. A previous study has shown that there exists a coordinated correlation between GbpA and mucin secretion in the intestine [12]. Thus, it is evident that GbpA is utilized by *V. cholerae* for colonizing the intestinal epithelium using mucin as the stage for early adherence. Secreted and bacteria-bound GbpA play an important role in the coordinated interaction of intestinal mucus secretion and *V. cholerae* attachment. This interaction causes an increase in mucus secretion by the intestinal epithelial cells and induces an innate immune response. The up-regulation of mucus secretion is considered to be a marker for innate immune response [8].

Epithelial cells play a role in initiating early mucosal inflammatory signals through the release of pro-inflammatory cytokines. *V. cholerae* infection has been linked to the induction of intestinal pro-inflammatory responses [13], specifically responses leading to IL-8 secretion by intestinal epithelial cells [14]. Along with other modulators from *V. cholerae*, flagellin has been identified as a key factor in inducing pro-inflammatory responses, especially the production of IL-8 [15–18]. GbpA, known for its role in increasing mucin secretion, has been shown to stimulate IL-8 secretion and enhance mucus production in the intestine [12,19]. In this study, the mechanism by which GbpA induces IL-8 secretion was investigated. GbpA induced IL-8 secretion through a lipid raft complex involving TLR1/2, CD14, and GbpA. This complex activated downstream kinases, with NFκB and AP-1 identified as the major transcription factors. The overall cascade involved myeloid differentiation response protein 88 (MyD88), tumor necrosis factor receptor associated factor 6 (TRAF6), jun-N-terminal kinase (JNK1/2), and inhibitor of nuclear factor-κB (IκB) kinase (IKK), ultimately leading to the secretion of IL-8 in intestinal epithelial cells.

As a secreted protein, GbpA likely plays an important role in the host response mechanisms, although further research is needed to fully understand the extent of the impact. The present study revealed the mechanism by which GbpA induced IL-8 secretion through the formation of a lipid raft complex and subsequent activation of transcription factors, with several key signaling molecules involved in the process.

2. Materials and methods

2.1. Bacterial cells and culture

V.cholerae N16961 (O1 El Tor Inaba), N1RB1 (ΔGbpAN16961), and N1RC1 [12] were cultured and maintained in Luria Bertani medium. For construction of deletion and mutant IL-8 promoter constructs, *E coli* JM109 and XL-1 Blue were used. *V. cholerae* strains N1RB1 (ΔGbpAN16961) and N1RC1 (GbpA complemented) were generously gifted by Dr. Nabendu S. Chatterjee, ICMR-NICED, Kolkata, India.

2.2. Cell culture, treatment, and transfection

Various human cell lines, including HT29, HEK293, INT407, DLD1, CaCO2, T84, and HEK293-TLR2, were cultured using specific media, McCoy's 5A (Sigma, St Louis, USA), Dulbecco's modified Eagle Medium (DMEM), and minimal essential medium (MEM) (Sigma, St Louis, USA), supplemented with 10 % fetal calf serum (Eurobio, Paris, France), non-essential amino acids, penicillin-streptomycin (MP Biomedicals, Irvine, CA, USA), and Ham F12 (T84 cells). The HEK293-TLR2 cell line was cultured in the same manner as the HEK293 cell line but 100 μg/mL blasticidin (MP Biomedicals, Irvine, CA, USA) was added. Culture conditions were maintained in 75 cm^2 tissue culture flasks. Confluent cell monolayers were subjected to overnight starvation in incomplete medium with 0.5 % fetal calf serum before treatment with different recombinant GbpA proteins.

Transfection of the cells was performed using lipofectamine (Invitrogen, Massachusetts, USA) according to the manufacturer's protocol. Briefly, pGL3-Basic vector (Promega, Madison, Wisconsin, USA) was used to clone a putative promoter region of the IL-8 gene. This putative promoter region was identified and selected using the software programmes Genomatix and Alibaba 2.1. The probable transcription factor binding sites, i.e., binding sites for AP-1, NFκB, and c/EBP, were also selected using the programmes mentioned above.

The pGL3-Basic vector was cut using two restriction endonucleases, Mlu1 and BglII. The selected portions of the IL-8 promoter (–1481 to +44) were cloned into the pGL3-Basic vector using different sets of primers (Bioserve, India). The primers used here and for the site-directed mutagenesis of the specific nucleotides in the AP1, c/EBP, and NFκB binding site are given in the Supplementary Table S1.

These primers were used to prepare the specific mutant sites in the pGL3 IL-8 promoter-luciferase vectors using a

QuikChange II Site-Directed Mutagenesis Kit (Stratagene, San Diego, CA, USA) following the manufacturer's protocol.

These pGL3 IL-8 promoter-luciferase constructs were transfected in HT29 cells along with pRLTK (Rennila luciferase construct). Briefly, 6 μg each of the promoter constructs and 0.15 μg of pRLTK were transfected into 10^5 HT29 cells using lipofectamine following the manufacturer's protocol. siRNA against hTLR2, hTLR4, hTLR1, hTLR6, and GAPDH (Ambion, Austin, USA) were transfected using siPORT NEOFX^{TM} transfection reagent (Ambion, Austin, USA). Briefly, 0.5 ng of each of the siRNAs was transfected in 10^5 HT29 cells following the manufacturer's protocol.

The luciferase constructs for AP1, NFκB, and c/EBP were obtained from SA Biosciences and transfected into HT29 cells according to the manufacturer's protocol. Briefly, 150 ng of each of the AP-1, NFκB, and c/EBP reporter assay constructs along with appropriate positive and negative control vectors (supplied in the kit) were mixed with the specified amount of OptiMEM and the transfecting agent Surefect (SA Biosciences, MD, USA) diluted 1:10 in OptiMEM. The plasmid constructs and Surefect were mixed and added to the wells of a 96-well cell culture plate and incubated for 20 min. Approximately 15,000 cells were seeded in the wells containing the plasmid and transfecting agent. At this stage, the preparation was kept for 48 h at 37 ˚C with 5 % CO_2 under humidified conditions.

2.3. Expression and purification of recombinant GbpA

Different domains of recombinant GbpA and the full recombinant GbpA were expressed in pET 22b and pGEX 6P expression vectors, according to the previously described protocol of Wong et al. [5].

2.4. Induction of cytokine release in HT 29 cells

HT29 cells were subjected to induction with 250 ng of purified GbpA or GbpA distinct domains to investigate cytokine secretion. Additionally, induction experiments were conducted using culture supernatants from *V. cholerae* strains N16961, N1RB1, and N1RC1. The *V. cholerae* strains were cultured up to 10^7 CFU/mL, and the resulting supernatants were used for infecting HT29 cells with or without the use of cell culture inserts. Basolateral induction with the culture supernatants was performed to examine the involvement of TLR5 in IL-8 secretion, while apical induction was performed to assess the roles of TLR2, TLR1, and TLR6.

2.5. Quantification of cytokines

The quantification of the cytokines released as a result of GbpA treatment was performed using a colourimetric procedure by sandwich ELISA. The colourimetric assay was carried out with OptEIA kits (BD Biosciences, Franklin Lake, NJ, USA). In this process, the amounts of the different cytokines released were measured from a standard curve obtained using the provided standard cytokine samples reading the absorbance at 450 nm using a microplate reader (BioRad 550, CA, USA). The cytokines' expressions were also quantified using quantitative real time polymerase chain reaction (qRTPCR).

2.6. Lipid raft isolation and analysis

Lipid raft was isolated from treated and untreated HT29 cells following the previously described protocol by Yang and Reinherz [20]. Briefly, 5×10^6 cells were washed once in ice cold phosphate buffered salineand then resuspended in 0.8 mL of ice-cold MBS (25 mM 2-(N-morpholino) ethanesulfonic acid (MES), 0.15 M NaCl, 1 % triton X-100, supplemented with protease inhibitor cocktail). The cells were then incubated at 4°C for 1 h with shaking. The lysed cell preparation was gently mixed with 80 % sucrose in 1× MBS and loaded at the bottom of the tube. The sample was overlaid with 1.6 mL of ice-cold 30 % sucrose in 1× MBS and 0.8 mL of ice-cold 5 % sucrose in 1× MBS, successively. The sucrose gradient samples were then centrifuged at 180,000× *g* at 4°C for 20 h. After the centrifugation, 0.35 mL fractions were collected from the top of the tubes.

2.7. Luciferase assay

The luciferase assay was performed using a kit from Promega, following the protocol provided. In brief, HT29 cells were cultured in 12-well cell culture plates. Treated and untreated cells were scraped with ice cold 1× passive lysis buffer and were subjected to several rapid freeze-thaw cycles to lyse the cells completely. Total protein from the cells was isolated, an equal amount of the protein from each of the samples was mixed with 100 μL of luciferase assay reagent II, and the inducible firefly luciferase activity was measured using a luminometer (Berthelot). Then, 100 μL of Stop & Glo reagent was added to stop the luminescence of firefly luciferase and simultaneously the intensity of Renilla luciferase was measured.

2.8. Preparation of nuclear and cytoplasmic extracts

Nuclear and cytoplasmic extracts were prepared from HT29 cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, USA) using the following protocol. Approximately 40 mg of packed cells was placed in a clean microcentrifuge tube and 200 μL of ice-cold Cytoplasmic Extraction Reagent I was added. The reagent and cells were mixed and incubated on ice. After incubation, Cytoplasmic Extraction Reagent II was added and

the mixture was mixed, incubated on ice for 1 min, and then centrifuged at 16,000× *g* for 5 min. The supernatant as the cytoplasmic extract was transferred into a fresh pre-chilled tube and the pellet portion containing the nuclei was resuspended in Nuclear Extraction Reagent and incubated with shaking. The tube with the pellet was centrifuged at 16,000× *g* for 10 min and the supernatant was kept in a fresh pre-chilled tube as the nuclear extract. These extracts were used immediately or kept at –70˚C until further use.

2.9. Immunoprecipitation

Immunoprecipitation was used in the present study for several purposes. The requisite amount of Protein A Sepharose CL-4B beads were swelled overnight in PBS, precleared with bovine serum albumin, and finally resuspended in PBS after several washes with PBS. Specific primary antibody to the protein antigen was applied at a 1:250 dilution, or as recommended by the manufacturer, to 200–300 μL of lysate and the mixture was incubated overnight at 4˚C. Then 100 μL of pre-cleared bead slurry was added to the antibody-lysate mixture and the mixture was incubated with rocking at room temperature for 3 h, then washed with PBS-Tween-20 buffer. Beads were collected as a pellet and resuspended in SDS-PGE loading buffer and run into the gel for immunoblot analysis. For co-immunoprecipitation of two antigens, a similar protocol was followed using the primary antibody to one of the antigens to pull-down the complex and the primary antibody to the second antigen was used in the immunoblot analysis.

2.10. Fluorescence resonance energy transfer (FRET) study

The physical proximity of TLR1 and TLR2 on the cell surface during GbpA-mediated signaling initiation was determined using FRET. Both TLR1 and TLR2 were labeled with fluorochrome-tagged antibodies. The emission wavelength of the fluorochrome of the anti-TLR2 antibody was equal to the excitation wavelength of the fluorochrome of the anti-TLR1 antibody, and this radiation energy can be transferred from one to the other only when the two fluorochromes come within the Förster distance. The flow cytometer BD FACS ARIA II was used. Firstly, GbpA-treated and untreated cells were labeled with Alexafluor 488-tagged anti-TLR2 antibodies at a dilution of 1:150 and Alexafluor 594-tagged anti-TLR1 antibodies at a dilution of 1:100, respectively. Untreated cells were used as a control. Excitation energy was applied to the TLR2 antibodies and the emission was measured using TLR1 antibodies; a positive result for emission indicated FRET occurred between the fluorochrome-tagged antibodies when TLR1 and TLR2 came within the Förster distance.

2.11. Immunofluorescence study

HT29 cells were cultured on glass cover slips which were coated with poly-L-lysine by incubation with poly-L-lysine at room temperature for 1 h and then washed in sterile water. These cover slips were then sterilized by being placed under UV light for at least 4 h, and the cells were cultured on these slips. After optimum growth, cells were fixed in 4 % paraformaldehyde and washed. Then, the cells were permeabilized using 0.5 % saponin in PBS for 10 min, washed, and nonspecific sites were blocked with 1 % BSA in PBS for 30 min. After blocking, the cells were washed in PBS and primary antibody was applied and the cells were incubated in a humidified chamber for 2 h. The cells were washed well in PBS, secondary antibody was added with 1 % BSA solution and the cells were incubated for 45 min in the dark. Finally, the samples were washed in PBS, cover slips were mounted on the slides with DPX mounting medium, and imaged using a Zeiss AxioImager M1 (Carl Zeiss) microscope.

2.12. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was carried out to investigate the involvement of the transcription factors AP-1 and NFκB in increasing the secretion of IL-8. The ChIP assay was carried out using the ChIP-IT kit (Active Motif, USA) following the manufacturer's protocol. First, treated or untreated cells were fixed with incomplete medium containing 3 % formaldehyde for 10 min and then washed in PBS. The cells were scraped with cold PBS and harvested and then were resuspended in lysis buffer containing protease inhibitor cocktail and briefly homogenized in an ice-cold Dounce homogenizer. The cells were then resuspended in digestion buffer and incubated at 37˚C with Enzymatic Shearing Cocktail. This digestion reaction was stopped by addition of 20 μL of ice-cold EDTA. The specific antibodies (2–3 μg antibody/ChIP reaction) were then incubated with pre-cleared chromatin and resuspended protein G beads were added to the antibody-coupled chromatin, followed by incubation for 2–3 h at 4˚C.The bound DNA was eluted from the beads by adding elution buffer containing sodium bicarbonate. This eluted DNA was treated with proteinase K and incubated at 42˚C for 2 h to digest proteins remaining in the chromatin. Finally, the DNA was purified by column chromatography and eluted with DNAse free water for PCR analysis.

2.13. Curve fitting

Data fitting was performed using Kyplot Version 5.0 (64 bit) to obtain the best-fit curves and the dissociation constant (K_d). Values are the means of triplicate determinations from two separate experiments.

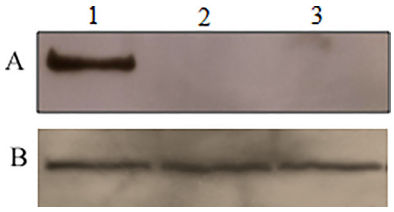

Fig. 1. Involvement of TLR2 in GbpA-mediated signaling initiation. GbpA-treated HT29 cells were incubated in cell lysis buffer and the total protein was extracted from each sample. Equal amounts of the total protein, i.e., 200 μg, was used for immunoprecipitation using anti-TLR2, anti-TLR4, and anti-TLR5 antibodies at a dilution of 1:100. The precipitates were then subjected to immunoblot analysis and detected with anti GbpA antibody. (A) 1. Lane 1, co-precipitated with TLR2; 2. Lane 2, co-precipitated with TLR4; 3. Lane 3, co-precipitated with TLR5. (B) β-Actin.

2.14. Statistical analysis

Where applicable, the results presented in this manuscript are the mean ± standard error (SE) of at least three separate experiments. Statistical differences were analyzed by ANOVA with the level of significance being set at 5 % ($p < 0.05$).

3. Results

3.1. Involvement of TLR2: Co-precipitation of TLR2 with GbpA

To explore the connection between non-invasive ligands triggering pro-inflammatory responses and GbpA-mediated IL-8 secretion, a co-immunoprecipitation study was conducted to identify the TLR implicated in this process. The results revealed that GbpA from HT29 cell lysates co-immunoprecipitated with TLR2, suggesting the involvement of TLR2 in GbpA-mediated IL-8 secretion. GbpA-treated HT29 cell lysate was co-precipitated with anti-TLR2, anti-TLR4, and anti-TLR5 antibodies. The precipitated samples were then subjected to immunoblot analysis using anti-GbpA antibody at a dilution of 1:3000. GbpA was found to be co-precipitated only with TLR2 (Fig. 1) and not with the other TLRs used in the study.

3.2. Specific binding of TLR2 with GbpA

To investigate the specific binding of GbpA with TLR2, a stably transfected HEK293 cell line was used that is known to express very low levels of TLR1 and TLR6, but which lacks TLR2 on the cell surface. These HEK293-TLR2 cells have the capability to initiate cell signaling in response to TLR2/1 and/or TLR2/6 agonists, as it has been demonstrated in previous studies [21,22]. HEK293 TLR2 cells were cultured in 96-well plates at a density of 10^3 cells per well. These cells were then incubated with varying concentrations of GbpA to investigate whether there was specific binding between GbpA and TLR2. The results of this experiment revealed there was

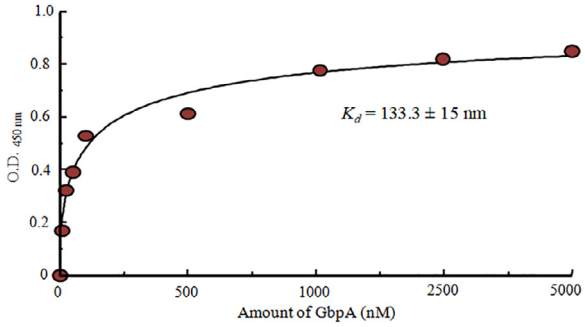

Fig. 2. Binding curve of HEK293 TLR2 and GbpA. HEK293 TLR2 cells were cultured in 96-well plates and fixed with 4 % paraformaldehyde, different amounts of GbpA were added, and the plates were incubated. The amount of bound GbpA in each case was quantified by an indirect ELISA method using anti GbpA antibody at a dilution of 1:500. The best-fitted binding curve was obtained using Kyplot Beta 5.0 (64 bit) software.

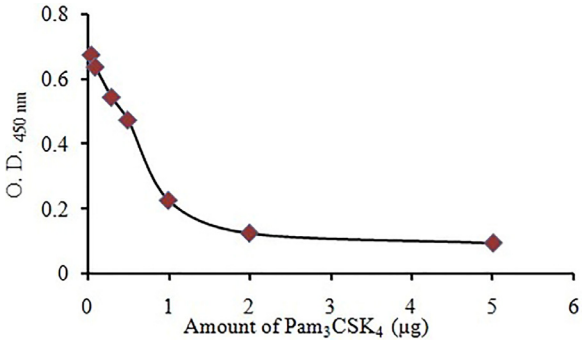

Fig. 3. Competitive binding of GbpA with HEK293 TLR2 in presence of Pam_3CSK_4. HEK293 TLR2 cells were incubated with increasing amounts (0.05, 0.1, 0.3, 0.5, 1.0, 2.0, and 5.0 μg) of Pam_3CSK_4 prior to the addition of a fixed amount of GbpA (0.2 μg in each well).

a concentration-dependent and saturable binding interaction between TLR2 and GbpA on the surface of HEK293 TLR2 cells.

A cell binding experiment with HEK293-TLR2 and GbpA at concentrations of 10, 25, 50, 100, 500, 1000, 2500, and 5000 nM was conducted. GbpA was observed to bind to the cells with a dissociation constant (K_d) value of (133.3 ± 15) nM (Fig. 2), indicating that GbpA exhibited specific binding to TLR2 on the HEK293 TLR2 cell surface, implying that there was an interaction between GbpA and TLR2.

For further validation that the binding of GbpA to HEK293-TLR2 cells was mediated through TLR2, a competitive binding assay was conducted using Pam_3CSK_4, a chemical agonist of TLR2 that is known to bind to TLR2 on the cell surface and initiate inflammatory signaling. The results of this assay indicated that there was a gradual reduction in the affinity of GbpA for HEK293 TLR2 cells as the concentration of pre-incubated Pam_3CSK_4 was increased (Fig. 3). In this experiment, 200 ng of GbpA was introduced to HEK293 TLR2 cells that had been pre-incubated with 0.05, 0.1, 0.3, 0.5, 1.0, 2.0, and 5.0 μg of Pam_3CSK_4. The observed decrease in the affinity of GbpA for the cells with increasing concentrations of Pam_3CSK_4

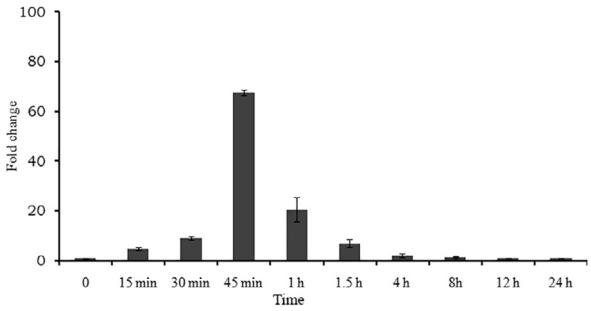

Fig. 4. Time-dependent expression of TLR2 mRNA from GbpA-treated HT29 cells. Total RNA was isolated from HT29 cells treated with GbpA for different time periods (15 min, 30 min, 45 min, 1.5 h, 2 h, 4 h, 8 h, 12 h and 24 h). A 2-μg sample of the total RNA was used for quantification of TLR2 mRNA by qPCR.

suggested that the binding of GbpA to HEK293 TLR2 cells was mediated through TLR2 and was competitively inhibited by Pam_3CSK_4.

3.3. Increase in TLR2 mRNA expression

Previous reports have shown that an increase in TLR expression can be induced by pro-inflammatory ligands. The variation in TLR2 expression upon induction by GbpA in HT29 cells was investigated. GbpA (250 ng) was incubated with 10^7 HT29 cells for various lengths of time and the total RNA was isolated. These RNA samples were then used to quantify the amount of TLR2 mRNA produced through GbpA induction using qRTPCR. The expression of TLR2 mRNA was found to increase with time and reached a maximum at 45 min after induction and then subsequently decreased (Fig. 4). HT29 cells generally express TLR2 at very low concentrations on the surface but here it was clear that after GbpA induction, TLR2 expression was enhanced on the HT29 cell surfaces.

3.4. Expression of TLR2 on HT29 cell surfaces

The expression of TLR2 on HT29 cells treated with a fixed amount of GbpA for various time intervals ranging from 0 to 1.5 h was investigated. The outer membrane of cells treated with GbpA were isolated, and each isolated outer membrane fraction was subjected to immunoblot analysis using an anti-TLR2 antibody at a dilution of 1:800. TLR2 became detectable at 20 min post-treatment, suggesting that GbpA treatment triggered the expression of TLR2 on the outer surface of HT29 cells. The expression of TLR2 continued to increase as the treatment time was increased. Specifically, the amount of TLR2 steadily rose with increasing duration of GbpA treatment. At 1 h post-treatment, the level of TLR2 expression reached the point of saturation. This result indicated that for periods greater than 1 h of GbpA treatment, the amount of TLR2 on the outer membrane of HT29 cells remained relatively constant, and further increases in the treatment time did not result in a proportional increase in TLR2 expression,

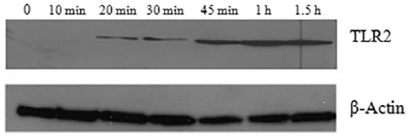

Fig. 5. Expression of TLR2 on the GbpA-treated HT29 cell surfaces. HT29 cells were treated with 250 ng of GbpA for different time periods (0–1.5 h). The outer membrane fraction was isolated from each sample and the amount of TLR2 expressed was detected by immunoblotting using anti-TLR2 antibody at a dilution of 1:800.

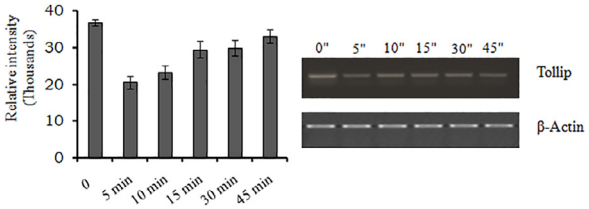

Fig. 6. Time-dependent expression of Tollip mRNA in GbpA-treated HT29 cells. The total RNA was isolated from GbpA-treated HT29 cells after 5, 10, 15, 30, and 45 min. A 2-μg sample of RNA was used for PCR with Tollip primers, and the PCR products were run on 1.5 % agarose gel. The intensity of the Tollip mRNA expressed was measured using Labworks software and plotted.

suggesting that GbpA treatment of HT29 cells induced the expression of TLR2, with a detectable increase starting at 20 min and reaching saturation at 1 h post infection (Fig. 5).

The mechanism for this increase of TLR2 mRNA expression was investigated and it was found that differential expression of Toll interacting protein (Tollip) was responsible for controlling the TLR2 expression. The mRNA level of Tollip in GbpA-treated HT29 cells was measured by semi-quantitative RT PCR. It was observed that Tollip mRNA expression was down-regulated at 5 and 10 min post infection compared with the untreated cells, and the expression increased again after 15 min (Fig. 6). Therefore, the decrease in Tollip expression was involved in the TLR2 up-regulation.

3.5. Role of the adapter molecule MyD88 and intermediate TRAF6 in GbpA-mediated IL-8 secretion

The involvement of MyD88 was investigated by immunoblot analysis of GbpA-treated HT29 cell lysates. GbpA (250 ng) was added to HT29 cells for 30 min, 45 min, 1 h, 1.5 h and 2 h. The treated samples were then incubated with cell lysis buffer and the total protein was isolated by leaving the cellular debris as pellets after centrifugation. Then, the total protein concentration was measured. The cell lysate from each sample having an equal amount of protein (100 μg) was immunoprecipitated using anti-TLR2 antibody and each sample was subjected to immunoblot analysis for detection by anti-MyD88 antibody (Fig. 7A). The immunoblot results suggested that the co-precipitation of MyD88 with TLR2 was increased with increasing time.

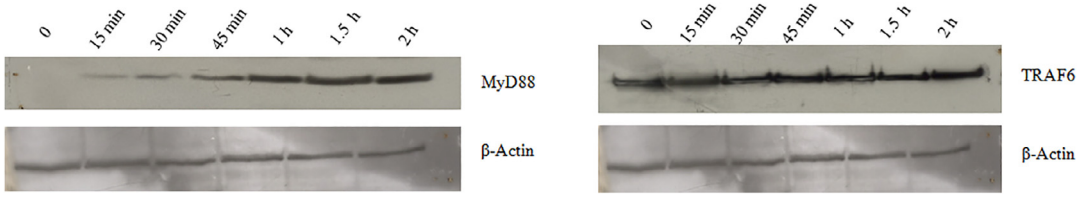

Fig. 7. Involvement of MyD88 and TRAF6 as intermediates in IL-8 induction in GbpA-treated HT29 cells. (A) Co-immunoprecipitation of MyD88 with TLR 2. Total protein was isolated from HT29 cells treated with GbpA for different time periods (15 min, 30 min, 45 min, 1 h, 1.5 h and 2 h). An equal amount of the total protein from each sample was immunoprecipitated using anti-TLR2 antibody and immunoblotted and detected with anti-MyD88 antibody. (B) Time-dependent TRAF6 activation in GbpA-treated HT29 cells. The total protein was isolated from HT29 cells treated with GbpA for different time periods (15 min, 30 min, 45 min, 1 h, 1.5 h and 2 h). An equal amount of the total protein from each sample was immunoblotted using anti-TRAF6 antibody at a dilution of 1:2500.

Further downstream from MyD88, the next intermediate signaling protein investigated was tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 is activated by the phosphorylation of interleukin-1 receptor-associated kinase 1 (IRAK1), and the active TRAF6 in turn activates IKK in the cytosol [23–25]. The phosphorylated IKK initiates the cytosolic degradation of inhibitor of nuclear factor κB (IκBα).GbpA-induced HT29 cells showed activation of TRAF6, as the amount of TRAF6 produced was increased upon GbpA induction in a time-dependent manner (Fig. 7B).

3.6. IL-8 secretion involves p38 MAPK-, JNK 1/2-, and ERK 1/2-dependent pathways

The cellular signaling pathway in GbpA-induced IL-8 secretion involved the activation of several MAP kinases. p38 MAPK was found to be activated within 0.5 h to 1 h after GbpA treatment, JNK1/2 was activated within 1 h after treatment, and ERK 1/2 was activated within 0.25–0.5 h after treatment, earlier than the two other kinases (Fig. 8A).

The involvement of these kinases in IL-8 secretion by GbpA was investigated using the inhibitors JNK1/2, MG132, and U0126. Activation of JNK1/2 played a major role in the IL-8 secretion because inhibition of this kinase decreased the IL-8 production by almost 75 % compared with the untreated sample. A 50 % decrease in IL-8 production was observed with MG132 treatment and treatment with U0126 decreased IL-8 secretion by 20 % (Fig. 8B).

3.7. IL-8 secretion is NFκB-and AP-1-dependent

In the search for the transcription factors involved in inducing IL-8 secretion in GbpA-treated HT29 cells, NFκB was shown to be translocated into the nucleus. IKK was found to be phosphorylated starting 0.5 h post-treatment and was most active at 0.75 h post treatment (Fig. 8A). In GbpA-treated HT29 cells, cytosolic IκBα was degraded, beginning at 1 h post treatment and within 2 h there was no detectable IκBα found in the cytosol (Fig. 9A). Nuclear translocation of NFκB started from 0.5 h post infection and was highest at 2 h post treatment (Fig. 9A). Fluorescence microscopy showed that NFκB was translocated into the nucleus of GbpA-treated HT29 cells (Fig. 9B). After addition of the proteasomal degradation inhibitor, MG132, GbpA-treated HT29 cells were shown to secrete lower levels of IL-8 ((377.4±14.5) pg/mL) compared with untreated cells ((883.5±34.8) pg/mL) (Fig. 9C). These results indicated the role of NFκB in GbpA-treated cells.

GbpA induced enhanced IL-8 expression in HT29 cells, as has been observed previously. IL-8 mRNA expression increased gradually with increasing GbpA incubation times and became highest at 8 h post infection and then gradually decreased (Fig. 10A). This experiment aimed to unveil the underlying transcription factors involved in increasing the IL-8 expression.

Promoter studies were conducted to investigate the role of AP-1 and NFκB transcription factors in GbpA-induced IL-8 secretion. These studies revealed that both AP-1 and NFκB are important transcription factors responsible for stimulating IL-8 secretion in HT29 cells after induction by GbpA. Specifically, when the AP-1 binding site was deleted, there was a notable decrease of approximately 50 % in the relative luciferase activity ($p < 0.05$). In contrast, deletion of the NFκB binding site led to a reduction in luciferase activity of approximately 35 %. These results strongly indicated that AP-1 plays a more crucial role compared with NFκB in GbpA-mediated IL-8 secretion (Fig. 10B).

To further investigate the involvement of AP-1 and NFκB in GbpA-mediated IL-8 secretion, the nucleotides of the binding sites of AP-1, NFκB, and c/EBP were mutated by site-directed mutagenesis. The results showed that mutation in the AP-1 and NFκB sites decreased the luciferase activity by up to 55 % and 40 %, respectively. Double mutants, with mutations in both the NFκB and AP-1 sites decreased the IL-8 promoter activity by almost 70 % ($p < 0.05$), whereas the c/EBP mutants with a second mutation at either the NFκB or AP-1 site, did not show decreased promoter activity, suggesting that c/EBP was the least involved of the three transcription factors in the process. The triple mutant decreased the promoter activity by up to 75 % of the wild type, which was comparable

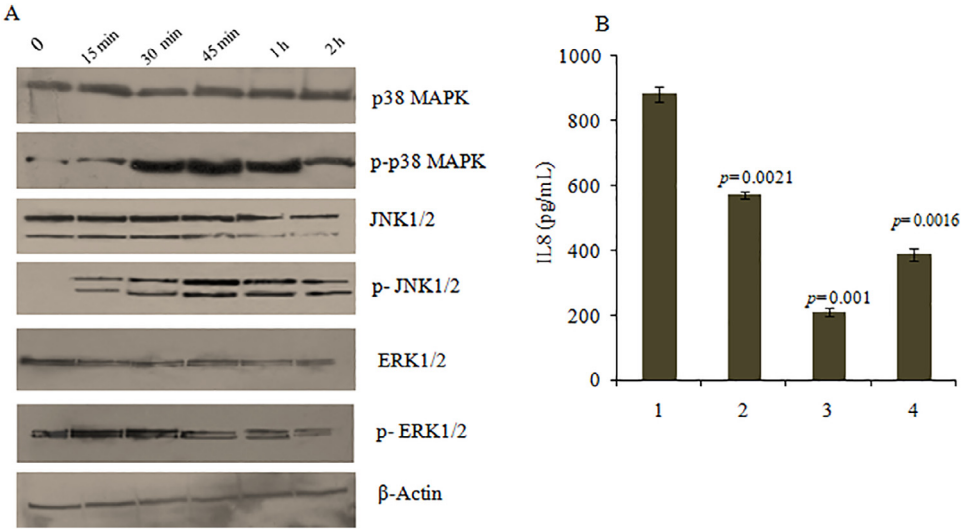

Fig. 8. IL-8 secretion in HT29 cells involves different cellular kinases. (A) Time-dependent activation of different cellular kinases. The total protein was isolated from HT29 cells treated with GbpA for different time periods (0–2 h). An equal amount of the total protein (30 μg) from each sample was used for immunoblot analysis using MAPK specific antibodies. The phospho-kinases were detected first and then nitrocellulose membrane dephosphorylated kinases were detected by stripping the previously tagged antibody by incubating the membrane in stripping buffer for 1 h. 1. Total p38 MAPK. 2. Phospho-p38 MAPK, activated within 30 min- to 1 h post infection. 3. Total JNK1/2. 4. Phospho-JNK1/2, activated within 45 min to 1 h post infection. 5. Total ERK1/2. 6. Phospho-ERK1/2, activated within 15 min to 30 min post infection, and 7. β-Actin. (B) Effect of the addition of inhibitors of cellular kinases on GbpA-induced IL-8 secretion in HT29 cells. HT29 cells were incubated with 5 μM of each inhibitor for 20 min prior to GbpA treatment. The cells were washed twice, GbpA was added, and the cells were incubated overnight. Secreted IL-8 was quantified from the culture supernatants of GbpA-treated cells by sandwich ELISA. 1. Uninhibited, 2. p38 MAPK inhibitor, 3. JNK 1/2 inhibitor, and 4. ERK 1/2 inhibitor.

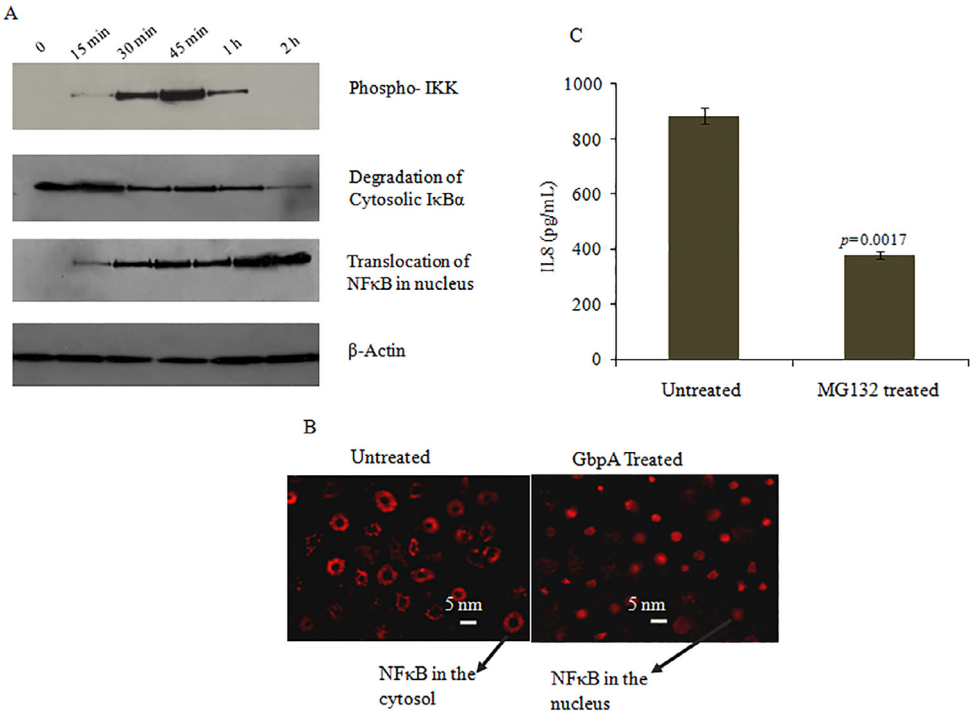

Fig. 9. IL-8 secretion involves NF-κB-dependent pathways. (A) Time-dependent activation of cytosolic IKK, degradation of cytosolic IκBα, and translocation of NFκB to the nucleus in GbpA-treated HT29 cells. 1. For detection of the time-dependent activation of IKK, the total protein was isolated from HT29 cells treated with GbpA for different time periods (0–2 h). An equal amount of total protein (30 μg) from each sample was subjected to immunoblot analysis and detected using anti-IKK antibody. For the detection of the degradation of cytosolic IκBα and the nuclear translocation of NFκB, the nuclear and cytosolic extracts of GbpA-treated HT29 cells were purified. An equal amount of protein from each sample was used for immunoblot analysis for the detection of cytosolic IκBα using anti-IκBα antibody and nuclear NFκB using anti-NFκB antibody. 2. Degradation of cytosolic IκBα; degradation started at 30 min post infection and within 2 h almost all the IκBα was degraded. 3. Translocation of NFκB into nucleus; the nuclear fraction of GbpA-treated HT29 cells showed that at 30 min post infection, NFκB became visible in the nucleus and the amount of NFκB increased in the nucleus over time. 4. β-Actin. (B) Nuclear translocation of NFκB in GbpA-treated HT29 cells. HT29 cells were treated with GbpA for 45 min and fixed with 4 % paraformaldehyde. The cells were permeabilized with 0.5 % saponin in PBS and treated with anti NFκBp-65 antibody at a dilution of 1:200 for 1 h, washed several times in PBS, and incubated with phycoerythrin-conjugated secondary antibody at a dilution of 1:500. In untreated HT29 cells, NFκB was present in the cytosol. In GbpA-treated HT29 cells, NFκB had migrated into the nucleus. (C) Effect of the application of a proteasomal degradation inhibitor of IκBα on IL-8 secretion. HT29 cells were incubated with 5 μM of MG132 for 20 min prior to GbpA treatment. The cells were washed twice, GbpA was added, and the cells were incubated overnight. IL-8 secretion was quantified from the culture supernatants of the GbpA-treated cells by sandwich ELISA.

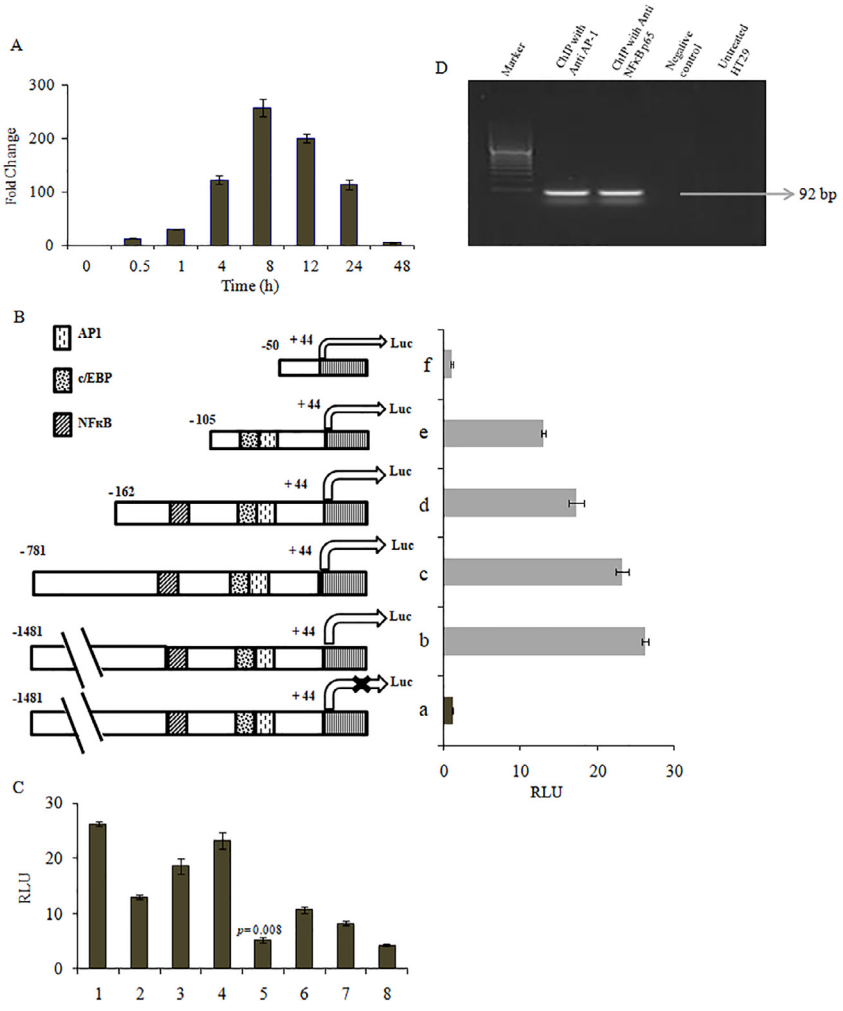

Fig. 10. Transcriptional activation of IL-8 secretion involves NF-κB and AP-1. (A) Upregulation of IL-8 mRNA in GbpA-treated HT29 cells. HT29 cells (10^6) were treated with 250 ng of GbpA and incubated for different time periods (0–48 h). The total RNA was isolated from each sample and the amount of IL-8 mRNA was quantified by qRT PCR. (B) Relative luciferase activity of IL-8 promoter constructs. The IL-8 promoter assay was performed by cloning the different portions with or without the binding sites for AP-1, c/EBP, and NFκB into a pGL3-Basic vector and the vector was transfected transiently into HT29 cells prior to GbpA treatment. Luciferase activity was measured by normalizing the transfection efficiency with pRLTK vector after subtracting the luciferase activity indicated by the empty pGL3 vector transfected into HT29 cells. a. Untreated, b–f. GbpA treated. (C) Relative luciferase activity of IL-8 promoter constructs with different point mutations. Mut AP-1 showed almost a 50 % decrease, MutNFκB showed almost a 30 % decrease, and Mut c/EBP showed very little decrease in luciferase activity. 1. Without treatment (WT), 2. Mut AP1, 3. MutNFκB, 4. Mut c/EBP, 5. Mut AP1+NFκB, 6. MutNFκB+ c/EBP, 7. Mut AP1+ c/EBP, and 8. Mut AP1+ c/EBP+ NFκB. (D) Chromatin immunoprecipitation of AP-1 and NFκB. PCR amplified product of the AP-1 and NFκB binding site. Immunoprecipitated portion of IL-8 promoter amplified by a specific primer.

with the promoter activity of the AP-1 and NFκB double mutant. These observations indicated that AP-1 was the most potent transcription factor in this inflammatory pathway followed by NFκB (Fig. 10C). These results were further confirmed by chromatin immunoprecipitation assay (ChIP asay) (Fig. 10D).

3.8. The TLR1/2/CD14 complex is involved in GbpA-mediated IL-8 secretion

TLR1/2/CD14 and GbpA are associated in a lipid-raft complex that mediates IL-8 secretion. TLR2 was found to be involved in GbpA-mediated IL-8 secretion in HT29 cells but not in HEK293 TLR2 cells. When HEK293 TLR2 cells were transfected with pUNOTLR1 (2 μg of construct/10^7 cells) and pUNOCD14 (2 μg of construct/10^7 cells) vectors separately, very little IL-8 mRNA was found to be expressed and a very small amount of IL-8 was secreted, which was less than the level of IL-8 secreted in HT29 cells. However, when these pUNO TLR1 and pUNO CD14 constructs were co-transfected in HEK293 TLR2 cells, the IL-8 mRNA expression was enhanced greatly and the IL-8 secretion was similar to the level of IL-8 secreted in GbpA-treated HT29 cells (Fig. 11A).

HT29 cells were treated either with GbpA, Pam_3CSK_4, or $MALP_2$ and the cell lysates were immunoprecipitated with anti-TLR2 antibodies. The immunoprecipitated samples were then subjected to immunoblot analysis with detection using anti-TLR1 or anti-TLR6 antibodies. The results showed that the GbpA-treated cells were positive for TLR1 and negative for TLR6 (Fig. 11B), suggesting that TLR1 interacted with TLR2 in signaling initiation process.

Gene silencing protocols also indicated the role of TLR2 in GbpA-mediated IL-8 secretion in HT29 cells. Use of a pZEROTLR2 (dn TLR2) construct was able to decrease the expression of IL-8 by up to 80 %, use of pZEROTLR1 by up to 75 %, and the simultaneous use of both dominant negative constructs decreased the IL-8 secretion by 80 % in HT29 cells (Fig. 11C).

TLR1 silencing reduced the amount of IL-8 mRNA by up to 30 % and the secretion of IL-8 was reduced by up to 40 %. TLR2 silencing resulted in the mRNA level of IL-8 being decreased by up to 70 % and the amount of IL-8 secretion by 80 % (Fig. 11D).

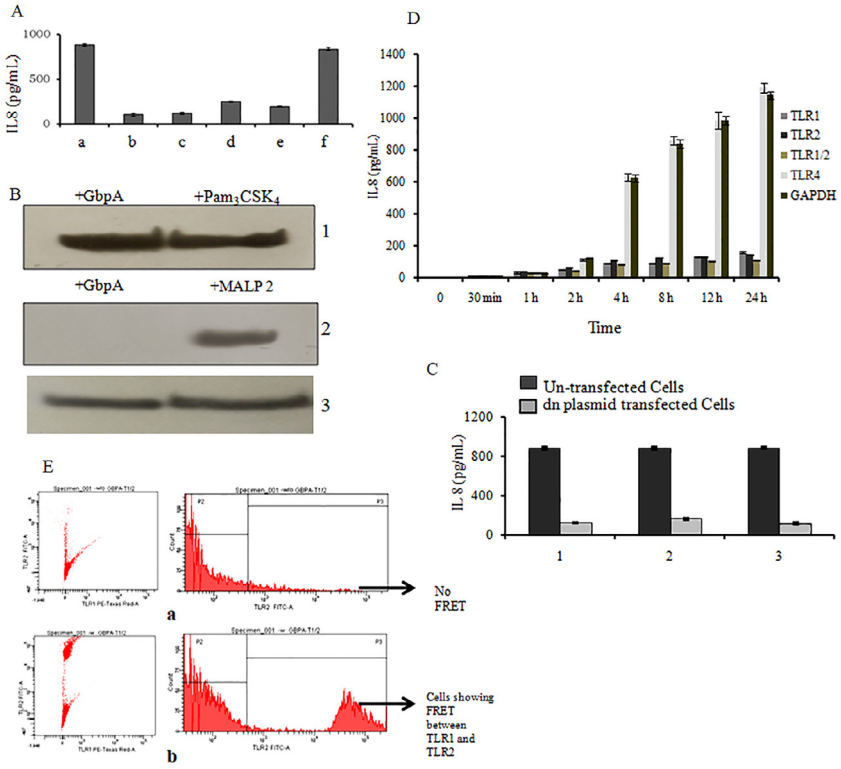

Fig. 11. TLR1/2/CD14 complex is involved in GbpA-mediated IL-8 secretion. (A) Involvement of TLR1 and CD14 as a co-receptor in GbpA-induced secretion of IL-8. a) HT29, b) HEK293, c) HEK293 TLR2, d) HEK293 TLR2 transfected with pUNOTLR1, e) HEK293 TLR2 transfected with pUNOCD14, and f) HEK293 TLR2 transfected with pUNOTLR1 and pUNOCD14. (B) Involvement of TLR1 and TLR2. 1. Immunoprecipitated samples were detected using anti-TLR1. Lane 1: cell lysate of HT29 treated with GbpA; Lane 2: cell lysate of HT29 treated with Pam_3CSK_4. 2. Immunoprecipitated samples were detected using anti-TLR6. Lane 1: cell lysate of HT29 treated with GbpA; Lane 2: cell lysate of HT29 treated with MALP2. 3. β-Actin. (C) Use of dominant negative constructs to investigate the involvement of TLR1 and TLR2. IL-8 secretion from GbpA-treated HT29 cells and HT29 cells co-transfected with dnTLR1 and dnTLR2 and singly transfected with dnTLR1 or dnTLR2 prior to GbpA treatment. HT29 cells were transfected with 1.5 μg of dnTLR1 (1), 1.5 μg of dnTLR2 (2), and 1.5 μg each of dnTLR1 and dnTLR2 (3) plasmids. (D) Use of siRNA in GbpA-treated HT29 cells to investigate the involvement of TLR1 and TLR2. Time-dependent expression of IL-8 mRNA and secretion of IL-8 from GbpA-treated HT29 cells transfected with siTLR1, siTLR2, siTLR1 and TLR2, siTLR4, and siGAPDH; 5 nm of each siRNA was transfected into HT29 cells using NEOFX siPORT reagent. (E) Physical proximity of TLR1 and TLR2 during initiation of IL-8 secretion. GbpA-treated and untreated cells were incubated with Alexafluor 488-conjugated anti-TLR2 and Alexafluor 594-conjugated anti-TLR1 antibodies at a dilution of 1:100 for 1.5 h. Cells were washed in PBS and loaded in a BD flow cytometer for the detection of FRET between TLR1 and TLR2 after treatment with GbpA. (a) Untreated HT29 cells showed that TLR1 and TLR2 did not come close as no FRET was observed. (b) In GbpA-treated HT29 cells, TLR1 and TLR2 came within the Förster distance and FRET occurred between the receptors.

For efficient initiation of TLR2-mediated signaling, TLR1 and TLR2 need to physically interact, and this interaction was studied using a FRET assay. HT29 cells were treated with GbpA and the cells were labeled with Alexafluor 488-conjugated anti-TLR2 and Alexafluor 594-conjugated anti-TLR1 antibodies. These cells were then assayed for FRET, i.e., if TLR1 and TLR2 come close during signaling initiation, the emission of Alexafluor 488 will be the excitation signal for Alexafluor 594 and FRET will be observed. In GbpA-treated HT-29 cells, FRET was observed to occur between TLR1 and TLR2 (Fig. 11E).

3.9. The TLR1/2/CD14 complex is associated in a lipid raft in GbpA-mediated IL-8 secretion

HT29 cells were treated with 250 ng of GbpA for 0.5 h and lipid raft complexes were isolated from these cells. TLR2, TLR1, CD14, and GbpA were detected in the raft fractions from GbpA-treated HT29 cells as depicted in the figure (Fig. 12A).

The need for the raft complex in GbpA-induced IL-8 secretion was investigated by the destabilization of micro-domains using methyl-β-D-cyclodextrin (MβCD). IL-8 secretion from MβCD-treated HT29 cells was found to be almost fourfold less than in the untreated control cells. In another set of experiments, after the addition of MβCD, 10 mM cholesterol was added and the cells were allowed to stabilize by regaining cholesterol. Cells which were incubated with cholesterol after MβCD treatment showed IL-8 secretion comparable with the untreated cells (Fig. 12B). The destabilization of micro-domains because of the treatment with MβCD was confirmed by an immunoblot study (Fig. 12C) using caveolin-1 as a marker.

3.10. Domain 4 of GbpA is involved in the interaction with TLR2 in IL-8 induction

To determine the role of the domain(s) of GbpA involved in the initiation of the IL-8 secretion signaling

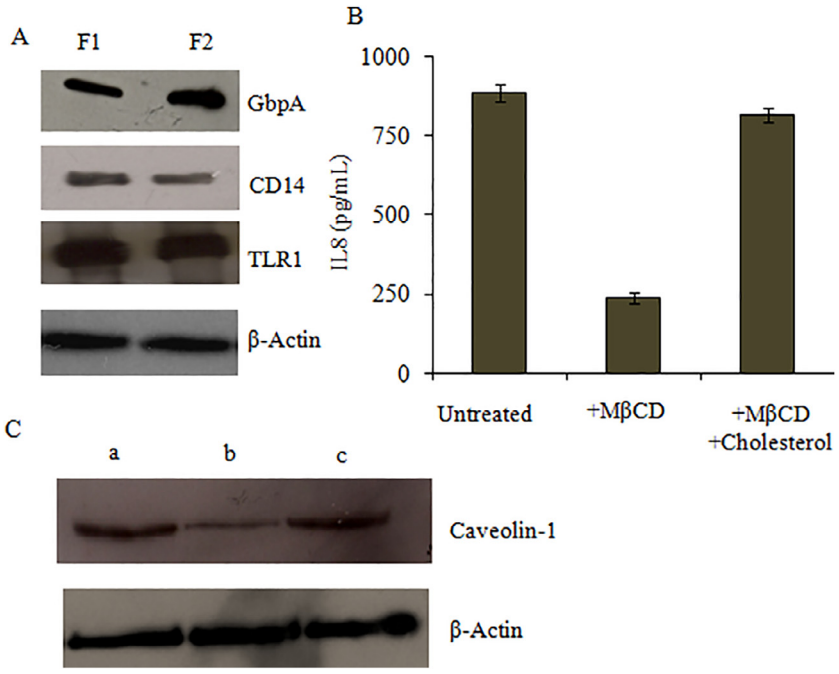

Fig. 12. Lipid raft-associated TLR1/2/CD14 complex initiates GbpA-mediated IL-8 secretion in HT29 cells. (A) Lipid raft in GbpA-treated HT29 cells associated with GbpA, TLR1, and CD14. TLR2 immunoprecipitated raft fractions (F1 and F2 indicate fraction 1 and fraction 2, respectively) showing the presence GbpA, CD14, and TLR1 in GbpA-treated HT29 cells. (B) Lipid raft association is essential for IL-8 secretion. IL-8 secretion in GbpA-treated HT29 cells incubated with MβCD or HT29 cells incubated with cholesterol after incubation with MβCD. (C) Effect of MβCD and cholesterol addition on lipid raft association. Caveolin-1 in lipid raft isolated from a. Untreated cells, b. Cells treated with 20 nM MβCD, and c. Cells incubated with 10 nM cholesterol after treatment with MβCD.

mechanism, HT29 cells were treated with different domains of GbpA. Domains 1, 2, and 3 were unable to induce IL-8 secretion, either alone or in combination. Only domain 4 alone could induce IL-8 secretion ((442.8 ± 32.8) pg/mL), which was comparable to the level of IL-8 induced by the full GbpA ((452.6 ± 27.8) pg/mL) (Fig. 13A). GbpA 4 alone induced IL-8 in a time- and dose-dependent manner, which was similar to the action of the full GbpA (Fig. 13B and C).

Domain 4 interacted directly with TLR2 as evidenced from a pull-down assay. GbpA 2-, GbpA 3-, and GbpA 4-treated HT29 cell lysates were pulled down using anti-TLR2 antibodies and assayed by immunoblotting with anti-GbpA antibody. Full GbpA and domain 4 were found to be precipitated with TLR2 (Fig. 13D). Domain 4 inhibited the binding of GbpA with TLR2, which further confirmed the previous observations. These results indicated that the binding of GbpA with HEK293-TLR2 cells was inhibited only by the purified domain 4 and the other domains of GbpA did not inhibit the binding of TLR2 (Fig. 13E).

4. Discussion

While the chitin-binding function of GbpA in host-pathogen interactions has been well studied, the specific role of GbpA in the host immune response remains less explored. The present study investigated the mechanism by which GbpA induces the production of IL-8. GbpA was found to bind specifically to TLR2, which acted as the primary receptor on the cell surface. Notably, despite previous indications that intestinal cells typically do not respond to the bacterial ligands of TLR2 [26], the present study revealed that TLR2 plays a central role in GbpA-mediated IL-8 secretion. Previous research has established that various secretory proteins from *V. cholerae* possess immunomodulatory properties, for example, cholera toxin could induce the production of tumour necrosis factor α (TNF-α) in mouse macrophages [27]. This observation was aligned with similar findings in other studies involving bacterial proteins and TLR2 as the primary receptor, emphasizing the importance of TLR2 in the cellular responses and cytokine secretion in various types of infections, such as has been found for the *Tannerella forsythia* secretory protein BspA [28]; *Helicobacter pylori* infections, where TLR2 activation was associated with chemokine expression; and the oral pathogen *Porphyromonas gingivalis* [29,30]. Additionally, the involvement of TLR5 in pro-inflammatory signaling pathways induced by *V. cholerae* flagellin has been observed, leading to the generation of IL-1β and IL-8 [18].

Generally, TLRs on intestinal epithelial cells are suppressed by Tollip, and the expression of Tollip is inversely correlated to TLR expression [31]. It has been previously observed that Tollip has an inhibitory effect on TLR2 expression [32]. However, in the present study, a transient decrease in Tollip expression was observed, alleviating the inhibition of TLR expression. Consequently, TLR2 was able to be expressed, facilitating the interaction with GbpA and initiating downstream signaling.

The present study revealed that TLR2 alone was insufficient for GbpA-mediated IL-8 secretion, and additional factors, such as TLR1 and the co-receptors CD14 or CD36, were required. It has been previously documented that TLR2 initiates inflammatory signaling in

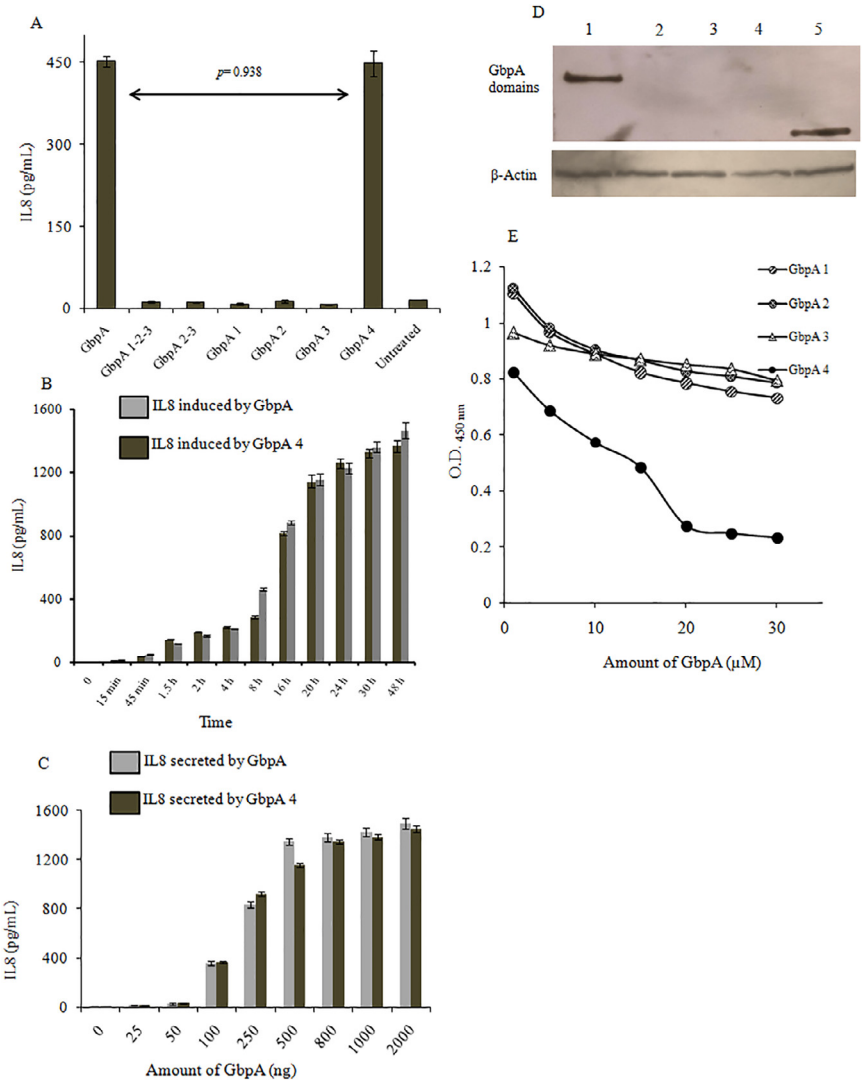

Fig. 13. Domain 4 of GbpA is involved in interacting with TLR2 in IL-8 induction. (A) Secretion of IL-8 in HT29 cells treated with different domains of GbpA. HT29 cells (10^6) were treated with 250 ng each of GbpA, GbpA 1-2-3, GbpA 2-3, GbpA 1, GbpA 2, GbpA 3, and GbpA 4 for 8 h. The amount of IL-8 secreted in the culture supernatants was estimated by sandwich ELISA. (B) Time-dependent secretion of IL-8 in HT29 cells treated with GbpA and GbpA 4. HT29 cells were treated with 250 ng each of purified GbpA and GbpA 4 for different time periods (0–48 h). The amount of IL-8 secreted was estimated by a sandwich ELISA method. (C) Dose-dependent secretion of IL-8 in HT29 cells treated with GbpA and GbpA 4. HT29 cells were treated with 0–2000 ng each of purified GbpA and GbpA 4 for 8 h. The amount of IL-8 secreted was estimated by a sandwich ELISA method. (D) Co-precipitation of GbpA 4 with TLR2. HT29 cells were treated with GbpA, GbpA 1, GbpA 2, GbpA 3, and GbpA 4. The cell lysate was pulled down using anti-TLR2 antibody (1:100) and pulled fractions were analyzed by immunoblotting. Lane 1. GbpA, Lane 2. GbpA 1, Lane 3. GbpA 2, Lane 4. GbpA 3, and Lane 5. GbpA 4. (E) GbpA-HEK293-TLR2 binding inhibition by GbpA 4. Increasing amounts (0–30 μM) of the purified domains of GbpA were incubated with HEK293-TLR2 and these cells were allowed to interact with purified full GbpA. The amount of bound GbpA was quantified by indirect ELISA using anti-GbpA antibody at a dilution of 1:400.

conjunction with either TLR1 and CD14 or TLR6 and CD36 [33]. Bacterial outer membrane proteins are recognized by either a TLR2-TLR1 or TLR2-TLR6 heterodimeric complex on the cell surface, and this interaction involves co-receptors, such as CD14 and CD36 [21]. GbpA engages with TLR2, forming a heterodimer with TLR1 and involving CD14 as a co-receptor. An interaction between TLR2 and TLR1 was demonstrated through co-precipitation and FRET analysis in the present study. In GbpA-treated HT29 cells, TLR1 and TLR2 were found in close proximity (within the Förster distance), as established by the detectable FRET signals. Although the physical closeness of CD14 in the TLR1/2 association could not be established, the role of CD14 as a co-receptor was evident because of the co-precipitation of CD14 with TLR2.

The participation of TLR1 and CD14 alongside TLR2 in instigating signaling for IL-8 secretion appears to be crucial. The absence of TLR1 on the cell surface, despite the presence of TLR2, led to a significant reduction in IL-8 secretion, as evidenced using dominant negative constructs. The initiation of GbpA-induced IL-8 secretion relies on the TLR1/2/CD14 receptor complex situated on the cell surface. This complex plays a pivotal role in signaling initiation. Previous reports have highlighted the collaborative role of CD14 and TLR2 in inducing cytokines, such as TNF-α and IL-1β. This phenomenon of inducing cytokines involving the role of TLR2 and CD14 has been documented in bacterial protein adhesins, including fimbrillin of *Porphyromonas gingivalis* and BspA of *Bacteroides forsythus*, in differentiated THP-1 cells [29].

A previous study by Nandakumar et al. in 2008 found that TLR4 did not play a role in the secretion of IL-8 during *V. cholerae* infection in HT29 cells, whereas the present study highlighted the importance of TLR2 in inducing IL-8 secretion [34]. In earlier research, TLR4 has been established as the primary receptor responsible for bacterial LPS recognition and initiating the IL-8 secretion signaling pathway involving MyD88 and NFκB [33]. However, in GbpA-mediated IL-8 secretion, TLR2 plays a pivotal role, initiating the signaling pathway in conjunction with MyD88, with NFκB later playing a crucial part

in the pathway. Therefore, these findings align with those of Nandakumar et al., suggesting that *V. cholerae* infection can indeed trigger IL-8 secretion in intestinal cells via TLR2 activation.

GbpA-mediated IL-8 expression was found to be associated with lipid raft micro-domains. TLR1, TLR2, and CD14 were detected within the liquid-ordered membrane segment, which was indicated by the presence of the lipid raft marker caveolin-1. This finding aligns with previous findings regarding TLR2 involvement in lipid raft micro-domains and inflammatory signaling in airway epithelial cells [35]. Other studies have demonstrated lipid raft-associated pro-inflammatory pathways in response to various stimuli, including *V. vulificus* elastase VvpE and lipoteichoic acid [36]. In addition, the TLR4-associated LPS mediated pro-inflammatory signaling cascade has been demonstrated to involve a lipid raft micro-domain as demonstrated by Płóciennikowska et al. [37], and lipid rafts have been shown to be involved in bacterial infection and inflammation [38]. The crucial role of lipid rafts was further supported by the results of experiments using MβCD, a compound which disrupts lipid rafts by removing cholesterol [39]. The disruption of lipid rafts led to a significant reduction in IL-8 secretion, emphasizing the essential role of lipid raft micro-domains in GbpA-mediated signaling.

In the present study, it was identified that MyD88 served as an adapter molecule and was co-precipitated with TLR2 in a time-dependent manner. Interestingly, earlier research has suggested that the inflammatory response in human intestinal cells triggered by *V. cholerae* was not reliant on MyD88. MyD88 in *V. cholera* OmpU has been shown to induce IL-6 and TNF-α production in murine macrophages and monocytes [40]. Another *V. cholerae* virulence factor, hemolysin, could induce TLR2 upregulation involving MyD88, TRAF6, and NFkB in mouse B1-a cells [41]. Furthermore, it has been demonstrated that the induction of the pro-inflammatory chemokine IL18, stimulated by the *V. cholerae* pore-forming toxin, hemolysin, was also independent of MyD88 [42].

In the present investigation, time-dependent activation of TRAF6, as a signaling intermediate, was observed; similar to what has been observed in *Helicobacter pylori* infection [43]. This activation of TRAF6 subsequently played a pivotal role in initiating the activation of NFκB. The activation of NFκB by TRAF6 has been similarly noted in *Helicobacter pylori* infection. The TRAF6-mediated activation then triggered downstream events, including the activation of IKK, which initiated the degradation of IκB. Consequently, the degradation of IκB facilitated the translocation of NFκB into the nucleus.

ERK1/2 and JNK1/2 were also involved in the GbpA-induced pro-inflammatory signaling pathway. Activation of these kinases was observed through their phosphorylation in GbpA-treated HT29 cells in a time-dependent manner. Activation of JNK1/2 resulted in the formation of an AP-1 complex, which moved into the nucleus and increased the IL-8 secretion. Activation of these MAP kinases has been shown to be involved in the IL-8 secretion induced by *V. cholerae* flagellin in T84 cells [18] and *V. cholerae* LPS [44].

In the *V. cholerae* infection of intestinal cells, IL-8 secretion involves NFκB and AP-1 activation, and a previous study has indicated that NFκB is the primary regulator [16,45]. A previous investigation by Bhowmick et al. in 2008 demonstrated the involvement of NFκB in the upregulation of intestinal mucin during GbpA-induced responses [12]. In the present study, it was aimed to elucidate the exact roles of AP-1 and NFκB in IL-8 secretion during GbpA treatment. ChIP assays indicated the direct involvement of both AP-1 and NFκB, with AP-1 exerting a more substantial influence, on IL-8 expression.

The pathway leading to IL-8 secretion was directly influenced by the GbpA domain 4, as evidenced using purified GbpA and truncated mutants. Only GbpA and GbpA domain 4 induced IL-8 secretion, emphasizing the focal role of domain 4 in the inflammatory response. TLR2 on HT29 cells specifically interacted with GbpA via domain 4, as demonstrated by co-precipitation experiments and binding inhibition studies. These results highlighted that domain 4 is the key contributor to initiating the inflammatory response by binding with TLR2.

In summary, GbpA functions as a secretory colonization factor for *V. cholerae*, contributing to the development of cholera. Moreover, GbpA plays a pivotal role in inducing pro-inflammatory signaling responses, highlighting the multifaceted involvement of GbpA in the pathogenic processes associated with *V. cholerae*.

The secretory colonization factor GbpA is a vital player in triggering the production of the pro-inflammatory cytokine IL-8 within intestinal epithelial cells by engaging the TLR2/1/CD14 complex, which is associated with lipid rafts on the cell surface. Notably, during *V. cholerae* infection, distinct pro-inflammatory host responses have been observed, with LPS and flagellins identified as instigators of the secretion of TNF-α and IL-8, respectively. GbpA is a noteworthy addition to the immunomodulatory components known to be employed by *V. cholerae*. The research presented herein sheds light on the involvement of GbpA in *V. cholerae* pathogenesis, particularly the role of GbpA in promoting inflammatory responses. Further investigations are needed to precisely identify the amino acids and the specific region within domain 4 of GbpA those participate in this process.

5. Conclusion

The *V. cholerae* secretory colonization factor, GbpA, triggers a pro-inflammatory response by amplifying IL-8

secretion. The intracellular signaling pathway involved in this process includes the binding of GbpA to TLR2, alongside TLR1, situated in the lipid raft micro-domain. This pathway requires various MAP kinases, ultimately culminating in the activation of NFκB and AP-1. Of these transcription factors, AP-1 emerged as the most important contributor to IL-8 secretion. The specific region identified as the instigator of this cell signaling response was domain 4 within the multi-domain GbpA protein.

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Author contributions

Avishek Ghosh: Conceptualization, Methodology, Data curation, Writing—original draft, Validation.

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Declaration of competing interest

The author declares no conflict of interest.

Data available statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics statement

Ethics approval was waived for this study because no patients' data were reported.

Informed consent

Not Applicable.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.imj.2024.100113.

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