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Functional characterization of *Helicobacter pylori* TlyA: Pore-forming hemolytic activity and cytotoxic property of the protein

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ABSTRACT

Helicobacter pylori is a human specific gastric pathogen. *H. pylori* pathogenesis process involves a number of well-studied virulence factors that include the 'vacuolating cytotoxin' and the 'cytotoxin associated gene A'. Analysis of the *H. pylori* genome, however, indicates presence of additional virulence factors that are yet to be characterized in molecular detail. For example, *H. pylori* genome harbors a gene that has potential to encode a protein with sequence similarity to those of the TlyA-like proteins of several pathogenic bacteria. Earlier studies have indicated potential association of this *H. pylori tlyA* gene in the virulence mechanism of the organism. Despite such notions, however, the TlyA-like protein of *H. pylori* has not been studied previously in molecular detail. In particular, purified form of *H. pylori* TlyA has never been studied before toward exploring its functional properties. Here, we report characterization of the *H. pylori* TlyA protein purified from the recombinant over-expression system in *Escherichia coli*. Purified form of the recombinant TlyA exhibits prominent hemolytic activity against human erythrocytes, presumably via formation of pores of specific diameter in the cell membrane. Purified TlyA also triggers prominent cytotoxic responses in human gastric adenocarcinoma cells. Altogether, our study establishes *H. pylori* TlyA as a potential virulence factor of the organism.

1. Introduction

The Gram-negative bacterium *Helicobacter pylori* is a human-specific pathogen that colonizes the gastric mucosal layer of the stomach. *H. pylori* infection is associated with gastritis, peptic ulcer, mucosa-associated lymphoid tissue lymphoma and gastric adenocarcinoma [1]. The 'vacuolating cytotoxin' (VacA) and the 'cytotoxin associated gene A' (CagA) are the two most extensively studied virulence factors produced by *H. pylori* [2–6]. However, the *H. pylori* genome encodes additional factors that may also potentially contribute toward the pathogenesis mechanism of the organism [7,8].

H. pylori genome harbors a gene (HP1086 in the genome of *H. pylori*, strain 26695) [8] that has been annotated to encode a protein with sequence similarity to the TlyA proteins of several pathogenic bacteria including *Mycobacterium tuberculosis* [9] and *Serpulina hyodysenteriae* [10] (Fig. 1A and B). This *H. pylori* HP1086 gene-product is therefore designated as a TlyA-like protein. TlyA proteins from *M. tuberculosis* and *S. hyodysenteriae* are indicated as potential virulence factors in terms of displaying potent hemolysin-like property [9,10]. Previous studies have indicated association of the *tlyA* gene in the hemolytic activity of *H. pylori* [11]. It has been shown that mutation in the *tlyA* gene in *H. pylori* partially compromises the in vitro hemolytic activity of the organism [11]. Moreover, introduction of the *H. pylori tlyA* gene into non-hemolytic *Escherichia coli* cells has been shown to confer hemolytic activity in the recombinant *E. coli* strain [11]. Despite such observation, however, the *H. pylori tlyA* gene-product has not been characterized in molecular detail. In particular, functional properties of the *H. pylori* TlyA protein, in its purified form, have not been explored yet. More importantly, *H. pylori* TlyA protein could not be purified previously in its hemolytically active form [11]. Therefore, it has not been established yet whether the hemolytic activity is an intrinsic property of the *H. pylori* TlyA protein, or whether the presence of the *tlyA* gene indirectly contributes toward the in vitro hemolytic property of the organism. In addition to these, it also remains to be tested whether the *H. pylori* TlyA protein could exert any cytotoxic effect on the human gastric epithelial cells.

In the present study, we report molecular characterization of the *H. pylori* TlyA protein. The *H. pylori* TlyA protein was recombinantly over-expressed in *E. coli*, purified to homogeneity, and tested for its functional activity against target eukaryotic cells that include human erythrocytes as well as human gastric adenocarcinoma (AGS) cells. In our study, purified TlyA was found to display pore-forming hemolytic activity against human erythrocytes. Purified form of *H. pylori* TlyA was also found to induce potent

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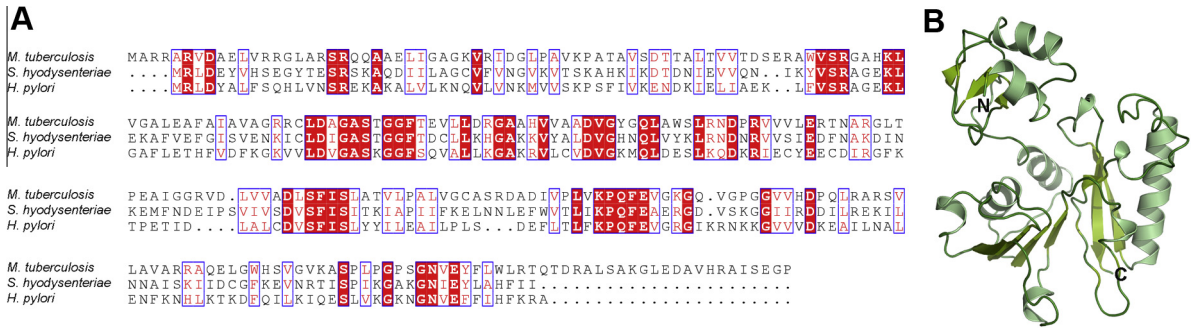

Fig. 1. (A) Amino acid sequence alignment of *H. pylori* TlyA with the TlyA-like proteins from *M. tuberculosis* and *S. hyodysenteriae*. Amino acid sequence of *H. pylori* TlyA shows 33% and 36% identity with those of the TlyA proteins from *M. tuberculosis* and *S. hyodysenteriae*, respectively. (B) Cartoon representation of the homology-based protein structure model of *H. pylori* TlyA. N- and C-termini of the protein are indicated.

cytotoxic responses in human AGS cells, thus confirming further its role as a cytotoxin protein. Altogether, our data established *H. pylori* TlyA protein as a factor potentially capable of contributing toward the pathogenesis process of the organism.

2. Materials and methods

2.1. Over-expression and purification of H. pylori TlyA

Nucleotide sequence encoding *H. pylori* TlyA was cloned into the bacterial expression vector pET14b (Novagen), and the recombinant plasmid was transformed into *E. coli* Origami B cells (Novagen). The pET14b expression vector allows incorporation of 6xHis-tag at the N-terminus of the protein, thus facilitating purification of the protein using Ni–NTA agarose (QIAGEN) affinity chromatography. A thrombin cleavage sequence between the 6xHis-tag and the cloning site also allows removal of the N-terminal His-tag via protease treatment. For large scale protein production, *E. coli* Origami B cells harboring the recombinant plasmid were grown in LB broth containing ampicillin (50 μg/ml) at 37 °C under constant shaking condition at 180 rpm to growth phase corresponding to A_{600} of ~1.0, and the cells were induced with 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG) for 3 h at 30 °C. Cells were harvested, re-suspended in a buffer containing 20 mM sodium phosphate, 150 mM NaCl (pH 7.0) (PBS) supplemented with bacterial protease inhibitor cocktail (Sigma), and were lysed using ultrasonic disruption method. Soluble fraction of the bacterial cell lysate was adjusted with 20 mM imidazole, passed through Ni–NTA agarose, and bound His-tagged TlyA protein was eluted with 200 mM imidazole in PBS. Eluted protein was diluted 5-fold with a buffer containing 10 mM Tris–HCl (pH 7.4), and passed through SP Sepharose cation-exchange resin (Sigma). Bound TlyA protein was eluted with 400 mM NaCl in 10 mM Tris–HCl buffer (pH 7.4). Purity of the protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie staining. Identity of the His-tagged TlyA protein was confirmed by Western blotting using anti-His antibody (QIAGEN). In order to remove the N-terminal His-tag, protein was treated with thrombin (Sigma) (1 unit of thrombin/500 μg of TlyA) for 4 h at 25 °C. Subsequently, thrombin was inactivated using 1 mM phenylmethylsulfonyl fluoride (PMSF), and processed TlyA was purified via SP Sepharose cation-exchange chromatography. Purity of the protein was analyzed by SDS–PAGE/Coomassie staining. Protein concentration was estimated by monitoring absorbance at 280 nm based on the theoretical extinction coefficient calculated from the primary structure of the recombinant TlyA (0.21 for 1 mg/ml His-tagged TlyA protein; 0.23 for 1 mg/ml TlyA protein lacking the His-tag). Functional properties of *H. pylori* TlyA were characterized using both the forms of the recombinant protein (i.e. with or without the His-tag). Presence or absence of the His-tag did not affect the functionalities of the recombinant TlyA protein.

2.2. Anti-TlyA antiserum

Polyclonal anti-TlyA antiserum was raised in rabbit using the purified form of recombinant *H. pylori* TlyA protein as antigen. The antiserum was generated using the Custom Polyclonal Antibody Production Service of GeNei/Merck, Bangalore India.

2.3. Far-UV circular dichroism (CD) spectroscopy of TlyA

Far-UV CD spectrum of TlyA (protein concentration in the range of 2 μM) in 1 mM Tris–HCl buffer (pH 8.0) was acquired with a Chiracsan spectropolarimeter (Applied Photophysics, Leatherhead, UK) equipped with a Peltier-based temperature controller, using 5 mm pathlength quartz cuvette. Far-UV CD spectrum of the protein was corrected for the baseline by subtracting the buffer spectrum. The secondary structure content was estimated using CDNN [12].

2.4. Experiments with human erythrocytes

Experiments using human erythrocytes have been approved by the Bioethics Committee of IISER Mohali.

Binding of TlyA with human erythrocytes was estimated using a flow cytometry-based assay as described previously [13,14]. Briefly, human erythrocytes (cell suspension corresponding to A_{600} = 1.5 was used) in 100 μl PBS were treated with TlyA for 2 h at 25 °C. Cells were washed with ice-cold PBS, incubated with rabbit anti-TlyA antiserum [1:100 volume/volume dilution in PBS containing 0.1% weight/volume BSA; Sigma–Aldrich] for 30 min at 4 °C, and subsequently treated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody as described previously [13,14]. Cells were analyzed for FITC fluorescence by FACSCalibur (BD Biosciences) flow cytometer with an excitation wavelength of 488 nm, with an emission wavelength of 530 nm. Cells that were not treated with TlyA, but stained with anti-TlyA and anti-rabbit-FITC served as the negative control. Data were analyzed using the FlowJo software (www.flowjo.com).

For estimation of the hemolytic activity of *H. pylori* TlyA, human erythrocytes suspended in PBS were treated with His-tagged TlyA for 18 h at 25 °C. Lysis of erythrocytes was monitored by estimating the release of hemoglobin spectrophotometrically at 415 nm. Human erythrocytes concentrations in the reaction mixtures were adjusted such that complete lysis the cells corresponded to the OD_{415} in the range 0.8–0.9. The 100% lysis of erythrocytes was induced by treatment with 100 nM *Vibrio cholerae* cytolysin as the positive control [13,14].

2.5. Experiments with human AGS cells

Human AGS cell line was procured from the National Center for Cell Science (NCCS), Pune, India, and was maintained in DMEM-F12K medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 1% antibiotic and antimycotic solution (Himedia laboratories Pvt. Ltd., India) at 37 °C in a 5% CO_2 incubator with 95% humidity.

Effect of TlyA on human AGS cells was visualized by differential interference contrast (DIC) microscopy using Zeiss Axio Observer Z1 microscope. Human AGS cells (1×10^6) were treated with TlyA in 200 μl PBS for 4 h at 25 °C. After the treatment, 20 μl of the cell suspension was placed on a glass slide, covered with the square-shaped cover slip, and was sealed with transparent nail paint. Cells were examined at ×40 magnification. Human AGS cells without TlyA treatment were taken as control.

For cell viability and cytotoxicity assays, AGS cells were seeded at a density of 5×10^4 cells/100 μl/well in 96-well plates in phenol red-free DMEM media (Invitrogen) supplemented with 10% FBS and 1% antibiotic and antimycotic solution, incubated for 2 h at 37 °C in a 5% CO_2 incubator with 95% humidity, and then treated with various concentrations of His-tagged TlyA for 24 h under the same growth condition. Human AGS cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT)-based cell growth determination kit (Sigma–Aldrich) as per manufacturer's protocol. Cells without TlyA treatment served as the positive control for 100% cell viability, whereas media blank without any cell served as the negative control corresponding to 0% cell viability. For cytotoxicity assay, release of lactate dehydrogenase (LDH) in the cell culture medium was estimated quantitatively using the CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit (Promega) according to the manufacturer's protocol. Maximum LDH release (corresponding to 100% cytotoxicity) was induced by treating the cells with the lysis solution provided with the kit.

Binding of His-tagged TlyA with the human AGS cells were estimated using the flow cytometry-based assay. Briefly, human AGS cells (0.5×10^6 cells) in 100 μl PBS were treated with the protein for 2 h at 25 °C. Binding of His-tagged TlyA was analyzed following the method as described for human erythrocytes.

2.6. Amino acid sequence alignment

Amino acid sequence alignment of *H. pylori* TlyA with TlyA-like proteins from *M. tuberculosis* and *S. hyodysenteriae* was generated using the program ClustalW [15] within the Biology Workbench server available online at http://workbench.sdsc.edu [16]. The sequence alignment was rendered with the ESPript server (http://espript.ibcp.fr/ESPript/ESPript/) [17].

2.7. Homology-based structural model of H. pylori TlyA

Amino acid sequence of *H. pylori* TlyA was subjected to BLAST search in the NCBI server for obtaining the appropriate template with experimentally determined three-dimensional structure. The most appropriate template available in the Protein Data Bank (PDB) [18] as detected by BLASTP [19] was the crystal structure of a putative hemolysin from *Streptococcus thermophilus* (PDB Id: 3HP7; with 34% sequence identity). Homology-based structural model of *H. pylori* TlyA was generated based on the structural coordinate of 3HP7 using the SWISS-MODEL server (http://www.expasy.org/spdbv/) [20]. Protein structure model was visualized using PyMOL [DeLano WL, The PyMOL Molecular Graphics System (2002) found online (http://pymol.org)].

3. Results and discussion

3.1. Purification of H. pylori TlyA

In the present study, *H. pylori* TlyA with N-terminal hexahistidine-tag was recombinantly over-expressed in *E. coli* and purified to homogeneity (Fig. 2A). His-tagged TlyA expressed in the cytosolic fraction of the *E. coli* cells was initially purified by Ni–NTA agarose affinity chromatography, and was subsequently purified via SP Sepharose cation-exchange chromatography. The N-terminal His-tag was removed by proteolytic cleavage with thrombin, and the processed TlyA protein was further purified on SP Sepharose cation-exchange chromatography. Strong association of *H. pylori* TlyA with the cation-exchange chromatography resins was consistent with the overall net positive charge on the protein. Consistent with its primary amino acid sequence, the recombinantly generated TlyA protein displayed a monomeric molecular mass of ~27 kDa under the reducing SDS–PAGE condition (Fig. 2A). To examine the structural features of the recombinant TlyA, we monitored the far-UV CD spectra of the purified protein (Fig. 2B). Analysis of the far-UV CD profile estimated the presence of ~15% helix, and ~34% β-secondary structures in the protein.

3.2. Purified form of H. pylori TlyA shows pore-forming hemolytic activity against human erythrocytes

It has been reported previously that the *tlyA* gene product could be potentially associated with the hemolytic activity of *H. pylori*. However, purified form of the *H. pylori* TlyA protein has never been characterized earlier for its hemolytic activity. Therefore, in this direction, we explored whether the recombinant TlyA, in its purified form, could display any hemolytic activity against human erythrocytes. Our study showed that the purified TlyA could display concentration-dependent hemolytic activity against human erythrocytes over a protein concentration range of 2.5–20 μM, when incubated for a prolonged duration of at least 18 h at 25 °C (Fig. 3A). Under such experimental condition, 20 μM TlyA induced ~90% lysis of the human erythrocytes. It, therefore, appeared from our data that the purified form of recombinant *H. pylori* TlyA could display prominent hemolytic activity against human erythrocytes. We also explored the possibility whether the TlyA-induced hemolytic activity was the result of colloid osmotic lysis of the cells due to the specific pore-forming property of the protein in the erythrocyte cell membrane. For this, we monitored TlyA-mediated hemolysis of human erythrocytes in presence of saccharides with varying molecular sizes. If the hemolytic activity of TlyA is attributed to its ability to form transmembrane pores of specific

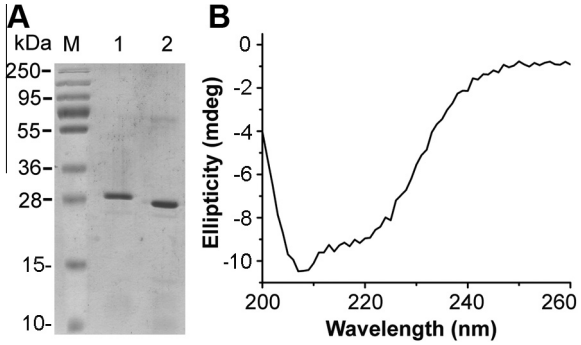

Fig. 2. (A) SDS–PAGE/Coomassie staining profile of purified form of the recombinant *H. pylori* TlyA protein. Lane 1, His-tagged TlyA; lane 2, purified form of TlyA after removal of the N-terminal His-tag; Lane M corresponds to the protein molecular mass standards. (B) Far-UV CD spectrum of the purified TlyA protein.

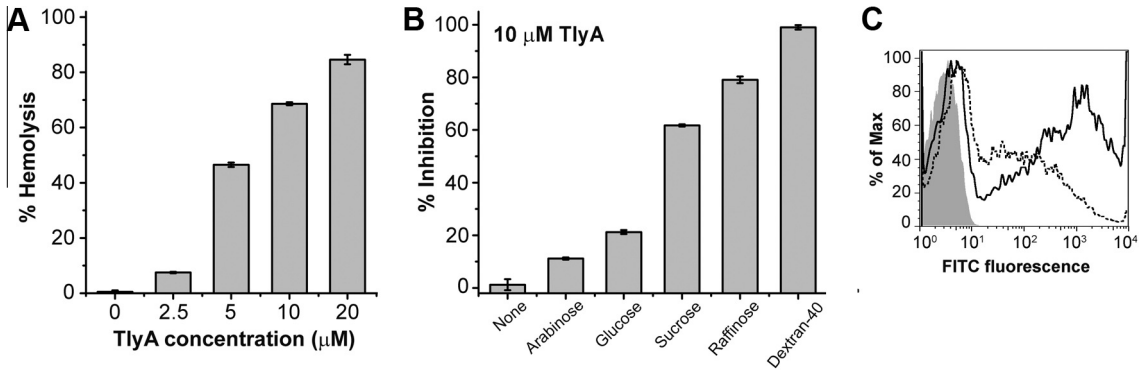

Fig. 3. (A) Hemolytic activity of *H. pylori* TlyA against human erythrocytes. The data shown are the average ± standard deviations of three independent measurements. (B) The % inhibition of hemolytic activity of *H. pylori* TlyA against human erythrocytes in presence of osmoprotectants. Dextran-40 was adjusted to 15% in the reaction volume. For other sugars, 50 mM concentration was used in the reaction mixture. The data shown are the average ± standard deviations of three independent measurements. (C) Binding of TlyA to human erythrocytes, as detected by the flow cytometry-based assay. Solid line, 5 μM TlyA; dashed line, 2.5 μM TlyA. Filled gray curve represents the negative control.

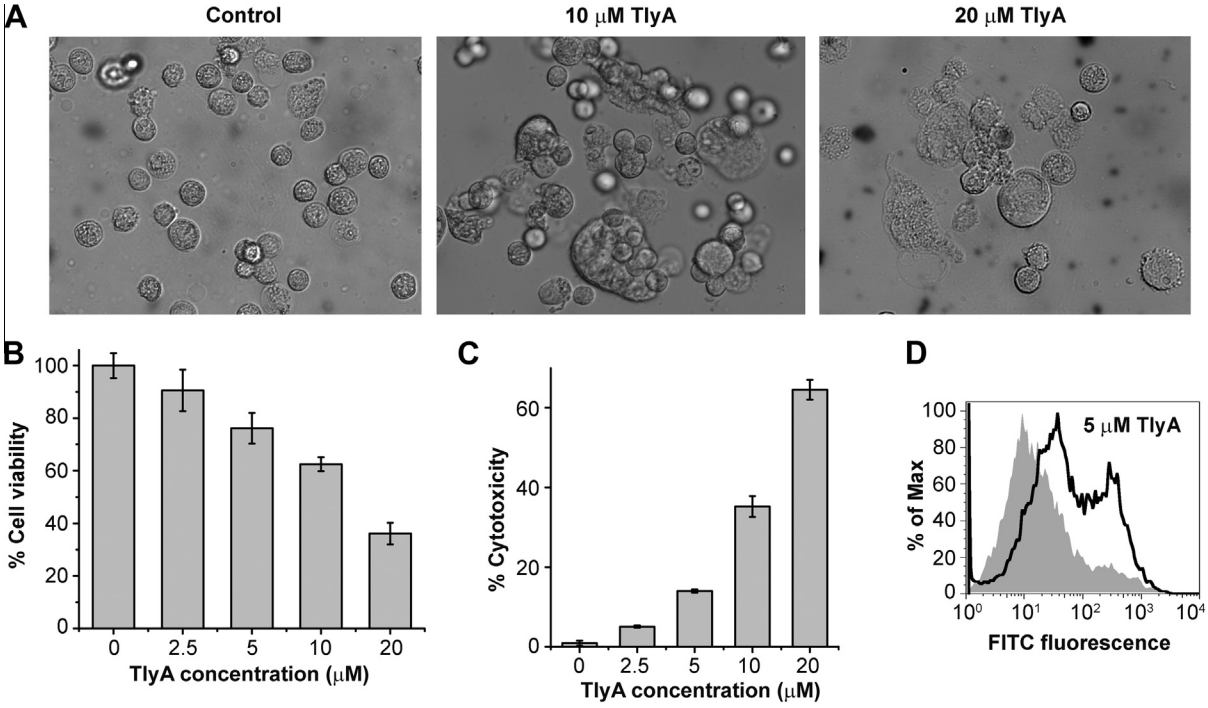

Fig. 4. (A) DIC images of human AGS cells upon treatment with purified form of TlyA at different protein concentrations. (B) Estimation of human AGS cell viability upon treatment with TlyA, as monitored by the MTT-based assay. The data are the average ± standard deviations, determined from three independent measurements. (C) Cytotoxic activity of TlyA against human AGS cells as monitored by LDH release assay. The data presented are the average ± standard deviations of three independent measurements. (D) Binding of TlyA to human AGS cells (solid line), as detected by the flow cytometry-based assay. Filled gray curve represents the negative control.

diameter, then the hemolysis would be expected to be inhibited in the presence of the saccharides having sizes larger than the pore diameter. Hemolytic activity of TlyA against human erythrocytes was found to be inhibited significantly by the saccharides having molecular diameter larger than 1 nm (sucrose, raffinose, and dextran-40), while a marginal inhibition of hemolysis was noticed in presence of smaller sacchardides (arabinose and glucose) [21] (Fig. 3B). Moreover, the extent of inhibition of hemolysis was found to be consistent with the order of the molecular diameter of the sacchardies: arabinose < glucose < sucrose < raffinose < dextran-40. These data, therefore, indicated that TlyA could induce lysis of human erythrocytes via formation of membrane pores having specific diameter, presumably in the range of <1.0 nm. We confirmed the binding of TlyA to human erythrocytes using a flow cytometry-based assay. In the concentration range of 2.5–5 μM, TlyA displayed prominent binding to erythrocytes (Fig. 3C).

3.3. Cytotoxicity of *H. pylori* TlyA against human AGS cells

In order to test further the functionality of the purified form of *H. pylori* TlyA, we studied the effect of the protein on human AGS cells. First, we employed DIC microscopy to qualitatively monitor the effect of TlyA on human AGS cells, when treated in suspension for 4 h at 25 °C. DIC images showed notable extent of alterations in cellular morphology/integrity upon TlyA treatment (Fig. 4A). Next, we monitored the viability of the human AGS cells upon treatment with purified TlyA protein using an MTT-based assay. MTT-based assay allowed quantitative estimation of the cell viability via

monitoring of the mitochondrial dehydrogenase activity in the living cells. Using this MTT-based assay, we observed that the purified form of TlyA, in the concentration range of 2.5–20 μM, could trigger marked decrease in the AGS cell viability, when incubated for 24 h at 37 °C (Fig. 4B). At a protein concentration of 20 μM, TlyA induced >60% decrease in the viability of the human AGS cells. We also monitored the release of LDH from the human AGS cells upon treatment with purified TlyA protein. LDH is a cytosolic enzyme, release of which in the cell culture supernatant could be interpreted as the result of cell lysis. Therefore, in our study, quantitative estimation of LDH release from the TlyA-treated human AGS cells was used as a measure of cytotoxic effect of TlyA on the human AGS cells. We observed that the TlyA protein could elicit cytotoxicity in the human AGS cells in a dose-dependent manner over the concentration range of 2.5–20 μM, when incubated for 24 h at 37 °C (Fig. 4C). At a protein concentration of 20 μM, TlyA could trigger ~65% cytotoxcity in the human AGS cells. We confirmed association of *H. pylori* TlyA with human AGS cells using the flow cytometry-based assay, as described for human erythrocytes. The flow cytometry-based assay showed marked binding of the TlyA protein (at a protein concentration of 5 μM) with the human AGS cells (Fig. 4D). Our data, altogether, confirmed potent interaction, and cytotoxic effect of *H. pylori* TlyA against human AGS cells.

In sum, in the present study, we have characterized the functionalities of the *H. pylori* TlyA protein in its purified form. Based on the data presented here, it is plausible to propose *H. pylori* TlyA as a potential virulence factor of the organism. However, more studies would be required in future to establish the role of TlyA in the virulence process of *H. pylori*. Also, detail studies would be required to elucidate TlyA mode of action in the context of *H. pylori* pathogenesis process, particularly focusing on the following aspects: (a) mechanism of delivery of the TlyA protein toward the target cell membrane, (b) mechanistic basis of the membrane pore-forming property employed by TlyA, and (c) mechanistic details of the cytotoxic responses evoked by TlyA in its target cells.

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