International Journal of Medical Microbiology 309 (2019) 274–282

Contents lists available at ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Effect of amyloid curli fibrils and curli CsgA monomers from *Escherichia coli* on in vitro model of intestinal epithelial barrier stimulated with cytokines

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ARTICLE INFO

Keywords:
Inflammation
Cytokines
Epithelium
Curli
Amyloid
Escherichia coli

ABSTRACT

Amyloid curli fibrils produced by *Escherichia coli* are well-known virulence factor influencing *E. coli* adhesion and biofilm formation. However, the impact of curli on intestinal epithelial barrier stimulated with proinflammatory cytokines is unknown. In the study, we examined the effect of curli produced by nonpathogenic *E. coli* K-12 and wild-type *E. coli* EC32 strains, and purified CsgA proteins on differentiated Caco-2 cell monolayers stimulated with a mixture of IL-1β, TNF-α, and INFγ cytokines as a model of 'inflamed intestinal epithelial barrier' in vitro. The results of the study indicated that curliated *E. coli* adhered better to polarized Caco-2 cells than their curli-deficient mutants and the adherence was further augmented by stimulation of epithelial cells with proinflammatory cytokines. Interestingly, curli reduced internalization but enhanced intracellular survival of the wild-type *E. coli* strain EC32 within intestinal epithelial cells. Curli-expressing *E. coli*, as well as purified CsgA proteins, attenuated IL-8 secretion by unstimulated Caco-2 cells, although the effect was barely observed on cytokine-stimulated cells. The findings of the study revealed that curli fibrils are an important virulence factor enabling curliated *E. coli* to effectively colonize intestinal epithelium especially in individuals with inflammatory intestinal disorders.

1. Introduction

Curli fibrils composed of amyloid protein curlin (CsgA) subunits are thin coiled fibers produced by many strains of *Enterobacteriaceae* family (Bokranz et al., 2005). Assembly of curli fibrils involves extracellular self-assembly of CsgA subunit, a soluble protein secreted by *Escherichia coli* into an environment and is dependent on minor curli subunit CsgB at the bacterial surface, that serves as a specific nucleator for CsgA polymerization (Gophna et al., 2001). The gene cluster encoding curli is commonly present and expressed in many pathogenic and non-pathogenic *E. coli* strains. Curli expression occurs optimally during the stationary phase of growth at low temperature and low osmolarity (Chirwa and Herrington, 2003). On the other hand, Bian et al. (2000) reported on curli production at 37° C in vitro by *E. coli* isolated from human blood cultures. These investigators have also shown anti-CsgA antibody in serum samples from convalescent patients with sepsis, providing evidence that curli fibrils are expressed *in vivo* as well. There are several reports on curli expression at 37° C by *E. coli* strains growing in a biofilm community or associated with animal diseases e.g. avian *E. coli* septicemia and bovine mastitis (Kikuchi et al., 2005; Bian et al., 2000; Provence and Curtis, 1992). Curli produced by *E. coli* are involved in bacterial aggregation and adhesion to abiotic surfaces, and biofilm formation (Kai-Larsen et al., 2010). Furthermore, curli allow *E. coli* strains to adapt to many niches in the host organism by binding several extracellular matrix and plasma proteins e.g. fibronectin, laminin, tissue plasminogen activator, plasminogen and H-kininogen, and so may contribute to pathologic processes (Gophna et al., 2001; Olsén et al., 1998). Bokranz et al. (2005) have shown that expression of curli at 37 °C is common among *E. coli* isolates colonizing the gastrointestinal tract of humans. Oppong et al. (2013) have demonstrated that curli produced by *Salmonella enterica* serovar Typhimurium enhanced intestinal epithelial barrier integrity and decreased interleukin-8 (IL-8) secretion by epithelial cells. On the other hand, however Chen et al. (2016) have shown that aged rats exposed to curli-producing bacteria displayed increased neuronal deposition of misfolded alpha-synuclein (AS), a prion-like protein, in the gut and brain. The study suggested that

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https://doi.org/10.1016/j.ijmm.2019.05.001
Received 24 October 2018; Received in revised form 4 May 2019; Accepted 10 May 2019

curli may function as a trigger to initiate AS aggregation through cross-seeding. Similarly, Lundmark et al. (2005) have demonstrated that curli can enhance amyloid protein A amyloidosis in mice via the cross-seeding mechanism. Furthermore, CsgA has been found to contain amyloidogenic peptide repeat motifs shared by human prions and AS (Chen et al., 2016; Evans and Chapman, 2014).

Inflammation of intestinal epithelium accompanies several intestinal disorders i.e. gastrointestinal tract infections and inflammatory bowel disease (IBD) (Martin et al., 2017). Proinflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α and interferon γ (IFNγ) play an important role in mucosal inflammation as chemoattractants for leukocytes and factors compromising epithelial barrier integrity via decreased expression of junctional proteins, epithelial cells apoptosis or junctional proteins endocytosis (Luissint et al., 2016;Bruewer et al., 2003). At present, little is known about the impact of curliated *E. coli* on the inflamed intestinal epithelium, therefore the aim of the study was to investigate the effect of curliated *E. coli* strains and purified CsgA proteins on cultured polarized Caco-2 cell monolayers stimulated with proinflammatory cytokines.

2. Materials and methods

2.1. E. coli strains, media and culture conditions

In the study, two *E. coli* strains were used: a wild-type *E. coli* strain EC32 and nonpathogenic *E. coli* strain C600 K-12, both carrying *csg* operon encoding curli synthesis. *E. coli* strain EC32 was derived from an archival collection of *E. coli* strains isolated from biopsy specimens of children with IBD. The collection is deposited in the Department of Microbiology of University of Medicine, Wrocław, Poland. The study was approved by the Research and Ethics Committee of the University of Medicine, Wrocław, Poland and children's parents provided written consent on behalf of all child participants (Permission number KB–292/2008). *E. coli* strains were stored at −80° C in Luria broth with 15% glycerol and kanamycin at a concentration of 0.3 mg/mL as appropriate. For optimal curli expression *E. coli* strains were grown for 48 h at a room temperature (RT) on colonization factor antigen (CFA) agar (10 g casamino acids, 15 g yeast extract, 50 mg $MgSO_4$, 5 mg $MnCl_2$, 2 g Bacto Agar per liter; pH 7.4) supplemented with 15 μg/mL kanamycin as appropriate.

2.2. Construction of the csgBA gene knockout

From both *E. coli* strains curli deletion mutants EC32Δ*csgBA::kan* (designated as EC32Δ*csgBA*) and K-12Δ*scgBA::kan* (designated as K-12Δ*csgBA*) were constructed according to Datsenko and Warner (2000) using primers listed in (Table S1) and the λ *red* recombination system. Primers *csgA* and *csgB* were used to amplify the kanamycin resistance cassette from plasmids pKD4 and pKD78. PCR products were purified and electroporated into *E. coli* strains EC32 and K-12 that had been induced to express the λ Red recombinase. *E. coli* cells were incubated in Luria broth (LB) supplemented with L-arabinose (10 mM) for 16 h and then plated on agar media containing kanamycin (15 μg/mL) and incubated overnight. Mutations were confirmed by PCR with primers pre-*csgB* and post-*csgARV* that included the region of recombination.

2.3. Detection of curli production

To evaluate curli production *E. coli* strains were cultured for 48 h at RT or at 37 °C on CR-CFA agar (CFA agar supplemented with 40 μg/mL Congo red dye [CR]) and on CFA agar with curcumin (80 μg/mL) according to McCrate et al. (2013). Curli-producing bacteria form red colonies on CR-CFA agar and yellow colonies on CFA-curcumin agar. Quantitative Congo red binding was performed according to Gophna et al. (2001). Bacteria cultured for 48 h on CFA agar without Congo red dye were scraped off from the agar plates and suspended in saline (0.85% NaCl) to a concentration of 9×10^8 bacteria per mL. A 1 mL of bacterial suspension was pelleted by centrifugation for 10 min at 14,000 rpm, then resuspended in a 1 mL of 0.002% Congo red solution in NaCl and incubated for 10 min at RT. Then, bacteria were pelleted again and OD of supernatants was measured at 500 nm against saline (background) to determine the reduction in OD of Congo red solution. Indirect immunofluorescence with rabbit anti-CsgA antibody was involved to detect curli on the surface of *E. coli* strains as described below. PCR reaction was performed to detect *csgA* and *csgD* genes encoding CsgA curli subunit and CsgD regulator of curli synthesis according to Bian et al. (2001) and Prigent-Combaret et al. (1999). Primers sequences and conditions of PCR reactions are presented in Table S2. All PCR amplifications were performed in a DNA-Engine PT200 thermal cycler (MJ Research Waltham, MA, USA). The PCR products were visualized after electrophoresis on a 2% agarose gel buffered with Tris-acetate-EDTA and staining with SYBR Green I Nucleic Acid Gel Stain.

2.4. Purification of curli amyloid protein

Amyloid proteins from *E. coli* EC32 and *E. coli* K-12 strains were isolated and purified according to Collinson et al. (1991) with some modifications. Bacterial colonies cultured on CFA agar for 48 h at RT were scraped off the medium and suspended in Tris buffer (pH 8.0) containing RNase A (0.1 mg/mL) and DNase I (0.1 mg/mL). Then bacterial cells were broken by sonication on ice for 30 min at 80 Hz and mixed with 1 mM $MgCl_2$ prior to incubation for 20 min at 37° C. After that 1 mg/mL of lysozyme was added and samples were incubated with shaking for 40 min at 37° C. The mixtures were treated with 1% sodium dodecyl sulfate (SDS) and incubated for 30 min at 37° C. The insoluble material remaining after centrifugation of lysates (12,000 rpm for 15 min at 25° C) was resuspended in 10 mL Tris buffer (10 mM; pH 8.0) and boiled for 10 min to remove agar present in the sample. The procedure was repeated twice to remove any residual agar. The resulting pellet was redigested with RNase A, lysozyme and DNase I under conditions described above. The obtained material was suspended in 2 mL of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer (10% glycerol; 5% β–mercaptoethanol; 2% SDS; 62.5 mM Tris HCl; pH 6.8), then boiled for 15 min and loaded onto polyacrylamide gel (3% stacking gel and 12.5% preparative gel). Electrophoresis was carried out for 5 h at 20 mA (1.25 mA/cm gel). The material that did not enter the stacking gel and remained in wells was collected, washed twice with deionized water, extracted twice with 95% ethanol solution and lyophilized (Vacuum centrifuge, JW Electronic). Next, the sample was resuspended in deionized water and sonicated at 50 Hz to break up large clumps. Then the material was extracted with 0.2 M glycine (pH 1.5) at 100° C for 10 min, pelleted (16,000 rpm for 10 min at 4° C), washed five times with deionized water and lyophilized. The lyophilized sample was mixed for a few seconds with 95% formic acid to depolymerize of curli into CsgA monomers and immediately frozen in liquid nitrogen. The formic acid was removed by using vacuum centrifuge (JW Electronic; Poland) operated at 45° C for 1 h. The lyophilized samples of CsgA monomers were stored at −20° C for further study. The total concentration of protein in depolymerized curli samples was measured spectrophotometrically (Picodrop Microliter UV/Vis Spectrophotometer) and by the bicinchoninic acid method of Smith et al. (1985) with bovine serum albumin (BSA) as the protein standard. Lyophilized CsgA monomers were dissolved in deionized water, dried overnight on a glass slide, stained with Congo red dye working solution (1% NaOH; 50 mL of Congo red stock solution: 0.3% Congo red dye; 0.3% NaCl; 80% ethyl alcohol) according to Ishii et al. (2003) or with 0.0125% thioflavin T (ThT) solution in 50% (v/v) ethanol and visualized under Olympus BX60 light microscope equipped with polarizer at magnification 20 x or Olympus BX51 fluorescence microscope at magnification 40x.

2.4.1. Preparation of immune serum

Purified CsgA protein (400 µg) obtained from strain EC32 was resuspended in PBS (pH 7.2), sonicated to complete dissolution and emulsified in Freund's complete adjuvant at a 1:1 (v/v) ratio. The sample was used to immunize a 1-month-old Tremont White rabbit by subcutaneous and intramuscular injections. The booster injections were performed after 2 and 4 weeks with samples emulsified in incomplete Freund's adjuvant at a 1:1 (v/v) ratio. One week after last vaccination the titers of the rabbit immune serum were determined by indirect immunofluorescence with CsgA monomers as the antigen and goat polyclonal anti-rabbit IgG antibody labeled with fluorescein isothiocyanate (FITC) as the second antibody. A 10 days after the last injection the rabbit was bled and immune serum was inactivated and frozen at −80° C. Rabbit immunization was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiment of the University of Medicine in Wrocław, Poland (Permit Number: KB-292/2008).

2.4.2. SDS-PAGE and Western blot analysis

Lyophilized CsgA protein was resuspended in a sample buffer (10% glycerol, 5% β-mercaptoethanol, 2% SDS, 0.004% bromophenol blue, 62.5 mM TRIS-HCl; pH 6.8), incubated at 100° C for 10 min and separated electrophoretically in 12.5% separating gel with a 5% stacking gel, with a double concentration of buffer used in the separation gel and the running buffer. A protein band with molecular mass of 17 kDa corresponding to CsgA monomers was observed by Coomassie blue staining. CsgA monomers were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P^{SQ}, Millipore) and detected by Western blotting with rabbit immune serum. The tank electroblotting was carried out in the CAPS (3-[cyclohexylamino]-1-propane sulfonic acid; pH 11.0) transfer buffer supplemented with 20% methanol for 2.5 h at 40 V. After transfer, the unoccupied membrane sites were blocked with non-fat milk (5%) in TBS-T buffer (20 mM Tris; 50 mM NaCl; 005% Tween 20; pH 7.5) solution and then incubated with rabbit immune serum (diluted 1:4 000 in TBS-T buffer) as the first antibody followed by labeling with goat anti-rabbit IgG immunoglobulin conjugated to horseradish peroxidase (diluted 1:10,000 in TBS-T buffer) as the second antibody. The protein bands were detected on the membrane after staining with DAB (3,3′-diaminobenzidine).

2.4.3. UPLC-Q-TOF/MS analysis

The CsgA protein obtained from EC32 strain was dissolved in acidic Milli-Q water (pH 2.0 adjusted with HCl); first, in 99% acetic acid, and then in Milli-Q water (in ratio 1:3) and in formic-acetic acid, diluting rapidly to < 10%, first in 99% acetic acid, and then in 95% formic acid (in ratio 1:1). The solutions were then analyzed by UPLC-Q-TOF/MS. Waters Acquity Ultra Performance LC system (Waters Corp., Milford, MA, USA) equipped with an ACQUITY UPLC® HSS T3 1.8 µm analytical column (50 mm x 1 mm; USA) was performed to separate the amyloid. The analytical column temperature was set to 40° C and the gradient elution program started with 90% solvent A (0.1% formic acid in water) and 10% solvent B (0.1% formic acid in acetonitrile). The column was eluted with a linear gradient of 10%–95% solvent B for 2–6 min; 95% solvent B was held for 1 min and then returned to 10% over 7.1–10 min at a flow rate of 300 µL/min. A Xevo G2-Q-TOF (Waters Corp., Milford, MA, USA) was performed to mass analysis. The spectrometer was equipped with a Z-Spray (electrospray ionization source). The capillary voltage was set at 3 kV and the cone voltage was set at 40 V. The cone gas and the desolvation gas were set at 85 and 800 (L/h), respectively. The source temperature was set at 90° C and the desolvation temperature was set at 500 °C. The positive ion mode was performed. The [Glu1]-Fibrinopeptide B human (Waters Corp., Milford, MA, USA) was used as the lock mass solution for accurate mass measurement. Data were collected from *m/z* 200 to *m/z* 2000. Deconvolution of mass spectra was performed with MaxEnt 1 (Waters Corp., Milford, MA, USA). The following deconvolution parameters were used for data processing: mass range, 10,000–20,000 Da; resolution, 0.5 Da, number of iterations, to convergence, minimum intensity ratio left and right, 33%; width at half height for uniform Gaussian model, 0.75.

2.4.4. Polymerization of CsgA

The kinetics of polymerization of CsgA monomers into amyloid fibrils was monitored by ThT fluorescence intensity. CsgA monomers (2 mg/mL) were diluted in 20 mM Tris-HCl buffer (pH 7.4) and centrifuged (25 000 x g for 10 s at 25 °C) to remove insoluble aggregates. A 100 µL of collected supernatant was mixed with ThT to the final concentration 20 µM in a 96-well opaque plate and incubated for 5 h at 37 °C in a SpectraMax Gemini XPS, Molecular Device reader. Fluorescence intensity was measured every 30 s after shaking for 5 s at 438 nm excitation and 495 nm emission with a 475 nm cutoff according to Dueholm et al. (2010). In order to determine the effect of rabbit antiserum on the polymerization of CsgA monomers, the serum was diluted 1:5 in the test sample. Tris-HCl buffer (pH 7.4) and bovine serum albumin (1% solution in PBS) were used as negative controls.

2.5. Interaction of curliated E. coli and CsgA with cytokine-stimulated and non-stimulated Caco-2 monolayers

2.5.1. Cell culture

Caco-2 cells (ATCC HTB-37™) were maintained in a minimal essential medium (MEM) with 10% fetal bovine serum (FBS), 2 mM stable L-glutamine (Glutamax), 1 mM nonessential amino acids (NEAA), 1 mM sodium pyruvate and antibiotics (penicillin 100 U, streptomycin 100 µg/ml) at 37° C in a humidified atmosphere with 5% CO_2. All cell culture reagents were from Biowest, Inc., US. Caco-2 cells were seeded onto polyethylene terephthalate (PET) cell culture Transwell membranes (0.4 µm pore size) in 12-well plates at a density of 1×10^5 cells per 1 cm^2 and cultured for 21 days to complete cell differentiation to an enterocyte-like phenotype. Cell culture medium was refreshed every other day. A day before experiments, cells were washed three times with phosphate buffered saline (PBS, pH 7.2) to wash out antibiotics and MEM with 1% FBS and 0.5% methyl-D-mannopyranoside (Sigma Aldrich, Germany) without antibiotics was added to apical and basolateral compartments. Nevertheless, antibiotics were added to the culture medium in all experiments with CsgA proteins. Proinflammatory cytokines (5 ng/mL of IL-1β, 50 ng/mL of TNF-α and 50 ng/mL of IFNγ; recombinant human, BioLegend) were added to the basolateral compartment and incubated for 24 h [24].

2.5.2. Adherence, internalization and intracellular survival of curliated E. coli in epithelial cells

E. coli strains were grown for 48 h on CFA agar at RT. The in vitro adherence assay to Caco-2 monolayers stimulated or non-stimulated with cytokines was performed according to Cravioto et al. (1991) in the presence of 1% methyl-α-D-mannopyranoside to exclude type-1 fimbria-mediated adhesion. The internalization assay of *E. coli* to epithelial cells was evaluated by the standard gentamycin protection test as described elsewhere (Falkow et al., 1987). Confluent Caco-2 monolayers were infected with 10^7cfu/mL of *E. coli* strains per insert at a multiplicity of infection of 100 (MOI = 100). After 3 h of incubation at 37 °C in an atmosphere supplemented with 5% CO_2, Caco-2 cells were washed three times with PBS and then incubated with fresh medium containing 100 µg/mL of gentamycin for 1 h to kill extracellular bacteria. After three washes in PBS, Caco-2 cells were lysed with 1% Triton X-100 in deionized water for 10 min. Adherence assay was performed like the invasion assay except that the gentamycin treatment was omitted. In order to assess the survival ability of internalized *E. coli* strains over a 24 h period, Caco-2 cells infected with *E. coli* for 3 h were washed and incubated with medium containing gentamycin at 20 mg/

mL for 24 h more. After, Caco-2 cells were washed and lysed with Triton X-100. Sample dilutions of cells lysates were plated onto MacConkey agar and incubated overnight at 37 °C to determine the number of bacterial colony forming units (CFU).

2.5.3. Transepithelial electrical resistance (TEER) measurement

The integrity of cultured Caco-2 cell monolayers was controlled at 7, 14 and 21 days by measurement of transepithelial electrical resistance (TEER) using Millicell ERS-2 Voltohmmeter (Millipore, Germany). TEER values of Caco-2 monolayers infected with *E. coli* strains or treated with CsgA monomers were measured at two different locations of the culture prior to infection (time 0) and 24 h post-infection. Before TEER measurement Caco-2 monolayers were washed two times with PBS and equilibrated in serum-reduced assay medium (MEM with 1% FBS and 0.5% methyl-D-mannopyranoside) for 20 min at RT.

2.5.4. Quantification of lactate dehydrogenase (LDH) release

The cytotoxicity of purified CsgA monomers and CsgA polymers, and *E. coli* strains to Caco-2 cells non-stimulated or stimulated with cytokines was assessed using the colorimetric cytotoxicity assay (CytoTox 96® Non-Radioactive Cytotoxicity Assay; Promega, US) that quantitatively measures LDH, a cytosolic enzyme that is released upon cell lysis or necrosis. The assay was performed according to the manufacturer's instruction. LDH released was measured in the cell-free culture medium from apical compartments after 3 h of incubation of Caco-2 monolayers with purified CsgA proteins or Caco-2 monolayers infected with *E. coli* strains. Before infection, the cell culture medium was changed to remove intrinsically produced LDH released to the medium. Caco-2 monolayers exposed for 3 h to 1% Triton X-100 in PBS served as a control for 100% cell lysis (positive control) while unstimulated and uninfected Caco-2 cells served as a negative control.

2.5.5. IL-8 assay

Non-stimulated or cytokine-stimulated Caco-2 monolayers were infected with curliated *E. coli* strains and their curli-deficient mutants or were treated with purified CsgA proteins (10 µg/mL in 20 mM Tris-HCl buffer; pH 7.4). Bovine serum albumin (BSA) at a concentration of 10 mg/mL dissolved in 20 mM Tris-HCl buffer was used as a negative control. A 24 h post-infection cell culture medium from the basolateral compartment was removed and centrifuged at 25 000 x g for 10 s to remove cellular debris. The concentration of IL-8 was determined using Human IL-8 ELISA MAX™ Standard (Biolegend; San Diego, US) according to the manufacturer's instruction.

3. Results

3.1. Wild-type E. coli strain produces temperature-independent curli

The ability of *E. coli* EC32 and K-12 strains to produce curli was assessed by the detection of *csgA* and *csgD* genes, Congo red and curcumin binding, and by immunostaining with rabbit immune serum. Both, *E. coli* strains EC32 and K-12 carried *csgA* and *csgD* genes of curli operon as confirmed by positive PCR reactions (Fig S1). Both *E. coli* strains grown on Congo red-CFA agar bound the dye and formed red colonies at RT (Fig. 1Aa). However, *E. coli* K-12 strain at 37° C switched to white phenotype in contrast to wild-type EC32 strain that produced a temperature-independent red phenotype (Fig. 1Ac). Both these strains bound curcumin and produced yellow colonies on CFA-curcumin agar at RT (Fig. 1Ab), but only wild-type strain EC32 bound curcumin at 37 °C (Figs. 1Ad). On the contrary, EC32Δ*csgBA* and K-12Δ*csgBA* derivatives did not bind curcumin and produced white colonies on CFA-curcumin agar (Fig. 1A). Since both mutants retained the ability to produce cellulose they grown on Congo red-CFA agar as a pink colonies characteristic to cellulose-producing bacteria. In a quantitative Congo red binding assay, wild-type *E. coli* strain EC32 cultured on CFA agar at RT and at 37 °C bound more Congo red dye than nonpathogenic *E. coli* K-12 (Fig. 1B). The immunostaining with rabbit immune serum was positive for both *E. coli* strains grown on CFA agar at RT, indicating that the serum cross-reacted with curli produced by *E. coli* K-12 (Fig. 1C). However, only wild-type strain EC32 cultured at 37° C showed positive immunostaining.

3.2. CsgA amyloid protein

The purity of CsgA proteins from both *E. coli* strains was assessed by western blot (Fig. 2C). Moreover, the purity of CsgA protein derived from wild-type *E. coli* strain EC32 was assessed by mass spectrometric UPLC-Q-TOF/MS analysis (Fig. 2B). The mass spectrometric analysis revealed the exact molecular mass of CsgA monomer as 13,093 Da for a sample dissolved in diluted acetic acid (Fig. 2Ba) and acidic Milli-Q water (Fig. 2Bb). This value was lower than that indicated in SDS-PAGE and western blot that was ˜17 kDa (Fig. 2Cb). The purified polymerized CsgA proteins derived from both *E. coli* strains bound amyloid-specific dye ThT (Fig. 2Ac) and showed specific to amyloid proteins yellow-green color under polarized light after staining with Congo red dye (Fig. 2Aa and Ab). The kinetic of curli polymerization in 20 mM Tris-HCl buffer was assessed by ThT fluorescence at 37 °C (Fig. 2D). Immediately after the start of measurements of ThT fluorescence intensity, a short lag phase corresponding to the oligomerization of CsgA monomers was observed. The lag phase last 10 min for *E. coli* K-12 and 15 min for EC32 strain. The increase in ThT fluorescence indicated that CsgA monomers started to fibrillate immediately after the short lag phase. Constant ThT fluorescence intensity observed after 2 h of incubation indicated elongation of curli fibers. The rabbit anti-CsgA antibody totally inhibited curli fibrillation. Since in all experiments, the epithelial Caco-2 cells were incubated at least for three hours with CsgA samples, the CsgA samples untreated with the immune serum that were mostly composed of oligomers and polymers of CsgA monomers are referred herein as CsgAp (polymers) whereas the CsgA samples pretreated with the immune serum inhibiting polymerization of CsgA monomers are referred herein as CsgAm (monomers).

3.3. Cytokine-stimulated epithelial cells enhance adherence of curliated E. coli

Both curliated *E. coli* strains adhered to unstimulated epithelial Caco-2 cells monolayer (Figs. 3A and 3B). Nonetheless, adherence of *E. coli* strain EC32 was more than 40-fold greater than that of *E. coli* strain K-12 ($p < 0.0001$). Both curli-deficient mutants were less adherent than the parental strains and for K-12Δ*csgBA* mutant the mean adherence rate decreased 1.4-fold compared with curliated strain ($p = 0.0001$), whereas EC32Δ*csgBA* mutant was 3.9 times less adherent than the wild-type strain ($p < 0.0001$). The results indicated that curliated *E. coli* strains adhered more efficiently to polarized unstimulated Caco-2 cells than their curli-deficient counterparts. Both, wild-type EC32 strain and *E. coli* K-12 demonstrated increased adhesion (2.5-fold and 7.8-fold, respectively; $p < 0.0001$) to cytokine-stimulated Caco-2 cells in comparison to unstimulated cells. Similarly, adherence of EC32Δ*csgBA* and K-12Δ*csgBA* mutants to cytokine-stimulated Caco-2 cells was higher (1.5-fold; $p = 0.0001$ and 3.4-fold, respectively; $p < 0.0001$) than to unstimulated epithelial cells. Nevertheless, the mean adhesion rates of both mutants to cytokine-stimulated Caco-2 cells were lower ($p < 0.0001$) than those of respective parental strains. The findings revealed that stimulation of epithelial cells with proinflammatory cytokines significantly increased adherence of both curliated *E. coli* strains, particularly non-pathogenic *E. coli* strain K-12.

3.4. Curli fibrils reduce E. coli invasion of epithelial cells

The invasion efficiency of curliated *E. coli* strain EC32 was lower than that for EC32Δ*csgBA* mutant, indicating that curli reduced

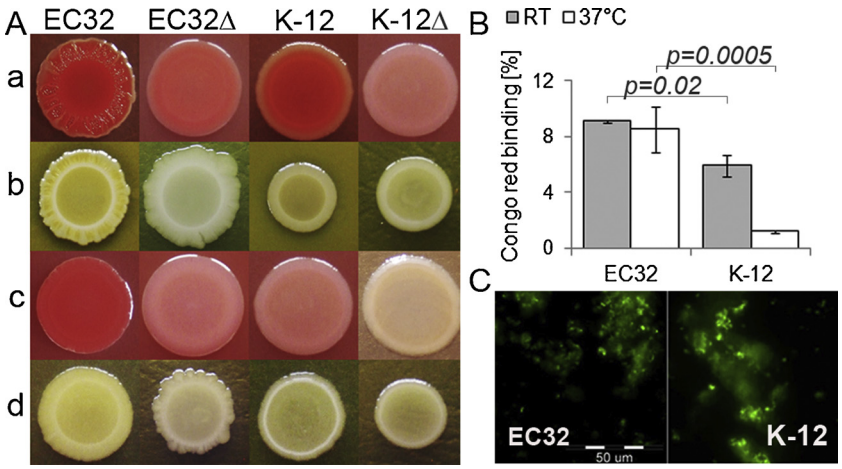

Fig. 1. Curli production. (A) Congo red and curcumin binding by *E. coli* strains and their curli-deficient EC32Δ*csgBA* (EC32Δ) and K-12Δ*csgBA* (K-12Δ) isogenic mutants cultured for 48 h on Congo red-CFA agar at RT (a) and at 37 °C (c), and on CFA-curcumin agar at RT (b), and at 37 °C (d). On Congo red-CFA agar, EC32Δ*csgBA* (EC32Δ) and K-12Δ*csgBA* (K-12Δ) mutants yielded pink colonies corresponding to the production of cellulose without curli. (B) Quantitative evaluation of Congo red binding by *E. coli* strains performed according to Gophna et al. (2002). P values were determined by Student's two-tailed *t* test with $p < 0.05$ considered statistically significant. (C) The positive reaction of curliated *E. coli* EC32 and *E. coli* K-12 strains cultured on CFA agar for 48 h at RT with rabbit anti-CsgA antibody as a primary antibody specific to curli produced by *E. coli* strain EC32 and FITC-conjugated goat anti-rabbit antibody as a secondary antibody; fluorescence microscope, magnification 100 × (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

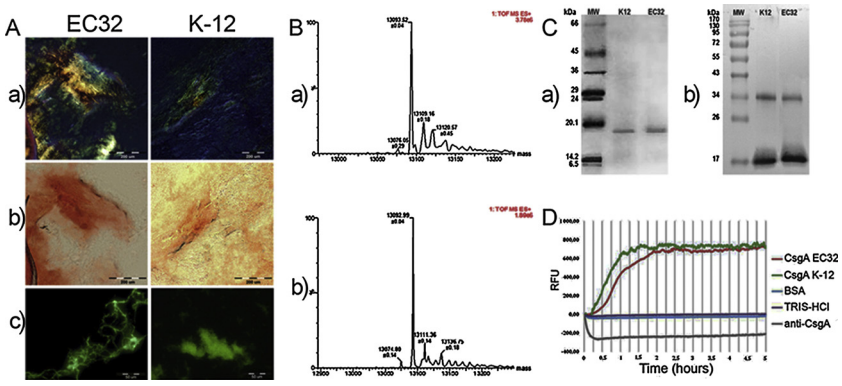

Fig. 2. Analysis of purity and polymerization of curli preparations. (A) Birefringence of Congo red stained CsgA proteins. CsgA samples from *E. coli* EC32 and K-12 strains were dried overnight on a glass slides, stained with Congo red, and observed under polarized light. CsgA with (a) cross and (b) aligned polarization; light microscope, magnification 40 x; (c) thioflavin T positive staining of CsgA samples from *E. coli* EC32 and *E. coli* K-12 strains; fluorescence microscope, magnification 40 × . (B) Deconvoluted molecular mass spectra of CsgA protein dissolved in (a) diluted acetic acid (b) and acidic Milli–Q water. The mass spectrometric analysis of purified CsgA from curli of EC32 strain revealed the exact molecular mass of CsgA monomer as 13,093 Da. (C) Purity of CsgA samples assessed by (a) SDS-PAGE and (b) Western blot analysis. CsgA subunits of ˜17 kDa (and their dimers of ˜34 kDa) from curli fibrils of *E. coli* EC32 and K-12 strains were visualized with primary rabbit anti-CsgA antibody and horseradish secondary anti-rabbit antibody (Cb). (D) polymerization of purified CsgA from *E. coli* EC32 and K-12 strains; RFU, relative fluorescence units; 2% bovine serum albumin (BSA) and Tris-HCl buffer were used as negative controls; anti-CsgA indicates CsgA monomers from curli fibrils of *E. coli* strain EC32 mixed with rabbit anti-CsgA antibody (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

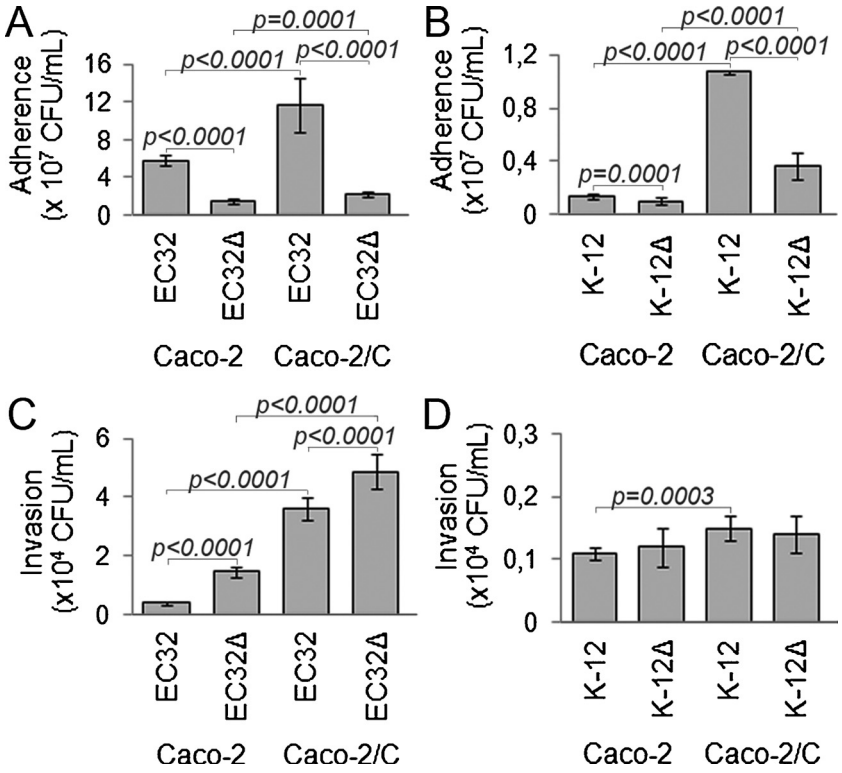

Fig. 3. Adherence and internalization of curliated and curli-deficient *E. coli* strains by polarized Caco-2 monolayers. Adherence rates of *E. coli* EC32 (A) and K-12 (B) strains, and their isogenic *csgBA* mutants EC32Δ*csgBA* (EC32Δ), and K-12Δ*csgBA* (K-12Δ) to unstimulated and cytokine-stimulated epithelial cells (Caco-2/C) 3 h postinfection. Internalization of *E. coli* strain EC32 (C) and *E. coli* K-12 (D), and EC32Δ*csgBA* (EC32Δ), and K-12Δ*csgBA* (K-12Δ) mutants by unstimulated and cytokine-stimulated Caco-2/C cells 3 h postinfection. P values were determined by Student's two-tailed *t* test with $p < 0.05$ considered statistically significant. Data are representative of three independent experiments.

internalization of the strain by unstimulated epithelial cells (Fig. 3C). This accords our earlier observation that *E. coli* strain EC32 cultured overnight in conditions unfavorable for curli synthesis (Luria broth at 37° C) was more invasive than the strain grown on CFA agar for 48 h at RT (Fig S2). The curliated EC32 strain entered cytokine-stimulated Caco-2 cells nine times more efficiently ($p < 0.0001$) than unstimulated cells. On the other hand, EC32Δ*csgBA* mutant was internalized more efficiently by unstimulated and cytokine-stimulated Caco-2 cells ($p < 0.0001$) than parental strain indicating that curli also reduce internalization of the wild-type strain by cytokine-stimulated epithelial cells. The ability of *E. coli* strain K-12 to invade differentiated epithelial cells is limited and generally, the strain is considered non-invasive and used as a negative control in an in vitro invasion assays into epithelial cells. In the study curliated *E. coli* K-12 strain entered unstimulated Caco-2 cells 3.5-fold less efficiently than the wild-type EC32 strain ($p < 0.0001$) (Fig. 3D). Moreover, there was no difference in the invasion efficiency of unstimulated ($p = 0.4$) and cytokine-stimulated ($p = 0.5$) Caco-2 cells by curliated K-12 strain and its curli-deficient K-12Δ*csgBA* counterpart. Nevertheless, the number of intracellular *E. coli* K-12 recovered from cytokine-stimulated Caco-2 cells was higher of 36.4% than from unstimulated Caco-2 cells ($p = 0.0003$). Altogether, the results showed that curli reduced invasion of the wild-type *E. coli* strain EC32 into unstimulated and cytokine-stimulated polarized epithelial cells. On the contrary, curli had no effect on internalization of the nonpathogenic *E. coli* K-12.

3.5. Curli promote survival of E. coli in epithelial cells

The ability of internalized EC32 and K-12 strains to survive within epithelial cells was assessed over 24 h period. The analysis revealed that the numbers of intracellular bacteria decreased over the period of 24 h from values observed at 3 h. However, the curli-deficient EC32Δ*csgBA* mutant was more rapidly killed in unstimulated Caco-2 cells than curliated wild-type strain EC32. The survival rate of EC32Δ*csgBA* 24 h post-infection was decreased to 40.8% and the wild-type EC32 to 57% ($p < 0.0001$) compared to the initial uptake 3 h post-infection (Fig. 4C). Curliated *E. coli* K-12 strain and its curli-deficient K-12Δ*csgBA* counterpart were more susceptible to intracellular killing as only 28.9% and 26.3% of the bacteria internalized, respectively, were recovered from unstimulated Caco-2 cells 24 h post-infection. In cytokine-stimulated Caco-2 cells the survival rates of curliated wild-type strain EC32 and its *csgBA* mutant were reduced to 6.5% and 8.3%, respectively, whereas the percentage of viable K-12Δ*csgBA* mutant and curliated *E. coli* K-12 strain decreased to 3.7% and 10.2%, respectively. Altogether, the results indicated that curli increased survival of *E. coli* in unstimulated Caco-2 cells. In contrast, stimulation of epithelial cells with proinflammatory cytokines significantly reduced intracellular survival rates of both, curliated and curli-deficient *E. coli* strains.

3.6. CsgA decreases IL-8 secretion by unstimulated intestinal epithelial cells

The baseline level of IL-8 measured 24 h after incubation of control uninfected Caco-2 cell monolayers in serum-reduced culture medium was 8.5 ± 0.8 pg/mL. Stimulation of control Caco-2 cells with a mixture of proinflammatory cytokines (5 ng/mL of IL-1β, 50 ng/mL of TNF-α and 50 ng/mL of IFNγ) resulted in a 77-fold increase (656.6 ± 41.8 pg/mL) in IL-8 secretion. Similar levels of IL-8 were observed when unstimulated and cytokine-stimulated Caco-2 cells were treated with BSA (10 μg/mL in 20 mM Tris-HCl buffer; pH 7.4) as a negative control for CsgAp and CsgAm. Infection of unstimulated Caco-2 monolayers with nonpathogenic curliated *E. coli* K-12 strain and its curli-deficient mutant did not have any significant effect on IL-8 secretion ($p > 0.05$). In contrast, when unstimulated Caco-2 cells were infected with the wild-type EC32 strain, IL-8 secretion increased by 47% compared to uninfected control ($p = 0.02$) (Fig. 4A). However, infection of Caco-2 monolayers with curli-deficient EC32Δ*csgBA* mutant increased IL-8 secretion by 66.4% compared to the parental wild-type strain ($p = 0.002$), indicating that curli reduced IL-8 secretion. Similar results were obtained with cytokine-stimulated Caco-2 epithelial cells (Fig. 4B). Wild-type EC32 strain increased the secretion of IL-8 by 21.4% ($p = 0.02$) whereas EC32Δ*csgBA* by 91% ($p = 0.001$) compared to uninfected control. Although the infection of cytokine-stimulated Caco-2 monolayers with non-pathogenic *E. coli* K-12 strain was associated with IL-8 release at the level similar to control cells ($p = 0.4$), infection with K-12Δ*csgBA* mutant increased IL-8 secretion by nearly 20% ($p = 0.03$) compared to negative control. Treatment of unstimulated Caco-2 cells with both polymers ($CsgAp_{EC32}$ and $CsgAp_{K-12}$) decreased IL-8 secretion by 54.4% ($p = 0.005$) and 39.4% ($p = 0.02$),

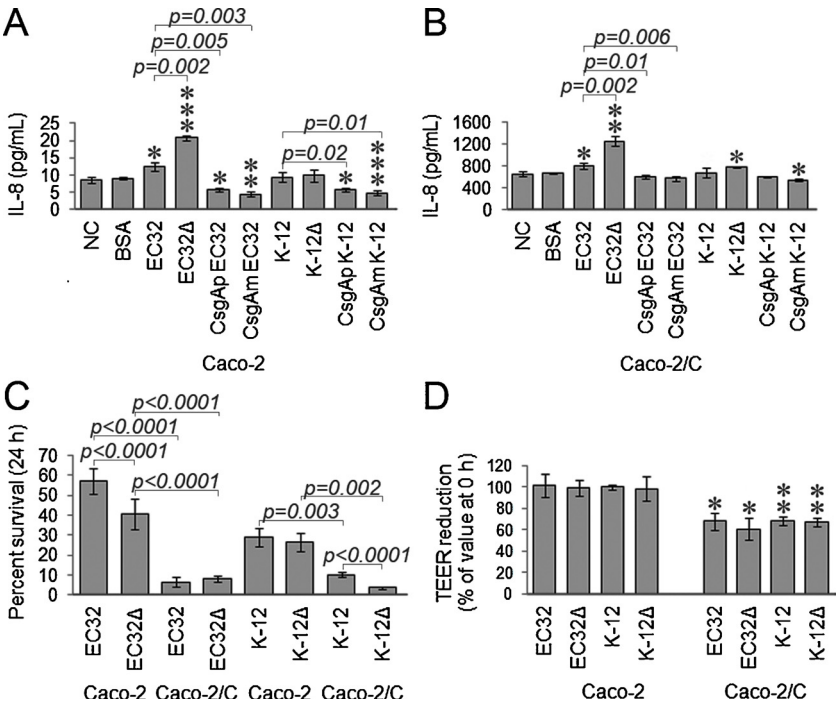

Fig. 4. IL-8 response, intracellular survival of *E. coli* strains and transepithelial electrical resistance (TEER) of unstimulated and cytokine-stimulated Caco-2 monolayers infected with *E. coli* strains. Caco-2 monolayers unstimulated (A) and stimulated (B) with proinflammatory cytokines (5 ng/mL of IL-1β, 50 ng/mL of TNF-α and 50 ng/mL of IFNγ; Caco-2/C) were incubated for 3 h with curliated *E. coli* strains (1×10^7 CFU/mL) and CsgAm, and CsgAp polymers (10 μg/mL in 20 mM Tris-HCl buffer). BSA solution (10 μg/mL in 20 mM Tris-HCl buffer) served as a negative control for CsgAm and CsgAp. Cell culture medium from the bottom wells was collected 24 h postinfection to determine IL-8 concentration using Human IL-8 ELISA MAX™ assay kit. NC, negative controls indicating the background levels of IL-8 after 24 h. The asterisks indicate values significantly different from the values of control cells (NC). (C) The percentage of *E. coli* EC32 and K-12 strains recovered from Caco-2 cells after 24 h of incubation in the presence of gentamycin (20 μg/mL) relative to the initial uptake 3 h postinfection (set as 100%). Caco-2/C, Caco-2 cells stimulated with cytokines. (D) Mean change in TEER as a percentage of initial TEER of Caco-2 monolayers unstimulated and cytokine-stimulated (Caco-2/C). All data are representative of three independent experiments ± standard error. The asterisks indicate statistically significant difference between TEER at t = 0 and t = 24 h determined by Student's two-tail *t*-test with $p < 0.05$ considered statistically significant, * $p < 0.05$; ** $p \leq 0.001$; ***$p \leq 0.0001$.

respectively, compared to curliated EC32 and K-12 strains and by 32.9% for both, $CsgAp_{EC32}$ and $CsgAp_{K-12}$, compared to control cells ($p = 0.0019$ and $p = 0.0018$, respectively). The $CsgAm_{EC32}$ and $CsgAm_{K-12}$ reduced IL-8 secretion by 64% ($p = 0.003$) and 49% ($p = 0.01$), respectively, compared to curliated EC32 and K-12 strains, and by 47% ($p = 0.0004$), and 43.5% ($p = 0.0001$), respectively, compared to control cells. Treatment of cytokine-stimulated Caco-2 monolayers with $CsgAp_{EC32}$ and $CsgAm_{EC32}$ insignificantly decreased the secretion of IL-8 by 7% ($p = 0.2$), and 12.9% ($p = 0.08$), respectively, compared to control cells, and by 23.4% ($p = 0.01$) and 28.3% ($p = 0.006$) compared to curliated EC32 strain. The $CsgAp_{K-12}$ and $CsgAm_{K-12}$ reduced IL-8 secretion by 7.7% ($p = 0.1$), and 17.2% ($p = 0.02$), respectively, compared to control cells and by 10.9% ($p = 0.2$), and 21.4% ($p = 0.1$), respectively, compared to curliated K-12 strain. This indicated that purified CsgAp and CsgA at the concentration 10 μg/mL attenuated IL-8 secretion by unstimulated Caco-2 cells more efficiently than curliated *E. coli* strains at the bacterial numbers applied herein. However, this anti-inflammatory effect of curli was barely observed in cytokine-stimulated intestinal epithelial cells.

3.7. CsgA decreases cytokine-compromised epithelial barrier function

Transepithelial electrical resistance (TEER) was applied in the study to examine the effect of curliated *E. coli* strains and their curli-deficient mutants on the integrity of differentiated Caco-2 cell monolayer as a model of intestinal epithelial barrier in vitro. The mean TEER of uninfected control Caco-2 cells used in the study was $868.8 \pm 60.7\ \Omega.cm^2$ indicating a tight epithelial barrier (Fig S3). The mean TEER ($440.7 \pm 35.2\ \Omega.cm^2$) of control Caco-2 cells stimulated with a mixture of proinflammatory cytokines (INFγ, IL-1β, and TNF-α) was two-fold lower than unstimulated cells indicating cytokine-mediated disruption of epithelial cell barrier. Infection of unstimulated Caco-2 monolayers with both curliated *E. coli* strains and their curli-deficient counterparts was not associated with any significant reduction in TEER (Fig. 4D). In contrast, infection of cytokine-stimulated epithelial cell monolayers with *E. coli* EC32 and K-12 strains reduced TEER by 31.8% ($p = 0.01$) and 31.6% ($p = 0.0005$), respectively, compared to the baseline TEER value. However, infection with EC32Δ*csgBA* and K-12Δ*csgBA* led to a similar fall in TEER (39.3%; $p = 0.002$ and 32.7%; $p = 0.0008$, respectively), indicating that the drop of TEER was unrelated to curli.

3.8. CsgA polymers and CsgA monomers are not toxic for intestinal epithelial cells

Cytotoxic effect of curliated *E. coli* strains and CsgAp, and CsgAm on the viability of unstimulated and cytokine-stimulated Caco-2 cells was evaluated 3 h post-infection based on the quantification of lactate dehydrogenase (LDH) release. In control, non-infected and non-stimulated Caco-2 cells spontaneous LDH release was $2.7 \pm 0.2\%$. Stimulation of control Caco-2 cells with proinflammatory cytokines (5 ng/mL of IL-1β, 50 ng/mL of TNF-α and 50 ng/mL of IFNγ) elicited a significant increase in LDH release (9.8 ± 3.5; $p = 0.01$). Similarly, LDH release was significantly greater in cytokine-stimulated Caco-2 cells infected with curliated *E. coli* EC32 and K-12 strains than in unstimulated Caco-2 cells with 3.8-fold ($p < 0.0001$) and 2.3-fold ($p = 0.03$) increases, respectively (Fig. 5). Both, unstimulated and cytokine-stimulated Caco-2 monolayers showed a similar profile of LDH release after infection with curli-deficient EC32Δ*csgBA* and *E. coli* K-12Δ*csgBA* mutants, indicating that the cytotoxicity was curli-independent. Nevertheless, the wild-type *E. coli* EC32 and EC32Δ*csgBA* strains were more cytotoxic to unstimulated and stimulated Caco-2 cells than *E. coli* K-12 strain and its curli-deficient mutant ($p < 0.05$). The viability of both, unstimulated and cytokine-stimulated Caco-2 cells treated with CsgA polymers and CsgA monomers (10 μg/mL) was similar to the viability of control cells indicating that both CsgA proteins were not cytotoxic for Caco-2 cells.

4. Discussion

Curli fibrils produced by *E. coli* and *S.* Typhimurium are one of best-known bacterial amyloid proteins. In a recent years there has been increased interest in bacterial amyloids due to their similarity to amyloid-forming proteins in the human body. Unlike human amyloid proteins, amyloids produced by microorganisms provide with necessary life functions and present different primary amino acid sequences. On the other hand, bacterial amyloids share common features with human disease-related amyloids, namely the ability to induce inflammation in the human body and the interaction with the same cellular receptors i.e. the TLR2/TLR1/CD141 heterocomplex (Tükel et al., 2010). The effect of curli on human organism remains poorly understood and contentious. According to Chen et al. (2016), the amyloid properties of curli might impact human amyloidosis. Others have reported that curli may play a critical role in maintaining homeostasis in the human body via the regulation of intestinal epithelial barrier (Oppong et al., 2013).

The present study was designed to investigate the in vitro effects of purified CsgA proteins and curliated *E. coli* strains on cytokine-stimulated intestinal epithelial cell barrier. It is well documented that adherence of *E. coli* to intestinal epithelial cells induces the secretion of proinflammatory cytokines (Ohkusa et al., 2009; Zhou et al., 2003). However, very little is yet known about the interaction of curliated *E. coli* with epithelial cells stimulated with proinflammatory cytokines. According to van de Walle et al. (2010) the use of a mixture of IL-1β, TNF-α, and INFγ cytokines accurately mimics 'inflamed' intestinal epithelial barrier in vitro. Therefore, in this study, the mixture of cytokines was used to stimulate polarized Caco-2 monolayers. Indeed, the cytokines induced LDH and IL-8 release and disrupted the integrity of an in vitro polarized Caco-2 monolayer. To evaluate the effect of curli on cytokine-stimulated epithelial cells, *E. coli* K-12 C600 weak curli-expressing strain, and wild-type *E. coli* EC32 strong curli-expressing strain, and their curli-deficient mutants were examined. Moreover, purified CsgA monomers and polymers derived from curli of both these strains were included in the study.

It has been reported, that curli expression contributes to adherence of *E. coli* and *Salmonella* species to epithelial cells (Saldaña et al., 2009; Bokranz et al., 2005; Kikuchi et al., 2005). In agreement with those studies, our results showed that both curliated *E. coli* strains adhered to unstimulated, polarized Caco-2 cells more efficiently than their curli-deficient mutants, confirming the important role of curli in the adherence of *E. coli* to differentiated intestinal epithelial cells. However, adherence of both these curliated strains to cytokine-stimulated Caco-2 monolayers was significantly higher than to unstimulated cells, indicating that cytokine-induced inflammation of intestinal epithelium increases adhesion of curliated *E. coli* strains. On the other hand, we observed that curli fibrils reduced invasion of the wild-type *E. coli* strain EC32 as the curli-deficient EC32Δ*csgBA* mutant was internalized more effectively by unstimulated and cytokine-stimulated epithelial cells. Although, it should be noted that the invasion rates of the wild-type *E. coli* strain to cytokine-stimulated Caco-2 monolayers were significantly higher than that to unstimulated epithelial cells. Opposite results are reported by Tükel and co-workers who found that wild-type curliated *S. Typhimurium* and its curli-deficient *csgBA* mutant invade cultured epithelial HT-29 cells equally well (Tükel et al., 2005). In this study, however, we also demonstrated that curliated nonpathogenic *E. coli* K-12 strain was not internalized more efficiently by unstimulated and cytokine-stimulated Caco-2 cells than its *csgBA* mutant. This appears to be consistent with Gophna et al. (2001) findings which showed that the *csg* gene cluster encoding curli and originating from the virulent *E. coli* O78 strain mediated a level of internalization significantly higher than that conferred by the gene cluster from nonpathogenic *E. coli* K-12 strain. Consequently, these results emphasize qualitative or quantitative differences in curli among curli-producing bacteria that may affect their pathogenic potential.

Intracellular localization provides intestinal pathogens with

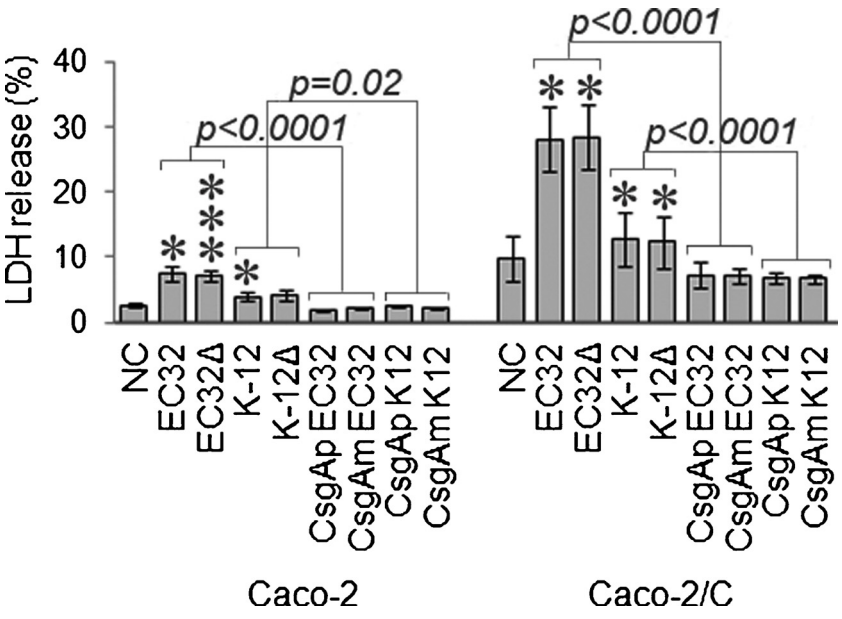

Fig. 5. LDH release from unstimulated and cytokine-stimulated Caco-2 monolayers (Caco-2/C) infected with *E. coli* strains and treated with CsgA samples. The viability of Caco-2 monolayers was assessed on the basis of LDH released to cell culture medium using CytoTox 96® Non-radioactive Cytotoxicity assay kit following 3 h infection with *E. coli* strains and 3 h treatment with CsgAp polymers, and CsgAm monomers. The results are expressed as the mean percentage of cellular viability of Caco-2 monolayers over cell monolayers treated with Triton X-100 as a positive cytotoxicity control. The asterisks indicate values significantly different from the values of control cells (NC). Data are representative of three independent experiments. P values were determined by Student's two-tail *t* test, * $p < 0.05$; *** $p \leq 0.0001$.

effective protection against innate immune response and elimination from the gut. Since curli fibrils form a layer of mesh on the surface of curli-producing bacteria, therefore, may mask surface-exposed antigens avoiding immune recognition. Indeed, in this study, curli increased survival rates of wild-type curliated *E. coli* strain within unstimulated epithelial cells in comparison to its curli-deficient mutant. Although, the survival rates for the nonpathogenic strain were much lower than those achieved by the wild-type strain. However, stimulation of epithelial cells with proinflammatory cytokines enhanced intracellular killing of both, curliated as well as curli-deficient *E. coli* strains. This implies that production of curli may be an important virulence strategy of *E. coli* strains colonizing human gut mucosa and able to produce curli at human body temperature.

Internalization of enteroinvasive pathogens induces in epithelial cells up-regulated expression and production of proinflammatory cytokines (Elewaut et al., 1999). In this study, we demonstrated that curliated wild-type *E. coli* strain and especially purified curli polymers and monomers attenuated the secretion of IL-8, a potent chemoattractant of polymorphonuclear cells. Although the infection of unstimulated and cytokine-stimulated Caco-2 cells with curliated wild-type *E. coli* strain EC32 induced the secretion of IL-8, the mean level of the chemokine released upon infection with curli-deficient EC32ΔcsgBA mutant was significantly higher, reflecting the role of curli in the attenuation of the inflammatory response. These results corroborate earlier findings (Oppong et al., 2013) that curli-deficient *csgBA* mutant of *S.* Typhimurium induced the secretion of higher levels of IL-8 than curliated wild-type strain. However, in this study, the effect of *E. coli* K-12 strain, a weak curli-producer, on IL-8 secretion by cultured Caco-2 monolayers was not as significant as in the case of the wild-type *E. coli*, suggesting that low-level of curli expression in some *E. coli* strains may be insufficient to modulate the immune response of epithelial cells. Indeed, CsgA polymers and CsgA monomers from both, wild-type and nonpathogenic *E. coli* strains at the same concentrations significantly reduced IL-8 secretion by unstimulated epithelial cells, confirming the direct effect of curli on the attenuation of intestinal epithelial cells inflammatory response, but also reaffirming that only the substantial amount of curli plays a protective anti-inflammatory role. The ability of curliated *E. coli* to reduce IL-8 secretion was also observed in Caco-2 cells stimulated with proinflammatory cytokines as both curli-deficient mutants induced the secretion of higher levels of IL-8 than their parental strains. In contrast, CsgA monomers and CsgA polymers from both *E. coli* strains barely attenuated IL-8 secretion by cytokine-stimulated Caco-2 cells. One reason for this could be an insufficient concentration of CsgA used in this study. Nevertheless, the ability of CsgA polymers and CsgA monomers to attenuate inflammatory response in unstimulated Caco-2 cells implies an important role of CsgA protein in *E. coli* colonization of intestinal epithelium.

Incubation of colonic epithelial cell monolayers with IFNγ alone or in combination with other cytokines elicits a dramatic reduction in TEER along with increased solute permeability (Watson et al., 2005). In this study, we observed that both curliated *E. coli* strains, as well as their curli-deficient EC32Δ*csgBA* and K-12Δ*csgBA* mutants, had no significant effect on the integrity of unstimulated Caco-2 monolayers. In contrast, Oppong et al. (2013) found that curliated wild-type *Salmonella* Typhimurium contrary to its *csgBA* mutant tighten the integrity of polarized T84 cell monolayers via activation of the TLR2/PI3K pathways. This apparent lack of correlation with our results can be attributed to differences in curli produced by *E. coli* and *Salmonella* spp. or to different cell lines (T84 vs. Caco-2 cells) used as models of intestinal epithelial barrier in vitro. However, in the current study, infection of cytokine-stimulated Caco-2 monolayers with curliated *E. coli* strains and their curli-deficient mutants led to significant, but similar TEER decreases suggesting that the effect was curli-unrelated.

It should be noted that, in this study, neither CsgA polymers nor CsgA monomers were cytotoxic for unstimulated and cytokine-stimulated Caco-2 cells supporting previous findings of Rapsiński et al. (2015) who showed that curli produced by *E. coli* and *S.* Typhimurium failed to cause LDH release and cell death of bone marrow-derived macrophages. On the contrary, in this study, both curliated *E. coli* strains as well as their *csgBA* mutants were cytotoxic for unstimulated epithelial cells. However, the cytotoxicity of the wild-type *E. coli* strain was significantly higher than that of *E. coli* K-12 strain and could be attributed to virulence factors other than curli. Similarly, significantly higher cytotoxicity of the wild-type *E. coli* and its curli-deficient mutant was observed in cytokine-stimulated Caco-2 cells in contrast to *E. coli* K-12 strain and its *csgBA* mutant that induced the release of LDH levels similar to values of control cells. Nonetheless, since the cytotoxicity of curliated *E. coli* strains and their curli-deficient mutants were at comparable levels these results affirmed that the cytotoxicity was curli-unrelated.

5. Concluding remarks

In the current study for the first time, we investigated the effect of

curli produced by nonpathogenic *E. coli* K-12 and wild-type *E. coli* EC32 strains on cytokine-stimulated polarized Caco-2 cells. The in vitro adherence and internalization assays demonstrated that curli fibrils significantly enhance the adherence of both curliated *E. coli* to cytokine-stimulated Caco-2 cells, but simultaneously reduce internalization of the wild-type *E. coli* EC32 in contrast to noninvasive curliated *E. coli* K-12 strain. Despite reduced internalization, curli fibrils increase survival rates of curliated wild-type *E. coli* EC32 within unstimulated Caco-2 cells. The effect seems to be associated with the amount of curli produced as survival rates of curliated *E. coli* K-12 strain were lower than that of the wild-type strain and similar to that of curli-deficient K-12Δ*csgBA* mutant. Although, we cannot exclude that other than curli virulence factors could affect the survival of both *E. coli* strains within intestinal epithelial cells. On the other hand, stimulation of Caco-2 monolayers with proinflammatory cytokines efficiently reduces the number of intracellular curliated *E. coli* strains and their curli-deficient mutants. Furthermore, the findings of the study revealed that curliated *E. coli,* as well as purified CsgA polymers and CsgA monomers, attenuate IL-8 secretion by unstimulated Caco-2 cells although, the effect was barely noticeable on cytokine-stimulated cells. Neither curli fibrils on the bacterial surface nor CsgA polymers, and CsgA monomers are cytotoxic for unstimulated and cytokine-stimulated Caco-2 cells. Moreover, curli fibrils had no influence on the integrity of unstimulated and cytokine-stimulated Caco-2 monolayers. The findings of this research provide new insight into the interaction of curliated *E. coli* and curli CsgA monomers with cytokine-stimulated intestinal epithelial cells.

Conflict of interest

Authors certify that they have no conflicts of interest to disclose.

Acknowledgement

The study was supported by grant No. ST917 from the Wroclaw Medical University,Wrocław, Poland.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ijmm.2019.05.001.

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