International Journal of Biological Macromolecules 62 (2013) 217–224

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

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

Effects of polysaccharide isolated from *Streptococcus thermophilus* CRL1190 on human gastric epithelial cells

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ARTICLE INFO

Article history:
Received 15 May 2013
Received in revised form 17 July 2013
Accepted 14 August 2013
Available online xxx

Keywords:
Streptococcus thermophilus
Cytoprotection
Exo-polysaccharide
Helicobacter pylori
Human gastric epithelial cells
Mucin

ABSTRACT

EPS1190 was isolated from skim milk fermented with *Stretococcus thermophilus* CRL1190. The polysaccharide consisted of 33% glucose and 66% galactose with 1,4- and 1,4,6-galactose residues as main building blocks beside a high amount of 1,4-linked glucose. The polymer was characterized additionally concerning viscosity and zeta potential. EPS1190 stimulated cellular vitality and proliferation of human stomach AGS cells and human buccal KB cells significantly. EPS1190 stimulated phagocytosis rate of murine macrophages RAW264.7 significantly. NO-release or anti-inflammatory effects by inhibition of LPS-induced NO release were not observed. Confocal laser scanning microscopy revealed that EPS1190 is partially internalized into AGS cells *via* endosomes. The bioadhesive absorption of FITC-labeled EPS1190 into the mucus layer on the apical side of the epithelium using histological tissue sections from human stomach was observed. Specific interaction of EPS1190 with mucin can be excluded as shown by microviscosimetry studies. EPS1190 increased the adhesion of *H. pylori* to AGS cells, which resulted in increased secretion of proinflammatory cytokines TNFa, IL-6 and IL-8. Summarizing, EPS1190 seems to stimulate epithelial cell regeneration and immunological innate defense mechanisms, which again can rationalized the use of this polysaccharide as cytoprotective compound in probiotioc preparations.

1. Introduction

Gastritis, one of the major gastrointestinal diseases caused by *Helicobacter pylori*, analgesic and anti-inflammatory drugs, alcohol, or stress is generally treated by specific pharmacotherapy according to clinical standard guidelines [1]. High recurrence rates and heavily increased antibiotic resistance of *H. plyori* force science into further strategies against this disease. Non-pharmacotherapy by cytoprotective probiotics and secondary products produced and secreted by these living microorganisms are more and more assessed to have health benefits on the host (for recent review see [2,3]. Many probiotics contain lactic acid bacteria with positive influence on gastritis [3] some of which produce high amounts of capsular polysaccharides or *exo*-polysaccharides (EPS) [4]. EPS from such lactic acid bacteria have been supposed to exert pharmacological effects as immunomodulation and anti-ulcer activity [5,6]. *Streptococcus thermophilus* CRL1190 is a strain isolated from homemade yoghurt, and is an efficient producer of *exo*- and capsular polysaccharides which confers viscosity to fermented milk. Therapeutic effect of *S. thermophilus* CRL1190-fermented milk on chronic gastritis has recently been shown [4,7,12]. The beneficial effect was related to the EPS; its administration induced mucus formation, increase of regulatory cytokines (IL-10) and a reduction of pro-inflammatory cytokines (INF-γ and TNF-α) [4,8].

The aim of this study was to determine the effect of EPS1190 under *in vitro* conditions to obtain more information on its potential mode of action. Therefore, the influence of the polysaccharide on two gut cell lines, the relationship with the macrophage activity, and the interaction with endogenous intestinal mucin was investigated.

2. Materials and methods

2.1. General experimentation procedure

If not stated otherwise, all chemicals were purchased by Sigma (Deisenhofen, Germany) and VWR (Darmstadt, Germany). The total fraction of acidic human milk oligosaccharides were

Abbreviations: CLS, confocal laser scanning microscopy; EPS, exo-polysaccharide; FITC, fluorescein isoisothiocyanate; MOI, multiplicity of infection.

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http://dx.doi.org/10.1016/j.ijbiomac.2013.08.011

obtained from Danone Research – Center for Specialized Nutrition (Friedrichsdorf, Germany). Mucin was a commercial sample (Sigma, Deisenhofen, Germany) from porcine stomach (type III, bound sialic acid 0.5–1.5%) and was purified as described below.

2.2. EPS1190 production and isolation

Exo-polysaccharide (EPS1190) was isolated from *S. thermophilus* CRL 1190 (EPS^+ and CPS^+ producer strain; CERELA culture collection, Tucumán, Argentina). The strain was cultured (10 mL/L inoculum) in LAPTg broth (peptone, 15 g/L; tryptone, 10 g/L; yeast extract, 10 g/L; glucose, 10 g/L; Tween®80, 1 mL/L) and subcultured at least twice in reconstituted skim milk (RSM, 100 g/L). Fermented milk (FM) was prepared in sealed bottles containing 2 L sterile RSM (sterilized at 115 °C for 20 min and cooled down to 37 °C) inoculated (1.0%, v/v) with an 16 h old culture of the EPS^+ strain *S. thermophilus* CRL 1190, incubated at 37 °C for 24 h and maintained at 4 °C prior to EPS1190 isolation. Once isolated [7,12], the EPS solution was deproteinised with trichloracetic acid (20%, w/v) at a ratio 1:1, incubated at 4 °C for 2 h and centrifuged (18,600 × *g*, 20 min, 4 °C). EPS1190 was precipitated from the clear supernatant by adding 3 volumes of cold ethanol 96% and incubating at 4 °C for 48 h. Precipitated EPS1190 was obtained by centrifugation (18,600 × *g*, 20 min, 4 °C). Deproteination and precipitation steps were made twice to totally eliminate remnant proteins. The polysaccharides were dissolved into bidistilled water and dialyzed (cellulose membrane, MWCO: 12.000, Sigma Chemical Co, St. Louis, MO) at 4 °C for 4 days. Finally, EPS1190 was lyophilized and stored at 4 °C until usage. 20.5 L of FM were processed with an approximate yield of 0.004% w/v. Analytical characterization of EPS1190 was performed according methods described in [36–39].

2.3. Viscosimetric measurements

The intrinsic viscosity of EPS was determined by anautomated rolling ball microviscosimeter (Anton Paar, Ostfildern, Germany) by adapting previously described protocols [9,10]. The intrinsic viscosity [η] was calculated with the well-known Huggins, Kraemer and "single point" equations.

2.4. Cell lines, bacteria, cultivation and tissue sections

AGS cells (ATCC CRL-1730) is an adherent human gastric adenocarcinoma epithelial cell line and were kindly provided by Prof. Dr. med. Winfried Beil (Medizinische Hochschule Hannover, Germany). Cells were cultured in RPMI 1640 (PAA Laboratories, Cölbe) supplemented with 10% FCS (PAA Laboratories, Cölbe) and 1% antibiotics (streptomycine/peniciline, PAA Laboratories, Cölbe), at 5% CO_2/37 °C. Passaging was performed once a week to a maximum of 20 passages.

KB cells (ATCC CCL-17), derived from a human oral epidermoid carcinoma, were kindly provided by Dr. S. Eick (University of Jena, Germany). Recent testing performed during ATCC accessioning has established that this line might be contaminated with HeLa cells. This finding is based on isoenzyme analysis, HeLa marker chromosomes and DNA fingerprinting (see www.atcc.org). The cells were cultured in Earl's minimum essential medium (EMEM) (Lonza, Basel, Switzerland) supplemented with 8% (v/v) heat-inactivated FCS (PAA Laboratories, Cölbe, Germany) and 50 μg/ml gentamicin (MP Biomedicals, Irvine, USA) at 5% CO_2/37 °C. Passaging was performed twice a week to a maximum of 15 passages.

Murine macrophage cell line RAW 264.7 (ATCC TIB-71) was cultivated and incubated in DMEM medium (high glucose with glutamine, supplemented with 10% FCS, Thermo Scientific, Waltham, USA) and 1% penicillin 10,000 U/mL/streptomycin 10 mg/mL (PAA Laboratories, Cölbe) in a humidified incubator at 8% CO_2/37 °C. Sub-cultivation was carried out every second or third day by gentle scraping when the cells had reached a confluence of about 80% [15]. For functional testing, passages 30 to 60 were used.

Helicobacter pylori ATCC 700824 (strain J99, identification for quality control by PCR for vacA, cacA genes) was cultivated for two or three passages to minimize the risk of phase-variable switching of OMP genes. Cultivation was performed according Niehues et al. [25].

Gastric tissue sections were kindly provided by Prof. Dr. Gerhard Faller, St. Vincentius Kliniken, Karlsruhe, Germany. All sections were obtained from *H. pylori* negative individuals; the respective mucosa in the gastric antrum region did not show any significant pathological changes.

2.5. Influence of EPS1190 on cellular vitality [13]

To determine the influence of test compounds on cell viability, AGS cells were cultured in 96-well plates (Translucent, Sarstedt, Nümbrecht, Germany) with 5×10^4 cells/well for 24 to 48 h at 37 °C. At 80% confluence the medium was discarded and cells were washed twice with PBS (100 μL/well). Test solution (100 μL/well) containing *exo*-polysaccharide EPS1190 (0.001 to 1000 μg/mL) was added, followed by incubation for 6, 12 and 24 h. Untreated control cells, cultivated only with fresh RPMI medium, served as negative control while cells supplemented with 10% FCS was used as positive control. For further details see also [11].

Similar conditions were used for assays with KB cells using EMEM medium supplemented with 10% FCS and 1% gentamicine at 37 °C/5% CO_2 as well as for macrophages RAW-264.7 in DMEM medium supplemented with FCS 10% and 1% penicillin/streptomycin (PAA Laboratories, Cölbe).

Test solutions were prepared by dissolving EPS1190 in medium and sterilized by filtration (0.2 μm, Corning Inc., Germany).

After incubation with EPS1190, the cell viability was determined by MTT assay [13]. The supernatant medium in the wells was discarded and cells were gently washed twice with 200 μL PBS/well. 50 μL MTT reagent (2.6 mg/mL in PBS) were added into each well and the plates were incubated for 4 h/37 °C. MTT reagent was removed from the plates and 50 μL DMSO/well were added to dissolve the insoluble formazan crystals. After 5 min the absorbance of the formazan was measured (Tecan Sunrise, Tecan Austria, Salzburg, Austria) at λ = 492 nm against reference wavelength λ = 690 nm.

2.6. Influence of EPS1190 on cellular proliferation by BrdU incorporation ELISA [17]

AGS and KB cells were cultured for 24 h in 96-well plates. Solutions of the test compound (100 μL/well) in FCS-free medium were added and cells were incubated for another 24 h at 37 °C. Cellular proliferation was performed by BrdU cell proliferation kit (Roche Diagnostics, Mannheim, Germany) according instructions of the manufacturer. The resulting color was measured photometrically (Tecan Sunrise, Tecan Austria, Salzburg, Austria) at λ = 450 nm, against reference wavelength λ = 690 nm.

2.7. Hemaglutination assay

Erythrocyte suspension (2%) from fresh human EDTA-stabilized blood (B−) in PBS was prepared. *H. pylori* suspension (25 μL) with an OD of 2.0 was transferred to 96-well-microtiter plates and 25 μL of PBS were added to each well. The suspension was serial diluted (1:2, 1:4, 1:8). 25 μL of the test solution were added to each well and the plate was incubated for 30 min, 37 °C). 50 μL of the erythrocyte suspension were added to each well; the plate was gently shaken for 5 min and incubated for 4–16 h at room

temperature. Hemagglutination was measured by MicroWin® 2000/4.38 (Microtek LabSystems, Overrath, Germany).

2.8. Agar difussion test

To exclude unspecific cell toxicity of test compounds against *H. pylori* a disk diffusion test was performed at different concentrations between 1 and 5 mg/mL of test compounds using BD Sensi-Disks (Becton and Dickinson, Heidelberg, Germany), placed on agar plates; positive control amoxicillin (0.5 μg per disk, MP Biomedicals, Irivine, U.S.A). The incubation time was between 3 to 4 days under microaerophilic condition (CampyGen Container System – Gaspak, Oxoid, Ltd., UK).

2.9. Anti-adhesive effect of EPS1190 against H. pylori on AGS cells

The assay was performed according to the method described by [25]. *H. pylori* was inoculated on Tryptic Soy Agar – Difco™ (TSA) plates (Merck, Darmstadt), supplemented with 5% defibrinated sheep blood (Oxoid, Ltd. Wesel) and incubated at 37 °C under anaerobic conditions during 48 h. AGS-cells grown for 48 h with 200,000 cells/well to a confluence of 80%, reaching after incubation time around 800,000 cells/well.

Cells at 80% confluence were incubated for 2 h with EPS1190 in RPMI medium (1, 2.5 and 5 mg/mL) at 37 °C/CO_2. Samples were analyzed by flow cytometry (FACS-Calibur, BD, Heidelberg, Germany).

2.10. Cytokine secretion of AGS cells after H. pylori infection

AGS cells were inoculated in 6 well plates at a cell density of 2×10^4 per well and incubated at 37 °C in CO_2 atmosphere for 48 h (confluence aprox. 80%). *H. pylori* in TSA plates were harvested and a bacterial suspension was diluted to obtain an OD of 0.05 in each well, with a final volume of 2 mL. The multiplicity of infection (MOI) was adjusted to 1:20, referred to the AGS cell-*H.pylori* ratio. For investigation on the influence of EPS1190 on *H. pylori* solutions of the test compound (1–1000 μg/mL) were added to the wells with infected AGS cells. After 24 h incubation time, the media were removed by centrifugation (5000 × g) for 5 min and stored at −20 °C for further analysis. IL-6, IL-8 and TNF-α were determined in these sample by ELISA kit (PeproTech, Hamburg Germany) according the instructions of the manufacturer.

2.11. Internalization of EPS1190 into AGS cells

Fluorescent-labbeling of EPS1190 with FITC was performed as described by [34]. For internalization experiments see [14,27]. AGS cells were cultivated on glass slides (Menzel GmbH, Braunschweig, Germany) within removable silicone chambers (Greiner bio-one, Frickenhausen, Germany) at 1×10^5 cells per chamber in 300 μL growth medium (RPMI 1640 supplemented with 10% FCS and 1% streptomycin/penicillin). Attachment of EPS1190 to mucus layer *in situ* human stomach tissue section.

FITC-EPS1190 was dissolved in blocking buffer (PBS, 0.2% BSA, and 0.05% Tween®20) at 100 μg/mL and then incubated under shaking at 37 °C for 4 h. For incubation with FITC-EPS1190 the tissue slides were deparaffinated (xylol, isopropanol, water, PBS). Deparaffinized sections were then incubated for 20 min with blocking buffer. 200 μL of FITC-EPS1190 were added to the tissue, followed by 1 h incubation at RT in the dark. After incubation the samples were washed 2 times with PBS and analyzed by fluorescent microscopy (Leica DMIL with camera Leica DFC 300FX, Software Leica IM50 4.0; Leica, Solms, Germany).

Table 1
Linkage analysis of partially methylated alditol acetates from EPS1190 after GC–MS evaluation.

Carbohydrate	Yield (mol%)	Carbohydrate	Linkage type (mol%)
1-Glucose	1	1-Galactose	7
1,4-Glucose	31	1,4-Galactose	27
1,6-Glucose	1	1,3-Galactose	7
		1,6-Galactose	3
		1,4,6-Galactose	22

2.12. Interaction with soluble porcin mucin fraction in dilute solution

Mucin from porcine stomach was firstly hydrated in water (5 mg/mL) under mild stirring for 3 h, then the soluble fraction was isolated by centrifugation at 25,000 × *g* for 50 min at 10 °C and the supernatant fraction was collected in aliquots of 15 mL (after addition of 0.2 mg/mL of sodium azide as preservative), lyophilized and subsequently stored at 4 °C until use. Clear solutions of mucin were obtained in water upon re-dissolving. Mixtures of this soluble mucin and EPS (relative viscosity, $\eta_{rel} \sim 2$) at pH 4.5 were measured at mucin/total mass ratio 0.5 and 0.9 in water and in 100 mM NaCl. The mixtures were kept at 37 °C for 20 min and the dynamic viscosity was measured at 37 °C in the microviscosimeter with an inclination angle of 50°. Percentual deviations in η_{rel} from the theoretical additive line or "line of non-interaction", following a protocol previously described to investigate polysaccharide–polysaccharide interactions in the dilute regime [10].

2.13. Phagocytosis of FITC-labeled zymosan particles: see [15]

2.13.1. Influence of EPS1190 on NO-release: see [15].

Statistical analysis were performed by using SPSS®. The experimental results are expressed as the mean ± SD. Data were assessed by analysis of variance. In case the analysis indicated significant differences between groups, each group was compared by Dunnett's *t*-test (two-sided) and $p < 0.05$ was considered to be statistically significant.

3. Results

An exo-polysaccharide EPS1190 was isolated from skim milk, fermented with *S. thermophilus* CRL1190, in yields of 39 mg/mL [12]. The hydrodynamic volume of the polymer was determined by GPC, corresponding to 1.782 kDa. Analysis of the monosaccharide composition of the polysaccharide after TFA hydrolysis and derivatisation to the respective alditol acetates revealed the presence of 33% glucose and 66% galactose. The polysaccharide was free of protein and did not contain any uronic acids. Linkage analysis after methylation analysis [16] indicated the presence of 1,4- and 1,4,6-galactose residues as main building blocks beside a high amount of 1,4-linked glucose (Table 1). ^{13}C NMR signals at 100.98 and 101.03 ppm were assigned to the α-anomeric C1 of linked Gal and Glc hexoses. The zeta potential (ζ) was determined to be – 5.4 ± 0.9 mV at pH 4.5. Intrinsic viscosity of EPS1190 in water ($[\eta]_{H_2O}$) was 351 mL/g and in NaCl 0.1 M ($[\eta]_{NaCl}$) 394 mL/g. The ratio $[\eta]_{H_2O}/[\eta]_{NaCl}$ found for EPS was 0.89, thus indicating that the hydrodynamic volume of the coils are essentially similar in water and in 0.1 M NaCl. The dimensionless index $[\eta]_{H_2O}/[\eta]_{NaCl}$, can be used as indicator of the ability of charged polymer coils to contract in presence of salt due to to its intrinsic chain felxibility and overall charge density. The low $[\eta]_{H_2O}/[\eta]_{NaCl}$ index of EPS1190 (*cf.* chitosan $[\eta]_{H_2O}/[\eta]_{NaCl} \sim 2.95$) confirms that the polysaccharide is a neutral polymer, consistent with the absence of uronic acids. The

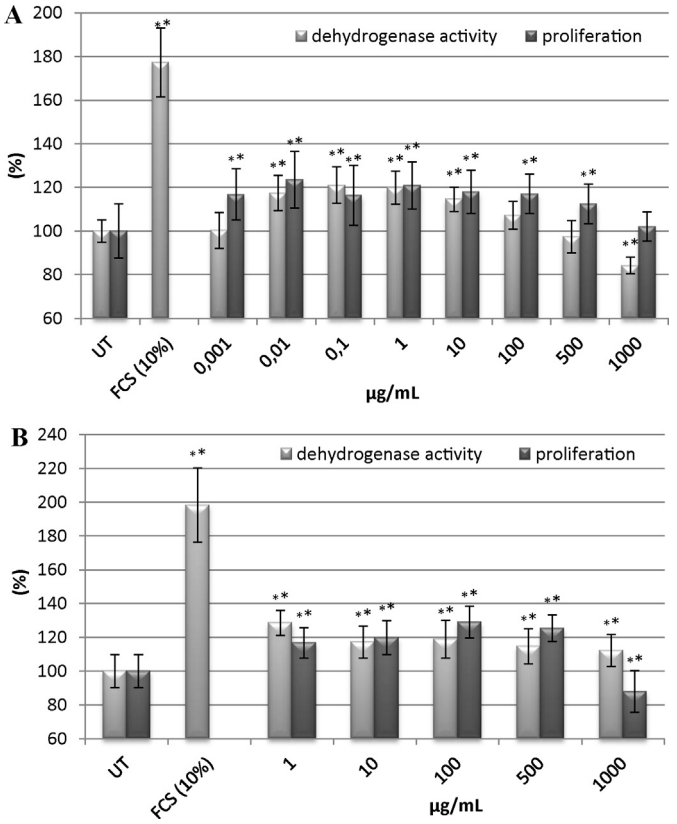

Fig. 1. Influence of EPS1190 on cellular viability (MTT assay) and proliferation rate (BrdU-ELISA) of human stomach AGS cells (A) and human epithelial buccal KB cells (B), incubated for 24 h with EPS1190 (0.001–1000, resp. 1–1000 μg/mL). Bars represent standard deviation (SD) with $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the untreated control group (UT); positive control 10% FCS.

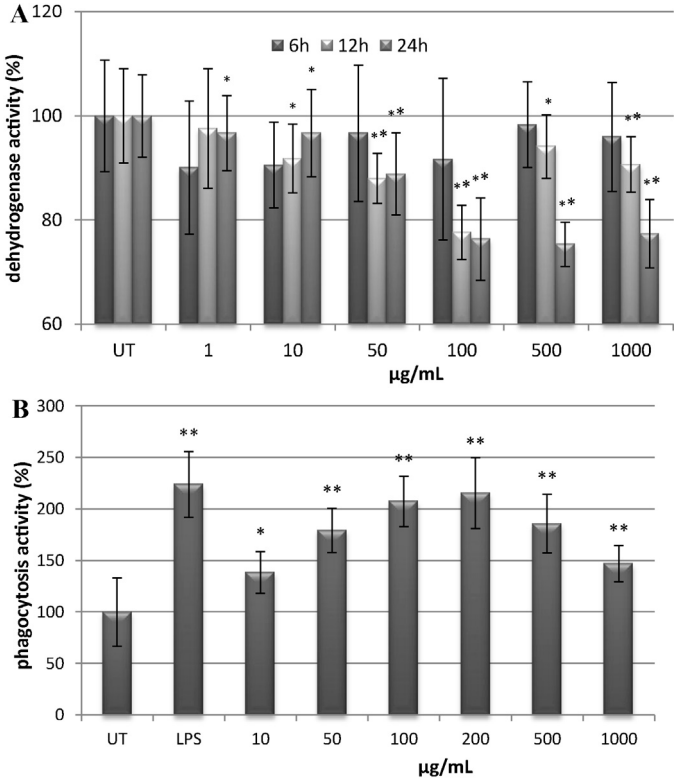

Fig. 2. Influence of EPS1190 on cell viability (A) and phagocytosis activity (B) of murine macrophages RAW 264.7. For determination of cell viability (MTT assay) RAW 264.7 cells were incubated with EPS1190 (1–1000 μg/mL) for 6, 12 and 24 h of incubation time. Bars represent standard deviation (SD) with $^{*}p < 0.05$, $^{**}p < 0.01$ compared to the untreated control group (UT). For determination of influence of EPS1190 on phagocytosis activity RAW 264.7 were incubated at concentrations of 10–1000 μg/mL for 4 h; for read-out FITC-labeled Zymoan particles were used. Positive control: lipopolysaccharide (LPS) from *E. coli*; negative control: untreated cells (UT).

low average value of zeta potential (ζ) at pH 4.5 also confirms its neutral character.

The influence of EPS1190 on eukaryotic cells at different concentrations and incubation periods was determined against human stomach cell line (AGS) and human buccal epithelial cells (KB) by quantification of succinate dehydrogenase activity (MTT assay) as marker for cellular vitality [13] and determination of respective proliferation rates by BrdU incorporation ELISA [17].

No significant effects of EPS1190 on AGS cells after 6 h of incubation time were observed. Significant increase of about 20% ($p < 0.01$) of mitochondrial activity at 1 and 10 μg/mL EPS1190 was noticeable after 12 h treatment of the cells. Higher concentrations of EPS were inactive. However, after 24 h of incubation significant stimulation of cellular vitality at EPS concentrations between 0.01 and 10 μg/mL was observed while higher concentrations of EPS diminished cellular activity (Fig. 1A). At the same time EPS1190 at 1 ng to 500 μg/mL triggered AGS cells toward a significantly increased proliferation rate (Fig. 1A).

Similar stimulating effects of EPS1190 were determined against KB cells (Fig. 1B). No significant effects were detected after 6 and 12 h of incubation time (data not shown) while increase in mitochondrial activity and cellular proliferation rates occurred after 24 h of incubation (1–500 μg/mL) (Fig. 1B). Again, higher concentrations diminished cellular activity and proliferation.

At this point, stimulating effects on epithelial cells from the gastrointestinal system were obvious, being in accordance with recently published cytoprotective effects of EPS1190 against stomach inflammation [4]. Therefore it seemed interesting to investigate if the polysaccharide is able to also influence the activity of immune cells. For that the effect of EPS1190 (1–1000 μg/mL) against murine macrophage cell line RAW 264.7 was determined. No significant results were observed after 6 h incubation time but a reduced vitality of the macrophages got obvious after 12 h, which was even higher after 24 h of treatment (Fig. 2A). On the other side, EPS1190 exerted a strong, dose-dependent stimulation of phagocytosis rate of the macrophages after 4 h of incubation time, which indicates immunomodulating potential of the polysaccharide (Fig. 2B). EPS1190 did not have any influence on macrophage NO-release and did not inhibit the LPS-induced NO formation (data not shown). This means that the polysaccharide does not stimulate NO-mediated inflammation and does not exert any influence on LPS-induced NO-release.

Due to the fact that many gastric ulcer are caused by *H. pylori* infections, EPS1190 was investigated concerning potential effects against this bacterium. Within agar diffusion assay no direct cytotoxicity of the polysaccharide against *H. pylori* in the concentration range of 1 to 5 mg/mL was observed. On the other side, several reports have been published recently on polysaccharides from different origins with strong antiadhesive activities against *H. pylori* [18–22]; in these cases antiadhesion will result in inactivation of bacterial outer membrane proteins which are responsible for recognition of host cell surface structure and binding to the eukaryotic host cells.

Potential antiadhesive effects of EPS1190 were investigated by an *in vitro* antiadhesion assay using FITC-labeled *H. pylori*, strain J99, and human stomach AGS cell line. Labeled bacteria are incubated with the eukaryotic cells and AGS cells with adherent bacteria are quantified after trypsinisation of the monolayer by flow

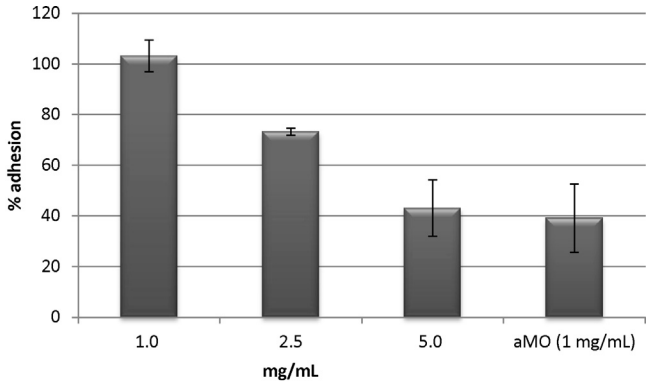

Fig. 3. Effect of 2 h pretreatment of AGS cells with different concentrations of EPS 1190 on the relative adhesion of FITC-labeled *H. pylori*. Relative adhesion values [%] are related to the bacterial adhesion to untreated AGS cells, which served as negative control (100%). Values are mean ± SD; acidic milk oligosaccharides aMO served as positive control [24,25].

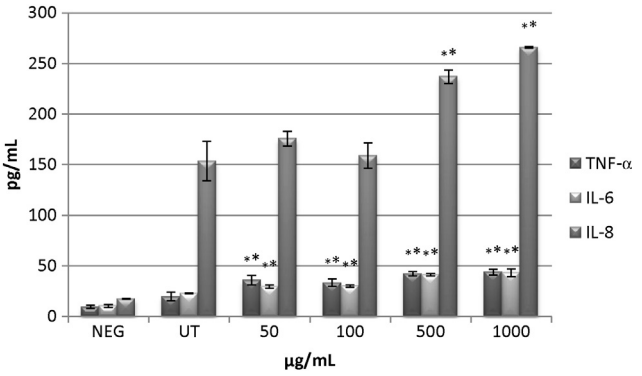

Fig. 5. Secretion of TNF-α, Il-6 and IL-8 by AGS cells after coincubation of AGS cells with *H. pylori* (MOI 1:150) in the presence of different concentrations (50–1000 μg/mL) of EPS1190 for 24 h. NEG: negative control, non-infected with bacteria, no EPS1190 treatment; UT: AGS cells, infected with bacteria, without EPS1190. Significances are calculated against respective values of UT control group, $^{**}p < 0.01$.

cytometry. Acidic human milk oligosaccharides which includes a variety of structures bearing a-2,3-linked sialic acids capable of inhibiting the adhesins SabA and HpaA served as positive control [23–25].

Pretreatment of AGS cells with different concentrations of EPS1190 for 2 h followed by addition of labeled *H. pylori* showed no signs for antiadhesive activity at doses >1 mg/mL. In contrast, higher concentrations exerted significant antiadhesive activity against *H. pylori* with inhibition rates of about 30% at 2.5 mg/mL and 60% at 5 mg/mL (Fig. 3). Although concentrations for obtaining the antiadhesive effects are quite high, they have to be assessed as being in a similar range to other antiadhesive compounds already investigated in clinical studies [26]. From these experiments it can be deduced that EPS1190 has an affinity to AGS cells, interacting with eukaryotic binding sides to the respective complementary bacterial adhesins.

Modification of the adhesion experiments by pretreatment of *H. pylori* with EPS1190, and subsequent incubation with AGS cells resulted in a drastic increased bacterial adhesion; similar results were obtained during cotreatment of bacteria together with AGS cells and with the polysaccharide (Fig. 4). This indicates strong affinity of the polymer to the bacterial surface, which again results in an increased interaction with the eukaryotic cells. The controversial findings in the different adhesion experiments can be explained as follows: pretreatment of AGS cells with EPS1190 results in the formation of a bioadhesive polymer layer on the cell surface, which can not be penetrated by the bacterium. Pretreatment of the bacteria with EPS1190 results in strong binding to the bacterial surface, which "glues" the coated bacteria to the AGS cells, leading to increased bacterial adhesion. From this point of view an unspecific interaction of the polysaccharide to cellular surfaces can be deduced. For verification of this increased adhesion the destructive process of *H. pylori* toward the infected AGS cells was investigated. Coincubation of AGS cells together with *H. pylori* and EPS1190 led – as expected – to significantly increased release of inflammatory cytokines from the eukaryotic cells: IL8 release and to a minor extent also IL6 and TNF-α secretion was stimulated significantly during pretreatment of bacteria with EPS1190 and also during cotreatment (Fig. 5).

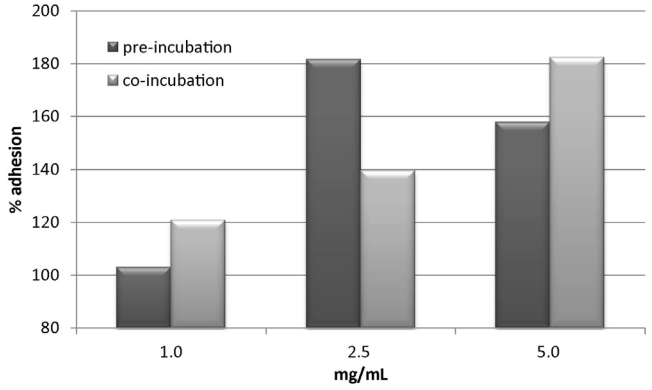

Fig. 4. Effect of 2 h pretreatment of *H. pylori* and 1 h cotreatment of *H. pylori* and AGS cells with EPS1190 (1, 2.5 and 5 mg/mL) on the adhesion of *H. pylori*. Values are related to untreated control groups (100%).

Because the major outer membrane proteins of *H. pylori* for bacterial adhesion are BabA and SabA, the influence of EPS1190 against the sialic acid-binding adhesin SabA was investigated by hemagglutination assay. *H. pylori* was pretreated with the polysaccharide, followed by incubation with a serial dilution of human erythrocytes. No influence on hemaglutination was visible (data not shown), which means that EPS1190 does not interact with SabA or the complementary ligands of the eukaryotic cell (Sialyl–Lewis^a, Sialyl–Lewis^x, Laminin, MUC7 and MUC5B).

For evaluation if EPS1190 interacts specifically with mucin (MUC) an automated falling ball microviscosimetric method with the purified soluble mucin of porcine stomach was used. No MUC-EPS1190 interaction was detected in this test system (data not shown). This indicates that the binding target of EPS1190 on cell surfaces is probably not the endogenous mucin layer of the tissue.

In order to investigate if EPS1190 forms bioadhesive polymer-layers on the surface of tissue histological sections of human stomach were used [19,20,22] which were incubated with FITC-labeled EPS1190 followed by intensive washing steps for removal of unbound polymer. As shown in Fig. 6A, enrichment of the labeled polysaccharide on the epithelial side of the stomach tissue was evident; higher magnification indicated absorption of the polymer into the mucin layer (Fig. 6B).

From these results and that obtained with AGS cells it is clear that the interaction between EPS1190 and eukaryotic cells finally leads to increased cellular activity and proliferation. At least two ways of interaction of the polysaccharide with the cell can be hypothyzed: (i) adhesion of the polymer to the cell surface and interaction with extracellular protein- and receptor-structures, and (ii) internalization of the polysaccharide into the cell *via* endosomal transport as described recently for some other high molecular weight polysaccharides [14,27].

For that, laser scanning microscopy of AGS cells incubated for 24 h with FITC-labeled EPS1190 was performed (Fig. 7A,

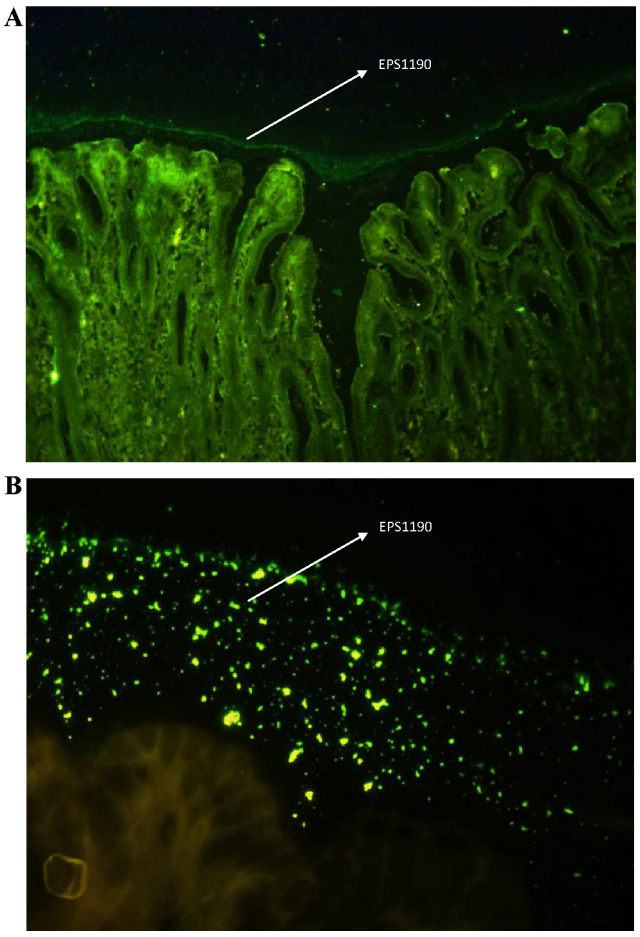

Fig. 6. Adhesion of FITC-labeled EPS1190 to human stomach tissue sections after fluorescent microscopy. (A) FITC-EPS1190 attached to mucus layer of the tissue (magnification 10×); (B) EPS1190 agglomerates absorbed in the mucus layer of the tissue.

yellow-green staining). Cell nuclei were stained with DAPI (Fig. 7B, blue staining). Endosomes were visualized with Dextran-TexasRed® (Fig. 7C, red staining). Overlay of the images indicated endosome-mediated uptake of EPOS1190 (Fig. 7D and E, orange mix color, resulting from green-labeled EPS1190 within the red-stained endosomes). Additionally green spots were still visible in many cells indicating the existence of non-internalized FITC-EPS on the cell outer surface (Fig. 7D and E).

Further internalization assays in the presence of Cytochalasin B, an inhibitor of endosoms, were carried out. As expected, no endosomes were visible and the FITC-labeled EPS1190 was exclusively found outside the cells (Fig. 7F and G).

From these findings we assume that EPS1190 interacts with the cellular membrane followed by a partial endosomal transport into the cell.

4. Discussion

In the last years, probiotics have become promising agents for prevention of several diseases [28] mainly those connected to gastrointestinal problems. Well established probiotic effects are prevention of virus or antibiotic-induced diarrhea, prevention of unspecific diseases of the gut, normalization of passing stool and stool consistency, and prevention of allergies, respiratory tract infections [28] and gastritis [28]. These beneficial effects are closely linked to the probiotic bacteria strain as itself or to the metabolites produced. Interaction of bacteria or metabolites with different molecular targets of the tissue are discussed, competing with the gastrointestinal microflora, leading to an increase of innate and specific immune response *via* activation of the gastrointestinal lymphocyte tissue. Direct interaction with potential pathogens are also described blocking the adhesion of pathogens with gastrointestinal tissue, regulating K^+/Na^+ pumps activity, or increasing the strength of gastrointestinal mucus layer. Prebiotics, as non-digestible food ingredients affect beneficially the host by selectively stimulating the growth and/or the activity of one or a limited number of bacteria in the colon and thus improve host health [3]. The probiotic strain *S. thermophilus* CRL1190 is a highly effective producer of *exo*-polysaccharide (EPS1190), which has not yet been investigated as a potential prebiotic compound neither its effect on gut cells. From the present study it was interesting to note that EPS1190 interacts with stomach cells under *in vitro* conditions, which leads to an increase of the cellular activity and proliferation rate (Fig. 1). This may be beneficial in case of inflamed or damaged stomach tissue caused by destructive effects during acute and chronic gastritis. These effects would be related to the capacity of this polysaccharide to attach to the cellular surface (Fig. 6). On the other side, the high molecular weight compound is effectively absorbed into the cells by endosomal transport (Fig. 7). This again may initiate the intracellular signaling to the subsequent cellular reactions. Such internalization processes and stimulation of cell viability status has been mostly related in old literature to the activation of receptor structures on the outer membrane of the cells on the basis that polysaccharides could not be internalized. Recent studies clearly indicate the rapid endosomal uptake of such polar and high molecular weight products into the cells [27], similar to the endosomal uptake of CRL1190 *exo*-polysaccharide in the present study. Besides, this interaction in murine stomach seems to be connected not only to the cellular activity of the stomach cells but also related to an increased cytokine regulation, especially by stimulation of IL-10 producer cells and inhibition of INF-γ and TNF-α producing cells [4,8]. In the present study EPS1190 did not influence murine macrophages after a 6 h incubation period while longer contact time decreased cellular activity significantly. In contrast, the phagocytosis activity was increasing enormously (Fig. 2). From these results it is assumed that the polysaccharide may interact with the immunological system.

Some pre- and probiotics interact with the bacterial adhesion of pathogens to the eukaryotic binding receptors throughout so called antiadhesive compounds [29–33]. In case of pretreatment of AGS cells with EPS1190 the polysaccharide was shown to inhibit *H. pylori* adhesion by interaction with the surface of AGS cells (Fig. 3). Pretreatment of *H. pylori* with the polysaccharide led to the interesting effect that it increased the bacterial adhesion of *H. pylori* to stomach cells significantly on the presence of AGS cells (Fig. 4), leading to an increased inflammatory response (Fig. 5). This can be explained with an unspecific binding to the bacterial cell wall, which forms a bioadhesive layer which again "glues" the bacterium to the mucin layer of the stomach cells [34]. This interaction may be related to binding between EPS and the glycoproteins of the mucus layer, mainly composed of the secreted human mucins MUC5AC produced from the superficial mucosa and MUC6 produced from the gland mucosa [35]. Both mucins are not part of the soluble fraction of the porcine mucine used for the mucin-EPS1190 interaction assay, which indicate no significant interaction between the two polymers. This interaction of EPS1190 with mucus layer could be not an important effect by itself; however, the presence of the polysaccharide in fermented milk may play a role in helping the bacteria together with milk proteins to maintain joining to stomach inner surface [4] as it was evinced by Rodriguez et al. [8]. From these results, we consider EPS1190 as a potential bioprotector in gastric troubles, which may be used as a dietary supplement during gastritis treatment.

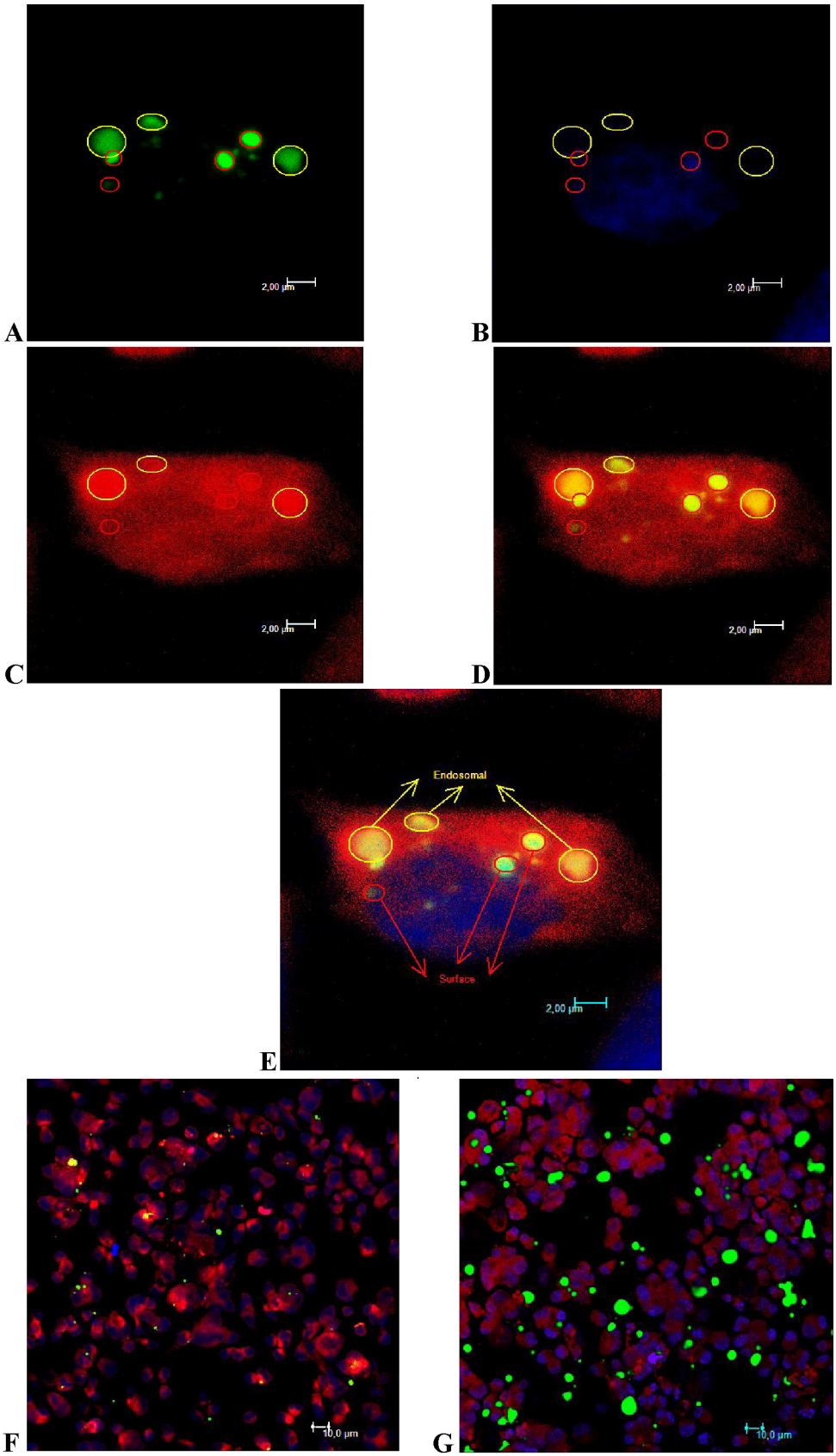

Fig. 7. Laser scanning microscopy of cellular localization of FITC-EPS1190 after 24 h incubation time in AGS cells. (A) Green-yellow flourescence of FITC-labeled EPS1190; (B) dark blue fluorescence of nuclei stained with DAPI; (C) red-orange fluorescence of endosomes stained with Dextran-TexasRed; (D) overlay image of A and C; (E) overlay images of A, B and C. (F) AGS cells incubated with FITC-labeled EPS1190, without (F) and with addition (G) of Cytochalasin B. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conflict of interest

The authors state that there are no are no conflicts of interest including any financial, personal or other relationships with other people or organizations within three years of beginning of the submitted work that could inappropriately influence, or be perceived to influence, their work.

Acknowledgements

Financial support by the German Academic Exchange Service DAAD providing an Exchange stipend to G. M. is acknowleged. Financial support by the German Research Foundation DFG (International Research Training Group 1549 Molecular and Cellular Glyco-Sciences MCGS, Münster Hyderabad) to A.H. J.M., F.M.G. and B.M is acknowledged.

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