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Further studies on immunomodulatory effects of exopolysaccharide isolated from *Lactobacillus rhamnosus* KL37C

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Abstract

Lactobacilli, bacteria of human microbiome and major probiotic bacteria, show immunoregulatory properties. The immunomodulatory effect can be achieved not only by whole bacteria but also by cell-wall components such as peptidoglycan and lipoteichoic acid (LTA). Other, biological active bacterial products are exopolysaccharides (EPS), major components of the lactobacilli biofilm. The aim of this study was to examine immunostimulatory potential of highly purified EPS derived from L. rhamnosus KL37 (EPS_{37}). We have examined the effect of EPS_{37} on some selected biological functions of major inflammatory cells (neutrophils, macrophages, dendritic cells) in vitro, and on OVA-specific humoral response in mice. We have shown weak antioxidant properties of EPS_{37}. It decreased the reactive oxygen species (ROS) production in neutrophils stimulated with zymosan, but had no effect on phagocytosis of dextran by macrophages. Interestingly, in contrast to LTA, pure EPS_{37} did not stimulate cytokine production by MΦ. Moreover, EPS_{37} did not induce synthesis of prostaglandin E_2 (PGE_2) and did not affect the expression of COX-2 protein in MΦ, while it slightly induced the production of IL-12p40 and of IL-6 but not IL-10, in dendritic cells. On the other hand, we have found that EPS_{37} diminished the production of OVA-specific IgG antibodies. Moreover, EPS_{37} inhibited the adjuvant effect of LPS. Therefore, our present results confirm the suppressor effect of EPS_{37} on antigen-specific humoral response in mice. These data extend our knowledge about the role of EPS in interaction between bacteria and the immune system. However, further studies are necessary to explain its role as a major component of biofilm matrix.

Key words: *biofilm, cytokines, exopolysaccharides, Lactobacillus rhamnosus, lipoteichoic acid, macrophages, dendritic cells, probiotics.*

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Introduction

Recently, an increasing number of studies have suggested that the immunoregulatory effect of probiotic bacteria can be achieved not only by using live or dead bacteria, but also by using components of the bacterial cell wall or their secretory products such as exopolysaccharides (EPS) excreted into the environment during growth of bacteria [1, 2]. Most lactic acid bacteria are capable of synthesizing and secreting EPS. *Lactobacillus*, *Streptococcus* and *Lactococcus* prevail among these genera but also it can be produced by some strains of *Bifidobacterium* [3-6]. Over 50 different exopolysaccharides derived from lactic acid bacteria have been described, and more than 30 of these bacteria belong to *Lactobacillus* [7].

EPSs are linear macromolecular carbohydrate biopolymers which potentially express biological activity [8]. *Lactobacillus* bacteria can both secrete EPS to the environment and synthesize its cell wall-bound type. EPS, which is the main component of biofilm, facilitates adhesion of bacteria to intestinal epithelium, enables bacterial cells to sur-

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vive in bile salts [9] and prevents infection of bacteria by the bacteriophage [10]. Genes encoding enzymes and proteins required for EPS synthesis have been discovered and described [11]. However, involvement of EPS in an interaction between *Lactobacillus* bacteria and MALT system is not fully understood. It is not known either whether a receptor for EPS exists.

In our previous study we have shown immunomodulating properties of crude EPS isolated form *Lactobacillus rhamnosus* KL37C [12]. We have compared the effect of EPS on production of inflammatory mediators by mouse peritoneal macrophages with the effect of lipopolysaccharide (LPS). EPS could effectively stimulate production of cytokines by macrophages, especially tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and IL-12. However, its stimulatory potential was significantly lower than that of LPS. Furthermore, our data demonstrated that exposure of macrophages to LPS induced a state of hyporesponsiveness, as indicated by a reduced production of TNF-α after restimulation with either LPS or EPS ('cross-tolerance'). Our results also indicated that inhibitors of both ERK and p38 MAPKs inhibit production of TNF-α induced by LPS or EPS. Elsewhere, we have shown that *Lactobacillus rhamnosus* exopolysaccharide ameliorates arthritis induced by a systemic injection of collagen and lipopolysaccharide in DBA/1 mice [13]. However, further studies with highly purified EPS are necessary to find out whether EPS can be clinically useful as an immunomodulatory agent.

In the present study we have focused on analyzing *in vitro* immunoregulatory properties of a highly purified exopolysaccharide from *L. rhamnosus* KL37C (EPS_{37}). The immunostimulatory potential of EPS_{37} was compared with that of lipoteichoic acid (LTA) isolated from the same bacteria and with LPS from *Escherichia coli.* We have examined the effect of EPS on some selected biological functions of major inflammatory cells, namely, neutrophils, macrophages and dendritic cells.

Material and methods

Mice

Inbred CBA/J mice and Balb/c mice (8-12 weeks of age, 18-22 g) were maintained in the Animal Breeding Unit, Chair of Immunology, Jagiellonian University Medical College, Krakow. All mice were housed in the laboratory room with water and standard diet *ad libitum.* The authors were granted permission by the Local Ethics Committee to use mice in this study.

EPS_{37} isolation

Exopolysaccharide was obtained from *L. rhamnosus* KL37C strain as described before [14]. The strain was isolated from feces of human newborns and then stored at –70°C in MRS broth supplemented with 10% glycerol. Bacteria were cultivated in supplemented MRS liquid broth (Oxoid, UK) under anaerobic conditions at 37°C for 48 h. Cells were harvested by centrifugation at 7300 g (4°C, 30 min) and washed twice with PBS. Freeze-dried bacterial mass was extracted with 10% TCA (25°C, 2 h) and then centrifuged at 14 500 g for 20 min. The pellet was discarded and EPS_{37} from the supernatant was precipitated with 5 volumes of cold 96% ethanol (4°C, 16 h) and collected by centrifugation at 23 500 g (4°C, 50 min). The pellet was suspended in water, dialyzed for 48 h against water and then lyophilized.

EPS_{37} purification

Freeze-dried preparation of crude EPS_{37} was dissolved in buffer (50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$) and treated with DNase and RNase (37°C, 6 h) and with protease from *Streptomyces griseus* (37°C, 16 h) (both Sigma-Aldrich, Germany), then dialyzed against water at 4°C for 24 h. Resuspended polysaccharide was purified by ion-exchange chromatography on 1.6 × 20 cm DEAE-Sephadex A-25 column (Pharmacia Fine Chemicals, Sweden). EPS_{37} was eluted with 20 mM Tris buffer pH 8.2. Fractions containing EPS_{37} were pooled, desalted by dialysis against water at 4°C for 24 h and lyophilized.

LTA isolation

Defrosted aliquot of bacteria was mixed with equal volume of *n*-butanol (Merck, Germany) under stirring for 30 min at RT. After centrifugation at 13 000 g for 20 min, the aquatic phase was collected and dialyzed against distilled water, lyophilized, resuspended with chromatography start buffer (15% *n*-propanol in 0.1 M ammonium acetate, pH 4.7) and centrifuged at 45 000 g for 15 min. The supernatant was subjected to hydrophobic interaction chromatography on 1.6 × 40 cm octyl-Sepharose CL-4B column (Pharmacia Fine Chemicals, Sweden). Unbound material was washed out with start buffer. Afterwards, bound materials were eluted with 35% *n*-propanol in 0.1 M ammonium acetate buffer (pH 4.7). Column fractions containing LTA were pooled and dialyzed against distilled water.

Macrophages

Peritoneal mouse (CBA/J) macrophages (MΦ) were induced by an intraperitoneal injection of 2.0 ml of thioglycolate (Sigma, USA). Cells were collected 72 h later by washing out the peritoneal cavity with 5 ml of DPBS containing 5 U heparin/ml (Polfa, Poland). Cells were centrifuged and red blood cells were lysed. Osmolarity was restored by addition of PBS. At least three mice were used as donors of peritoneal macrophages for each experiment.

Dendritic cells

Bone marrow derived dendritic cells (DC) were cultured from Balb/c mice. Briefly, bone marrow cells were flushed

out from femurs and grown at starting density of 2×10^6 cells/ml of IMDM (Cytogen, Germany). The medium was supplemented with HEPES (24 g/l), 50 nM of 2-mercaptoethanol (both Sigma-Aldrich, Germany), 10% FBS (PAA, USA), gentamicin (50 mg/ml, Krka, Slovenia) and 3 ng/ml GM-CSF (Invitrogen, USA). After 3 days, the medium containing most of non-adherent cells, was removed and replaced with the fresh one. None adherent DCs, released spontaneously from proliferating cell clusters, were recovered at 7th day of culture. Prepared cells were suspended in PBS containing 0.05% FBS and 2 mM EDTA and purified with Magnetic Advanced Cell Sorting system (MACS, Miltenyi Biotec, Germany). Firstly, cells were incubated on ice for 40 min with anti-CD11c antibodies. Then, cells were washed in PBS and passed through a column with iron wool placed in the magnetic field. First collected fractions of cells were CD11c negative. After removing the column from the magnetic field, CD11c-positive cells were collected. Thus, purified DC were suspended in the culture medium IMDM and used for further experiments.

Reactive oxygen species generation: luminol-dependent and lucigenine-dependent chemiluminescence assay

The effect of EPS_{37} on induction production of reactive oxygen species (ROS) by neutrophils was evaluated *in vitro* using luminol-dependent chemiluminescence or lucigenine-dependent chemiluminescence. Chemiluminescence was counted at 37°C in temperature-stabilized luminometer Lucy 1 (Anthos, Austria). Briefly, 18-hour peritoneal cells induced by thioglycolate (5×10^5/well), were preincubated with tested agents in Hank's balanced salt solution (60 min at 37°C in an atmosphere of 5% CO_2) on a 96-well flat-bottom black plate (Nunc, Denmark). Then, the cells were mixed with luminol (0.8 mg/ml) or lucigenine (0.25 mM) in 1 : 1 volume ratio (both Sigma-Aldrich, Germany) and incubated at 37°C for another 30 min. After incubation, the cells were quickly stimulated with yeast zymosan (200 µg/ml, Sigma-Aldrich, Germany) and photon emission over 75 min with 3-min intervals was measured. Results are expressed as relative light units (RLU) where photons were counted every 5 seconds.

HOCl-specific UV absorption spectra measurement

HOCl concentration was determined before each reaction by UV absorption spectra (λ = 200 to 400 nm) measured at 292 nm using extinction coefficient factor 350 $M^{-1}cm^{-1}$. HOCl and EPS_{37} were mixed in concentration 1 mM HOCl and 100 µg/ml EPS_{37} and incubated for 5 min in RT. Each reaction of HOCl with EPS_{37} was monitored by UV absorption spectra (λ = 200 to 400 nm) to estimate the influence of EPS_{37} on UV spectra of HOCl.

Macrophage culture and treatment

MΦ were cultured in 24-well flat-bottom cell culture plates (5×10^5/well) IMDM (Cytogen, Germany) supplemented with 5% FBS, and gentamicin at 37°C in atmosphere of 5% CO_2. After 1 h, non-adherent cells were removed, whereas the adherent cells were stimulated with indicated concentrations of EPS_{37}, LTA and LPS (*E. coli* 001:B4; Sigma-Aldrich, Germany) After 24 h, culture supernatants were collected and frozen at –80°C until used. All groups were investigated in duplicates.

Dendritic cell culture and treatment

Dendritic cell prepared as described above, were cultured on 96-well plates in IMDM supplemented with 5% FBS, HEPES and gentamicin at 37°C in atmosphere of 5% CO_2. Cells were stimulated with selected concentrations of examined bacterial structures, and after 24 h supernatants were collected and level of cytokines IL-6, IL-10 and IL-12p40 was estimated.

Cytokines determination

The concentration of cytokines in supernatants was determined using the ELISA method with some modifications, as described before [12]. 96-well plates (Costar, USA) were coated with antibody against the selected cytokine (TNF-α, IL-2, IL-6 and IL-12p40 – Bioscience, USA) and incubated for 24 h at 4°C. After blocking the plate with a solution of 3% skimmed milk (2 h), tested samples and standards for each of the cytokines were applied. After further 24 h of incubation at 4°C, biotinylated antibodies against the selected cytokine were added for 1 h. The ELISA was developed using horseradish peroxidase conjugated with streptavidin (Vector, USA) followed by o-phenylenediamine and H_2O_2 (both Sigma-Aldrich, Germany). After 30 min, reaction was stopped by addition of 3M H_2SO_4. The optical density of each sample was measured at 492 nm in microplate reader PowerWavec (Bio-Tek Instruments, USA). 0.05% Tween-20 in phosphate buffer was used as a washing solution. Exception to the above procedure was the measurement of IL-10, which was performed according to the manufacturer's instructions (BD Biosciences, USA).

Phagocytosis

To evaluate the effect of EPS on phagocytosis of dextran by macrophages, cells were resuspended in 1% FBS in PBS and then incubated with EPS_{37} or LPS and RITC-dextran (Sigma-Aldrich, Germany) for 30 min at 37°C. The reaction was stopped by addition of cold 1% FBS in PBS. Cells were washed three times using the same buffer, and then macrophages were prepared for analysis by flow cytometry. Cells were stained with allophycocyanin-conjugated anti-mouse F4/80 monoclonal antibody (eBioscience, USA). Nonspecific binding was blocked using monoclonal 2.4G2 antibody (BD Pharmingen, USA). Cells

were incubated with antibodies for 40 min, at 4°C in the dark, then washed twice with 1% FBS in PBS and suspended for analysis in the same buffer. To exclude dead cells, propidium iodide was added just before analysis (Sigma-Aldrich, Germany). Cells were analyzed using FACSCalibur flow cytometer (Beckton Dickinson, USA) and CellQuest Pro software (Beckton Dickinson, USA).

PGE_2 determination

PGE_2 concentration in supernatants was determined by Prostaglandin E_2 Monoclonal EIA kit (AssayDesigns, USA) according to the manufacturer's instructions.

Western blot analysis: evaluation of COX-2 expression

The level of induction of COX-2 protein was determined by the Western blot technique. MΦ were lysed in lysis buffer containing mixture of protease inhibitors (Sigma-Aldrich, Germany). The total protein concentration in resulting lysates was determined using a bicinchoninic acid protein assay kit (Sigma-Aldrich, Germany). Samples containing equal amounts of protein (14 mg) were suspended in loading buffer (reagents Sigma, USA), in a 2 : 1 ratio and denatured for 4 min at 100°C. Samples were applied to polyacrylamide gel with 10% SDS and were separated electrophoretically in the Laemmli system using Mighty Small II apparatus (Amersham Biosciences, USA) [15]. Separated proteins were transferred to the nitrocellulose membrane (Bio-Rad, USA) using Hoefer TE22 transfer equipment (Amersham Biosciences, USA). After overnight incubation with protein blocking solution at 4°C (3% skimmed milk), membranes were incubated for 2 h at RT with rabbit polyclonal anti-COX-2 antibodies (Cayman, USA) and mouse monoclonal anti-β-actin antibodies (Sigma-Aldrich, Germany). Then, membranes were incubated for 2 h with secondary antibodies, anti-rabbit IgG (Sigma-Aldrich, Germany) and goat IgG conjugated with phosphatase alkaline (Sigma-Aldrich, Germany) at RT. The bands were detected with phosphatase alkaline substrate BCIP/NBT (Sigma, USA). Protein bands were scanned and analyzed using freeware Scion Image for Windows (Scion, USA). Results are presented as a ratio of optical density of protein to β-actin, which is constitutively expressed in cells.

Mice immunization with OVA

Mice were immunized intraperitoneally with OVA (200 μg/mouse) in the presence of LPS (1 μg/mouse; both Sigma-Aldrich, Germany), or/and of EPS_{37} (50 μg/mouse) or/and of LTA (50 μg/mouse) twice, on days 0 and 14. Seven days after the second immunization mice were bled and serum was processed further.

Measurement of serum OVA-specific antibody titers

The level of antibodies specific to OVA in mouse serum was determined using the ELISA test as described before [13]. Briefly, plates were coated with OVA (5 μg/ml) overnight. Serial dilutions of mouse serum in PBS were applied to antigen-coated wells for 1 h at RT. Levels of IgGs were determined using biotin-conjugated antibodies against IgG (Sigma-Aldrich, Germany), IgG1 (MP Biomedicals, USA), or IgG2a (Southern Biotech, USA). The amount of antibody was expressed in arbitrary ELISA units calculated from anti-OVA titer: 1 Unit = 1/100 titers of antibodies specific to OVA.

Statistical analysis

Statistical significance between two groups were tested using Student's *t* test. For more groups, comparison one-way ANOVA and Tukey *post hoc* tests were used. Results are expressed as a mean ± SEM values. A p value < 0.05 was considered statistically significant. Analysis was performed using GraphPad Prism ver. 5.01 program (GraphPad Software, USA).

Results

Influence of EPS_{37} on the production of ROS by neutrophils

To answer the question whether exopolysaccharides released from bacteria may affect ROS production by inflammatory cells, non-stimulated and zymosan-stimulated neutrophils were incubated with EPS_{37}. The effect was compared with that of LTA and LPS (Fig. 1). In order to determine the effect of tested substances on an intracellular and extracellular level of ROS, luminol-dependent (Fig. 1A) or lucigenine-dependent (Fig. 1B) chemiluminescence was measured, respectively. The cells generated and secreted significant amounts of ROS in response to zymosan. In contrast, EPS_{37}, LTA and LPS did not induce ROS production when added alone to non-stimulated neutrophils. Interestingly, EPS_{37} and LTA added to neutrophils before stimulation with zymosan inhibited formation of ROS by about 40%. The effect was observed only in the case of luminol-dependent chemiluminescence (Fig. 1A). What is more, incubation of EPS_{37} with HOCl resulted in scavenging of HOCl as indicated from alteration of the HOCl-specific UV absorption curve (Fig. 1C).

Effect of EPS_{37} and LTA on release of cytokines from macrophages

In order to determine immunostimulatory properties of pure EPS_{37} and LTA, peritoneal macrophages were cultured for 24 h with selected concentrations of examined factors. The results clearly indicate strong stimulatory properties of LTA. LTA at a concentration of 30 μg/ml and 100 μg/ml LTA significantly induced the production of all tested cytokines (TNF-α, IL-6, IL-12p40 and IL-10), while EPS_{37} had no effect, as it is shown in Fig. 2.

Effect of EPS_{37} on phagocytic activity of macrophages

To answer the question whether EPS_{37} can affect the phagocytic activity of macrophages, the cells were incubated with EPS_{37} and then with RITC-Dextran. The effect was compared with that of LPS. As shown in Fig. 3, neither EPS_{37} nor LPS altered phagocytosis of dextran molecules by macrophages.

Effect of EPS_{37} on COX-2 expression and PGE_2 synthesis by macrophages

To examine the relative potency of EPS_{37}, LTA and LPS in induction of eicosanoids production by macrophages, the cells were cultured with the tested agents. The expression of COX-2 was estimated in the cell cytosol and the level of PGE_2 was determined in supernatants, as described in Material and methods. Non-stimulated macrophages neither expressed COX-2 protein nor released PGE_2, the major eicosanoid of macrophages, as shown in Fig. 4. LTA and LPS-stimulated macrophages expressed high levels of COX-2 and produced a significant amount of PGE_2. In contrast, the pure EPS_{37} did not induce expression of COX-2 protein and the level of secreted PGE_2 was comparable to that observed in non-stimulated macrophages.

Effect of EPS_{37} and LTA on release of cytokines from dendritic cells

Dendritic cells were stimulated with selected concentrations of EPS_{37} to determine whether EPS_{37} affects cytokines production by antigen presentation cells. The effect was compared with that of LTA and LPS (Fig. 5). LPS was the strongest inducer of all tested cytokines. There was also a massive release of IL-12p40 in response to LTA, while the influence of LTA on IL-6 and IL-10 production was negligible. In contrast to LPS and LTA, EPS_{37} did not stimulate the production of any of the tested cytokines.

Effect of EPS_{37} on humoral immune response in mice

Previously we have shown that pure EPS inhibited the *in vivo* production of total IgG specific to OVA. In the present study we have extended the observation to other IgG isotypes to examine the hypothesis considering polarization of the immune response by bacterial products. The results shown in Fig. 6 clearly indicate that EPS_{37} is able to inhibit humoral response to OVA in mice. EPS_{37} injected simultaneously with the antigen decreased markedly the production of anti-OVA IgG (65% of inhibition), IgG1 (90%) and IgG2a (82%) antibodies. Interestingly, EPS_{37} given together with LPS diminished its adjuvant properties by inhibiting OVA-specific IgG production, especially anti-OVA IgG2a (87%) (Fig. 6C).

Discussion

Lactobacilli represent bacteria of human microbiome and are most commonly used for probiotic therapy [16, 17].

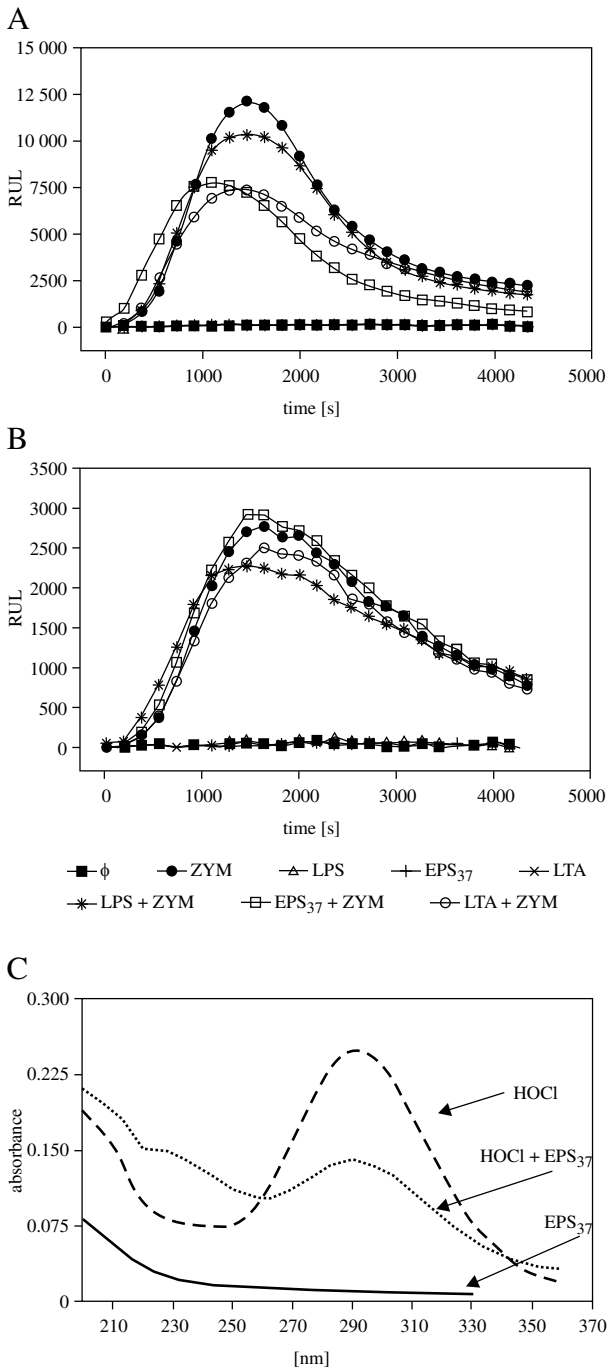

Fig. 1. The effect of EPS_{37} on production of ROS by neutrophils. LPS (0.1 μg/ml) or EPS_{37} (100 μg/ml) or LTA (100 μg/ml) was added to either non-stimulated or zymosan-stimulated neutrophils (ZYM, 0.2 mg/ml). The level of ROS was measured by chemiluminescence dependent either on luminol (A) or lucigenine (B), as described in Methods. The experiment was repeated three times, the graph shows one representative experiment. RLU – relative light units. (C) The UV absorption spectra of HOCl after incubation with EPS_{37}

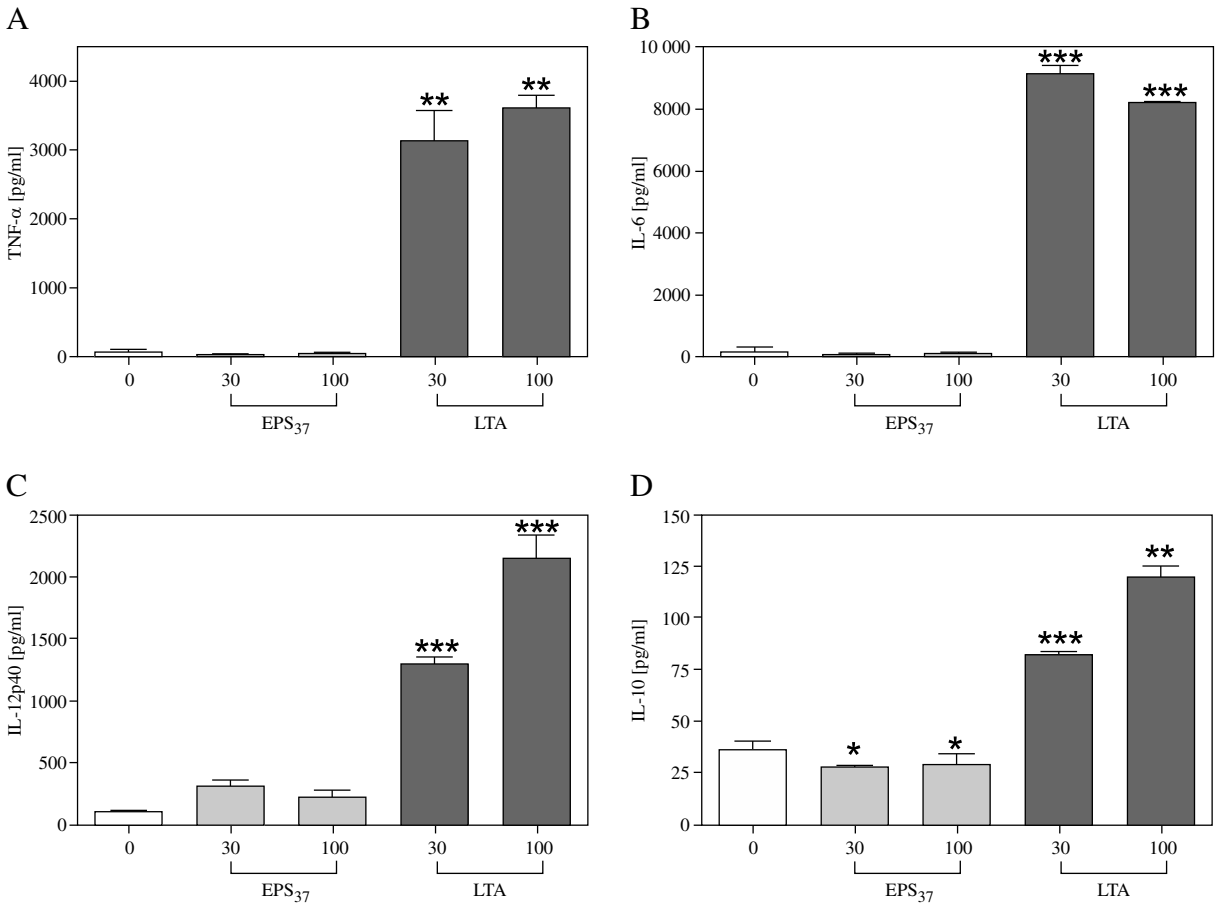

Fig. 2. The effect of EPS_{37} and LTA on cytokine secretion by macrophages. Peritoneal macrophages (5×10^5/ml) were incubated with EPS_{37} or LTA (30 and 100 μg/ml). The levels of TNF-α (A), IL-6 (B), IL-12p40 (C) and IL-10 (D) were measured in cultures supernatants after 24 h of incubation by the ELISA test. Results are expressed as a mean of three independent experiments ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ non-stimulated cells versus stimulated cells

Our previous study indicates that immunoregulatory properties of lactobacilli are strain specific [18]. In addition, other authors have shown that this effect may be achieved using bacterial components such as LTA or peptidoglycan [19, 20]. Much less is known about the immunoregulatory effect of EPS. However, we have previously shown that crude EPS from *Lactobacillus rhamnosus* KL37C exerts an immunostimulatory effect on mouse macrophages *in vitro* [12]. It is of great interest to explain a contribution of EPS in bacteria – immune system cross-talk, especially in bacteria which synthesize EPS as a major component of biofilm matrix [21].

In this study we have investigated the effect of pure EPS_{37} on the selected functions of major cells of acute inflammation, namely: neutrophils, macrophages and dendritic cells. We have found that EPS_{37}, and LTA significantly decrease the level of ROS in mouse neutrophils stimulated by zymosan. This can indicate an antioxidant capacity of pure EPS_{37} similar to that of LTA. Antioxidant properties of EPS have been also described by Kodali *et al.* They have demonstrated that EPS from *Bacillus coagulans* RK-02 has antioxidant properties and may act as a scavenger of free radicals. These properties were comparable with those of standard antioxidants such as vitamin C and E [22]. Further, we investigated the influence of EPS_{37} on the phagocytic function of macrophages. The ability of cells to phagocyte dextran molecules in the presence of pure EPS_{37} was not inhibited. In addition, pure EPS_{37}, in contrast to the crude form described before [12], did not stimulate murine peritoneal macrophages to produce cytokines. Furthermore, there was no effect of EPS_{37} on PGE_2 synthesis and COX-2 protein expression in macrophages. This suggests that pure EPS_{37} is not a key component of *Lactobacillus rhamnosus* responsible for its immunostimulating properties. Therefore, we compared the effect of EPS_{37} with LTA isolated from the same bacteria. LTA showed a strong stimulatory effect. Macrophages stimulated with high concentrations of LTA produced a significant level of both pro-and anti-inflammatory cytokines. LTA also stimulated macro-

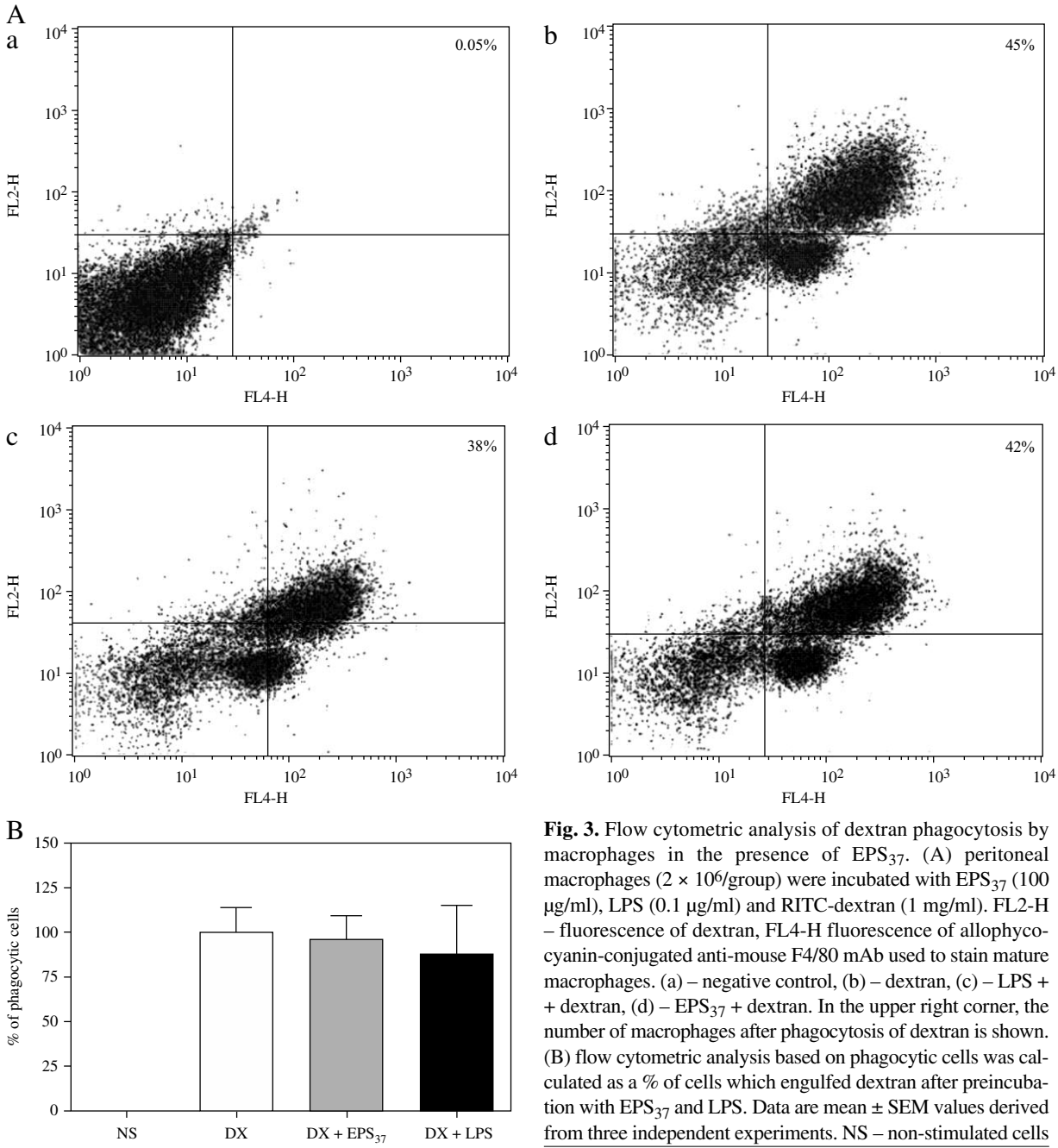

Fig. 3. Flow cytometric analysis of dextran phagocytosis by macrophages in the presence of EPS_{37}. (A) peritoneal macrophages (2×10^6/group) were incubated with EPS_{37} (100 µg/ml), LPS (0.1 µg/ml) and RITC-dextran (1 mg/ml). FL2-H – fluorescence of dextran, FL4-H fluorescence of allophycocyanin-conjugated anti-mouse F4/80 mAb used to stain mature macrophages. (a) – negative control, (b) – dextran, (c) – LPS + + dextran, (d) – EPS_{37} + dextran. In the upper right corner, the number of macrophages after phagocytosis of dextran is shown. (B) flow cytometric analysis based on phagocytic cells was calculated as a % of cells which engulfed dextran after preincubation with EPS_{37} and LPS. Data are mean ± SEM values derived from three independent experiments. NS – non-stimulated cells

phages to synthesize PGE_2 and COX-2 protein. These data are consistent with other studies where LTA, derived from Gram (+) bacteria, exerts also immunostimulatory effects [23-25]. LTA can strongly induce synthesis of PGE_2 and COX-2 not only in immune cells. For example, PGE_2 secretion was observed in human lung epithelial cells and neurons of the cerebral cortex in rats after stimulation with LTA [26, 27]. As immunostimulatory effects depend on both, bacterial cell-wall components and on target cells, we have also examined effects of LTA and EPS_{37} on cytokine production by dendritic cells. We have observed a significant production of IL-12p40 and small amounts of IL-6 in 24-hour DC cultures stimulated with tested agents. There are very few data showing effects of probiotic bacteria components on DC functions and they mainly concern whole bacteria. Weiss *et al.* have shown that probiotic bacteria can interact with DC in two ways. Such strains as *Lactobacillus acidophilus*, *Lactobacillus gasseri*, *Lactobacillus casei* and *Lactobacillus plantarum* induced murine myeloid DC to synthesize IL-12 and TNF-α. They observed the oppo-

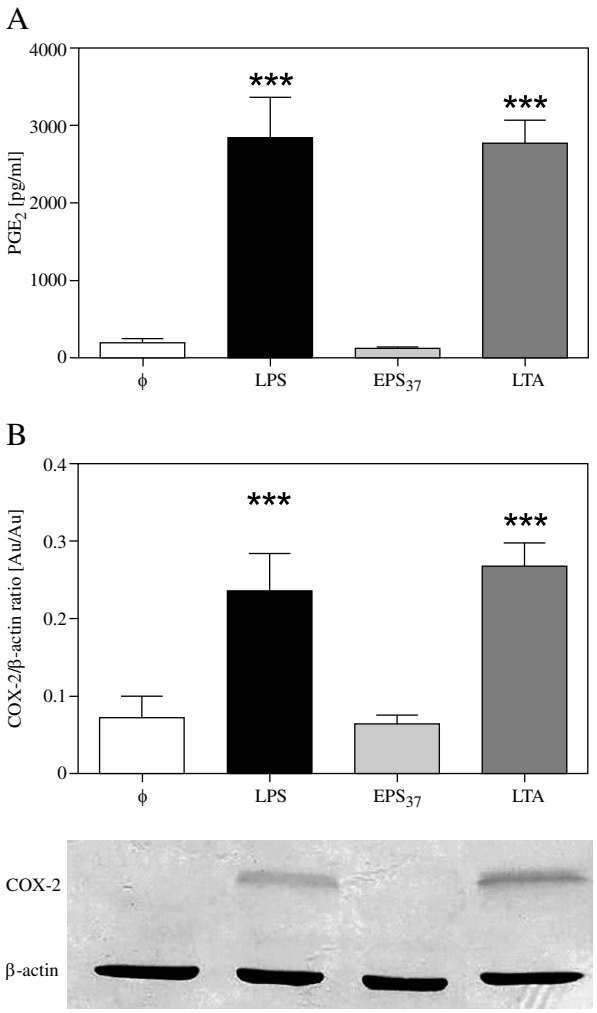

Fig. 4. The effect of EPS_{37} on production of PGE_2 and expression of COX-2 in macrophages. Cells (5×10^5/ml) were cultured in the presence of LPS (0.1 μg/ml), EPS_{37} (100 μg/ml) and LTA (100 μg/ml). After 24 h of stimulation PGE_2 level (A) in culture supernatants and COX-2 level (B) in macrophages were determined as described in Material and methods. Results are expressed as a mean of three independent experiments ± SEM. $*p < 0.05$, $***p < 0.001$ non-stimulated cells versus stimulated cells. NS – non-stimulated cells. Picture shows one selected, representative Western blot analysis

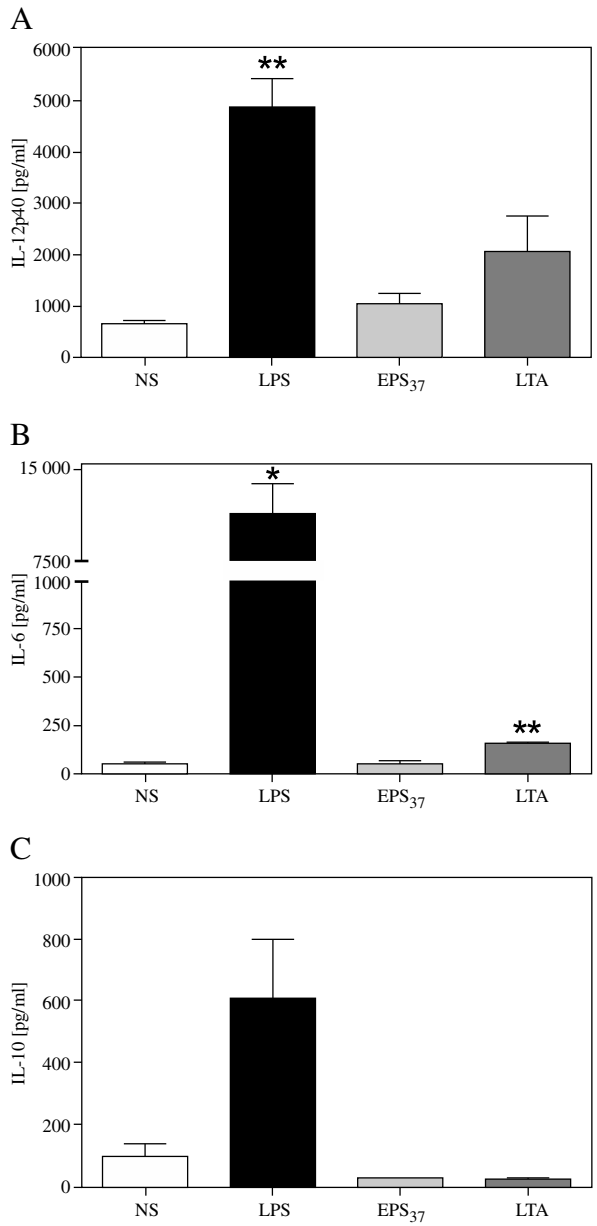

Fig. 5. The effect of EPS_{37} on cytokine production by DC. Cells (6×10^4/ml) were cultured in the presence of LPS (0.1 μg/ml), EPS_{37} (100 μg/ml) and LTA (100 μg/ml). The concentrations of IL-12p40 (A), IL-6 (B) and IL-10 (C) were measured by ELISA in culture supernatants after 24 h of incubation. NS – non-stimulated cells. Results are expressed as a mean of three independent experiments ± SEM. $*p < 0.05$, $**p < 0.01$ non-stimulated cells versus stimulated cells

site effect in the case of use of *Bifidobacterium* bacteria [28]. Evrard *et al.* showed that *L. rhamnosus* Lcr35 increased synthesis of the Th1/Th17 cytokine profile, but had no significant effect on the level of IL-10 [29]. Previously, we have demonstrated the impact of EPS_{37} on humoral immune response in mice. We have shown that EPS_{37} inhibited synthesis of IgG specific to OVA. The effect of EPS was opposite to the effect of LPS. LPS was a strong adjuvant and significantly enhanced OVA-specific response [30]. In this study, we expand our investigations to other OVA-specific IgG isotypes to examine the hypothesis considering polarization of the immune response by the bacterial product. We have found that EPS_{37} diminished the serum level of OVA-specific IgG1 and IgG2a antibodies to the same degree. Moreover, EPS_{37} also inhibited the adju-

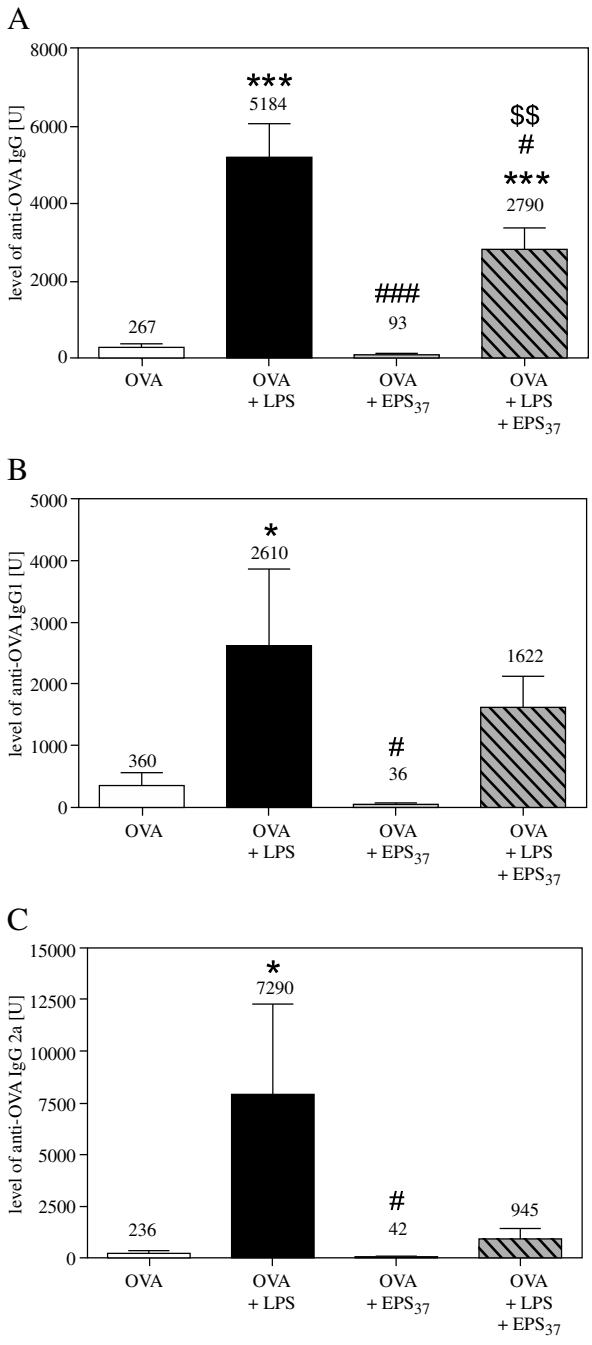

Fig. 6. The effect of EPS_{37} on OVA-specific humoral response. Mice were immunized intraperitoneally with 200 μg OVA together with LPS (1 μg/mouse) or EPS_{37} (50 μg/mouse) on days 0 and 14. The levels of IgG (A), IgG1 (B) and IgG2a (C) specific to OVA were measured by ELISA 7 days after the second immunization. The results are expressed in arbitrary units: 1U = 1/100 titers of IgG anti-OVA ± SEM (n = 10). Results are a mean of two independent experiments. *$p < 0.05$ vs. OVA; ***$p < 0.001$ vs. OVA; #$p < 0.05$ vs. LPS; ###$p < 0.001$ vs. LPS, $$$p < 0.01$ vs. EPS_{37}. The levels of IgGs are shown in numbers

vant effect of LPS in the same way. Therefore, our present results did not show polarizing properties of EPS_{37}, while confirmed its suppressor effect on antigen-specific humoral response in mice.

In conclusion, the present study extends our knowledge concerning biological properties of bacterial EPSs. However, some fundamental questions are still unanswered. We can suggest that pure EPS, at least EPS from *L. rhamnosus* KL37C, shows a very weak, immunostimulatory action if compared with that of LTA (major component of lactobacilli cell wall). One may suggest that it is the effect of lack of EPS-specific PRR receptor (not yet found). On the other hand, EPS is the main component of biofilm matrix. It may suggest its protecting role against immune attack on bacteria hidden in biofilm. However, further studies are necessary to investigate the role of EPS in the interaction between bacteria and the immune system cells. Proper understanding of these interactions may open new strategies for probiotic therapies. Importantly, due to a large heterogeneity of bacterial EPSs, biological properties of each individual bacterial strain must be taken into account. Indeed, we have shown that potential of EPS is strain-dependent. EPSs isolated from three different strains of *Lactobacillus* (*L. reuteri*, *L. johnsonii* and *L. animalis/murinus*) stimulated the murine macrophage to produce both pro- and anti-inflammatory cytokines in a different manner [31].

The authors declare no conflict of interests.

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