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Immunosuppressive effect of systemic administration of *Lactobacillus rhamnosus* KL37C-derived exopolysaccharide on the OVA-specific humoral response

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Abstract

Lactobacilli, including Lactobacillus rhamnosus, commonly used probiotic bacteria, show the ability to produce biofilm. Whole lactic acid bacteria, as well as their cell-wall components show strain specific immunomodulatory properties. Exopolysaccharides (EPSs) are major components of extracellular polymeric substances and the lactobacilli biofilm matrix. However, in contrast to peptidoglycans or teichoic acids, the role of EPS in the modulation of the immune system by lactobacilli is still obscure. Previously, we have shown that crude EPS (cEPS) isolated from L. rhamnosus KL37C can effectively stimulate production of inflammatory mediators by macrophages in vitro. Moreover, EPS inhibits the production of anti-collagen specific immunoglobulin G (IgG) in mice. Interestingly, the reduction of anti-collagen antibodies correlated with the amelioration of collagen-induced arthritis. However, the mechanism of EPS immunosuppressive activity remains unknown. In this paper we examine the ability of EPS to inhibit humoral response to ovalbumin (OVA). The experiments were performed in CBA mice, by immunizing animals with OVA in the presence of EPS or lipopolysaccharide (LPS). The results showed that EPS inhibited humoral response to OVA. Exopolysaccharide injected simultaneously with the antigen decreased the production of anti-OVA IgG, IgG1 and IgG2a antibodies. Interestingly, EPS given together with LPS diminished its adjuvant properties by inhibiting OVA-specific IgG production. Therefore, our data indicate that EPS may exert opposing effects on antibody production in vivo to the adjuvant-like effect of whole lactic bacteria strains, as reported by others. Moreover, this study expands our understanding of a role of EPS in the "cross-talk" between biofilm forming bacteria and the immune system.

Key words: *exopolysaccharides, humoral response, Lactobacillus rhamnosus, LPS, ovalbumin, probiotics.*

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Introduction

Exopolysaccharides (EPSs), major components of extracellular polymeric substances, are produced by bacteria during formation of biofilm [1]. However, in contrast to well-known receptors of the bacteria cell-wall structures, such as Toll-like receptor 4 (TLR-4) for lipopolysaccharide (LPS) of Gram-negative bacteria or TLR-2 for peptydoglycans of Gram-positive bacteria [2], it is not clear whether EPS can be recognized by the pathogen recognition receptors (PRRs). On the other hand, the immunoregulatory potential of EPS from various bacteria has been demonstrated in a number of papers [3-6]. Moreover, it is well established that EPS, a component of biofilm matrix, plays an important role in bacteria immune evasion and resistance to anti-bacterial agents, such as antibiotics and antiseptics [7-9].

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It has been shown that EPSs from different *Lactobacillus* strains not only effectively alter the production of inflammatory mediators by the cells of innate immune system but can also affect antigen-specific humoral response [10, 11].

In our laboratory *Lactobacillus rhamnosus* KL37C, the high-level-EPS producer, have been selected out of several tested lactobacilli for investigations of EPS immunoregulatory properties. Recently, we have shown that crude EPS (cEPS) significantly ameliorates arthritis in the active model of collagen-induced arthritis (CIA) in mice, especially when LPS was used as adjuvant [11]. Independently, other reports have shown that oral administration of *L. rhamnosus* attenuates CIA and inhibits arthritogenic autoantibodies [12]. Therefore, it was reasonable to check whether systemic administration of cEPS affects the development of CIA and depresses the production of anti-collagen IgG. Indeed, cEPS strongly reduced the serum level of total collagen-specific IgG and of both measured subclasses of IgG, namely IgG1 and IgG2a [11]. The inhibitory effect of EPS on the humoral response was also confirmed by others [13].

The main aim of this study was to further evaluate the immunosuppressive potential of crude EPS derived from *L. rhamnosus* KL37C in the OVA-model of antigen-specific antibody production in CBA mice. The effect of crude EPS was compared with that of lipopolysaccharide (LPS) and the selected polysaccharides derived from other sources. Finally, we compared the effect of crude EPS (extracellular polymeric substances) with that of purified form of EPS (EPS_{37}).

Material and methods

Mice

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

EPS isolation

Exopolysaccharides were obtained from *L. rhamnosus* KL37C strain isolated from the faeces of the human newborns and from *Lactobacillus reuteri* 133 from the gastrointestinal tract of Gαi2-deficient mice which showed clinical symptoms of colitis [14, 15]. Briefly, bacteria were 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 8000 rpm (4°C, 30 min) and washed twice with phosphate buffer solution (PBS). Bacterial mass was suspended in water (10 ml) and sonicated three times for 5 min, in an ice bath. After centrifugation at 4000 rpm (30 min, 4°C), the supernatant was centrifuged twice at 12 000 rpm, 4°C for 1 h and then precipitated with five volumes of cold ethanol (–20°C, overnight). The precipitated material was recovered by centrifugation at 12 000 rpm 4°C for 20 min and freeze-dried. Isolation of EPSs was performed by gel filtration on the TSK HW-50 column (1.6 × 100 cm) in 0.05 M aqueous pyridine acetate buffer (pH 5.6). The eluate was monitored with a Knauer differential refractometer. The first fraction, eluted in the void volume, contained polysaccharide (crude EPS) and was the subject of the present investigation.

Purification of EPS_{37}

Purification of crude EPS from *L. rhamnosus* KL37C was performed as described before [16]. The freeze-dried preparation of cEPS 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) with protease from *Streptomyces griseus* (both Sigma-Aldrich, Germany) (37°C, 16 h), then dialyzed against water at 4°C for 24 h. The resuspended polysaccharide was purified by ion-exchange chromatography on DEAE-Sephadex A-25, 1.6 × 20 cm, column (Pharmacia Fine Chemicals, Sweden). The pure EPS_{37} was eluted with 20 mM Tris buffer, pH 8.2. The fractions containing EPS_{37} were pooled, desalted by dialysis against water at 4°C for 24 h and lyophilized.

Mice immunization with OVA

If not stated otherwise, mice were given intraperitoneally injection of OVA (200 mg/mouse) in the presence of LPS (*Escherichia coli* 011 : B4; 1 μg/mouse; both Sigma-Aldrich, Germany), or/and with cEPS or EPS37 or EPS from *L. reuteri* 133 (cEPS133); 50 μg/mouse twice, on day 0 and 14. Seven days after the second immunization mice were bled and serum was collected. Level of OVA-specific antibodies was measured by ELISA.

Measurement of serum OVA-specific antibody titer

The level of antibodies specific to OVA in mouse serum was measured by ELISA test. Briefly, plates were coated overnight with OVA (5 μg/ml). Serial dilutions of mouse serum in PBS were applied to antigen-coated wells for 1 h at room temperature. Immunoglobulins in tested serum samples were detected 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 of differences between groups was analyzed using one-way ANOVA, followed, if significant, by a Tukey test for *post hoc* comparison. Results are expressed as mean ± SEM values. A p-value < 0.05 was con-

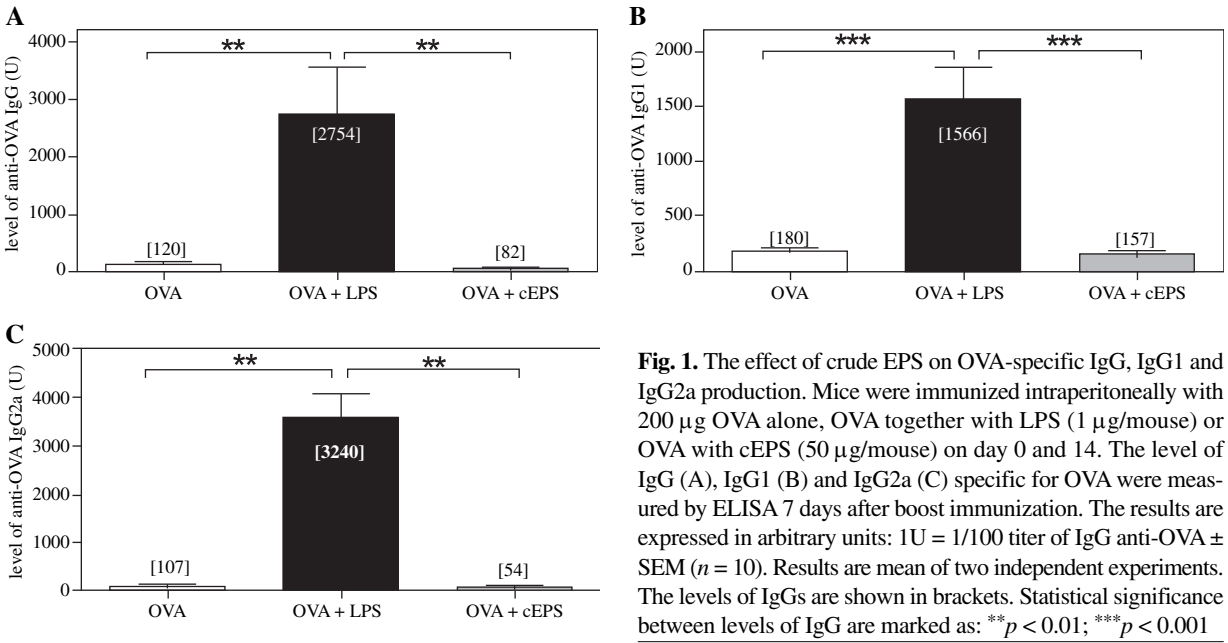

Fig. 1. The effect of crude EPS on OVA-specific IgG, IgG1 and IgG2a production. Mice were immunized intraperitoneally with 200 μg OVA alone, OVA together with LPS (1 μg/mouse) or OVA with cEPS (50 μg/mouse) on day 0 and 14. The level of IgG (A), IgG1 (B) and IgG2a (C) specific for OVA were measured by ELISA 7 days after boost immunization. The results are expressed in arbitrary units: 1U = 1/100 titer of IgG anti-OVA ± SEM (n = 10). Results are mean of two independent experiments. The levels of IgGs are shown in brackets. Statistical significance between levels of IgG are marked as: $^{**}p < 0.01$; $^{***}p < 0.001$

sidered statistically significant. Analysis was performed using Graphpad Prism v. 5.01 (GraphPad Software, Inc., USA).

Results

Effects of crude EPS on the level of OVA-specific IgG

To examine the immunosuppressive potential of crude EPS on humoral response in mice we used the protocol of OVA-specific antibody production and the effect of cEPS was compared with that of LPS. In comparison to the preliminary studies we extended our present investigation on a measurement of IgG subclasses [11]. Lipopolysaccharide, a well-known bacterial stimulator of B cell proliferation [17] behaved as a strong adjuvant in all our experiments, enhancing significantly OVA-specific IgG of all tested subclasses (10-20-fold enhancement). Crude EPS, in contrast to LPS, slightly inhibited the serum concentration of OVA-specific IgG (total IgG, IgG1 and IgG2a), as shown in Fig. 1. We did not find any effect of cEPS on anti-OVA IgM production in our experimental set-up (data not shown). On the other hand, cEPS not only reduced the production of OVA-specific antibodies when injected with OVA alone, but also abolished the adjuvant effect of LPS. Indeed, when cEPS was given simultaneously with antigen and LPS, the serum concentration of anti-OVA IgG was markedly reduced (Fig. 2).

Different routes of EPS injection and the production of OVA-specific IgG

To exclude the effect of local interactions between crude EPS and OVA, the antigen and cEPS were administered by different routes. The results shown in Fig. 3 clearly indicate that cEPS injected intraperitoneally (i.p.) or intravenously (i.v.) leads to reduction of the production of anti-OVA IgG. However, the strongest suppression was observed after intravenous administration of cEPS. By contrast, subcutaneous injection (s.c.) of cEPS only slightly increased the serum level of OVA-specific IgG. In all experiments OVA was applied intraperitoneally.

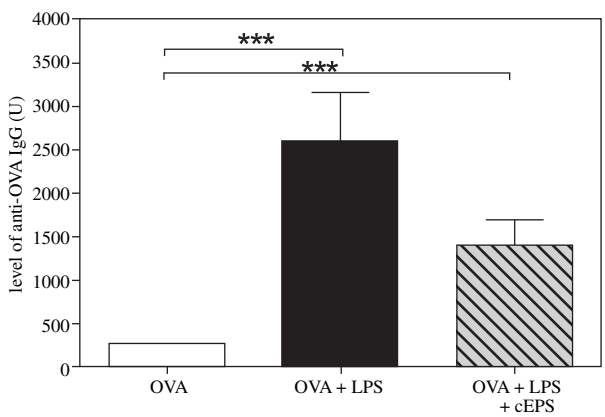

Fig. 2. Anti-adjuvant effect of cEPS. Mice were immunized intraperitoneally with 200 μg OVA alone, OVA together with LPS (1 μg/mouse) or OVA together with LPS and cEPS (50 μg/mouse) on day 0 and 14. The level of OVA-specific IgG was measured by ELISA in individual sera collected 7 days after boost immunization. The results are expressed in arbitrary units representing 1U = 1/100 titer of IgG anti-OVA ± SEM (n = 15). Results represent three independent experiments. Statistical significance between levels of IgG are marked as: $^{***}p < 0.001$.

The ability of EPS to inhibit OVA-specific humoral response is not restricted to cEPS derived from human L. *rhamnosus* KL37C

Previously we have shown that the ability of cEPS from *L. rhamnosus* KL37C to stimulate the production of cytokines by macrophages differs from that of EPSs derived from other *Lactobacillus* strains [10]. To examine whether EPS-dependent suppression of humoral response is also strain-specific we compared the effect of cEPS from *L. rhamnosus* KL37C with that of other crude EPS isolated from mice *L. reuteri* 133 and human *L. rhamnosus* GG. As shown in Fig. 4, the production of total IgG anti-OVA was markedly inhibited by $cEPS_{133}$. The similar suppressor effect was achieved when we used EPS from *L. rhamnosus* GG (data not shown). Nevertheless, the strongest reduction of serum OVA-specific IgG was caused by cEPS from *L. rhamnosus* KL37C.

Effects of crude and purified EPSs on the OVA-specific IgG production

Finally, we compared the suppressor potential of purified EPS (EPS_{37}) with that of the cEPS to examine whether the polysaccharide, but not the other components of crude EPS is responsible for the reduction of OVA-specific IgG production. Administration of EPS_{37} together with OVA during the primary and secondary immunization resulted in the inhibition of antigen-specific humoral response to a similar extent to that observed after administration of cEPS. Moreover, EPS_{37} showed also anti-adjuvant effect by neutralization of the LPS-dependent enhancement of serum anti-OVA IgG (Fig. 5). Thus, these data suggest that the contribution of other components of extracellular polymeric substances, such as proteins, nucleic acids, are negligible in the observed suppression of OVA-specific humoral response.

Discussion

Lactic acid bacteria, the major probiotic bacteria, show strain-specific intrinsic immunoregulatory properties as was reported in a number of papers [18, 19]. Wide spectrum of lactobacilli immunoregulatory activities includes a modulation of effector and regulatory T cell subsets, interactions with dendritic-cells, macrophages and B cells, with the mucosal tissue-associated cells especially [19-23]. Indeed, it has been demonstrated that the oral administration of some lactobacilli strains can induce oral tolerance. Namely, total and the antigen-specific IgE production was inhibited in atopic dermatitis model NC/Nga mice [24]. On the other hand, some lactobacilli strains were used as adjuvants to enhance the production of IgA in the MALT system [25, 26]. Moreover, lactobacilli have been shown to increase the immunogenecity of orally administered vaccines (e.g. rotavirus, cholera vaccines) [27, 28]. Such distinct and even

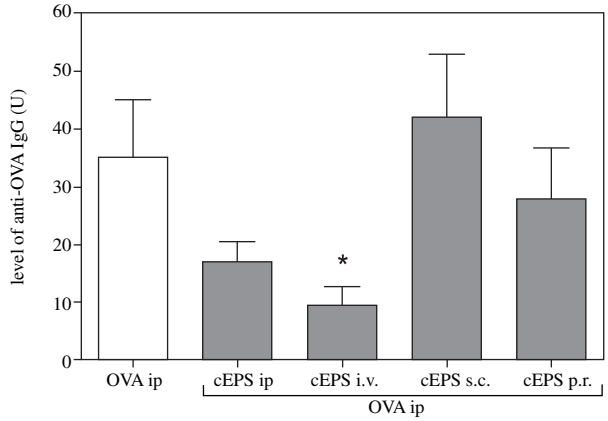

Fig. 3. Effects of crude EPS on the level of OVA-specific IgG – alternative routes of cEPS injection. Mice were immunized with 200 μg OVA alone or OVA together with cEPS (50 μg/mouse) on day 0 and 14. cEPS was administered intraperitoneally (i.p.), intravenously (i.v.), subcutaneously (s.c.) or rectally (p.r.). In all cases OVA was administered intraperitoneally. The level of OVA-specific IgG in serum from individual mice was measured by ELISA 7 days after boost immunization. The results are expressed in arbitrary units representing 1U = 1/100 titer of IgG anti-OVA ± SEM ($n = 5$). The results show one representative experiment. $^{*}p < 0.05$ OVA vs. OVA + cEPS i.v.

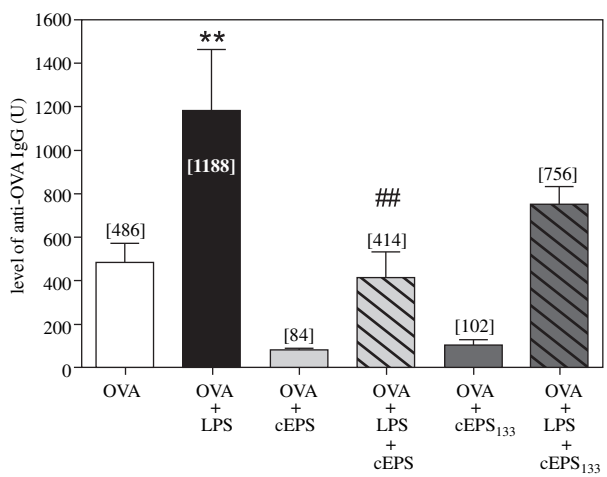

Fig. 4. The ability to inhibit OVA-specific humoral response is not restricted to cEPS from *L. rhamnosus* KL37C. Mice were immunized intraperitoneally with 200 μg OVA alone, OVA together with LPS (1 μg/mouse) or OVA with cEPS (50 μg/mouse) or OVA with cEPS133 (50 μg/mouse) on day 0 and 14. The level of IgG specific for OVA were measured by ELISA 7 days after boost immunization. The results are expressed in arbitrary units representing 1U = 1/100 titer of IgG anti-OVA ± SEM ($n = 5$). The results show one representative experiment. The levels of IgG are shown in brackets $^{**}p < 0.01$ OVA vs. LPS; $^{\#\#}p < 0.01$ OVA + LPS vs. OVA + LPS + cEPS

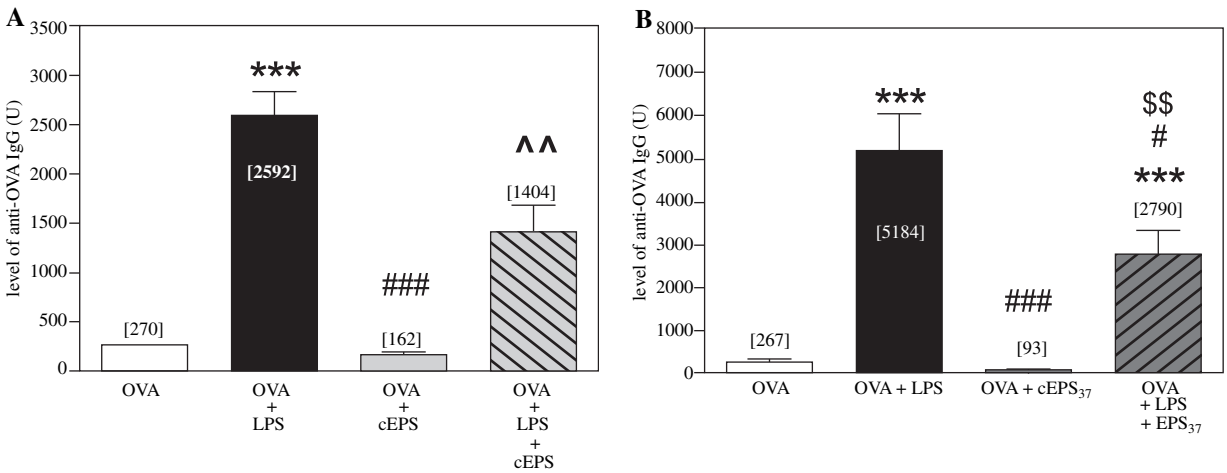

Fig. 5. Comparison of the effect of crude EPS (A) and purified EPS_{37} (B) on OVA-specific antibody production. Mice were immunized intraperitoneally with 200 μg OVA alone, OVA together with LPS (1 μg/mouse) or OVA with cEPS (50 μg/mouse) or OVA with EPS_{37} (50 μg/mouse) on day 0 and 14. The level of IgG specific for OVA were measured by ELISA 7 days after boost immunization. The results are expressed in arbitrary units representing 1U = 1/100 titer of IgG anti-OVA ± SEM ($n = 10$). Results represent the mean of two independent experiments. The levels of IgG are shown in brackets. $^{***}p < 0.001$ vs. OVA; $^{\#}p < 0.05$ vs. LPS; $^{\#\#\#}p < 0.001$ vs. LPS; $^{\wedge\wedge}p < 0.01$ vs. cEPS; $^{\$\$}p < 0.01$ vs. EPS_{37}

opposite immunomodulatory effects (enhancement vs. suppression of humoral response) of various lactobacilli may be explained by an extreme structural diversity of the cell wall components, especially due to the presence of different sugar monomers [29, 30]. Interestingly, it has been documented that EPSs, the major components of lactobacilli biofilm matrix, show a significant impact on lactic bacteria immunoregulatory properties, including enhancement of the humoral response [3]. Moreover, to our knowledge, apart from our investigations [11], only a few reports have described the immunosuppressive effects of bacterial EPSs [5, 31]. All these data support a common opinion that biological functions of lactobacilli (EPSs) are not only strain-specific but the final effect also depends on a route of bacteria (EPS) administration, i.e. versus oral administration [32]. However, detailed mechanism of action of EPSs remains unclear.

In the present study we examined the suppressor activity of crude EPS derived from *L. rhamnosus* KL37C in the OVA-model of antigen-specific antibody production in CBA mice. In our basal experimental system mice were injected intraperitoneally with the antigen (OVA), adjuvant (LPS) and cEPS. Therefore, in contrast to other experimental models, restricted to oral administration of both EPS and antigen [33, 34] we could observe the effects of EPS on the systemic immune system. Importantly, we used the same route of OVA immunization, as the one we used previously for collagen to induce the CIA. Our present data indicate that cEPS inhibits the production of OVA-specific IgG in a similar way as it suppressed the production of anti-collagen IgG in the CIA [11]. In both unrelated antigenic systems the strongest reduction of serum level of antigen-specific antibodies was observed in IgG2a subclasses. It suggests that a polarization of T helper subsets occurs under the influence of cEPS. Moreover, the present study confirmed "anti-adjuvant" properties of cEPS as it was able to neutralize the LPS-dependent enhancement of OVA-specific IgG production.

Nevertheless, it is still not known how cEPS attenuates the activity of LPS and reduces the serum level of antigen-specific IgG. We can speculate that at least two independent mechanisms may be responsible for the cEPS-dependent suppression of OVA-specific IgG production. Firstly, cEPS may interact directly with LPS reducing its contact with the antigen presenting cells. Secondly, cEPS may induce T regulatory cells (e.g. T suppressor cells in the spleen) and indirectly inhibit LPS-dependent proliferation of B cells. However, to confirm the above hypothesis we need stronger experimental data.

Importantly, in the present study we have shown for the first time that purified EPS_{37} exerted the same inhibitory properties as the crude EPS. It is of importance for further investigations as it would be easier to determinate receptor(s) specific for extracellular polysaccharides, especially for those which are components of a biofilm matrix, using a purified EPS.

In conclusion, further studies are necessary to confirm the hypothesis on the mechanism of cEPS-dependent suppression, to decide whether biofilm components such as EPS interact with PRRs of the cells of innate immune system and whether EPS may be used as immunomodulator with defined properties. Finally, in our opinion, a major gap

in our knowledge about mechanisms of immunomodulation by probiotics (including biofilm EPS) concerns their diversity and their fate *in vivo*. Therefore, in the future, immunoregulatory functions of bacterial (probiotic) polysaccharides of a well-characterized structure should be examined *in vitro* and then confirmed in animal experimental models.

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