

Short Communication

Exopolysaccharide from *Lactacaseibacillus rhamnosus* induces IgA production in airways and alleviates allergic airway inflammation in mouse model

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The currently observed high prevalence of allergic diseases has been associated with changes in microbial exposure in industrialized countries. Defined bacterial components represent a new strategy for modulating the allergic immune response. We show that intranasal administration of exopolysaccharide (EPS) isolated from *Lactacaseibacillus* (*L.*) *rhamnosus* LOCK900 induces TGF- β 1, IgA, and regulatory FoxP3⁺ T-cells in the lungs of naïve mice. Using the ovalbumin mouse model, we demonstrate that intranasal administration of EPS downregulates the development of allergic airway inflammation and the Th2 cytokine response in sensitized individuals. At the same time, EPS treatment of sensitized mice, similar to EPS-induced responses in naïve mice, significantly increased the level of total, OVA-specific, and also bacteria-specific IgA in bronchoalveolar lavage and the number of IgA-producing B-cells in the lung tissue of these mice. Thus, EPS derived from *L. rhamnosus* LOCK900 can be considered a safe candidate for preventing the development of allergic symptoms in the lungs of sensitized individuals upon exposure to an allergen.

Keywords: Allergic airway inflammation • Exopolysaccharide • IgA antibody • Immunomodulation • *Lactacaseibacillus*



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Airway allergies and asthma affect more than 30% of people in Western and highly industrialized countries. The rapid increase in prevalence has been related to lifestyle changes and improved

sanitation over the past 70 years. This fact has been put forward by the so-called “hygiene hypothesis”, which states that our immune system develops inadequately or detrimentally with poor regulatory mechanisms due to the reduced exposure to bacterial antigens [1]. Microbiota dysbiosis or lack of certain bacterial species such as lactobacilli have been reported in allergic patients [2]. This observation creates rationale for supplementation of these bacteria to allergic patients in order to dampen the aberrant immune responses. While there are few reports on the

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beneficial effects of orally administered probiotics on the ongoing allergic response in the airways, bacterial therapy that directly targets the immunoregulatory mechanisms in the lung mucosa seems to be more promising [3]. In contrast to the use of live bacteria which could induce adverse immune responses, bacteria-derived defined molecules have been shown to be promising candidates for the management of immunoallergic responses in mouse models of airway inflammation [4–6].

Microbiota-induced secretory IgA plays an indispensable role in maintaining mucosal tolerance by attenuating immune responses, suggesting its protective role in the allergic inflammatory response in both human patients and mouse models [7]. Impaired IgA-microbiota interactions in the gut or even IgA deficiency have been associated with increased risk of allergy and asthma in children [8, 9]. While the mechanisms of interactions between microbiota and IgA in the intestinal mucosa have been extensively described [10], the formation of IgA in the airway mucosa by commensal bacteria or their antigens is not well documented.

We have recently investigated the effect of *L. rhamnosus* LOCK900 and LOCK908 and *L. paracasei* LOCK919 on allergic sensitization in gnotobiotic mouse model showing that colonization by three lactobacilli-mixture dampens the allergic sensitization by stimulation of TGF- β 1 and IgA responses [11]. Next, we have isolated two exopolysaccharides (L900/2; 830 kDa and L900/3; 18 kDa) produced by *L. rhamnosus* LOCK900 strain, determined their structures, and proved their potential to modulate immune response to a bystander antigen in bone marrow-derived dendritic cells [12] and in a mouse model of ovalbumin (OVA) sensitization [13]. Here, we investigate the potential of intranasal administration of exopolysaccharide (EPS) L900/2 to stimulate the regulatory response in the lung and prevent the development of an allergic immune response in the airways after allergen challenge in sensitized mice.

Results and discussion

Intranasal EPS administration induces cellular and humoral immune responses in lungs

BALB/c mice were intranasally treated by 20 μ g of EPS (EPS group) or PBS (SHAM group) for four consecutive days. Mice were sacrificed 24 h after the last treatment (Fig. 1A), and cell infiltration and IgA production were analyzed in bronchoalveolar lavage (BAL) and lung tissue. Histological analysis of lung tissue (Fig. 1B) revealed minor cellular infiltration in perivascular space in EPS-treated mice (Fig. 1B). By cell differential analysis of BAL, we detected elevated levels of neutrophils, lymphocytes, and monocytes/macrophages, suggesting the induction of cellular immunity in response to EPS intranasal administration (Fig. 1C). Flow cytometry analysis of cells from lung digests confirmed the elevated presence of neutrophils (Supporting information Fig. S1A and B), dendritic cells (DCs; Fig. 1D, Supporting information Fig. S1C), and T-lymphocytes (Supporting information Fig. S1D

and E). Interestingly, besides upregulating DC counts, EPS treatment also increased the expression of MHCII molecules on DCs (Fig. 1E, Supporting information Fig. S1C), suggesting the activation of antigen presentation process to T- and B-lymphocytes. It was shown that microbiota or bacteria-derived antigens were able to activate the process of DC maturation to regulatory state with abundant production of IL-10 or TGF- β 1 cytokines [14–16]. Using flow cytometry, we detected twofold elevated amount of regulatory CD4⁺FoxP3⁺ T-cells (Fig. 1F, Supporting information Fig. S2A) with significantly upregulated number of TGF-latency-associated peptide (LAP, form of TGF- β 1)-expressing Treg cells (Fig. 1G, Supporting information Fig. S2A). Moreover, we detected significantly increased levels of TGF- β 1 in BAL fluid and lung cell cultures from EPS-treated mice (Fig. 1H and I). Our results are in line with other authors [15] showing that polysaccharide CSGG isolated from different bacterial species *Bifidobacterium bifidum* is able to induce the generation of FoxP3⁺ Treg cells via a DC-dependent mechanism.

It is widely documented that intestinal microbiota is able to upregulate the production of TGF- β 1 from Treg and other cells to promote the IgA class-switching of B-cells within the intestinal mucosa [17]. Here, we found that the percentage but not the absolute numbers of B-lymphocytes from CD45⁺ cells was downregulated (Supporting information Fig. S1F). On the other hand, in line with the increased TGF- β 1 after EPS administration (Supporting information Fig. S1D and F), we found increased numbers of IgA-positive B-cells (Fig. 1J and Supporting information Fig. S1D) in lung tissue. Concomitantly, the level of total IgA in BAL fluid (Fig. 1K) as well as in supernatants from culture of lung cell suspension (Fig. 1L) was significantly increased in EPS-treated mice. These results corroborate with the study of Ruane et al. [16] showing that microbial stimuli induced TGF- β 1 production by lung DCs affected the IgA class-switch recombination on B-cells in lungs. To find out the specificity of EPS-induced IgA in lungs, we cultured cells from lung tissue homogenates isolated from naïve specific-pathogen-free (SPF) and germ-free (GF) mice with increasing concentrations of EPS. Although total IgA levels increased in supernatants of cultures from SPF mice in response to EPS dose, we observed only negligible IgA stimulation in naïve cells from GF mice (Fig. 1L), confirming previous results that intranasally immunized GF mice failed to generate specific IgA, unlike SPF age-matched controls [16]. The fact that we could not detect *L. rhamnosus* in the feces of the experimental mice (Supporting information Fig. S3) may suggest that the IgA response to EPS requires the prior encounter with a complex commensal microbiota that ensures the maturation of the immune system.

Intranasal EPS administration downregulates allergic airway inflammation

Having established the EPS immunomodulatory properties, we next tested the ability of EPS administration to alleviate allergic Th2 inflammation in lungs in mouse model of airway allergy. Female BALB/c mice were sensitized by two intraperitoneal

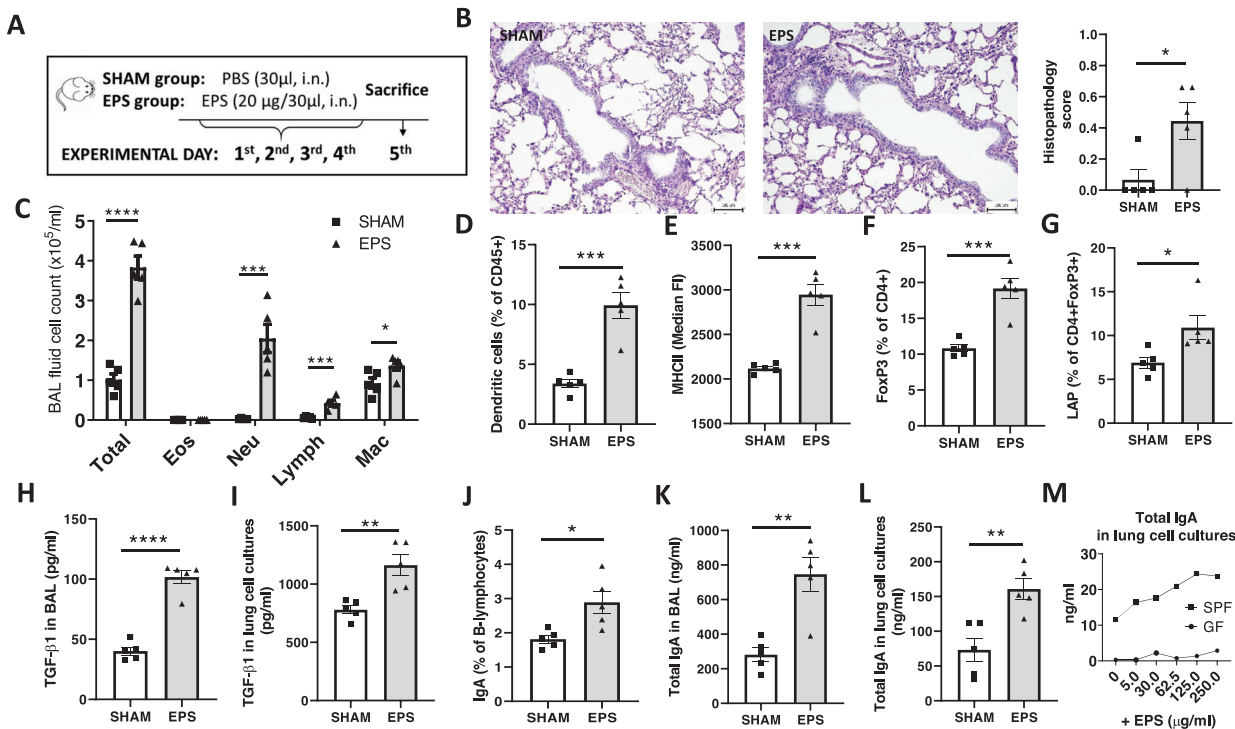


Figure 1. Intranasal administration of EPS stimulates regulatory cellular and humoral immune responses in lung tissue. (A) Experimental design of EPS intranasal administration. BALB/c mice received intranasally 20 µg of exopolysaccharide from *Lactocaseibacillus rhamnosus* L900 (EPS group, $n = 5$) in 30 µL PBS for four consecutive days. SHAM controls ($n = 5$) received 30 µL PBS. (B) Histological evaluation of changes in lung tissue (magnification 200 $\times$, scale bar 100 µm). (C) Analysis of total and differential cell count in bronchoalveolar lavage (BAL). (D) Flow cytometry analysis of dendritic cells as % of CD11c⁺MHCII⁺ cells from CD45⁺ cells. (E) MHCII expression on CD11c⁺MHCII⁺ cells expressed as median fluorescence intensity (FI). (F) Percentage of FoxP3⁺ T-cells analyzed by flow cytometry analysis and gated from CD4⁺. (G) Flow cytometry analysis of latency associated peptide (LAP, latent form of TGF-β1)-expressing cells gated from CD4⁺FoxP3⁺ cells. (H) Level of TGF-β1 cytokine was determined in BAL by ELISA and expressed as pg/mL. (I) Level of TGF-β1 cytokine was determined in supernatants of lung cell cultures by ELISA and expressed as pg/mL. (J) Percentage of IgA-expressing B-lymphocytes determined by flow cytometry in lung cell homogenates. (K) Level of total IgA determined in BAL by ELISA and expressed as ng/mL. (L) Levels of total IgA in cultures of lung cell homogenates. (M) Lung cells were isolated from SPF ($n = 3$) and germ-free (GF) ($n = 3$) mice, pooled and stimulated by increasing concentration of EPS (µg/mL) for 72 h. Levels of total IgA were analyzed by ELISA and expressed as ng/mL in supernatants of cultures. All data are biological replicates expressed as mean $\pm$ standard error of mean (SEM) from one experiment. Unpaired t-test was used for comparison between experimental groups of mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

injections of OVA/Alum mixture, and airway inflammation was induced by four intranasal OVA challenges. Four hours before each challenge, mice received intranasally either EPS or saline (Fig. 2A). Levels of total and OVA-specific IgE and IgG antibodies in sera were unaffected by EPS treatment (Supporting information Fig. S4A and B), suggesting that the EPS administration was not able to reverse the sensitization-established Th2 systemic humoral response. On the other hand, we observed markedly downregulated OVA-specific Th2 cytokines (IL-4, IL-5, IL-13) in splenocyte cultures (Supporting information Fig. S5). Directly in lung tissue, intranasal EPS treatment was able to reduce eosinophilia without affecting other cell populations (Fig. 2B). Histological analysis of lung tissue showed a reduction in leukocyte infiltration in the perivascular, peribronchiolar, and alveolar spaces and a reduction in mucus-producing cells in the airways of EPS-treated mice (summarized in the histopathological evaluation in Fig. 2C). Similar to the cytokine recall response in splenocyte cultures, EPS treatment downregulated the Th2 cytokines (IL-4, IL-5, IL-13) in the lungs, both at the level of gene expression (Fig. 2E) and protein concentration in BAL fluid (Fig. 2F).

Interestingly, EPS treatment did not induce IFN- γ (Th1) or IL-17 (Th17) cytokine responses (Supporting information Fig. S6A and B), but it increased TGF-β1 gene expression and cytokine production in lung tissue (Fig. 2E and F). These results are reminiscent of the recently reported intranasal administration of EPS isolated from *Bifidobacterium longum* 35624, which was able to downregulate Th2 immune response as well as lung eosinophilia in mouse model of airway inflammation [5]. Along the same lines, EPS-deficient variant of *Bacillus subtilis* failed to prevent eosinophilia in murine house dust mite allergy model [18], emphasizing the important role of polysaccharide bacterial surface component in modulation of Th2 response.

EPS administration activates IgA response in the lungs of sensitized mice

The levels of total and OVA-specific IgE in lungs of mice were affected only by OVA sensitization and challenge and not by EPS administration (Supporting information Fig. S7A and B). On the

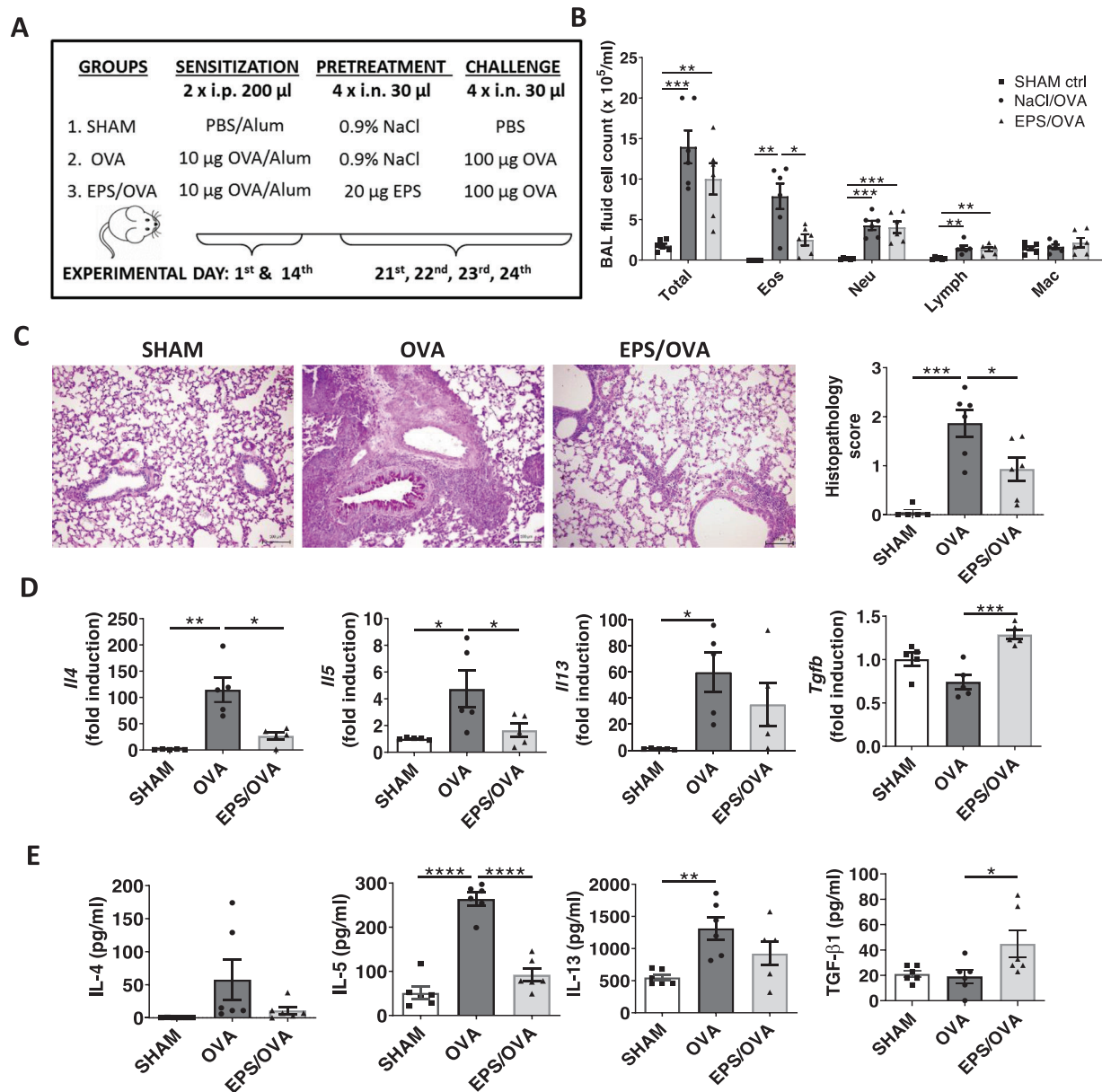


Figure 2. Intranasal administration of EPS during challenge phase downregulates allergic inflammation. (A) Timeline for OVA sensitization, EPS treatment, and OVA challenge. Eight-week-old BALB/c mice were intraperitoneally (i.p.) sensitized with 10 µg of OVA + Alum on day 1 and 14 of experiment. Seven days after second immunization, the intranasal (i.n.) challenge with 100 µg of OVA was performed for four consecutive days. Saline (SHAM, $n = 6$; OVA, $n = 6$) or exopolysaccharide from *Lactocaseibacillus rhamnosus* LOCK900 (EPS/OVA, $n = 6$) was administered intranasally 4 h before each OVA-challenge. (B) Total and differential cell counts were determined in bronchoalveolar lavage. (C) Representative periodic acid-Schiff staining of lung sections from SHAM, OVA-sensitized, and challenged mice pre-treated with saline or EPS (scale 100 µm). Histopathological analysis of airway inflammation was performed in lung tissue. (D) Gene expression of cytokines (IL-4, IL-5, IL-13, and TGF-β1) in lung tissue was normalized using the $2^{-\Delta\Delta C_t}$ method, and data are expressed as fold induction to SHAM control ($n = 5$ per group). (E) Levels of IL-4, IL-5, IL-13, and TGF-β1 cytokines were determined in bronchoalveolar lavage by ELISA ($n = 6$ per group). Three independent experiments were performed with similar results. All data are biological replicates expressed as mean $\pm$ standard error of mean (SEM) from one representative experiment. One-way ANOVA with Tukey's multiple comparison test was used for comparison between experimental groups of mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

other hand, similarly to naïve mice, EPS treatment significantly increased the production of total IgA (Fig. 3A), as well as OVA-specific and bacteria-specific IgA in BAL fluid (Supporting information Fig. S7C and D). Moreover, we detected increased IgA levels in the supernatants of lung cell cultures (Fig. 3B) as well

as percentage of B-lymphocytes from CD45⁺ cells (Fig. 3C) in lungs of EPS-treated mice compared to controls. By immunohistochemistry, we detected twice as many IgA-producing cells in the lung tissue in the peribronchial space of EPS-treated mice compared with both the SHAM and the allergic controls (Fig. 3D

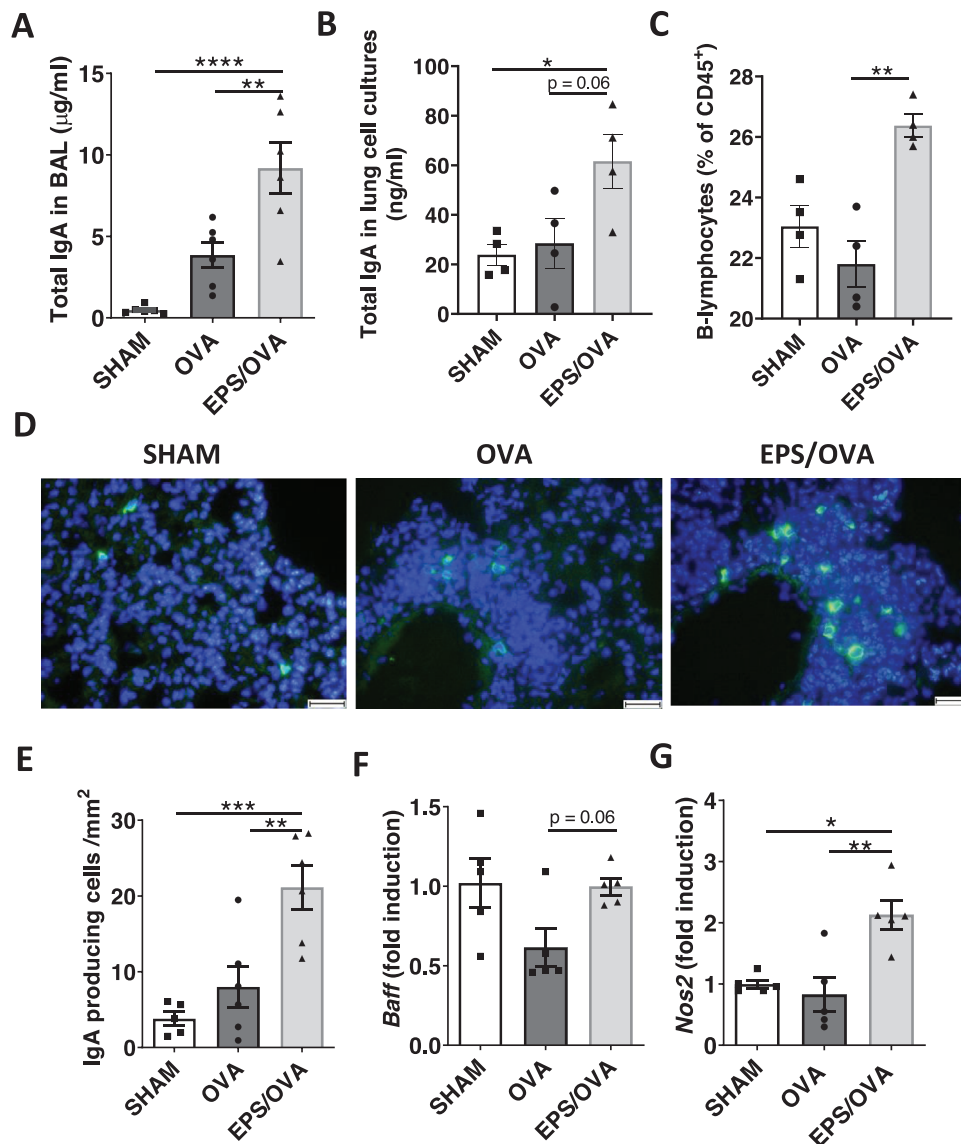


Figure 3. Intranasal administration of EPS activates polyspecific IgA response in Th2-primed lung tissue. (A) Total IgA responses were determined in bronchoalveolar lavage ($n = 5$ per group) and (B) supernatants of lung cell cultures of mice by ELISA ($n = 4$ per group). (C) Number of B-lymphocytes were analyzed in lung cell homogenates of mice by flow cytometry and shown as % from CD45⁺ cells ($n = 4$ per group). (D) Immunohistochemical analysis of IgA-producing cells. Representative lung tissue cryosections (scale 40 μ m) were stained with anti-mouse IgA-FITC antibody (green) and nuclei were counterstained by DAPI (blue). (E) IgA-positive cells were quantified per mm² of cryosections. (F) B-cell activating factor of the TNF family and (G) NOS2 costimulatory factors were determined by qPCR analysis in lung tissue; data were normalized using the $2^{-\Delta\Delta C_t}$ method and expressed as fold induction compared to SHAM control ($n = 5$ per group). Three independent experiments were performed with similar results. All data are biological replicates expressed as mean $\pm$ standard error of mean (SEM) from one representative experiment. One-way ANOVA with Tukey's multiple comparison test was used for comparison between experimental groups of mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

and E). Increased levels of IgA together with amelioration of allergic inflammation corroborate with findings by Fagerås et al., who showed that high SIgA levels were associated with less development of allergic symptoms in sensitized Swedish children [19]. Recently, Oh et al. showed that mucosal IgA secretion was observed after local (intranasal) but not systemic immunization with influenza virus in murine model and that the IgA secretion is provided by established lung-resident antibody-secreting cells [20]. Given the short time of intranasal EPS administration (only 4 days before sacrifice), the EPS-stimulated produc-

tion of polyspecific IgA happened more likely through a T-cell-independent (TI) pathway rather than through specific *de novo* class switch recombination [17]. B-cell activating factor of the TNF family and inducible nitric oxide synthase (NOS2) has been described as costimulatory factors of the TI pathway [17]. Along these lines, we found that both B-cell activating factor of the TNF family and NOS2 were upregulated in EPS-treated mice compared to allergic control (Fig. 3F and G). These data, together with increased TGF- β 1 production in lungs, support the TI pathway of IgA production as a result of EPS stimulatory activity.

Concluding remarks

Here, we show for the first time that intranasal administration of exopolysaccharide isolated from commensal *Lactocaseibacillus* strain stimulates the regulatory immune response and activates the polyspecific IgA production in the airways. Moreover, the EPS intranasal administration is able to downregulate the Th2-biased immunity in lungs of already sensitized individuals to protect against the allergic inflammation induced by intranasal allergen challenge.

Materials and methods

Bacteria and exopolysaccharide preparation

Isolation and purification of EPS from slime of *L. rhamnosus* LOCK900 was performed according to the methods described in Gorska et al. [12]. For the determination of *L. rhamnosus*-specific IgA, the *L. rhamnosus* strain was cultivated aerobically at 37°C for 48 h in MRS broth (Oxoid, UK) and inactivated in 1% formaldehyde-phosphate buffered saline (PBS) as described previously [11]. Bacterial concentration of 1×10^6 CFU/ well was used to coat the ELISA plates.

Animals

SPF female BALB/c mice (6–8 weeks old) were maintained in individually ventilated cages (IVC, Tecniplast, Italy). GF female BALB/c mice (8 weeks old) were kept under sterile conditions in Trexler-type plastic isolators, and the absence of aerobic and anaerobic bacteria, molds, and yeasts was confirmed every 2 weeks using standard microbiological procedures. SPF and GF mice were maintained on a 12-h light–dark cycle with free access to water and sterile irradiated diet (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany), irradiated bedding (Safe, Rosenberg, Germany) with enrichment nestlets (Ancare, NY, USA), or mouse house (Tecniplast, Italy). Animal experiments were approved by the Committee for the protection and use of experimental animals of the Institute of Microbiology, The Czech Academy of Sciences (Nr. 5/2019 and 91/2019).

Experimental design

To evaluate the humoral and cellular immune response to intranasal administration of EPS, female BALB/c mice (8 weeks of age) were anesthetized with 3% isoflurane and 20 µg of EPS in 30 µL of PBS (EPS group, $n = 5$) or 30 µL of PBS (SHAM group, $n = 5$) was administrated intranasally for four consecutive days. Next day were mice sacrificed and bronchoalveolar lavage and lung lobes for histopathological and flow cytometry analysis were harvested (Fig. 1A).

The mouse model of airway inflammation was performed as described previously [21]. Mice ($n = 4$ –10 per group) were divided into three experimental groups: SHAM, OVA, and EPS/OVA. Mice were two times sensitized by intraperitoneal (i.p.) injections of 10 µg OVA (Sigma Aldrich, grade V, USA) mixed with PBS and adsorbed on 0.65 mg of Alum (SERVA Electrophoresis GmbH, Germany) in a final volume of 200 µL on day 1 and 14. Seven days after the second immunization, mice were anesthetized with 3% isoflurane and challenged by intranasal (i.n.) administration of 100 µg OVA in PBS (OVA and EPS/OVA groups) or PBS alone (SHAM) at a dose of 30 µL. The procedure was repeated each day for four consecutive days. Four hours before each OVA-challenge application, mice were anesthetized with 3% isoflurane and 30 µL of 0.9% NaCl (SHAM and OVA groups) or 20 µg EPS mixed with 0.9% NaCl (EPS/OVA group) were applied intranasally (Fig. 2A). Mice were anesthetized with 3% isoflurane and euthanized the day after the fourth challenge and samples (serum, spleen, bronchoalveolar lavage [BAL], and lung lobes) were collected. The experiment was repeated three times yielding similar results; data from a representative experiment are shown.

Analysis of bronchoalveolar lavage

BAL fluid was collected from each animal by cannulation of the exposed trachea and gentle flushing of the lungs with 1 mL of sterile Dulbecco's Phosphate Buffered Saline (D-PBS, Gibco, USA). BAL fluid was centrifuged ($400 \times g$, 7 min, 4°C), and supernatant was collected and stored at -40°C . The cell pellet was recovered for cellular analysis by 200 µL of RPMI medium (Sigma-Aldrich). After cell counting, cells were spun down onto a microscope slide, fixed with methanol, and stained with the Dip-Quick-stain kit according to manufacturer's instructions (Medical Products, Czech Republic). Differential cell count was determined using standard morphologic criteria via light microscopy. The levels of IL-4, IL-5, IL-13, IL-17, and IFN- γ were determined using a Mouse Cytokine/Chemokine Multiplex Immunoassay (Millipore-Sigma, MO, USA), and TGF- β 1 was determined using ELISA kit (R&D Duoset System, Minneapolis, MN, USA) according to the instructions of the manufacturer.

Histopathological evaluation of lung inflammation

Small lung lobes were fixed in 4% paraformaldehyde for 24 h and stored in 80% ethanol. After embedding in paraffin, 5-µm-thick sections were treated with periodic acid-Schiff (PAS) staining. Histological pathology scoring was evaluated using light microscopy (scale 100 µm, magnification 200 $\times$) according to Pyclik et al. [21]: (i) perivascular and peribronchiolar inflammation (grade: 0 = no changes; 1 = few perivascular and peribronchiolar inflammatory cells; 2 = moderate numbers of cell infiltrations on several perivascular and peribronchiolar sites; 3 = large number of diffuse infiltrated cell cuffs), (ii) the presence of leukocytes in alveolar spaces (grade: 0 = no cells; 1 = 2–4 cells; 3 = 4–10 cells;

3 = more than 10 cells), and (iii) number of PAS positive cells per 50 counted bronchoalveolar epithelial cells (0 = no cells; 1 = <12; 2 = 12–25; 3 = >25). The histopathological score is expressed as a sum of single scores divided by 3. Altogether five fields of the slide per mouse was analyzed and mean was used for statistic analysis.

RT-qPCR analysis

Total RNA was isolated from lung samples using the NucleoSpin RNA kit (Macherey-Nagel, Germany) according to the manufacturer's instruction. Note that 1 µg of isolated RNA was reverse transcribed using random primers and SuperScript II RT (Thermo Fisher Scientific, USA). RT-qPCR reaction was performed using Limunaris HiGreen Fluorescein qPCR Master Mix (Thermo Fisher Scientific, USA) with IQ5 Realtime PCR Bio-rad Thermal Cycler (Biorad Laboratories, USA). Expression of target genes was normalized to tubulin-binding protein (*Tbp*) using the $2^{-\Delta Ct}$ method, and data were shown as fold induction to SHAM control. Primers are listed in Supporting Information Table S1.

Lung tissue cell homogenates

The right lung lobe was harvested from mice to 3 mL of incomplete RPMI medium (2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, free of FCS; Sigma-Aldrich, USA) and kept on ice until processing. The lung lobe was rigorously washed in incomplete RPMI, medium was removed, the tissue was minced, and incubated in incomplete RPMI containing 0.05 mg/mL Liberase TL (Roche, Mannheim, Germany) and 0.5 mg/mL DNase (Sigma-Aldrich) for 45 min. Digested lung tissue was forced through a 0.70 µm cell strainer, and red blood cells were lysed with ammonium-chloride-potassium buffer. After lysis, cells were resuspended in complete RPMI (10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin; Sigma-Aldrich) and plated out in 96-well plates (5×10^5 cells/well). Cells were incubated for 72 h (37°C/ 5% CO₂ atmosphere) in 200 µL of complete RPMI. For analysis of total IgA, pooled naïve lung tissue cells from SPF ($n = 3$) and GF ($n = 3$) mice were stimulated by EPS at increasing concentrations for 72 h (37°C/5% CO₂ atmosphere).

Flow cytometry analysis

The number of cells in lung cell homogenates was analyzed by flow cytometry and expressed as % of CD45-positive cells. The absolute numbers of B- and T-lymphocytes were calculated from cells gated on CD45⁺/FSC-H (Supporting information Fig. S1A). From the CD45⁺ cells, the number of CD3⁺CD19⁺ and CD3⁺CD19[−] cells (Supporting information Fig. S1D) were evaluated (Supporting information Fig. S1E and F). Briefly, purified cells were resuspended and incubated with Fc-Block (anti-

CD16/CD32; clone 93; eBioscience, USA). Samples were then incubated with anti-mouse CD45-PE-Cy7 (clone 30-F11), Ly6G-APC (clone 1A8-Ly6g), CD11c-FITC (N418), MHCII-APC (clone M5/114.15.2), CD3-PE (clone 145-2C11), CD19-APC (clone eBio1D3), CD4-FITC (clone RM4-5), FoxP3-PE (clone FJK-16s), LAP-PerCP-eFluor710 (clone TW7-16B4), and IgA-FITC (clone mA-6E1) (all eBioscience, USA). After the staining, cells were fixed with fixation buffer (Invitrogen). Analysis was performed using FACSCalibur (Becton Dickinson, San Jose, CA). Electronic compensation was used to eliminate residual spectral overlaps between individual fluorochromes. Potential contamination with doublets was eliminated by rigorous FSC/SSC gating and reanalysis, which proved that positive events always represented a compact subset.

Immunohistochemical staining of IgA-producing cells

Lung tissue segments were embedded in O.C.T. Tissue-tek (Sakura Finetek, Alphen aan den Rijn, NL) and frozen in liquid nitrogen. Acetone-fixed cryosections (5 µm thick) were used for immunohistochemical staining with goat anti-mouse IgA-FITC antibody (Invitrogen, USA) diluted 1:50 in PBS/Saponin at 4°C overnight in humidity chamber. The cell nuclei were stained with Slow Fade TM Gold Antifade reagent with DAPI (Invitrogen, USA). Samples were viewed under Olympus BX 40 microscope equipped with an Olympus Camedia DP 70 digital camera, and the images were analyzed using Olympus DP-Soft.

Quantification of cytokine production by splenocytes

Spleen cell suspensions (5×10^6 cells/mL) were cultivated on 96-well flat-bottom plates in 200 µL of complete RPMI (Sigma-Aldrich, USA). Cells were stimulated with OVA (100 µg/well; Endograde; Hyglos GmbH, Germany) or left unstimulated and cultured for 72 h at 37°C/ 5% CO₂. Supernatants were collected and stored at −40°C until further analysis. Levels of IL-4, IL-5, and IL-13 were determined using a Mouse Cytokine/Chemokine Multiplex Immunoassay (Millipore-Sigma) and analyzed with a Luminex 200 System (Bio-Rad Laboratories). TGF-β1 was determined using ELISA kit (R&D Duo Set System, USA) according to the manufacturer's instructions.

Allergen-specific antibody responses: ELISA and basophil release assay

Ovalbumin-specific serum IgG1, IgG2a, and IgA levels were determined by ELISA as previously described [11]. Briefly, 96-well microtiter plates (Nunc MaxiSorp, Thermo Fisher Scientific) were coated with OVA (5 µg/mL). Serum samples were diluted 1/10,000 for IgG1, 1/100 for IgG2a, and 1/10 for IgA. BAL fluid samples were diluted five times for *L. rhamnosus*- and OVA-specific IgA analysis. Rat anti-mouse IgG1, IgG2a, and IgA antibodies (1 µg/mL, Pharmingen, San Diego, CA, USA) were

applied, followed by peroxidase-conjugated mouse anti-rat IgG antibodies (1/1000; Jackson, Immuno Labs., West Grove, PA, USA) for detection. Antibody levels were reported as OD (wavelength 405 nm). Allergen-specific IgE levels in sera and BAL fluid were quantified by degranulation of rat basophil leukemia (RBL-2H3) cells, as described previously [21]. RBL-2H3 cells were plated in 96-well tissue culture plates (4×10^4 cells per well) and passively sensitized by incubation with mouse sera (final dilution of 1/270) and BAL fluid (undiluted) for 2 h. Results are reported as percentage of total β -hexosaminidase release from cells after disruption with 1% Triton X-100.

Total IgA and IgE responses

The levels of total IgA and IgG were measured in the sera and BAL fluid by mouse ELISA quantification kit (Bethyl, USA), and total IgE was determined by BD OptEIA Mouse IgE ELISA Set (BD Biosciences, San Diego, USA) according to the manufacturer's instructions. Sera were diluted 1/800 for IgA and 1/10,000 for IgG and 1/10 for IgE measurement. BAL fluid was diluted 1/20 for total IgA and 1/1 for total IgE analysis. Supernatants from lung cell cultures were diluted twice to determine the level of total IgA (Bethyl, USA).

Statistical analysis

Data are expressed as mean $\pm$ standard error of mean (SEM). Unpaired *t*-test or one-way ANOVA with Tukey's multiple comparison test was used for comparison between experimental groups of mice. A significant difference was considered to exist when the *p*-value was <0.05 . Statistical analysis was performed using Graph-Pad Prism 8.1 Software (San Diego, CA, USA): *****p* < 0.0001 ; ****p* < 0.001 ; ***p* < 0.01 ; **p* < 0.05 .

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Conflict of interest: The authors declare that there is no commercial or financial conflict of interest.

Author contributions: D.S. performed the experiments and drafted the manuscript. S.G. isolated and purified the exopolysaccharide from *L. rhamnosus* LOCK900. H.K. designed the in vivo experiments on mice and edited the manuscript. P.P.H. performed the antibody analysis and drafted the manuscript. T.N. performed qPCR analysis. T.H. performed the histology and immunohistochemistry analysis. T.S. performed BAL fluid cell differential counting, and M.Si. performed flow cytometry analysis of B-lymphocytes. M.Sch. coordinated and conceived the study, and drafted the manuscript. All authors read and approved the final manuscript.

Ethics statement: All mouse experiments were approved by the Committee for the protection and use of experimental animals of the Institute of Microbiology, Czech Academy of Sciences (protocols No. 5/2019 and No. 91/2019).

Data availability statement: The data are available from the corresponding authors upon reasonable request.

Peer review: The peer review history for this article is available at <https://publons.com/publon/10.1002/eji.202250135>

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- Abbreviations:** **EPS:** exopolysaccharide · **GF:** germ-free · **LAP:** latency-associated peptide · **SPF:** specific pathogen-free
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