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ORIGINAL ARTICLE **Experimental Models of Allergic Disease**

Neonatal supplementation of processed supernatant from *Lactobacillus rhamnosus* GG improves allergic airway inflammation in mice later in life

H. Harb[1]*, E. A. F. van Tol[2,3], H. Heine[4]*, M. Braaksma[5], G. Gross[2,3], K. Overkamp[5], M. Hennen[1]*, M. Alrifai[1]*, M. L. Conrad[1]*, H. Renz[1]* and H. Garn[1]*

[1]Institute for Laboratory Medicine and Pathobiochemistry - Molecular Diagnostics, Philipps University of Marburg, Marburg, Germany, [2]Mead Johnson Nutrition, Evansville, IN, USA, [3]Mead Johnson Nutrition, Nijmegen, The Netherlands, [4]Section of Immunoregulation, Research Center Borstel - Leibniz-Center for Biosciences and Medicine, Borstel, Germany and [5]Microbiology and Systems Biology, TNO, Zeist, The Netherlands

Clinical & Experimental Allergy

Summary

Background Oral supplementation with probiotic bacteria can protect against the development of allergic and inflammatory diseases.

Objective The aim of this study was to investigate potential immunomodulatory and allergy-protective effects of processed *Lactobacillus rhamnosus* GG (LGG)-derived supernatants early in life in neonatal mice.

Methods *In vitro*, RAW264.7 mouse macrophages were stimulated with viable LGG, LGG-derived supernatants, prepared from different growth phases, and different size fractions thereof, and pro- and anti-inflammatory cytokine production was analysed. Supernatant fractions were also treated with protease, DNAse or carbohydrate-digesting enzymes to define the nature of immunomodulatory components. *In vivo*, neonatal Balb/c mice were orally supplemented with differentially processed LGG supernatants. Starting at 4 weeks of age, a protocol of ovalbumin-induced acute allergic airway inflammation was applied and protective effects of processed LGG supernatants were assessed.

Results Incubation of RAW264.7 cells with LGG-derived supernatants significantly increased TNFα and IL-10 production. These effects were not restricted to a particular molecular size fraction. Treatment with protease, but not with DNAse or carbohydrate-digesting enzymes, completely abolished the immunomodulatory activities. Incubation of TLR/NOD-transfected cells with LGG-derived supernatants revealed that recognition and signalling of bioactive components is mediated by TLR2 and NOD2. *In vivo* supplementation of newborn mice with processed LGG-derived supernatants resulted in pronounced protective effects on the allergic inflammatory response as reflected by reduced eosinophil numbers, modified T helper cell cytokine production, significantly less lung inflammation and reduced goblet cell numbers in comparison with sham-treated controls.

Conclusion LGG-derived supernatants exert immunomodulatory activities, and neonatal administration of specifically processed supernatants may provide an alternative to viable probiotics in reducing allergic inflammatory responses.

Keywords asthma, LGG, neonatal animal model, prevention, soluble factors

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Correspondence:
Dr. H. Garn, Institute for Laboratory Medicine and Pathobiochemistry - Molecular Diagnostics, Biomedical Research Center, Philipps University of Marburg, Hans-Meerwein-Strasse 2, D - 35043 Marburg, Germany.
E-Mail: garn@staff.uni-marburg.de

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Introduction

Asthma is the most common chronic inflammatory disorder of the airways characterized by airflow obstruction, increased mucus production and bronchial hyperresponsiveness [1]. In industrialized countries, asthma prevalence has been steadily increasing over the last decades, in parallel with decreasing occurrences of infectious disease [2, 3]. These observations have given rise to the hygiene hypothesis suggesting that early-life exposure to microbial components may educate the developing immune system in such a way that reduces susceptibility for allergies, including allergic asthma, later in life [4–7]. Thus, early colonization with

*Members of the German Center for Lung Research

commensal bacteria not only prevents the overgrowth of potentially harmful bacteria by competing for limited resources [8] but may also provide important exogenous triggers of immunoprogramming by interaction with both the developing innate and adaptive immune systems [9].

In line with this concept, perinatal supplementation with probiotic bacteria such as *Lactobacillus rhamnosus GG* (LGG) or *Bifidobacterium lactis* has been demonstrated to significantly reduce the risk for the development of allergic disorders in several human studies [10–13]. Probiotic supplementation may also lead to tolerance induction in children with cow's milk allergy [14, 15]. However, overall results from clinical studies are inconsistent, which may be caused by differences in bacterial strains used, treatment regimens and dosage per study [16].

Animal models revealed that supplementation with probiotic bacteria resulted in reduction in the major features of allergic airway inflammation in a murine model of experimental asthma [17]. Protective effects were also transferred to the offspring when mothers were supplemented with LGG before and during pregnancy and weaning [18]. Moreover, supplementation with heat-killed lactobacilli was shown to suppress airway hyperresponsiveness (AHR) in mice [19], suggesting that allergy-protective effects are not dependent on viable bacteria. It was hypothesized that soluble factors excreted by probiotic bacteria into the culture medium may exert similar effects.

The aim of this study was to assess the immunomodulating and allergy-protective effects of viable LGG and LGG-derived soluble mediators *in vitro* and *in vivo*. For the latter, a mouse model including neonatal supplementation of compounds and subsequent induction of allergic airway inflammation was developed. Protective effects of specifically processed LGG supernatant preparations were studied to assess potential future applications for the dietary management of allergic disease.

Materials and methods

Bacterial strain and culture conditions

Stock cultures of *Lactobacillus rhamnosus GG* (originally obtained from Valio, Finland) were maintained at −80°C in 20% (v/v) glycerol. From the glycerol stock, individual colonies were streaked on a plate of MRS Agar [20] which was incubated at 37°C for 48 h. For pre-culture, 1 L of culture medium was inoculated with three colonies and incubated overnight at 37°C until OD_{600nm} = 10, which was then used for inoculation of a 200-L fermentor. Modified MRS broth was used as culture medium containing 60 g/L glucose, 10 g/L sodium acetate trihydrate, 2.6 g/L NH_4Cl, 4.8 g/L trisodium citrate dihydrate, 4.0 g/L K_2HPO_4, 0.4 g/L $MgSO_4$ heptahydrate, 0.08 g/L $MnSO_4$ monohydrate and 46 g/L yeast extract (Gistex LS powder; DSM, Delft, The Netherlands).

Supernatant collection and processing

For initial experiments, a laboratory-scale LGG culture was performed and supernatant was collected at the late-exponential growth phase referred to as LGG–SN. All subsequent experiments were performed with supernatants obtained from 200-L batch cultivations. Samples were withdrawn from the fermentor at three different time points: during mid-exponential growth phase (MEG), late-exponential growth phase (LEG) and stationary growth phase (SG). In these samples, OD_{600nm} was determined. Cells were separated from the medium by centrifugation at 14000 **g**, 4°C in a fixed-angle rotor for 15 min; the cell pellet was discarded and the supernatant was stored at −20°C until further processing.

A part of these supernatant samples was desalted using a Sephadex G25 column (GE Healthcare Europe GmbH, Diegem, Belgium) resulting in > 6-kDa fractions. These fractions were further fractionated by Superdex 75 column (GE Healthcare Europe GmbH) gel filtration into a 6- to 30-kDa fraction, a 30- to 50-kDa fraction and a > 50-kDa fraction.

For *in vivo* studies, the LEG supernatant (protein content 189 mg/L) and its > 6-kDa fraction was filtrated to remove any remaining bacterial cells using a Sartobran P 0.2-μm rated filter cartridge (Satorius Stedium Biotech, Göttingen, Germany). The > 6-kDa fraction was subsequently lyophilized and is referred to as LEGa. Part of the LEG supernatant was ultrafiltered against 50 mM sodium acetate (NaAC) buffer, pH 5.5; the resulting > 5-kDa fraction was lyophilized and is referred to as LEGb. All samples were stored at −20°C until testing. Prior to use, lyophilized samples were resolved in distilled water to the original volume resulting in comparable protein concentrations of 156 mg/L and 162 mg/L for LEGa and LEGb respectively.

Enzyme treatment of the LGG-derived supernatant

- One millilitre LEG supernatant was treated with 10 μL of protease K (52 Unit/mg) (Sigma, Hamburg, Germany) for 10 min at a temperature of 37°C. The protease activity was stopped with 20 μL protease inhibitor (15 mg/mL) (Roche, Mannheim, Germany).
- One millilitre LEG supernatant was incubated with 100 μL 10 × TURBO DNase Buffer and 1 μL TURBO DNase (Ambion Inc., Austin, TX, USA) at 37°C for 25 min. After incubation, DNase was inactivated by the addition of resuspended DNase inactivation reagent (100 μL) and incubation at room temperature for 2 min.

• A volume of 0.3 L of LGG-derived supernatant (LEG; 0.3 L) was incubated for 8 h at 50°C with 1 mL of cellulase complex (NS50013; Novozymes Biomass Kit, Bagsvaerd, Denmark), 1 mL of β-glucosidase (NS50010, Novozymes Biomass Kit) and 1 mL of multienzyme complex (NS50012, Novozymes Biomass Kit) to digest the carbohydrates. As a control, a similar volume of the LGG-derived supernatant was incubated under the same conditions without the enzymes.

In vitro *stimulation of RAW264.7 cells with LGG and LGG-derived supernatant*

RAW264.7 mouse leukaemic monocyte macrophage cells were seeded in 96-well plates (Nunc, Wiesbaden, Germany) at a concentration of 1×10^6 cells in 1 mL cell culture medium [Dulbecco's modified Eagle medium (DMEM) high glucose with stable glutamine (Biochrom, Berlin, Germany) + 1% penicillin/streptomycin (PAA Laboratories, Pasching, Austria), + 10% inactivated fetal calf serum (FCS) (PAA Laboratories)] per well. After 24 h, LGG (at a final concentration of 1×10^8 CFU/mL), different LGG-derived supernatants or lipopolysaccharide (LPS) (extracted from *Escherichia coli* O111:B4; final concentration 10 ng/mL; Sigma) as positive control were added each in a volume of 10 μL, and cells were incubated for another 24 h at 37°C in a 5% CO_2 and 95% humidified atmosphere. Plates were centrifuged at 350 **g** for 10 min and the supernatant was analysed for pro-inflammatory cytokine levels by ELISA. In some experiments, total cellular RNA was additionally prepared for subsequent RT-PCR analyses.

Stimulation of transiently transfected HEK cells

At 24 h before transfection, HEK293 cells were plated at a density of 5×10^4/mL in 96-well plates in DMEM (PAA Laboratories) supplemented with 10 U/mL penicillin, 100 μg/mL streptomycin and 10% FCS (Biochrom) and transiently transfected using Lipofectamine (Invitrogen, Karlsruhe, Germany). The expression plasmid coding for human CD14 was a kind gift of D.T. Golenbock (Worcester, MA, USA), and the Flag-tagged version of human Toll-like receptor 2 (TLR2) was a kind gift from P. Nelson (Seattle, WA, USA). Flag-tagged human TLR4 (P. Nelson) was further subcloned into pREP9 (Invitrogen). The human MD-2 expression plasmid was a kind gift from K. Miyake (Tokyo, Japan). NOD1 (nucleotide-binding oligomerization domain 1) and NOD2-coding pcDNA3 plasmids were a kind gift of Philip Rosenstiel (Kiel, Germany) and were further subcloned into the V5 and His–tag-containing Gateway-vector pcDNA-DEST40 (Invitrogen). Plasmids were used at 100 ng (10 ng for CD14 and MD-2) per transfection. After 24 h, cells were stimulated for another 18 h with either LGG or LGG-derived supernatants. Pam3 (500 nM, EMC microcollections GmbH, Tübingen, Germany), LPS (100 ng/mL; LPS from *Salmonella enterica* sv. Friedenau was a kind gift of Dr. Helmut Brade, Research Center Borstel), iDAP (500 ng/mL; Invivogen, Toulouse, France) and MDP (500 ng/mL; Invivogen) served as positive controls for TLR2, TLR4, NOD1 and NOD2 stimulation respectively. Subsequently, the IL-8 content of the supernatants was quantified by ELISA (Invitrogen).

Measurement of cytokines in RAW264.7 cell supernatants using ELISA

IL-6, IL-10 and TNFα were measured in RAW264.7 cell supernatants by ELISA (BD Pharmingen, San Diego, USA) according to manufacturer's manuals.

TNFα and IL-10 mRNA expression analysis by RT-PCR

RNA from LGG-derived supernatant-stimulated RAW264.7 cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and contaminating DNA was digested by deoxyribonuclease I (amplification grade; Invitrogen) treatment. Reverse transcription (RT) was performed using the Omniscript Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Subsequently, quantitative PCR was carried out using Quanti Tect SYBR Green PCR Master Mix (Qiagen) and a Rotorgen 3000 (Qiagen) instrument. Respective primers were for TNFα (sense: 3′–AGCCCACGTCGTAGCAAA CC-5′, antisense: 3′-TACAACCCATCGGCTGGCAC-5′), for IL-10 (sense: 3-'GCATGGCCCAGAAATCAAGG–5′, antisense: 3′-TCTTCACCTGCTCCACTGCC-5′) and for the housekeeping gene Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) (sense: 3′-AACTTTGGCATTGTGGA AGG-5′, antisense: 3-'ACACATTGGGGGTAGGAACA-5′). CT values of TNFα and IL-10 were normalized to CT values of the house keeping gene by ΔCT method [21]. Data are expressed as relative expression compared to ΔCT values of unstimulated control samples.

Animal experiment

Female pregnant Balb/c mice, aged 6–8 weeks, were obtained from Harlan Winkelmann (Borchen, Germany) and housed with two mothers per cage in a 12/12-h light/dark cycle with food and water available *ad libitum*. Newborn offspring received supplementation of LGG suspension [1 x 10^8 CFU/mL in phosphate-buffered salt solution (PBS)], LGG supernatants (LEG, LEGa and LEGb) or PBS (sham-treated control) starting at 2 days of age for 6 weeks with 50 μL applied orally every second day in the first

week, 100 μL orally in the second week and 200 μL by intragastric (i.g.) application from the third week onwards (18 i.g. administrations in total). At 4 weeks of age, mice were separated from their mothers and the protocol was continued with the female mice only. At the age of 42, 49 and 56 days, these animals were sensitized to ovalbumin (OVA) by subcutaneous injection of 10 μg OVA (grade VI; Sigma, Deisenhofen, Germany) without adjuvant in a total volume of 200 μL PBS. On days 68, 69 and 70, mice were placed in a Plexiglas chamber and exposed to an aerosolized OVA challenge (1% wt/vol diluted in PBS) for 20 min [22]. Control mice received PBS only for sensitization and challenge.

Airway reactivity was measured on day 71. Animals were killed on day 72 and samples for further analyses were collected. All experimental procedures were approved by the local animal ethics committee (08–150), and met German and international guidelines.

Measurement of OVA-specific antibodies in serum samples

Blood was obtained from mice on the day 72 and OVA-specific immunoglobulins IgE, IgG1 and IgG2a were quantified in the serum as previously described [23].

Bronchoalveolar lavage and cell differentiation

At 48 h after the last aerosol challenge, mice were killed by an overdose mixture of rompun 2% (Bayer, Germany) and ketamine 50 mg/mL (Insera Arzneimittel GmbH, Freiburg, Germany). Bronchoalveolar lavage (BAL) was performed as described previously [24] and BAL fluid was used for differential cell counts and cytokine analyses.

Measurement of BAL cytokines using cytometric bead array (CBA-Flex) technology

Cytokines characteristic for the activation of different T helper cell subpopulations (i.e. IL-5, IL-9, IFNγ, IL-10 and IL-17A) were measured in cell-free BAL fluids using cytometric bead array (CBA)-Flex technology (BD Pharmingen). Standards and samples were prepared according to the manufacturer's protocol. Assay diluent alone was used as a negative control. 40 μL of a cytokine capture bead mix were transferred to a 96-well plate with 50 μL of the standards or samples, and incubated for 1 h in the dark. Plates were washed, and 40 μL of PE detection reagent was added per well and incubated for 2 h in the dark. After incubation, the plates were washed once with 200 μL washing buffer. Then, samples were suspended in 180 μL washing buffer and measured using a FACS-Array Bioanalyser (Becton Dickinson). A total of 300 events were acquired per cytokine and analysis was performed using CBA analysis FCAP software. The minimum detection level for each cytokine was 0.5 pg/mL.

Lung histology

Sections of paraformaldehyde (4 % wt/vol)-fixed, agarose-embedded lung tissues were cut and stained with periodic acid/Schiff (PAS) as described previously [25]. PAS-stained sections were viewed and random images collected under 20 × objective. The number of goblet cells in the bronchial epithelium was quantified and expressed as cell number per mm basement membrane. Inflammation score was assessed by 1–3 score under the microscope by an investigator blinded to the experimental design.

Lung function analysis

Lung function analysis was carried out using the non-invasive method of head-out body-plethysmography. The mid-expiratory airflow (EF_{50}) was measured to assess bronchial responsiveness to increasing concentration of aerosolized β-methacholine (MCh) at 24 h after the last OVA aerosol challenge as described previously [26].

Statistical analysis

All numerical data are expressed as mean ± SD and analysed for significance using Student's unpaired *t*-test. Calculations were performed by use of GraphPad prism software, version 4.0 (San Diego, CA, USA).

Results

LGG whole bacteria and LGG supernatant exert comparable immunostimulatory activity in vitro

To test whether LGG-derived supernatant (LEG) exerts immunostimulatory activity, adherent RAW264.7 cells were stimulated with either LGG or LGG-derived supernatant (LGG-SN) for 24 h. After stimulation, TNFα, IL-10 and IL-6 levels were measured using ELISA. LPS was applied as a positive control and resulted in the release of the respective cytokines in the following ranges: TNFα 2,300–10 000 pg/mL, IL-10 4,000–9,000 pg/mL and IL-6 80–150 pg/mL (representative data from multiple experiments). Stimulation by both LGG and LGG-SN resulted in a significant production of TNFα and IL-10, whereas only small amounts of IL-6 were induced (Fig. 1). Cytokine induction was comparable between both preparations,

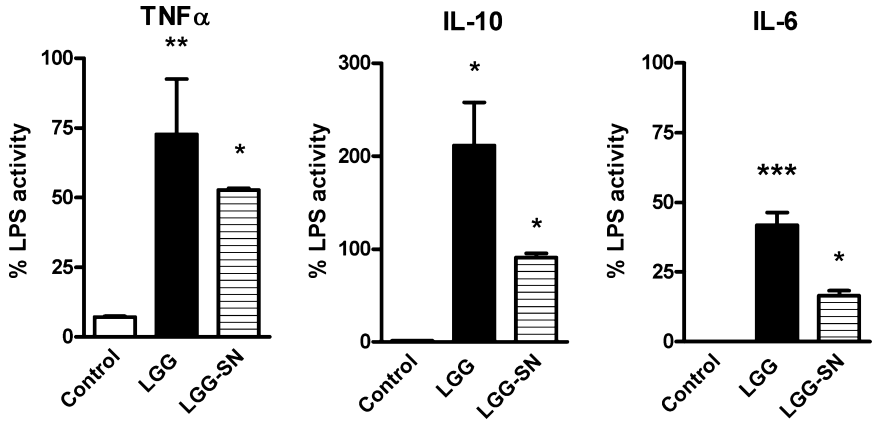

Fig. 1. Effect of *Lactobacillus rhamnosus GG* (*LGG*) and LGG-derived crude supernatant (LGG-SN) on the production of cytokines TNFα, IL-10 and IL-6 in RAW264.7 cells *in vitro*. Cells were incubated with either LGG or LGG-SN for 24 h and cytokines were measured in cell-free supernatants. Data are calculated relative to lipopolysaccharide (LPS) (10 ng/mL)-induced cytokine release, and results of one of three independently performed experiments are shown as mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control

suggesting that presence of viable LGG is not a prerequisite for the induction of immunostimulatory activities and those soluble mediators in LGG-derived supernatants may cause similar effects.

Moreover, a dose-dependent response to the LGG supernatant was demonstrated with the most prominent stimulation of RAW cells caused by a 1 : 8 dilution of the supernatant (data not shown).

Late-exponential growth phase supernatant causes the most pronounced in vitro *immunostimulatory effects*

As a next step, it was assessed whether the bacterial growth phase has an impact on the immunomodulatory activity of the derived supernatants. For this purpose, three different time points of the LGG culture were compared: the MEG, the LEG and the stationary growth phase (SG). Although supernatants from all culture states induced cytokine secretion in RAW cells as compared with controls, the LEG was found to be superior in stimulating TNFα and IL-10 secretion (Fig. 2a).

Combination of late-exponential growth phase subfractions causes the most prominent effects in RAW cells in vitro

To further investigate whether a specific fraction of the LEG supernatant was responsible of its immunomodulatory activity, LEG was further fractionated according to molecular weight, and fractions were analysed in the RAW264.7 cell system. As demonstrated in Fig. 2b, the activity of LEG was not restricted to a single fraction of defined molecular weight, but rather determined by a combination of factors contained in the whole > 6-kDa supernatant. However, as the 6- to 30-kDa subfraction exerted the lowest activity, components with a higher molecular weight are likely to be in particular responsible for the immunostimulatory effects.

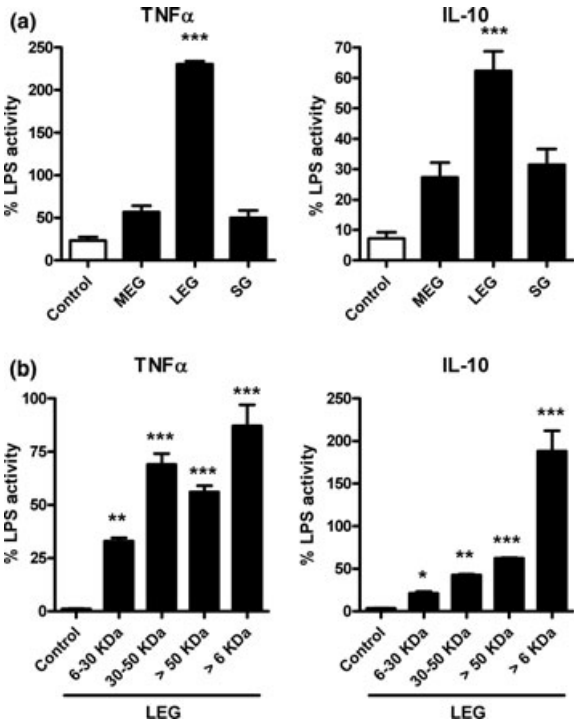

Fig. 2. (a) Effect of supernatants derived from mid-exponential growth phase (MEG), late-exponential growth phase (LEG) and stationary (SG) bacterial growth phases and (b) different subfractions of the LEG supernatant on the production of TNFα and IL-10 by RAW264.7 cells. Cells were incubated in the presence of the respective supernatant preparations for 24 h, and cytokine levels were measured. Data are calculated relative to lipopolysaccharide (LPS) (10 ng/mL)-induced cytokine release, and results of one of three independently performed experiments are shown as mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ vs. control

Protease-sensitive factors are responsible for the immunomodulatory activity of LGG supernatant in vitro

To gain first insights into the nature of the active compounds in LGG-derived supernatant, LEG supernatant was treated with protease, DNase or carbohydrate digesting enzymes prior to testing the immunostimulatory activity of the supernatant.

First, RAW264.7 cells were stimulated with the protease-treated supernatant and initially the viability of the cells was tested to exclude that remaining protease activity may affect the cells. It could be demonstrated that treated cells retained their full viability. Interestingly, protease treatment completely abolished the immunostimulatory activity of LEG (Fig. 3a) and LEG subfractions (data not shown). Similar results were obtained by ELISA and RT-PCR measurements, confirming the finding that protease-sensitive compounds are involved in the immunostimulatory activity of LEG (Fig. 3a).

DNase treatment or incubation with carbohydrate-digesting enzymes did not affect the capacity of LEG to induce cytokine production in RAW cells (Fig. 3b and c). These observations suggest that neither DNA nor carbohydrates play a significant role in the immunomodulatory activity of LGG-derived supernatant.

TLR2 and NOD2 are important receptors for bioactive components of LGG-derived supernatants

To gain first insights into the mechanisms of action underlying the immunostimulatory effects of the LGG supernatant preparations, HEK293 cells transfected with plasmids coding for either TLR2, TLR4, NOD1 or NOD2 were incubated for 18 h with either LGG, LEG, LEGa or LEGb and subsequently IL-8 was measured as indicator of HEK cell stimulation. As shown in Fig. 4, viable LGG and also LEG, LEGa and LEGb showed a significant induction of IL-8 production in both TLR2- and NOD2-transfected HEK293 cells (Fig. 4), indicating that these innate immune receptors are mainly involved in the recognition and signalling of LGG and bioactive components in LGG-derived supernatants. On the other hand, there was no induction of IL-8 in TLR4/CD14/MD2-cotransfected cells and only a very slight induction in NOD1-transfected cells with any of the supernatants (data not shown).

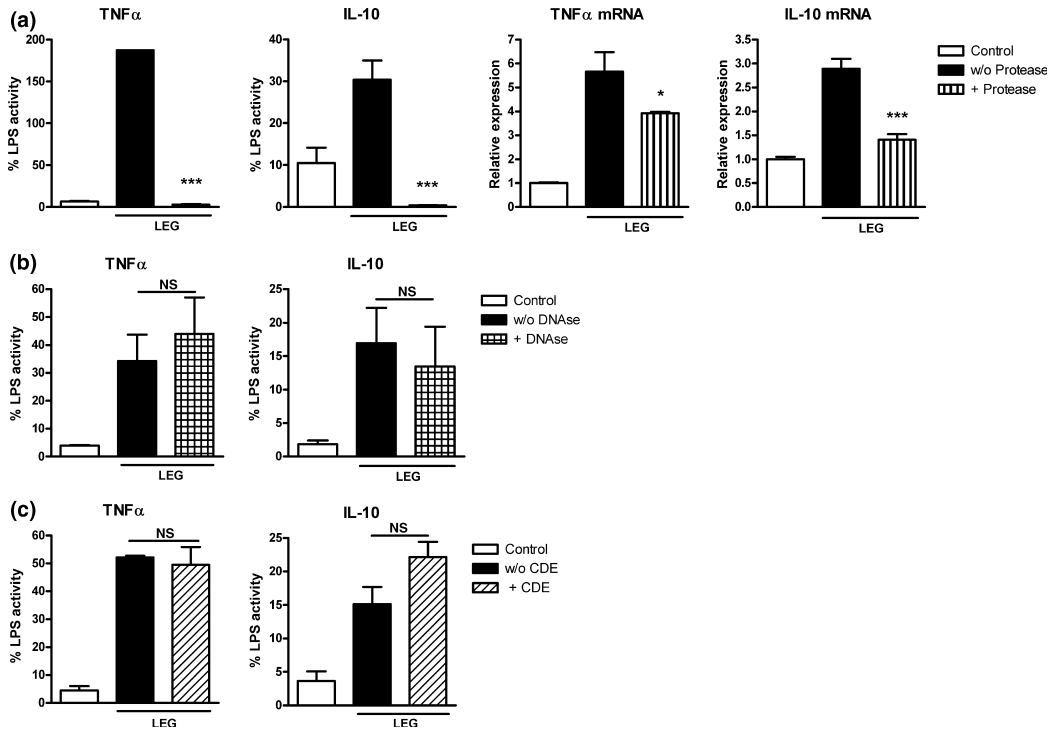

Fig. 3. Influence of protease, DNAse or carbohydrate-digesting enzymes treatment on the immunostimulatory activity of *Lactobacillus rhamnosus* GG (LGG)-derived supernatant. Late-exponential growth phase LGG supernatant (LEG) was pre-treated with either protease (a), DNAse (b) or a mixture of carbohydrate-digesting enzymes (CDE; c). RAW264.7 cells were incubated with enzyme-treated supernatants for 24 h, and TNFα and IL-10 protein levels were measured by ELISA. Data are calculated relative to lipopolysaccharide (LPS) (10 ng/mL)-induced cytokine release, and results of one of three independently performed experiments are shown as mean ± SD. In addition, TNFα and IL–10 mRNA expression was analysed by quantitative PCR. $*P < 0.05$, $***P < 0.001$ vs. untreated supernatant, n.s. not significant.

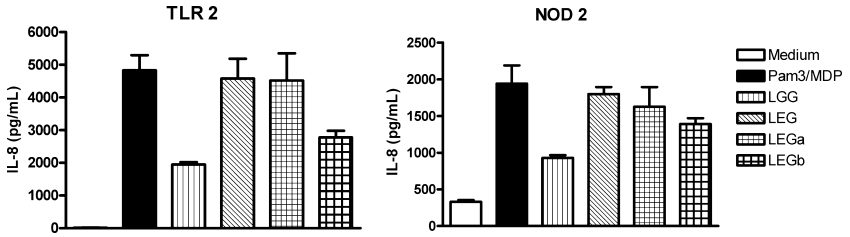

Fig. 4. Stimulation of TLR2- (left) and NOD2 (right)-transfected HEK293 cells by *Lactobacillus rhamnosus* GG (LGG) and LGG-derived supernatants. HEK293 cells were transiently transfected with human TLR2- or NOD2-coding plasmids and then stimulated for 18 h with LGG, late-exponential growth phase (LEG), LEGa or LEGb as well as TLR2- or NOD2-specific ligands as controls. HEK293 activation was assessed by production of IL-8 measured using ELISA.

Neonatal supplementation with processed LGG supernatants reduces characteristic parameters of allergic airway inflammation in vivo

The immunomodulatory effects of neonatal application of LGG-derived supernatants were evaluated *in vivo* using a mouse model of allergic airway inflammation. In control experiments without OVA sensitization, it was confirmed that neonatal *LGG* and LGG supernatant supplementation did not induce any signs of lung inflammation as compared with untreated controls (data not shown).

In the actual experiment including OVA sensitization and challenge, the effects of supplementation with viable LGG bacteria, LEG supernatant and two differentially processed LEG variants (LEGa and LEGb) were compared to PBS sham-treated control animals. OVA-specific immunoglobulins were measured in the serum 48 h after the last challenge. There was no significant effect of the different LGG-derived supernatants on OVA-specific immunoglobulin (IgE, IgG1 and IgG2a) levels in comparison to the sham-treated OVA group, while supplementation with LGG bacteria significantly reduced OVA-specific IgG1 levels (Fig. 5).

BAL cell counts were significantly increased in the unsupplemented OVA-sensitized/challenged group in comparison to sham-sensitized/challenged mice, with a preferential influx of eosinophils as a characteristic feature of this experimental asthma model. Both supplementation with viable LGG and LEGa supernatant caused a significant decrease in total BAL cell counts and lymphocyte numbers (Fig. 6a). Significantly decreased eosinophil numbers were observed in all LGG and LGG-derived supernatant-supplemented groups as compared with unsupplemented control animals. In contrast, neutrophil (Fig. 6a) and macrophage (data not shown) numbers were not affected by any of the supplementations.

With respect to Th2 cytokines, unsupplemented OVA-sensitized/challenged mice showed the expected increase in BAL IL-5 levels, which was significantly reduced by supplementation with LGG, but not by LGG-derived supernatants. In general, levels of cytokines characteristic for other T helper cell subpopulations (IFNγ and IL-17A) were very low. IFNγ was not influenced by any of the treatments; however, there was a substantial reduction of IL-17A levels in animals supplemented with LGG or LEGb supernatant. The latter also resulted in a significant decrease in BAL IL-9, paralleled by a significant increase in BAL IL-10 levels as compared with unsupplemented animals (Fig. 6b).

For analyses of histological parameters, lung sections were stained with PAS to investigate inflammation and goblet cell hyperplasia. The OVA sensitization/challenge

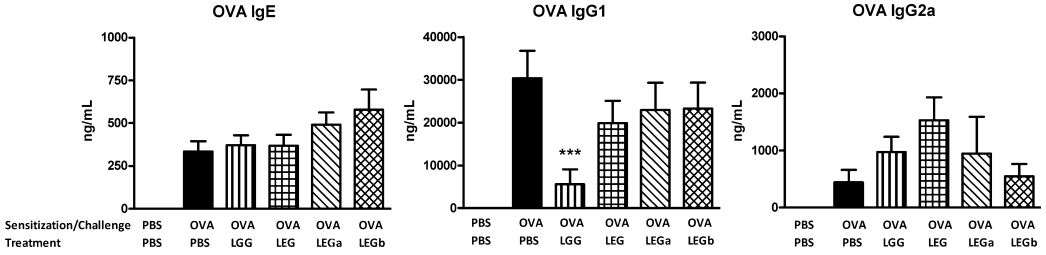

Fig. 5. Effects of orally supplemented *Lactobacillus rhamnosus* GG (LGG) and LGG-derived supernatant preparations on allergic sensitization. Mice were treated intragastrically with either LGG or the LGG-derived supernatant preparations late-exponential growth phase (LEG), LEGa or LEGb and then subjected to an acute experimental asthma protocol. Ovalbumin (OVA)-specific immunoglobulin (IgE, IgG1 and IgG2a) levels were measured in serum. $n = 8$ mice per group. ***$P < 0.001$ vs. untreated OVA-sensitized/challenged control group

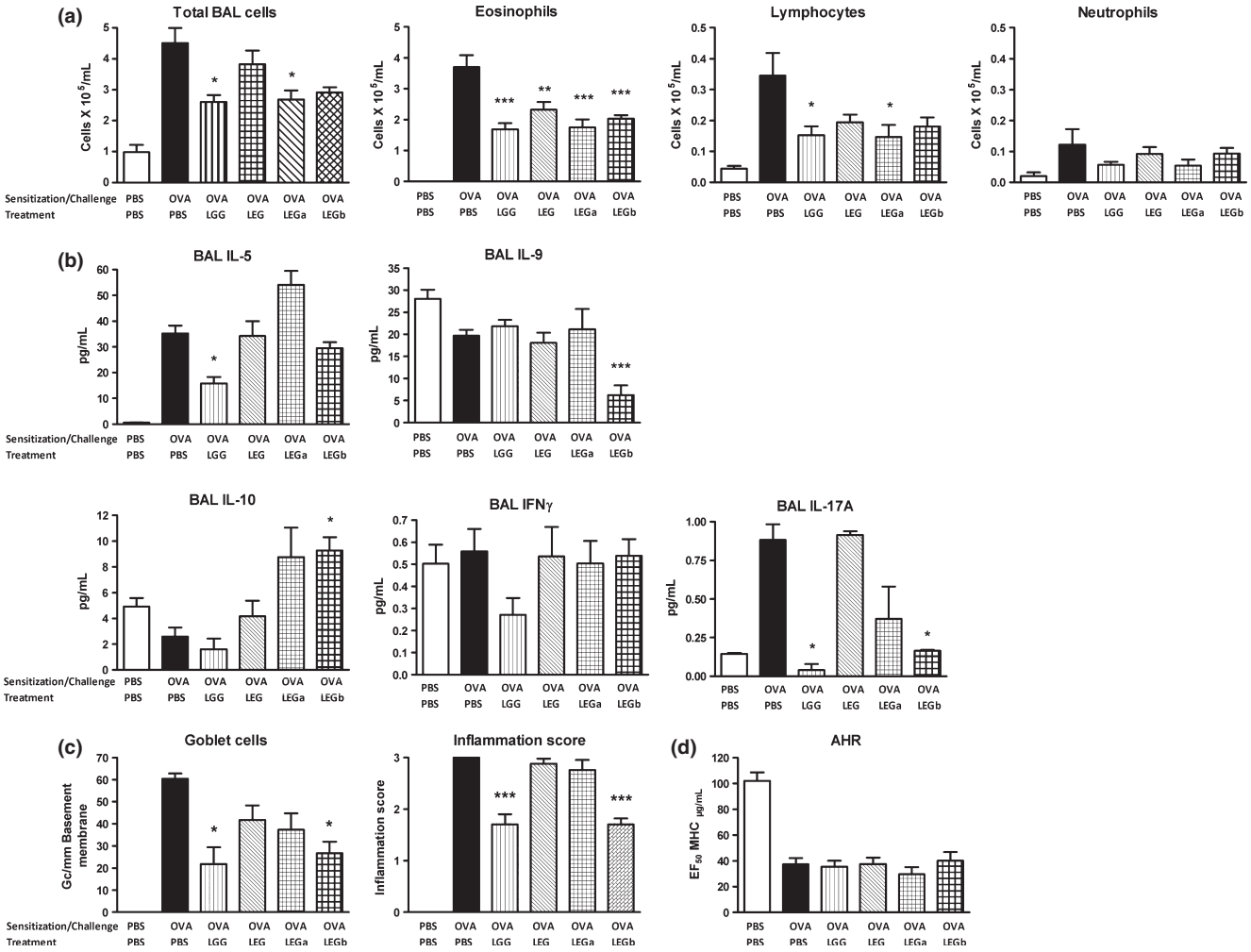

Fig. 6. Effects of orally applied *Lactobacillus rhamnosus* GG (LGG) and LGG-derived supernatant preparations on features of allergic airway inflammation. Mice were treated intragastrically with either LGG or the LGG-derived supernatant preparations late-exponential growth phase (LEG), LEGa or LEGb and then subjected to an acute experimental asthma protocol. Total cell, eosinophil, lymphocyte and neutrophil numbers (a) and cytokine concentrations for IL-5, IL-9, IFNγ and IL-10 (b) were analysed in the Bronchoalveolar lavage (BAL) fluid. Tissue inflammation and goblet cell numbers were quantified in periodic acid/Schiff-stained histological specimens (c). Airway hyperresponsiveness to methacholine was measured using head-out body-plethysmography (d). $n = 8$ mice per group. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. untreated ovalbumin (OVA)-sensitized/challenged control group

protocol led to an increase in both tissue inflammation and goblet cell numbers. Supplementation with viable LGG and LEGb supernatant significantly decreased peribronchial/perivascular inflammation and goblet cell scores, whereas LGG-derived supernatants LEG and LEGa supernatants did not exert comparable effects (Fig. 6c).

In addition, lung function was measured using head-out body-plethysmography. OVA-sensitized/challenged mice showed a significant hyper-reactivity to methacholine as indicated by reduced EF_{50} values as compared with non-allergic mice. Supplementation with neither viable LGG bacteria nor LGG-derived supernatants had an impact on OVA-induced AHR, indicating that these supplementations did not directly affect lung function (Fig. 6d).

Discussion

This study demonstrates that soluble factors secreted by the probiotic bacterium LGG into the culture medium exhibit immunostimulatory and allergy-protective effects in *in vitro* cell culture assays as well as after neonatal supplementation in an *in vivo* animal model of experimental asthma. Initial attempts to define the biochemical nature of the bioactive components suggest these factors to be protease sensitive, but resistant to DNAse and carbohydrate-digesting enzymes. In addition, well-defined cell-free supernatants harvested at the late-exponential bacterial growth phase were found to be most effective in terms of immunostimulatory capacity *in vitro*. Furthermore, the activity of the higher molecular weight fractions (30–50 kDa and > 50 kDa)

seemed to be more pronounced than that of the 6- to 30-kDa fraction. However, the exact nature of the bioactive components has not been identified yet.

Initially, a cell culture-based bioassay was developed to screen LGG-derived supernatants for immunomodulatory activities to allow for further evaluation of the differentially processed LGG supernatant samples. Based on the concept that innate immune mechanisms are essential for the systemic action of probiotics [27], this assay is based on the well-established macrophage-like cell line RAW264.7, with TNFα and IL-10 production as read-outs. TNFα was selected because of its pro-inflammatory activities [28, 29] and has been previously used as an indicative parameter for the evaluation of biological activities of drugs, allergens and toxins [30]. TNFα is induced by activation of important signalling pathways involving NFκB, JNK and MAP kinases. Although these pathways exhibit a high degree of overlap with the signalling cascade of other important pro-inflammatory and macrophage-derived cytokines, such as IL-1, IL-6, and IL-12, the results from our *in vivo* experiments support the selection of TNFα as a useful biomarker for immunological activities present in culture supernatants derived from probiotic bacteria. Moreover, Won et al. investigated inflammatory mediators produced by RAW cells in response to LGG, and observed that LGG did not lead to any IL-12p40 release, but induced significant amounts of IL-10 [31]. There is ample evidence that IL-10 plays a role in the suppression and prevention of (chronic) inflammatory events including inhibition of allergic reactivities [32]. Thus, this biomarker seems to be of particular relevance for the selection of bioactive compounds directed against the development and/or maintenance of Th2-driven immune responses as present in allergic diseases. A further reason to use IL-10 as an additional marker is the involvement of distinct signalling pathways [32, 33], contrary to those involved in TNFα induction. Indeed, with TNFα and IL-10 as read-outs, it could be clearly illustrated LGG-derived supernatants exhibit differential patterns of immunomodulating activities. For example, the 30- to 50-kDa fraction from LEG exhibits a stronger stimulatory activity on TNFα than on IL-10 production and generally, IL-10 induction seems to require the combined action of bioactive components from a broad molecular weight range.

Probiotic bacteria, including LGG have been demonstrated to exhibit a variety of health-promoting properties in different disease-related animal models. Due to the primary occurrence of probiotic bacteria in the gut, these activities were initially mainly noted in intestinal inflammatory conditions [34]. Meanwhile, it became evident that effects can also extend beyond the intestinal mucosa [27, 35, 36] as preventive effects were also demonstrated for inflammatory disease conditions outside the gut [9, 18]. With regard to underlying mechanisms of action, probiotic bacteria can exhibit their activities by direct interaction with cell-surface components present on epithelial or immune cells of the host. There is accumulating evidence for direct cell-mediated recognition of probiotic bacteria, particularly by pattern recognition receptors on intestinal cells [37–39]. In addition, probiotic bacteria can interfere indirectly with components of the host immune system via secreted factors that are present in the supernatant of bacterial culture medium, as shown in cell culture-based analyses [40, 41] and very recently also *in vivo* [42, 43]. In this study, we were able to show that TLR2 and NOD2 play a central role in the recognition and signalling of bioactive components in the LGG-derived supernatants. As those components have been demonstrated to be protease-sensitive, it may be speculated that peptidoglycans – that are known to signal via TLR2 – are involved in the biological activity of the supernatants. Activation of TLR2 by lipopeptides can induce IL-10 production by innate immune cells [44] and a shift in T helper cell differentiation towards IFNγ- and IL-10-producing phenotypes [45] – both phenomena were observed in our study. This is further supported by findings of van Hoffen et al., demonstrating that UV-killed LGG as well as TLR2 and NOD2 ligands induced a Th1 phenotype in PBMCs *in vitro* [46]. Moreover, lack of appropriate NOD2 signalling has been associated with increased susceptibility to inflammatory diseases including allergy and asthma [47, 48]. On the other hand, Yan and coworkers identified two LGG-derived proteins (p40 and p75) that improved intestinal epithelial cell survival and protected mice from intestinal inflammation *in vivo*. The authors suggested these proteins may be involved in the activation of the transcription factor Akt resulting in protection from pro-inflammatory cytokine-induced apoptosis [43, 49, 50]. Interestingly, the molecular weights of these proteins correspond to the molecular weight of immunomodulatory active fractions as shown in this study in neonatal mice. However, whether these molecules are responsible for the effects observed here by activation of comparable signalling pathways remains to be addressed in future investigations.

In this study, oral/intragastric probiotic supplementation was started in neonates on the second day of life to mimic the natural route of probiotic ingestion as well as to take into account the *window of opportunity* in early life, which is crucial for potential allergy-protective immunomodulation [9, 18, 51, 52]. In this model, LGG supernatants did not significantly affect allergic sensitization as reflected by the allergen-specific antibody response in contrast to viable LGG bacteria that had an impact on IgG1 levels. A possible reason for this finding may be the difference in doses of (yet unidentified)

bioactive components in the LGG supernatants or that the protocol of systemic sensitization (even though an adjuvant-free sensitization protocol was applied) was too strong to enable sufficient pharmacological modification of antibody responses by these compounds. Data obtained here are in contrast to observations by Feleszko et al. [9] who observed an impact of LGG supplementation on OVA-specific IgE levels. However, in that study, LGG was applied throughout the whole experimental protocol including the sensitization period, whereas in our protocol, LGG supplementation was discontinued before the first sensitization. Thus, differences in duration and/or timing of treatment may account for differential effects on sensitization parameters.

However, the local inflammatory reaction in the lung following allergen challenge was markedly improved by the supplementation with processed LGG-derived supernatant LEGb as well as viable LGG bacteria. Particularly, the influx of eosinophils, development of goblet cell hyperplasia and the production of inflammatory cytokines was markedly inhibited. Although the LEGb supernatant did not lead to increased levels of the Th1-related cytokine IFNγ, it did strongly stimulate IL-10 and reduced the IL-17. This observation is in line with the findings from our *in vitro* bioassay and points to the importance of IL-10 as a relevant anti-inflammatory and anti-allergic immune pathway, triggered by soluble factors of LGG.

It is important to note that in the current model, the development of AHR remained unaffected in all experimental groups, similar to a previous study employing prenatal LGG exposure [18]. Indeed, a clear dissociation between inflammatory cascades on one side and mechanisms controlling airway responsiveness on the other side has been shown in various animal model systems [26, 53, 54]. Development of AHR is controlled by several independently operating mechanisms involving pro-inflammatory pathways as well as non-immune neuronal signalling cascades. It is likely that LGG-derived supernatants selectively impact on the level of inflammation, leaving the neuronal pathways unaffected and therefore, do not significantly interfere with the development of AHR in the chosen experimental setting.

To gain first insights into the impact of processing conditions as applied for the production of nutritional formulations on bioactivity, LGG supernatants used in the *in vivo* animal model were processed with relevant procedures such as ultrafiltration, column chromatography and lyophilization. Interestingly, more pronounced protective effects were achieved with processed preparations LEGa and LEGb as compared with the original LEG supernatant. This observation indicates that bioactivity may not only be retained but even enhanced depending on the process conditions, possibly by concentration of bioactive components. Thus, our results clearly demonstrate that specifically processed LGG-derived supernatants exert beneficial effects in a neonatal mouse model of allergic airway inflammation. These findings may serve as the basis for an alternative probiotic strategy for the dietary management of allergic disease.

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