Colonization of Germ-Free Mice with the Mixture of Three *Lactobacillus* Strains Enhances the Integrity of Gut Mucosa and Ameliorates Allergic Sensitization to Main Birch Pollen Allergen Bet v 1

Hana Kozakova[a], Martin Schwarzer[a], Ludmila Tuckova[a], Dagmar Srutkova[a], Elzbieta Czarnowska[b], Ilona Rosiak[b], Tomas Hudcovic[a], Irma Schabussova[c], Petra Hermanova[a], Zuzana Zakostelska[a], Tamara Aleksandrzak-Piekarczyk[d], Anna Koryszewska-Baginska[d], Helena Tlaskalova-Hogenova[a], Bozena Cukrowska[b]

[a]Laboratory of Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i.

[b]Department of Pathology, The Children's Memorial Health Institute, Warsaw, Poland

[c]Institute of Specific Prophylaxis and Tropical Medicine, Medical University of Vienna, Austria

[d]Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw, Poland

Running title: Probiotic *Lactobacillus* mixture ameliorates allergic sensitization

Correspondence to:

Schwarzer Martin, PhD.

Laboratory of Gnotobiology, Institute of Microbiology, Academy of Sciences of the Czech Republic, v.v.i.

Doly 183

54922 Novy Hradek

Czech Republic

e-mail: schwarzer@biomed.cas.cz

Tel. +420491418537

Abbreviations

AJ, adherens junction

BM-DC, bone marrow-derived dendritic cells

Ctrl, unstimulated cells

CV, conventional

DE, desmosomes

GF, germ-free

HEK293, human embryonic kidney cell line 293

IFN, interferon

Ig, immunoglobulin

IL, interleukin

L, *Lactobacillus*

L900, *L. rhamnosus* LOCK0900

L908, *L. rhamnosus* LOCK0908

L919, *L. casei* LOCK0919

Lmix, Lactobacilli mixture

MLN, mesenteric lymph nodes

NOD2, Nucleotide-binding oligomerization domain-containing protein 2

PBS, phosphate-buffered saline

RBL, rat basophil leukemia cells

TGF, transforming growth factor

Th, T helper lymphocytes

TJ, tight junction

TLR, Toll-like receptor

Treg, T regulatory lymphocytes

TW, terminal web

ZO-1, zonulin-1

Abstract

Increasing numbers of clinical trials and animal experiments show that probiotic bacteria are a promising tool in allergy prevention. Here we analyzed the immunomodulatory properties of three selected lactobacilli strains and the impact of their mixture on allergic sensitization to Bet v 1 using gnotobiotic mouse model. We have shown that *Lactobacillus* (*L.*) *rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908, and *L. casei* LOCK0919 are recognized via TLR2 and NOD2 receptors and stimulate bone marrow-derived dendritic cells to cytokine production in a species- and strain-dependent manner. Colonization of germ-free (GF) mice with the mixture of all tree strains (Lmix) improved intestinal barrier by strengthening the apical junctional complexes of enterocytes and by restoring the structure of microfilaments extending into the terminal web. Mice colonized with Lmix and sensitized to Bet v 1 allergen showed significantly lower level of allergen-specific IgE, IgG1, IgG2a and elevated levels of total IgA in sera and intestinal lavages as well as increased levels of TGF-β compared to sensitized GF mice. Splenocytes and mesenteric lymph node cells of Lmix-colonized mice showed significant up-regulation of TGF-β after *in vitro* stimulation with Bet v 1. Our results show that Lmix colonization improved gut epithelial barrier and reduced allergic sensitization to Bet v 1. Furthermore, this was accompanied by increased production of circulating and secretory IgA and regulatory cytokine TGF-β. Thus the mixture of three lactobacilli strains shows a potential to be used in prevention of increased gut permeability and onset of allergies in humans.

Key words: *Lactobacillus*, probiotics, allergic sensitization, germ-free, intestinal barrier

Introduction

Humans, as all vertebrates, are essentially born germ-free (GF). The GF status changes rapidly during and after delivery and the subsequent interaction between the host and colonizing microbiota plays a crucial role in the development and function of the immune system as well as maintenance of intestinal homeostasis (1, 2). Perturbation in the colonizing microbiota leads to breakdown of equilibrium between commensal and pathogenic microbes. This dysbiosis has been linked to the increased permeability of the epithelium (3, 4) and with the development of chronic inflammatory diseases such as allergies or inflammatory bowel disease (5-7).

Allergies have become a serious health burden in developed countries. In line with the general hypothesis from Strachan (8) that the rapid increase in allergic diseases in humans is dependent on microbial deprivation early in life, reduced bacterial diversity and lower counts of lactobacilli and bifidobacteria were found in gut of allergic children (9, 10). This finding has been the rationale for administration of probiotic bacteria in prevention and/or therapy of allergy (11-13).

Probiotic lactobacilli and bifidobacteria are non-invasive and non-pathogenic Gram-positive bacteria possessing immunomodulatory properties which are strictly strain-dependent (14). They have been documented to compete with pathogens and toxins for adherence to the intestinal epithelium, promote intestinal epithelial cell survival, enhance barrier function and directly interact with cells of the immune system such as dendritic cells (DC) (15). Through engagement of innate receptors such as TLRs, NODs or C-type lectin receptors, probiotic lactobacilli and bifidobacteria induce distinct innate responses and cytokine profiles that subsequently shape the type of T-helper cell responses (16-18). There is accumulating evidence that certain strains possess intrinsic Th1-type immunomodulatory properties (18, 19) while others are able to induce regulatory responses (17, 20, 21).

TGF-β is present at high concentrations in the intestine and has a crucial involvement in modulating the immune response (22). It has been shown to inhibit the proliferation and differentiation of both B- and T-cells (23) and altered TGF-β signaling has been linked to the development of allergic disease (24). Furthermore, TGF-β is an initial trigger for production of mucosal IgA, which has a regulating role in mucosal integrity (25). Along these lines, we have previously shown that *Lactobacillus paracasei* stimulated production of regulatory cytokine TGF-β from bone marrow-derived DC in a TLR2/4-deppendent manner (21).

Among the inhalant allergens, pollen of the white birch (*Betula verrucosa*) is one of the most important sources responsible for eliciting allergic symptoms (26). In an experimental model, we have shown that oral application of *L. paracasei* to pregnant mothers prevented allergy development in their offspring in a mouse model of birch pollen allergy (21). Similarly, intranasal application of probiotic bacteria reduced allergic poly-sensitization in adult mice (27). Although the majority of studies use single strains the supplementation with probiotic mixtures might have greater efficacy (28).

Germ-free animals represent a unique tool to study the interaction of the host with one specific probiotic strain or with defined probiotic mixture and to investigate their impact on the development of the immune system (6, 29). Using a mouse model of allergic sensitization to major birch pollen allergen Bet v 1 we have previously shown that neonatal colonization of GF mice with *Bifidobacterium longum* is able to prevent allergic sensitization (20), but the underlying mechanism of host-bacteria interaction in gnotobiotic models is still far from being elucidated.

Recently, we have selected three lactobacilli strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 out of twenty four strains isolated from the stool of healthy infants (30). These selected strains showed properties required for probiotic bacteria, e.g. the resistance to gastric acids and bile salts and inhibitory activity against

bacterial pathogens (30). Moreover, the mixture of these strains (Lmix) showed synergistic effects in induction of anti-allergic Th1-type cytokines and regulatory cytokine TGF-β in human whole blood cell cultures compared to the levels induced by each single strain alone (31). Our pilot study showed that supplementation of children presenting the first symptoms of allergy (atopic dermatitis) with the Lmix reduced the serum levels of IgE and IL-5 and diminished severity of the disease (Cukrowska, unpublished data).

Based on these observed effects, the aims of this study were to further characterize immunomodulatory properties of individual lactobacilli strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 as well as their mixture Lmix *in vitro*; and to investigate the effect of Lmix on the development of allergic sensitization to allergen Bet v 1 in a gnotobiotic mouse model.

Materials and Methods

Bacterial strains

L. rhamnosus LOCK0900 (32), *L. rhamnosus* LOCK0908 (33) and *L. casei* LOCK0919 (34) were obtained from the Pure Culture Collection of the Technical University of Lodz, Poland (LOCK). Overnight cultures in MRS broth (Oxoid, UK) were centrifuged, washed in sterile phosphate-buffered saline (PBS) and concentration was adjusted to 10^9 CFU/ml. For *in vitro* experiments, single bacterial strains were inactivated with 1 % formaldehyde-PBS for 3 h at room temperature, washed twice with sterile saline (PBS), and stored at – 40 ^{o}C.

Stimulation of HEK293 cells stably transfected with TLR2, NOD2 and TLR4

Human embryonic kidney cell line HEK293 stably transfected with plasmid carrying human (h)TLR2/CD14 gene were kindly provided by M. Yazdanbakhsh (Leiden, Netherlands), cells transfected with hTLR4/MD2/CD14 were a gift of B. Bohle (Vienna, Austria) and cells

158 transfected with hNOD2 were purchased from InvivoGen (InvivoGen, USA). Cells were
159 stimulated with formalin-inactivated single strains or their mixture in concentration 10^7
160 CFU/ml. TLR2 ligand Pam3CSK4 (PAM3; 1 μg/ml, InvivoGen, USA), NOD2 ligand
161 muramyl dipeptide (MDP; 100 ng/ml, InvivoGen, USA) and TLR4 ligand ultrapure LPS-EB
162 (LPS; 1 μg/ml, Invivogen, USA) were used as positive controls. After the 20-h incubation
163 period, culture supernatants were harvested and concentration of human IL-8 was analyzed by
164 ELISA (Thermo Scientific, USA) according to the manufacturer's instructions.
165
166 ***Preparation and activation of bone marrow-derived dendritic cells***
167 Mouse bone marrow-derived DC (BM-DC) were prepared as previously described (21).
168 Briefly, the bone marrow precursors were isolated from femurs and tibias of conventional
169 (CV) BALB/c mice. Cells were cultured at 4×10^5/ml in bacteriological Petri dishes in 10 ml
170 culture medium with GM-CSF (20 ng/ml; Sigma–Aldrich, USA). Fresh medium was added at
171 days 3 and 6 and BM-DC were used on day 8 of culture. BM-DC (10^6 cells/ml) were
172 stimulated with 10^7 CFU/ml of inactivated *L. rhamnosus* LOCK0900, *L. rhamnosus*
173 LOCK0908, *L. casei* LOCK0919 and their equal part mixture (Lmix) for 18 h. As controls,
174 BM-DC were incubated with Pam3CSK4 (PAM3; 1 μg/ml) or ultrapure LPS-EB (LPS,
175 1 μg/ml). Levels of IL-10, TGF-β, and TNF-α in culture supernatants were determined by
176 ELISA Ready-Set-Go! kits (eBioscience, USA) according to manufacturer's instructions.
177 Levels of IL-12p70 were measured with matched antibody pairs (BD Pharmingen, USA).
178
179 ***Animals***
180 GF inbred BALB/c mice were born and housed under sterile conditions and fed a sterile
181 standard pellet diet (ST1, Bergman, Kocanda, Czech Republic, 59 kGy irradiated for 30
182 minutes) and sterile water *ad libitum*. Animals were kept in a room with a 12 h light-dark

cycle at 22°C. Fecal samples were weekly evaluated for the presence of aerobic and anaerobic bacteria, molds and yeast by standard microbiological methodology. Conventional (CV) BALB/c mice (n=5) were fed with the same sterile diet as GF counterparts. Animal experiments were approved by the Committee for Protection and Use of Experimental Animals of the Institute of Microbiology. v.v.i., Academy of Sciences of the Czech Republic (approval ID: 50/2013).

Experimental design

Eight-week old GF mice (n = 12) were divided into two groups. Mice were colonized by intragastric tubing with 2×10^8 CFU of equal parts of overnight cultures of *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 in 0.2 ml sterile PBS (group 1). The second group served as GF control. Three weeks after colonization, *Lactobacillus*-colonized mice as well as GF controls where three times intraperitoneally (i.p.) immunized with 1 μg of recombinant birch pollen allergen Bet v 1 (Biomay, Austria) adsorbed to 2 mg aluminum hydroxide (Alum; Serva, Germany) in 10-day intervals as described before (35). Mice were sacrificed seven days after the last immunization by cervical dislocation (Fig. 4 A). Blood was collected and sera stored at -40° C until analysis. Samples of terminal ileum were removed for immunohistochemistry, Western blot and electron microscopy analysis, and the rest of the small intestine was excised for determination of total IgA with lavages performed as previously described (36). Mesenteric lymph nodes (MLN, pooled per group) and spleen were aseptically removed and prepared for *in vitro* cytokine assays. Briefly, after gentle crushing, meshing through the 70 μm cell strainer (BD Falcon, USA) and the lysis of red blood cells (180 mM NH_4Cl, 17 mM Na_2EDTA, pH 7.3; Sigma-Aldrich, Germany), mononuclear cells were resuspended in complete RPMI-1640 medium (Sigma-Aldrich, USA)

containing 10% fetal calf serum, 2 mM glutamine, 100 U penicillin, and 100 µg/ml streptomycin.

Bacterial colonization

The bacterial colonization of the mice was evaluated on the first two days and then at weekly intervals throughout the experiment. Feces were pooled per group, diluted (1:9, w/v) in sterile PBS and excessively vortexed with sterile glass beads. Volumes of 1 ml of appropriate 10-fold dilution were plated into MRS agar (Oxoid, UK) and cultivated in triplicate at 37°C for 48 h. On the species level, bacteria were distinguished on the basis of colonies morphology: the strain *L. casei* LOCK0919 formed small, white, non-mucosal colonies, whereas the strains *L. rhamnosus* LOCK0900 and LOCK0908 formed larger white-gray colored mucosal colonies. To distinguish between *L. rhamnosus* strains we isolated DNA from feces of colonized mice and performed strain specific qPCR (Supplementary Material and Methods).

Immunohistochemical detection of IgA producing cells

Segments of the terminal ileum were embedded in Tissue-Tek (Sakura Finetec Europe B.V., Netherlands) and frozen in liquid nitrogen. Cryosections (5 mm thick) of acetone-fixed colon were used for immunocytochemistry. Immunostaining was performed by goat anti-mouse IgA-FITC antibody (Invitrogen, USA). Samples were viewed under an Olympus BX 40 microscope equipped with an Olympus DP 70 digital camera. Photographs were taken by Camedia Master 2.5 and DP-Soft (Olympus, Germany).

Transmission electron microscopy

The ileum tissues were cut into small pieces (1 × 1 mm) and immediately fixed in 2.5% glutaraldehyde in PBS for 90 minutes. After fixation in 1% osmium tetroxide (Sigma-Aldrich,

USA) for 1 h and washing in 0.1 M cacodylate buffer samples were successively dehydrated in 35, 70, 96 and 100% ethanol and propylene oxide (EMS, USA). Subsequently, the segments were embedded in Epon resin (EMS, USA). Ileum areas chosen according semi-thin sections were cut into 65 nm ultra-thin sections (Leica Ultracut Uct52), stained with uranyl acetate and lead citrate, and examined under electron microscope (Jem 1011, Jeol, USA). Images of the ultra-structural features of ileum structures and junctions were visualized under magnification ranged from 3000 × to 100 000 ×. Specimens were obtained from 5 mice of each group. The widths and lengths of intracellular junctions were measured using the morphometric program iTEM (Olympus, Germany) at magnification of 100 000 ×. For each specimen 10-15 measurements were performed, and results were presented in nm.

Western blot analysis of ZO-1 and occludin

The terminal ileum was homogenized on ice in protein extract buffer with a protease inhibitor cocktail (Pierce, USA) for 10 min and sonicated. Samples were centrifuged at 10,000× rpm for 10 min at 4°C and stored at −80°C until use. Protein concentrations were measured using the BCA Protein Assay Kit (Pierce, USA). Western blotting was performed as described previously (37). The membranes were blocked with 2% (w/v) dry milk in 0.05% PBS-Tween-20 for 1 h at room temperature and incubated overnight at 4°C with antibodies against occludin (1:1000) (Invitrogen, USA), ZO-1 (1:1000) (ZYMED Laboratories Inc., USA) and β-actin (1:5000) (Abcam, USA). After incubation with the respective primary antibodies, secondary staining was conducted using horseradish peroxidase-conjugated species specific antibodies (1:1000) (ZYMED Laboratories Inc., USA) for 1 h at room temperature. The reactions were developed using the SuperSignal Weat Femto Maximum Sensitivity Substrate (ThermoScientific, USA) and the signal intensities were measured on the G:BOX (Syngene, UK) and processed with ImageJ (38).

Allergen-specific antibody responses: ELISA and basophil release assay

Allergen-specific serum IgG1, IgG2a and IgA levels were determined by ELISA as previously described (39). Briefly, 96-well microtiter plates were coated with Bet v 1 (2 μg/ml). Serum samples were diluted 1/10000 for IgG1, 1/100 for IgG2a and 1/10 for IgA. Rat anti-mouse IgG1, IgG2a and IgA antibodies (1 μg/ml, Pharmingen, USA) were applied, followed by peroxidase-conjugated mouse anti-rat IgG antibodies (1/1000; Jackson Immuno Labs, USA) for detection. Antibody levels were reported as optical density. Allergen-specific IgE levels in sera were quantified by degranulation of rat basophil leukemia (RBL-2H3) cells as previously described (40). 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 in a final dilution of 1/30 for 2 hours. After washing, Bet v 1 (0.3 μg/ml) was added for 30 min at 37°C to induce degranulation. Supernatants were incubated with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (Sigma-Aldrich, USA) for analysis of β-hexosaminidase using a fluorescence microplate reader (λ_{ex}: 360 nm/λ_{em}: 465 nm) Infinite M200 (Tecan Group Ltd., Austria). Results are reported as percentage of total β-hexosaminidase release from cells after disruption with 1% Triton X-100.

Total IgA and IgE responses

Total IgA and IgE were measured in sera and gut lavages (IgA only) by mouse IgA and IgE ELISA quantification kit (Bethyl, USA) according to manufacturer's instruction. Sera were diluted 1/400 for IgA and 1/10 for IgE measurement, for IgA determination in gut lavages 1/2500 dilution was used. Antibody levels are reported as μg/ml for sera and μg/g for gut lavages.

Cytokine production

Spleen cells and pooled MLN cell suspensions were cultured in 48-well flat bottom plates at concentration 5×10^6 cells in 500 µl of complete RPMI 1640 medium. Cells were cultivated with/without Bet v 1 (10 µg/well) restimulation at 37ºC under 5% CO_2 for 48 h. After cultivation supernatants were collected and stored at -40°C until analysis. IL-4, IL-5, IL-10 and interferon (IFN)-γ were determined by the Mouse cytokine/chemokine multiplex Immunoassay (Lincoplex, Millipore, USA) according to manufacturer's instructions and analyzed with the Luminex® 200™ System (Bio-Rad Laboratories, USA) with sensitivities < 0.3 pg/ml for IL-4; < 0.3 pg/ml for IL-5; < 10.3 pg/ml for IL-10; and < 0.7 pg/ml for IFN-γ. TGF-β was measured in culture supernatants and in 1/10 diluted sera by ELISA kit (R&D Duoset Systems, USA) according to the manufacturer's instruction with detection limit < 4 pg/ml.

Statistical Analyses

Non-parametric Mann-Whitney test was used for comparison between two groups and for comparison between multiple groups ANOVA with Tukey's multiple comparison test was performed with GraphPad Prism 5.02 software. Values of $P < 0.05$ were considered statistically different. All data are expressed as the mean ± standard error of the mean (SEM) unless stated otherwise.

Results

TLR2 and NOD2 but not TLR4 are involved in the recognition of all three investigated Lactobacillus strains

To specify pattern recognition receptors involved in *Lactobacillus* signaling pathways, single strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908, *L. casei* LOCK0919 and their equal part mixture (Lmix) were incubated with HEK293 cells transfected either with TLR2, TLR4 or NOD2. The IL-8 cytokine was measured as an indicator of cell stimulation via specific receptor. The level of IL-8 was significantly increased in supernatants of HEK293/TLR2 cells incubated with *L. rhamnosus* LOCK0900, and of HEK/NOD2 exposed to *L. casei* LOCK0919 and Lmix (Fig. 1A, B). There was no IL-8 stimulation in HEK293/TLR4 with any single lactobacilli strain or their mixture (Fig. 1 C).

Strain-specific profile of cytokines produced by stimulated BM-DC

Activation of bone marrow-derived dendritic cells (BM-DC) with single strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908, *L. casei* LOCK0919 and their mixture showed a trend in the increased induction of regulatory cytokine TGF-β independently of applied bacterial strain. On the other hand, the production of IL-10, IL-12p70 and TNF-α was strictly species and strain dependent and stimulation of cytokine production by Lmix corresponded to the average value of all applied bacterial strains (Fig. 2).

Colonization with Lmix improves the intestinal barrier

To evaluate the effect of Lmix colonization on the intestinal barrier, the ultrastructural analyses of apical part of ileal enterocytes were performed. In mice reared in conventional conditions, brush borders were regular, straight and contained microfilaments extending into the terminal web (TW) (Fig. 3 A). The apical junctional complex: tight junction (TJ),

adherens junction (AJ), and desmosome (DE) were well organized. In contrast, enterocyte brush borders of GF mice were irregularly arranged and exhibited a deficit of cytoskeletal microfilaments without elongation into the TW. As documented in Table 1, the AJ region was significantly broader and shorter in GF mice when compared to CV and Lmix-colonized mice. Interestingly, incomplete apical junctional complexes with the lack of DE were observed approximately in 30% of enterocytes of GF mice (Fig. 3 B). Lmix colonization of GF mice led to more organized arrangement of enterocyte microvilli with cytoskeletal microfilaments anchored in the TW, similarly to CV mice (Fig. 3 C). In these mice, in comparison to GF mice, DE were found in each apical junctional complex. Moreover, AJ in Lmix-colonized mice were significantly elongated and narrow as compared to GF mice and resembled those found in CV mice (Table 1). Western blot analysis of terminal ileum further confirmed the results observed in electron microscopy. The levels of ZO-1 (Fig. 3 G) were significantly increased in CV and Lmix-colonized mice compared to GF controls. Concomitantly, the levels of occludin were significantly higher in CV mice and there was a trend towards an increase in the Lmix-colonized mice (Fig. 3 H).

Colonization of GF mice with the Lmix

Stability of colonization with the Lmix was evaluated throughout the experiment. By plating feces on MRS agar we were able to distinguish the bacteria on the species level. As shown in Fig. 4 B, starting from the second day after colonization, concentration of *L. casei* reached levels between 3.3 – 5.0 x 10^9 CFU/g of feces, while *L. rhamnosus* strains were detected in concentration of 0.2 – 8.0 x 10^8 CFU/g. To distinguish between the two *L. rhamnosus* strains, we isolated the DNA from the stools and by qPCR we showed that LOCK0908 strain was more abundant compared to the LOCK0900 strain (Fig. 4 C).

Colonization by Lmix suppresses Bet v 1-specific antibody production

To analyze the effect of Lmix-colonization on allergic sensitization, our recently published mouse model (20, 41) was applied and specific antibody and cytokine production were evaluated. Lmix-colonized and GF mice were immunized intraperitoneally with recombinant birch pollen allergen Bet v 1 in 10-days intervals starting three weeks after the bacterial colonization (Fig. 4 A). Colonization with Lmix significantly reduced Bet v 1-specific IgE ($P < 0.03$), IgG1 ($P < 0.03$) and IgG2a ($P < 0.03$) serum antibodies, compared to aged-matched Bet v 1-sensitized GF controls (Fig. 4 D-F). No differences were found in Bet v 1-specific IgA antibodies between both groups (GF: 0.187 ± 0.44 OD, Lmix: 0.167 ± 0.027 OD; p = 0.857).

Colonization with Lmix reduced systemic IgE and induced systemic and local IgA production

Colonization of GF mice with Lmix resulted in decreased level of total IgE in serum (Fig. 5 A) while total levels of IgA in serum ($P < 0.013$) and small intestinal lavages increased significantly ($P < 0.04$) in comparison with Bet v 1-senzitized GF controls (Fig. 5 B, C). In the Lmix-colonized group, the induction of activated IgA-secreting plasma cells in lamina propria of terminal ileum was confirmed by immunofluorescence staining (Fig. 5 D). On the other hand, no IgA producing cells were found in age-matched GF controls (Fig. 5 D).

Lmix colonization reduced Bet v 1-specific IL-4 and IL-5 cytokine production

To investigate the impact of Lmix on Th1 and Th2 cytokine production, splenocytes and pooled MLN cell from Bet v 1-sensitized mice were co-cultured with Bet v 1 *in vitro*. We observed significantly reduced secretion of Th2 cytokine IL-4, trend in the reduction of IL-5 and slightly increased level of Th1-type cytokine IFN-γ in spleen cells supernatants from

Lmix-colonized mice compared to GF controls (Fig. 6 A-C). No IL-4 production was detected in pooled MLN cell cultures and the levels of both IL-5 (Fig. 6 D) and IFN-γ (GF: 5.56 pg/ml, Lmix: 1.30 pg/ml) were lower in supernatants from Lmix-colonized mice compared to GF controls.

Colonization with Lmix stimulated TGF-β production

To evaluate the effects of Lmix colonization on regulatory cytokine response, the level of TGF-β was determined in sera and supernatants of spleen or MLN cells co-cultured with Bet v 1 *in vitro*. A significant upregulation of TGF-β in sera was detected in mice colonized with Lmix compared to GF controls ($P < 0.009$) (Fig. 7 A). We observed significantly increased levels of TGF-β in supernatants of Bet v 1 stimulated splenocyte cultures of Lmix-colonized mice compared to GF controls (Fig. 7 B). A similar tendency was detected in supernatants of MLN cells isolated from Lmix-colonized mice (Fig. 7 C). There was no difference between Lmix-colonized and GF control groups in IL-10 production in any of cell culture supernatants (data not shown).

Discussion

In the present study, we aimed to investigate the ability of the Lmix, a mixture of three lactobacilli strains *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919, to modulate allergic sensitization in a gnotobiotic mouse model. We showed that colonization with Lmix ameliorates Bet v 1-specific allergic responses both on humoral and cellular levels. Furthermore, Lmix colonization improved the barrier function of the gut, which was immature in GF mice.

Modulation of the immune responses by single bacterial strain or by mixtures of different probiotic strains was documented in mouse models as well as in human trials (42, 43). This modulation occurs either by promoting Th1-type responses (44) or by induction of regulatory cells and cytokines (20, 45). Using gnotobiotic mouse model we show that colonization with Lmix reduced serum levels of both Th2-related Bet v 1-specific IgE and IgG1 antibodies as well as Th1-related IgG2a antibody, implicating the involvement of regulatory mechanisms. This was further supported by significantly higher level of TGF-β in serum. After *in vitro* restimulation of splenocytes or MLN cells with Bet v 1, we observed alteration in Th2/Treg cytokine production. We detected downregulation of Th2 associated cytokines IL-4 and IL-5 and upregulation of TGF-β production in Lmix-colonized group, suggesting that Lmix-colonization induced immunoregulatory mechanisms. Previously, Feleszko et al. (45) demonstrated that oral delivery of probiotic bacteria led to suppression of allergic sensitization and airway inflammation by TGF-β-producing Treg cells, which could be found in MLN. It has also been shown that peripheral conversion of CD4+ T cells to Treg cells occurs primarily in gut-associated lymphoid tissue in the presence of TGF-β and retinoic acid (46). In line with these findings we suppose that colonization with lactobacilli mixture induces upregulation of TGF-β production in intestine and generation of Treg cells.

In correlation with increased production of TGF-β we found a significant increase in gut and serum IgA level in Lmix-colonized mice. Secretory IgA has been shown to play a crucial role in maintaining bacterial homeostasis in the gut (reviewed in (47)). These results are in accordance with previous findings that colonization of GF mice with probiotic bacteria induces activation of IgA production, and that the mixture of probiotic strains is more effective in the development of plasmablasts in the gut compared to single strains (48).

The intestinal barrier is immature in GF mice (49), and Lmix significantly improves this condition. Enterocyte brush borders of GF mice were irregularly arranged and exhibited the deficit of cytoskeletal microfilaments without elongation into the terminal web. Adherens junctions in Lmix-colonized mice were significantly elongated and narrow as compared to GF mice and resembled those found in CV mice. This fortification of the intestinal barrier was further evident from the increased levels of ZO-1 and occludin proteins in Lmix-colonized and CV mice. To our knowledge this is the first report documenting the effect of lactobacilli colonization on the ultrastructure of brush border and apical junctional complexes of enterocytes in gnotobioitc mice. Along these lines, increased gut permeability was found in children with food allergy (50) and recently it was also detected in asthmatic patients (51). The homeostasis of the intestinal epithelium is maintained by a complex interplay of multiple regulatory mechanisms (52). *In vitro* studies presented that pro-allergic cytokine IL-4 contributes to barrier impairment, in contrast to TGF-β, which enhances the barrier function and activates the expression of proteins comprising the intercellular junctions (53). In our study the improvement of gut barrier in Lmix-colonized mice was accompanied by reduced secretion of pro-allergic cytokines and significant enhancement of TGF-β.

There is increasing evidence that probiotic bacteria can exhibit their activities by direct interaction with pattern recognition receptors. In this study we showed that TLR2 plays an important role in the recognition of *L. rhamnosus* LOCK0900 and NOD2 in the recognition of

L. casei LOCK0919. In contrast, *L. rhamnosus* LOCK0908 was poorly recognized by both these receptors. Interestingly, the significant feature of *L. rhamnosus* LOCK0908 strain is its high exopolysaccharides (EPS) production (33). Fanning et al. (54) recently showed that bifidobacterial strain producing surface EPS failed to elicit a strong immune response compared with EPS-deficient variants. It is thus tempting to speculate that the lack of TLR2, TLR4 and NOD2 activation by *L. rhamnosus* LOCK0908 could be caused by exopolysaccharides covering the bacterial surface and masking bioactive components, which play a role in binding to pattern recognition receptors.

Previously we have shown in human blood cell cultures that application of *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 strains together as a mixture has a synergistic effects in induction of anti-allergic Th1-type cytokines compared to the levels induced by each single strain alone (31). By using the mouse BM-DC we were not able to confirm these findings and we did not observe any synergistic effect in cytokine production. This discrepancy may be explained by the different way of bacterial inactivation (heating vs. formalin inactivation) (55), but also by the different donor species and cell type used.

By evaluating the bacterial colonization we were able to show that all three bacterial strains were detectable in feces till the end of the experiment. Two days after the colonization, *L. casei* LOCK0919 became the dominant strain in the feces of colonized mice. This finding can be related to the recent analysis of the complete genome sequence of *L. casei* LOCK0919 which revealed the presence of factors relevant to the colonization and persistence in the human gut, including proteins with the role in the adhesion to host cell structures (34). However, further experiments are needed to test, whether the observed *in vivo* effects could be achieved by colonization of mice by *L. casei* LOCK0919 alone. Although the *L. rhamnosus* strains compose a minority in feces of colonized mice, we cannot exclude that

they could play an important part in the immunomodulating outcome of the mixture and that they are necessary for the successful reduction of allergic sensitization. This argument is supported by our recent finding that EPS produced by *L. rhamnosus* LOCK0900 strain can modulate cytokine production of BM-DC induced by another bacteria (56).

To conclude, we have shown that three lactobacilli strains in Lmix - *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 were able to reduce sensitization to Bet v 1. The specific serum IgE and IgG as well as production of proallergic cytokines IL-4 and IL-5 by splenocytes and MLN cells was also reduced. This suppression was accompanied by upregulation of regulatory cytokine TGF-β and by improvement of epithelial gut barrier. These results clearly point out the beneficial role of selected lactobacilli strains in the process of allergic sensitization and support their use in the early prevention of allergies.

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Disclosures

The authors declare that there are no conflicts of interest.

Author contributions

H.K., M.S. and B.C. designed the experiments. H.K., M.S., D.S., I.S. and P.H. performed the experiments and analysed the data. E.C., I.R. and B.C. performed and analyzed the electron microscopy mikrographs. Z.Z. performed and analyzed the Western blot experiments. T.A.-P., K.A.-B. performed and analyzed the qPCR experiments. T.H. performed and analyzed the immunohistochemistry. H.K., M.S., L.T., I.S., H.T.-H. and B.C. wrote the manuscript.

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Table 1 Effect of bacterial colonization on the width (W) and length (L) of apical intracellular junctions in the ileum of conventional (CV), germ-free (GF) and Lmix-colonized mice

Group	**Tight Junctions (nm)**		**Adherens Junctions (nm)**	
	W	L	W	L
CV	10 ± 1	336 ± 40	30 ± 2	226 ± 50
GF	10 ± 1	203 ± 50*	40 ± 10*	181 ± 40*
Lmix	11 ± 3	236 ± 80	30 ± 7	234 ± 70

Values are means ± SEM (nm) out of 10-15 measurements per sample, n=5 samples were taken per group. *$P < 0.05$, significant difference of GF group versus Lmix and CV groups

Figure legends

Fig.1 ***Stimulation of HEK293 TLR2-, NOD2- and TLR4-transfected cells with Lactobacillus strains***

Human embryonic kidney cells (HEK293) stably transfected with an expression vector for human TLR2 (293-hTLR2) (A), NOD2 (pUNO-hNOD2) (B) and TLR4 (293-hTLR4/MD2/CD14) (C) were cultured for 20 h with 10^7 CFU/ml of formalin-inactivated *L. rhamnosus* LOCK0900 (L900), *L. rhamnosus* LOCK0908 (L908), *L. casei* LOCK0919 (L919) or equal mixture of these strains (Lmix). Pam3CSK4 (PAM3; 1 μg/ml), muramyl dipeptide (MDP; 10 μg/ml) and ultra-pure lipopolysaccharide from *E. coli* (LPS; 1 μg/ml) were used as positive controls for TLR2, NOD2 and TLR4, respectively. Unstimulated cells (ctrl) were used as control. Stimulation was evaluated by measurement of IL-8 production; results are expressed as mean ± SEM. Pooled values of at least three experiments are shown. a – significantly different from unstimulated control, *P < 0.05, **P < 0.01, ***P < 0.001.

Fig.2 ***Stimulation of bone marrow-derived dendritic cells with Lactobacillus strains***

Bone marrow-derived dendritic cells (BM-DC) were cultured with 10^7 CFU/ml of formalin-inactivated *L. rhamnosus* LOCK0900 (L900), *L. rhamnosus* LOCK0908 (L908), *L. casei* LOCK0919 (L919) strains and equal mixture of these strains (Lmix) for 18 h. As positive controls, Pam3CSK4 (PAM3; 1 μg/ml) or ultra-pure lipopolysaccharide from *E. coli* (LPS; 1 μg/ml) were applied. Unstimulated cells (ctrl) served as negative controls. Levels of IL-10, TGF-β, IL-12p70 and TNF-α in culture supernatants were determined by ELISA and expressed as mean values ± SEM. Pooled values of three experiments are shown. a – significantly different from unstimulated control, *P < 0.05, **P < 0.01, ***P < 0.001.

Fig.3 ***The effect of Lmix-colonization on architecture of the apical junctional complex of enterocytes and production of ZO-1 and occludin***

Electron microscopy micrograph of the apical surface of ileal enterocytes in conventional (CV), germ-free (GF) and Lmix-colonized mice (Lmix). The epithelial surface is covered by microvilli. Microfilaments extend from the microvilli into apical cytoplasm and filamentous terminal web (TW), which was lacking in the GF animals and restored in Lmix-colonized mice (A-C). The epithelial cell junctional complex contains the tight junctions (TJ), adherens junctions (AJ) and desmosomes (DE). DE were absent in 30 % of junctional complexes in GF mice (D-F). Representative micrographs out of 10-15 measurements per sample, n=5 samples per group. Western blot analysis of ZO-1 (G) and occludin (H) in ileum. Representative mouse per group is shown (3-4 mice per group were analyzed,). Quantification of the signals was done using the ImageJ. Data are expressed as mean ± SEM of 3-4 mice per group. *P < 0.05, **P < 0.01.

Fig.4 ***Sensitization of GF and Lmix-colonized mice with major birch pollen allergen Bet v 1***

(A) Experimental design: eight-week old germ-free (GF) mice (n = 12) were divided into two groups. The first group (Lmix) received equal parts (2×10^8 CFU/ ml) of *L. rhamnosus* LOCK0900, *L. rhamnosus* LOCK0908 and *L. casei* LOCK0919 by intragastric tubing. The second group was kept GF. Mice were sensitized three times intraperitoneally (i.p.) with recombinant Bet v 1 (1µg in alum) on days 77, 87 and 97. One week after the last immunization (day 104) tissue samples were collected for further analyses. Bacterial colonization of the Lmix-colonized mice was evaluated on the first two days and then at weekly intervals throughout the experiment. On the species level bacteria were distinguished based on different colony morphology by cultivation of appropriate serial dilution of feces

(B). *L. casei* LOCK 919 (full circles, solid line), *L. rhamnosus* LOCK 900 and LOCK 908 (open squares, dotted line). *L. rhamnosus* strain-specific discrimination was performed by qPCR on DNA isolated from feces at indicated time points (C). *L. rhamnosus* LOCK 900 (grey bars), *L. rhamnosus* LOCK 908 (black bars). Data are shown as percentage of each strain from all detected *L. rhamnosus* bacteria at given day after colonization. Bet v 1-specific antibodies were measured in sera of GF (white bars) and Lmix-colonized mice (black bars). IgE was measured by Bet v 1-mediated β-hexosaminidase release from rat basophil leukemia cells (D). Levels of IgG1 (E) and IgG2a (F) were evaluated by ELISA and expressed as optical density (OD) units. Data are shown as mean values ± SEM. One representative out of two experiments is shown, n = 6/ group. *P < 0.05, **P < 0.01.

Fig.5 ***Local and systemic humoral responses in sensitized GF and Lmix-colonized mice***

Levels of total IgE (A) and total IgA (B) in sera and total IgA (C) in gut lavage were measured by ELISA. Germ-free mice (GF, white bars) and Lmix-colonized mice (Lmix, black bars). Data are shown as mean values ± SEM. One representative out of two experiments is shown, n = 6/ group. *P < 0.05, **P < 0.01. The IgA-positive plasmocytes in lamina propria of terminal ileum were visualized by FITC labeled anti-IgA antibody (D).

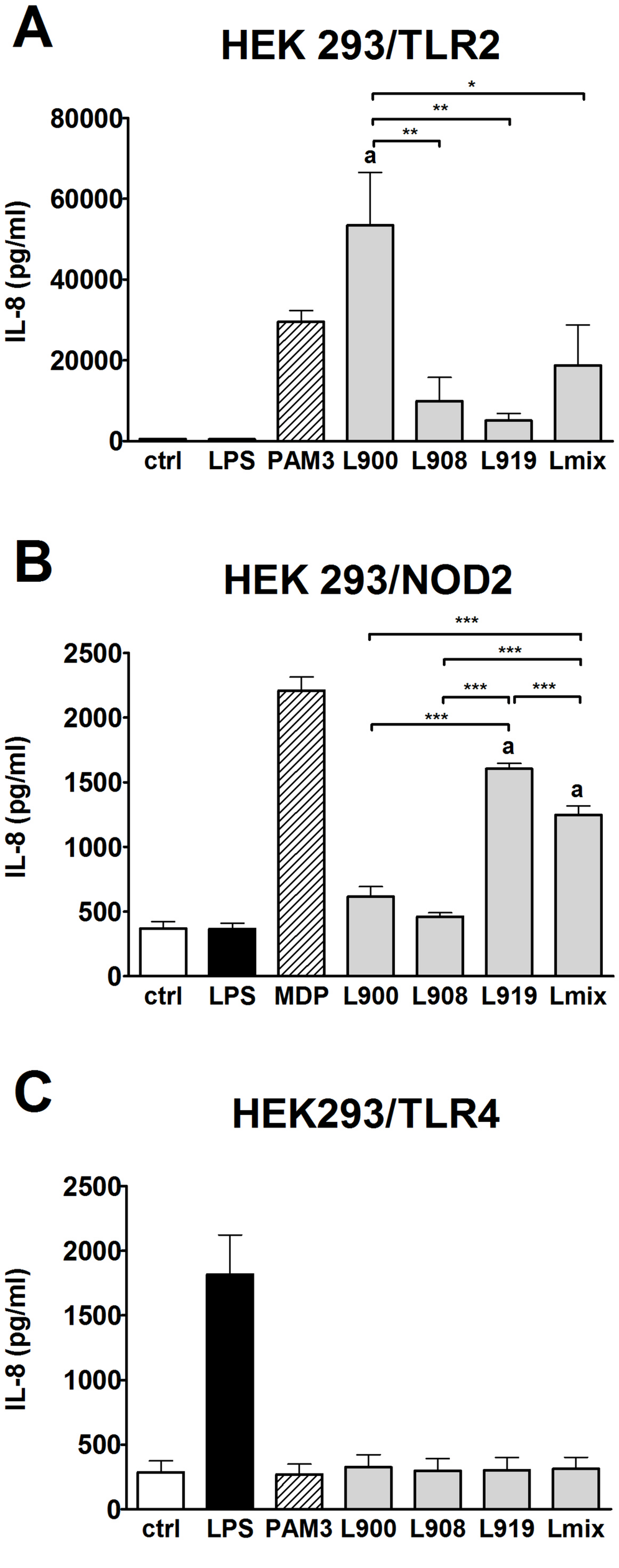
Fig.6 ***The effect of Lmix colonization on cytokine production in vitro***

Spleen and pooled mesenteric lymph node (MLN) cells of Bet v 1-sensitized GF (white bars) and Lmix-colonized mice (black bars) were re-stimulated with Bet v 1 (10 µg/well) for 48 hours. The levels of IL-4 (A), IL-5 (B) and IFN-γ (C) in spleen cell cultures and IL-5 (D) in pooled MLN were determined by ELISA. Results are expressed after subtraction of cytokine

levels measured in supernatants of non-stimulated cell cultures. One representative out of two experiments is shown, n = 6/ group. *P < 0.05.

Fig.7 ***The effect of Lmix colonization on systemic and local TGF-β production***

The level of TGF-β in Bet v 1-sensitized GF (white bars) and Lmix-colonized mice (black bars) in sera (A) and in supernatants from Bet v 1-re-stimulated spleen cell (B) or pooled mesenteric lymph node (MLN) cell (C) cultures was determined by ELISA. Results are expressed after subtraction of cytokines measured in supernatants of non-stimulated cell cultures. One representative out of two experiments is shown, n = 6/ group. **P < 0.01, *P < 0.05.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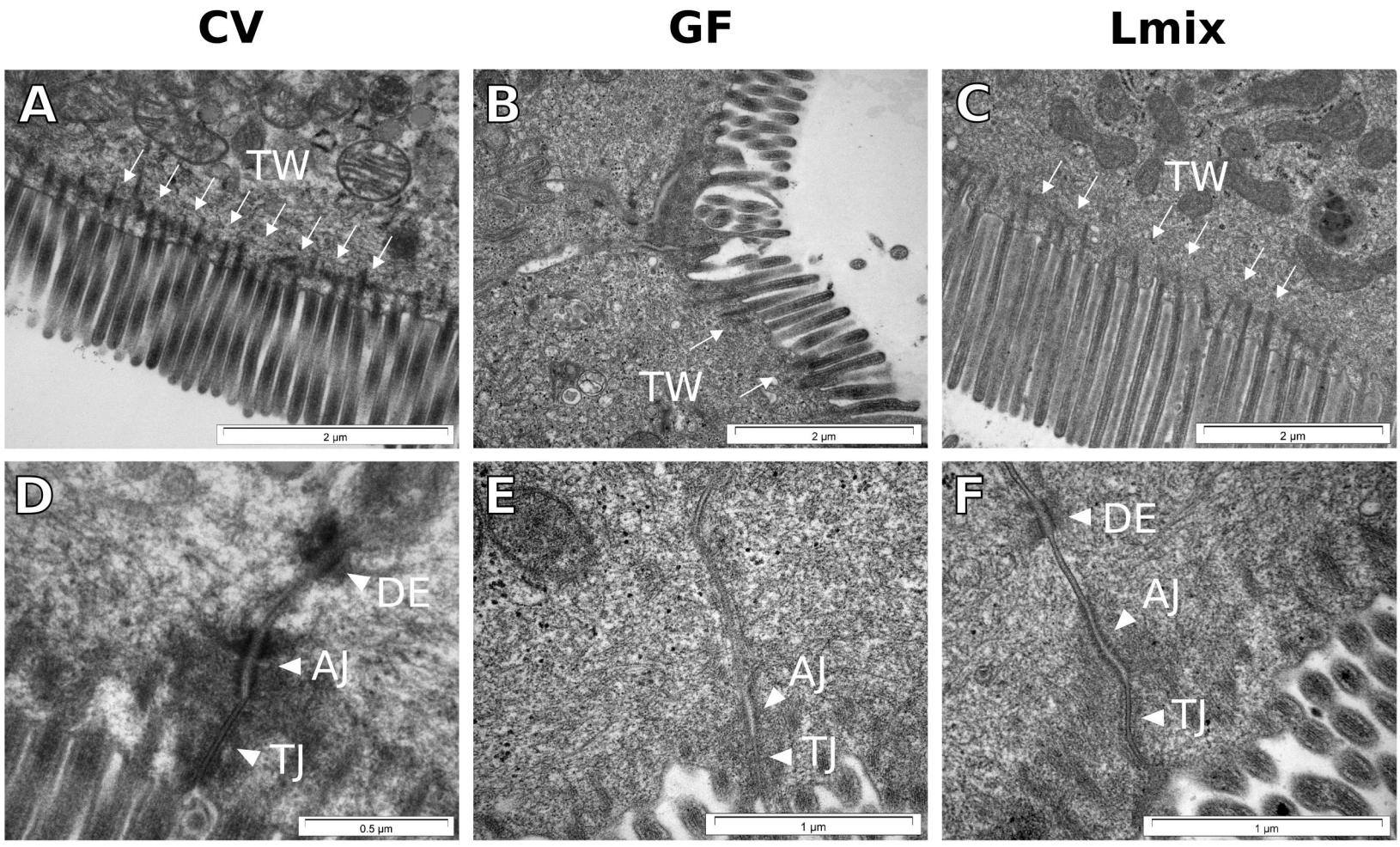
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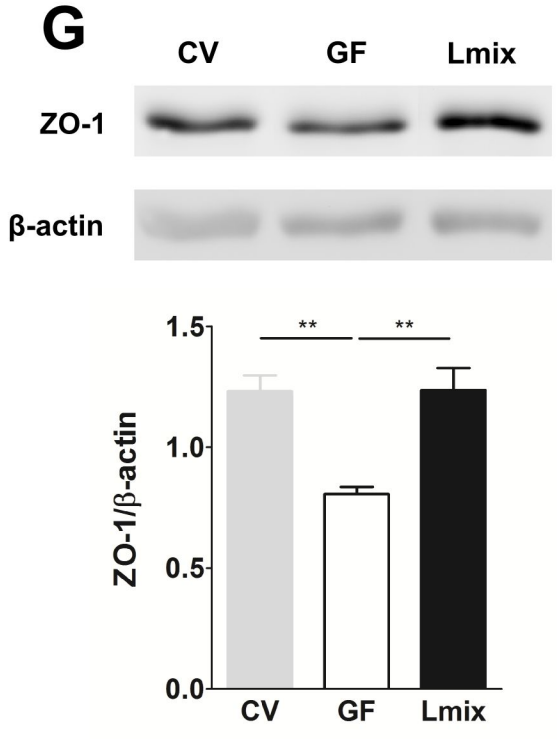
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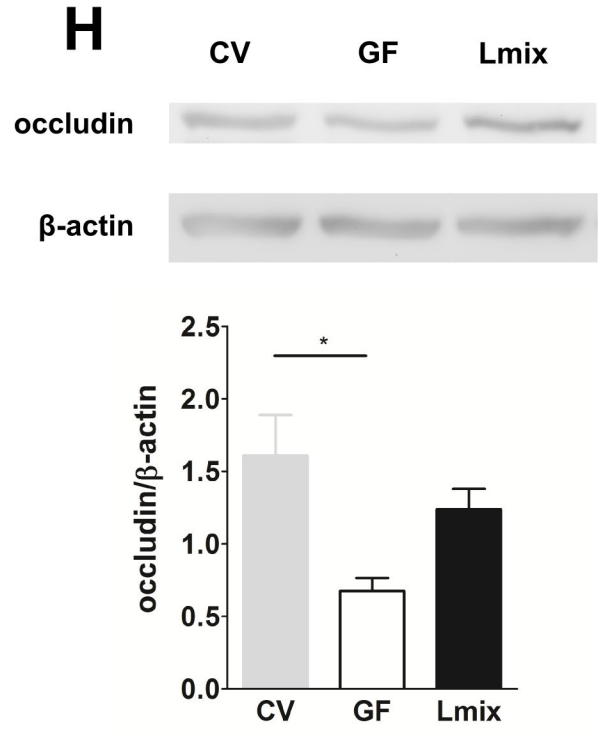
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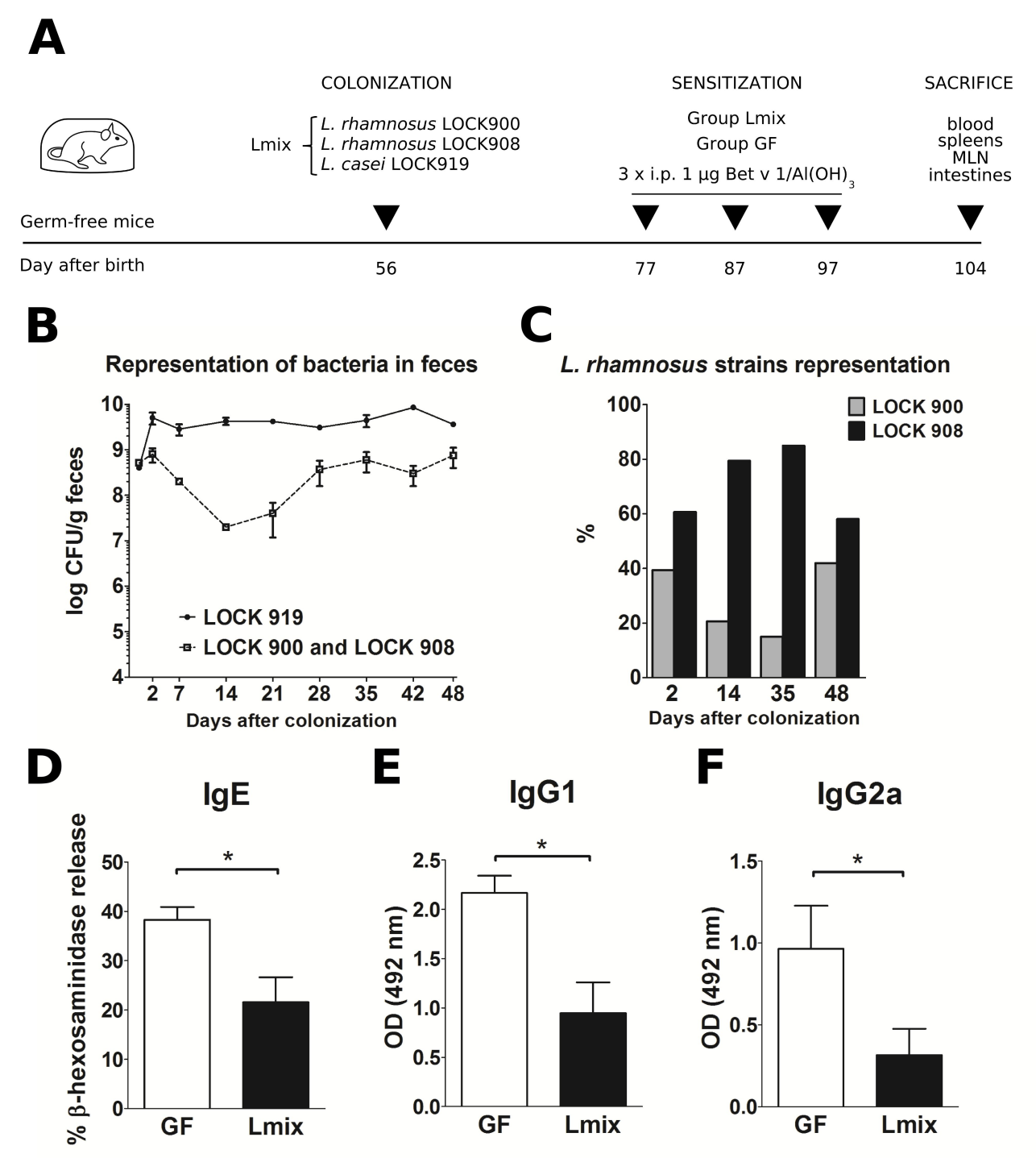
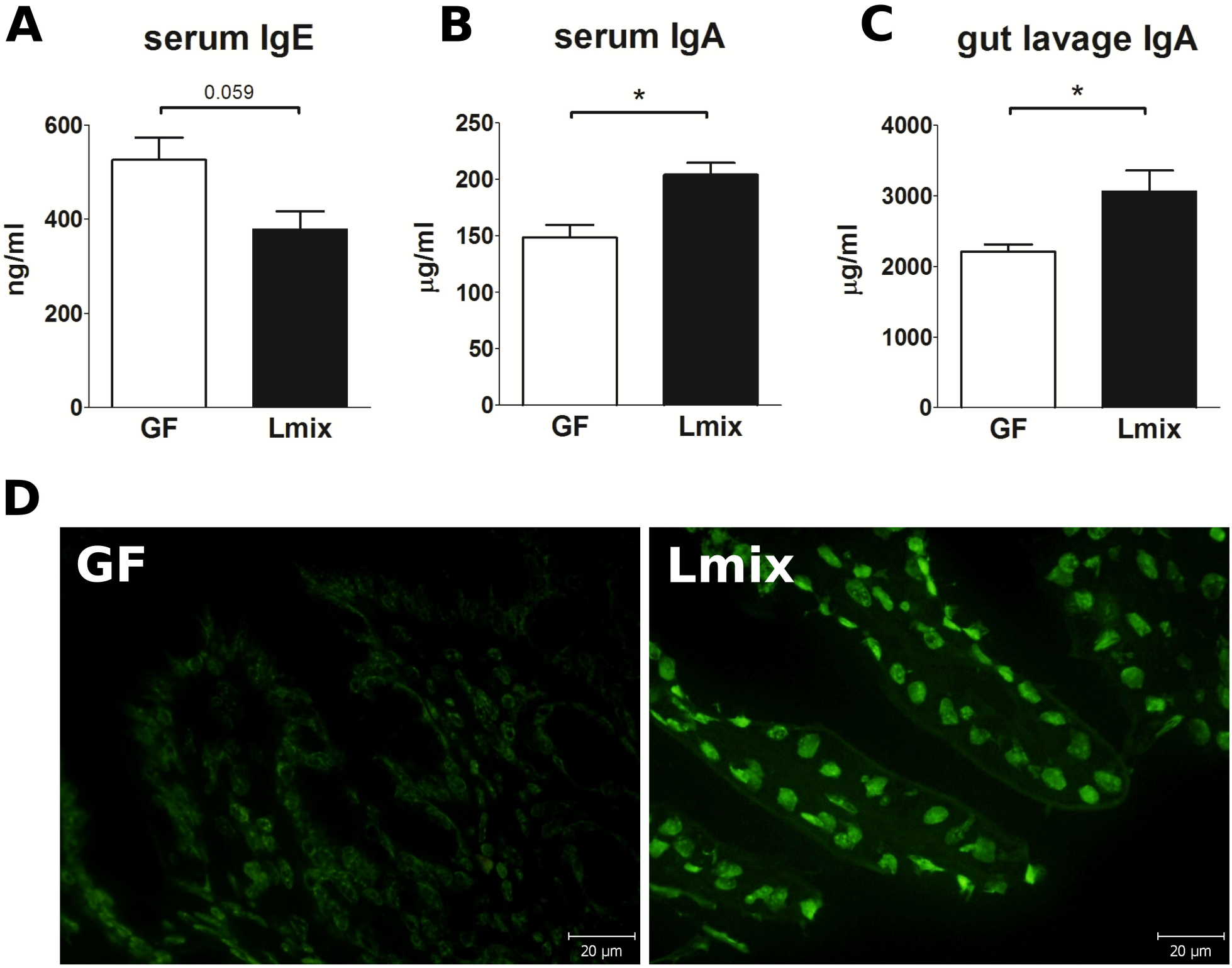

A
COLONIZATION
SENSITIZATION
SACRIFICE
Lmix
L. rhamnosus LOCK900
L. rhamnosus LOCK908
L. casei LOCK919
Group Lmix
Group GF
3 x i.p. 1 µg Bet v 1/Al(OH)3
blood
spleens
MLN
intestines
Germ-free mice
Day after birth
56
77
87
97
104
B
Representation of bacteria in feces
log CFU/g feces
LOCK 919
LOCK 900 and LOCK 908
Days after colonization
C
L. rhamnosus strains representation
LOCK 900
LOCK 908
%
Days after colonization
D
IgE
% β-hexosaminidase release
GF
Lmix
E
IgG1
OD (492 nm)
GF
Lmix
F
IgG2a
OD (492 nm)
GF
Lmix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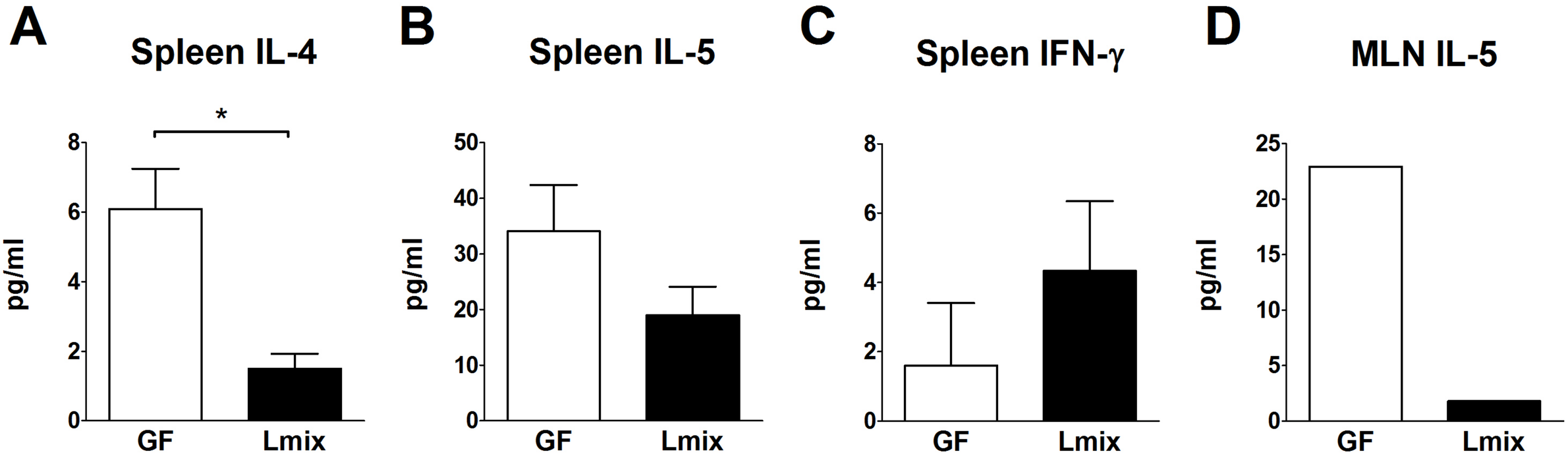
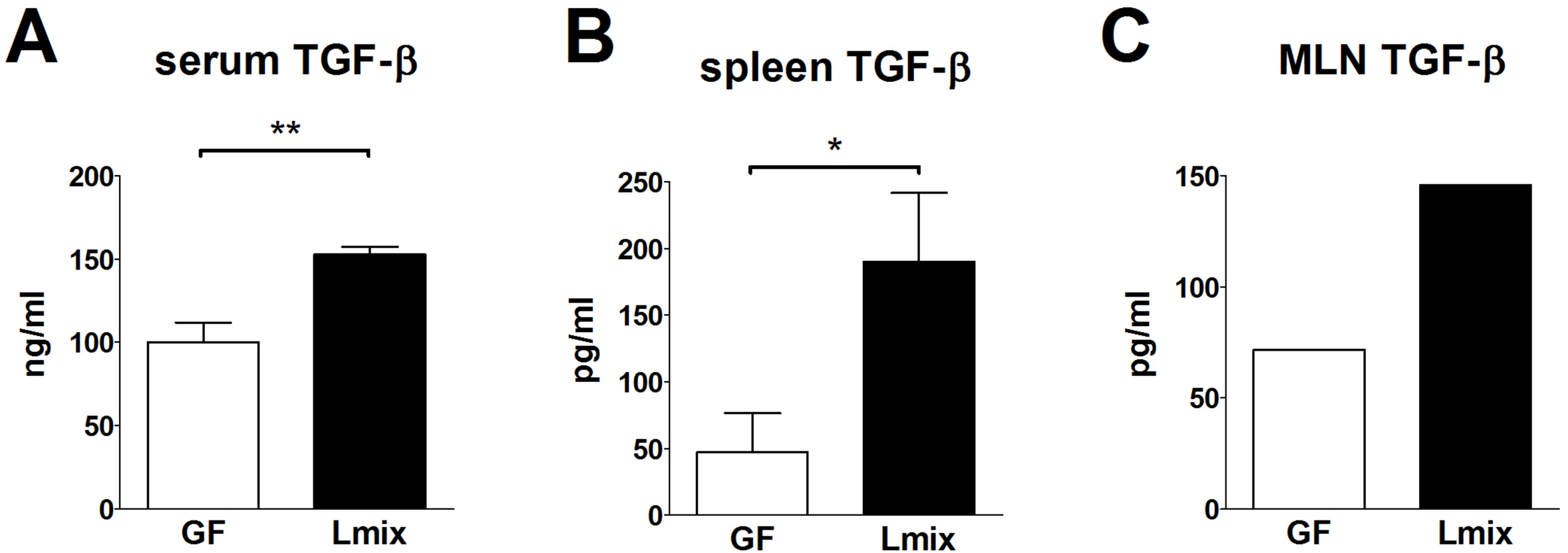
0.059
600
400
200
0
ng/ml
GF
Lmix
B
serum IgA
*
250
200
150
100
50
0
µg/ml
GF
Lmix
C
gut lavage IgA
*
4000
3000
2000
1000
0
µg/ml
GF
Lmix
D
GF
20 µm
Lmix
20 µm


A
Spleen IL-4
*
pg/ml
8
6
4
2
0
GF
Lmix
B
Spleen IL-5
pg/ml
50
40
30
20
10
0
GF
Lmix
C
Spleen IFN-γ
pg/ml
8
6
4
2
0
GF
Lmix
D
MLN IL-5
pg/ml
25
20
15
10
5
0
GF
Lmix


A
serum TGF-β
**
200
150
100
50
0
ng/ml
GF
Lmix
B
spleen TGF-β
*
250
200
150
100
50
0
pg/ml
GF
Lmix
C
MLN TGF-β
150
100
50
0
pg/ml
GF
Lmix