

ORIGINAL PAPER

Probiotic-induced suppression of allergic sensitization and airway inflammation is associated with an increase of T regulatory-dependent mechanisms in a murine model of asthma

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Clinical and Experimental Allergy

Summary

Background Microbial intestinal colonization in early in life is regarded to play a major role for the maturation of the immune system. Application of non-pathogenic probiotic bacteria during early infancy might protect from allergic disorders but underlying mechanisms have not been analysed so far.

Objective The aim of the current study was to investigate the immune effects of oral application of probiotic bacteria on allergen-induced sensitization and development of airway inflammation and airway hyper-reactivity, cardinal features of bronchial asthma.

Methods Newborn Balb/c mice received orally 10^9 CFU every second day either *Lactobacillus rhamnosus* GG or *Bifidobacterium lactis* (Bb-12) starting from birth for consecutive 8 weeks, during systemic sensitization (six intraperitoneal injections, days 29–40) and airway challenge (days 54–56) with ovalbumin.

Results The administration of either Bb-12 or LGG suppressed all aspects of the asthmatic phenotype: airway reactivity, antigen-specific immunoglobulin E production and pulmonary eosinophilia (mean: 137 vs. 17 and 13 cells $\times 10^3$ /mL, respectively). Antigen-specific recall proliferation by spleen cells and T-helper type 2 cytokine production (IL-4, IL-5 and IL-10) by mesenteric lymph node cells also showed significant reduction, while TGF production remained unchanged. Oral LGG administration particularly suppressed allergen-induced proliferative responses and was associated with an increase in numbers of TGF- β -secreting CD4⁺/CD3⁺ T cells in mesenteric lymph nodes (6.5, 16.7%) as well as nearly 2-fold up-regulation of Foxp3-expressing cells in peribronchial lymph nodes.

Conclusions Neonatal application of probiotic bacteria inhibits subsequent allergic sensitization and airway disease in a murine model of asthma by induction of T regulatory cells associated with increased TGF- β production.

Keywords allergy, asthma, bifidobacterium, Foxp3, immunomodulation, lactobacillus, mice, probiotics, regulatory T cell

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Introduction

A common explanation for the increased incidence rates of childhood allergy and asthma observed in industrialized countries during the past few decades is the 'hygiene

hypothesis', stating that a separation of the immune system from microbial exposure during a critical time-period early in life raises the risk of developing allergic diseases [1]. In support of this idea, epidemiologic studies point to a positive correlation between allergic diseases and changed microbial exposure patterns in western societies. One example for this is provided by data on colonization with certain gram-positive gut flora bacteria, such as lactobacilli and bifidobacteria [2, 3] correlating

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with lower incidence rates of allergic sensitization. It has been hypothesized that these microbes stimulate gut-associated lymphoid tissues, thus instructing the developing immune system to reverse the pre-natal Th2 bias into more balanced immune responses, including tolerance to not-dangerous ubiquitous antigens [4]. On the cellular level this hypothesis is based on the assumption of impaired tolerance induction due to deficient regulatory T cell (Treg) activity in the absence of microbial stimuli [5], resulting in exaggerated proliferation and cytokine production particularly by effector T cells [6].

Among the diverse probiotic strains, lactobacilli and bifidobacteria are the most promising candidates to naturally affect Treg cell development as they are part of the commensal gut flora of infants [7, 8]. For the same reason, they are the most frequently used probiotic strains for clinical intervention studies [8–10]. Administering probiotics to pregnant and lactating mothers improved the immunoprotective effects of breastmilk, assessed by increased amounts of the immunosuppressive cytokine, TGF- β [11]. In the same line, lactobacilli species were shown to modulate dendritic or T cell responses *in vitro* by boosting the production of immunosuppressive cytokines, IL-10 and TGF β [12–15]. On the basis of these descriptive studies it has repeatedly been hypothesized that orally delivered probiotics might regulate pulmonary immune responses by driving the T cell regulatory network [4, 16–18]. To further assess the cellular mechanisms of probiotic intervention, the present study delineated the immune modulatory activity of two strains (*Bifidobacterium lactis* Bb-12 and *Lactobacillus rhamnosus* GG) using a murine model. Here, we show that oral administration of probiotics, in a time-dependent matter, prevented the development of allergen-induced sensitization and airway disease by inducing T regulatory activities.

Materials and methods

Animals

Pathogen-free, female Balb/c mice (Harlan Winkelmann), 8 weeks of age at the time of mating, and their offspring were housed in a specific pathogen-free facility in individually ventilated and filtered cages under positive pressure (Tecniplast Deutschland GmbH, Hohenpeissenberg, Germany) on ovalbumin (OVA)-free diets and water *ad libitum*. All experiments were performed according to institutional approved guidelines.

Probiotics

Lyophilizate *Lactobacillus rhamnosus* GG (ATCC 53103) was provided by Valio Ltd. (Helsinki Finland), *Bifidobacterium lactis* (strain Bb-12) was provided by Chr. Hansen (Czastków Maz., Poland).

Experimental Procedures

In preliminary studies, kinetics (timing and dosage) of probiotic treatment was delineated using different sensitization protocols and age groups of mice. Based on these data, the following protocol was chosen for further analysis: probiotic bacteria were orally applied by a Neoflon[®] cannula (Becton Dickinson, Helsingborg, Sweden) every second day for a total of 8 weeks, starting on the first day after birth, with increasing volumes to reach the dose of 10^9 CFU/day within 1 week.

Experimental animals were divided into four groups on the first day after birth:

(I) Bb-12- and (II) *Lactobacillus rhamnosus* GG (LGG)-fed animals (Bb-12/OVA and LGG/OVA, respectively) received probiotics and were subsequently sensitized by six intraperitoneal injections of 10 μ g OVA grade VI (Sigma Aldrich, Steinheim, Germany) on days 29, 31, 33, 36, 38 and 40 of life. (III) The negative control group (H₂O/PBS) consisted of animals that were sham treated with corresponding volumes of water and sham sensitized with PBS. (IV) Positive controls (H₂O/OVA) were fed with water and sensitized with OVA. All animals were challenged via the airways by aerosolized OVA (50 μ g grade VI in 50 μ L PBS) or PBS on days 54–56, daily for 20 min.

In vivo airway reactivity

On day 57, *in vivo* lung function measurement was performed by whole-body plethysmography (Buxco[®], EMKA Technologies, Paris, France), as previously reported [19]. Airway reactivity (AR) was expressed as fold increase (FI) of enhanced pause (Penh) values for each concentration of methacholine (Mch) relative to baseline (PBS) Penh values.

Bronchoalveolar lavage

On day 58, lungs were lavaged twice with 0.8 mL of PBS as previously described [20]. Cytospin preparations were stained with Diff Quik (Dade Behring AG Dürdingen, Switzerland) and differentiated according to standard morphologic criteria by counting 200 cells via light microscopy.

Immunoglobulin serum levels

On day 58, blood was drawn via the tail vein and serum levels of total and OVA-specific Ig were measured by means of ELISA, as previously described [21]. Detection limits were: total IgE: 3.9 ng/mL, OVA-specific IgE: 3.1 EU/mL, OVA-specific IgG2a: 2.8 EU/mL.

Proliferation assay and cytokine production

On day 58, mesenteric lymph node (MLN) mononuclear cells were isolated and cultured with OVA (50 μ g/mL) at 1×10^6 cells/well as previously described [21]. Cytokines in supernatants were assessed using commercially

available ELISA kits. OptEia (Pharmingen, Hamburg, Germany) for IL-4, IL-5, IL-10, IFN- γ and DuoSet (R&D Systems, Wiesbaden, Germany) for TGF- β . Detection limits were IFN- γ 3.95 pg/mL, IL-4 15.6 pg/mL, IL-5 15.1 pg/mL, IL-10 7.8 pg/mL, TGF- β 3.9 pg/mL. Proliferative responses of spleen MNCs (0.2×10^6 cells/well) cultured with medium or OVA (concentrations as above) were determined after addition of $^3\text{[H]}$ -thymidine (0.5 $\mu\text{Ci}/200 \mu\text{L}$, Amersham, Buchter, Braunschweig, Germany) for the last 16 h of a 72 h culture period. Data were expressed as FI over incorporation by cells cultured with medium alone.

Flow cytometry analysis

Surface molecule expression of CD4, CD8, CD3, CD25, by spleen and MLN cells was analysed by flow cytometry (unless stated otherwise, mAb were provided as a kind gift from R.A. Kroczeck, Robert-Koch-Institute, Berlin, Germany). To prevent unspecific binding of mAb, all samples were pre-incubated with anti-Fc-RII/III mAb (2.4G2; 100 $\mu\text{g}/\text{mL}$; American Type Culture Collection) and purified rat IgG (200 $\mu\text{g}/\text{mL}$; Nordic Immunology Laboratories (Tilberg, Netherlands)) 10 min before and during staining.

Cytoplasmic cytokine staining of IL-4, IL-5, IL-10, IFN- γ and TGF- β in spleen MNCs after culture with PMA (20 ng/mL) and Ionomycin (500 ng/mL) was performed with the BD GolgiPlug[®] kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's instruction. FACS analysis on a minimum of 50 000 live cells was performed on a BD FACS Calibur[®] (BD Biosciences), data were analysed via CellQuest[®] software (BD Biosciences).

Reverse transcriptase polymerase chain reaction

On day 58, FoxP3 RNA expression by peribronchial lymph node (PBLN) cells was analysed by real-time PCR. PBLN cells were harvested on day 58, transferred into liquid nitrogen immediately and stored frozen at -80°C . For Foxp3 analysis total RNA was prepared from 30 mg cryoconserved tissue using Invisorb Spin Tissue RNA Mini Kit (Invitex, Berlin, Germany). Reverse transcription was done with 2 μg of total RNA, 0.5 μL Random Hexamer Primer (3 $\mu\text{g}/\text{mL}$), 5 μL MML V RT $5 \times$ reaction buffer, 2.0 μL dNTP (10 mM) and 0.5 μL MML V RT (200 U/ μL) in a final volume of 25 μL . Subsequently, cDNA (10 ng) was mixed with $10 \times$ PCR buffer, dNTP (10 mM), MgCl_2 (2.0 mM final), primers and Taq Gold DNA polymerase (5U/ μL) in a final volume of 25 μL . PCR was done in a DNA thermal cycler for 40 cycles.

The expression of Foxp3 and β -Actin was quantified by real-time PCR with Mx3000P System (Stratagene, La Jolla, CA, USA). We used the ABI TaqMan[®] Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) as well as the following primers and Sybr Green as fluorescence: Foxp3 5'-GGCCCTTCTCCAGGACAGA-3' and 5'-GCTGATCATGGCTGGGTGT-3'. For both targets

Foxp3 and β -actin mRNA quantification, each sample was run in triplicate. Foxp3 mRNA was normalized to β -actin mRNA for each sample.

Statistics

Unless indicated otherwise, all experiments were performed in two independent sets with $n = 6-8$ mice/group. Data from two independent experiments were combined and expressed as means $\pm$ SEM. Comparisons between groups were performed with the two-tailed Mann-Whitney U -test (InStatTM version 3.00, GraphPad Software Inc., www.graphpad.com). In the AR experiments the scores per group were compared with Kruskal-Wallis test (non-parametric ANOVA) and $P < 0.05$ was considered to be statistically significant.

Results

Probiotic bacteria suppress allergen-specific immunoglobulin production

Systemic sensitization and local airway challenges with allergen (OVA) elicited OVA-specific immunoglobulin production, pulmonary inflammation dominated by eosinophils and airway hyper-reactivity in positive controls ($\text{H}_2\text{O}/\text{OVA}$). Oral administration of both, Bb-12 and LGG resulted in a marked depression of the production of total IgE antibodies (436 ± 104 vs. 218 ± 52 and 208 ± 42 ng/mL, respectively) (Fig. 1a). When OVA-specific immunoglobulin levels were analysed, even stronger differences were present showing a significant inhibition of both allergen-specific IgE and IgG2a production in animals fed with probiotic microorganisms before sensitization (Figs. 1b and c). These data suggested that probiotic treatment resulted in a general effect on allergen-specific immunoglobulin production, thus not only inhibiting Th2-dependent (IgE) but also suppressing Th1-skewed (IgG2a) immune responses.

Oral probiotic application reduces eosinophilic airway inflammation and airway hyper-reactivity

BAL fluid analysis was performed to examine the effects of oral probiotic application on the development of allergen-induced airway inflammation and hyper-reactivity. In animals that were fed with Bb-12 and LGG before and during allergen sensitization and airway challenges, a significant suppression of total cell counts was found (70 ± 19 and 76 ± 13 vs. 251 ± 35 cells $\times 10^3/\text{mL}$, respectively; $P < 0.05$) (Fig. 2a), that was especially prominent for the eosinophil influx into the airways (mean: 17 ± 6 and 13 ± 4 vs. 137 ± 38 cells $\times 10^3/\text{mL}$, respectively, $P < 0.005$, Mann-Whitney U -test). Additionally, the development of, *in vivo* airway reactivity to increasing doses of inhaled Mch in OVA-sensitized and challenged mice

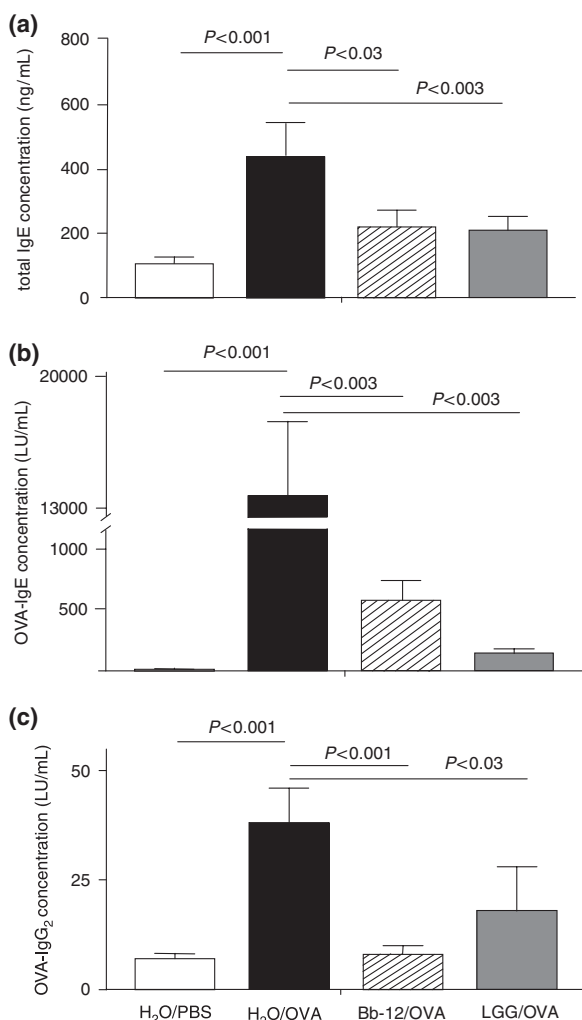


Fig. 1. Probiotic bacteria suppresses allergen-specific immunoglobulin production. Serum Ig levels from non-treated, sham-sensitized (H₂O/PBS, white bars), sham-treated and ovalbumin (OVA) sensitized (H₂O/OVA, black bars) and probiotic-treated and OVA sensitized mice (*Bifidobacterium lactis* strain Bb-12 /OVA, dashed bars; LGG/OVA, grey bars). (a) Total IgE, (b) OVA-specific IgE, (c) OVA-specific IgG₂. Data from four independent experiments were pooled, $n = 6-9$ /group.

was significantly reduced in mice receiving probiotic bacteria ($P = 0.0067$, Kruskal-Wallis non-parametric ANOVA) (Fig 2b). Airway reactivity in animals receiving probiotic bacteria was reduced by approximately 60% in comparison to OVA-sensitized and challenged mice, whereas the latter showed marked increases in AR compared with negative controls (Fig. 2b; $P < 0.05$, Mann-Whitney *U*-test).

Allergen-specific recall proliferation of spleen cells is reduced following probiotic application

Stimulation of spleen MNCs from allergen-sensitized and challenged animals (H₂O/OVA) with OVA resulted in enhanced proliferative responses compared with non-

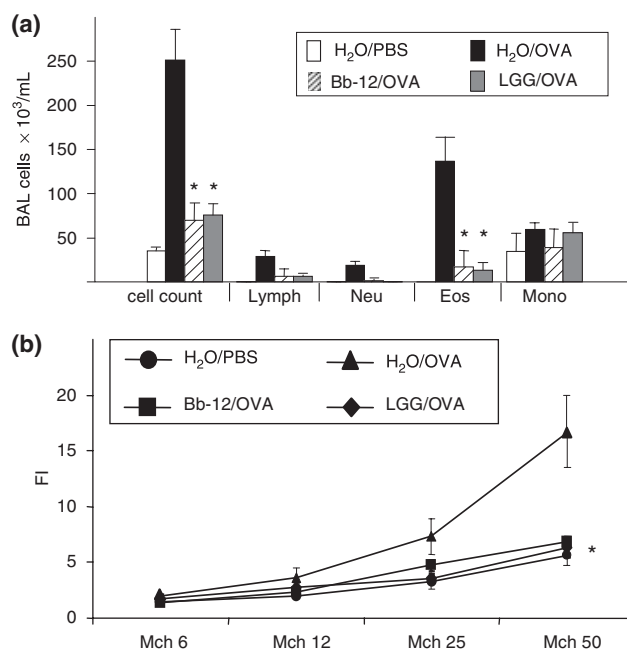


Fig. 2. Application of probiotic bacteria reduces eosinophilic airway inflammation and Mch-induced airway hyper-reactivity. (a) Ovalbumin (OVA) treatment (H₂O/OVA, black bars) induced significant eosinophilic inflammation compared with negative controls (H₂O/PBS, white bars) which was greatly reduced in animals fed with either *Lactobacillus rhamnosus* GG (LGG) or *Bifidobacterium lactis* Bb-12 (Bb-12) (Bb-12/OVA, dashed bars; LGG/OVA, grey bars) (lymph, lymphocytes; Neu, neutrophils; Eos, eosinophils; Mono, monocytes). Data from four independent experiments were pooled. (b) Bb-12 (Bb-12/OVA) or LGG (LGG/OVA) reduces airway reactivity by 2.5-fold as compared with positive control (H₂O/OVA). Expressed are the fold increase (FI) in Penh of negative controls (H₂O/PBS, black circles) vs. OVA-sensitized mice (H₂O/OVA, black triangles; Bb-12/OVA, black squares; LGG/OVA, black rhombus). * $P < 0.05$ compared with positive controls (H₂O/OVA) (Mann-Whitney test).

sensitized controls, which proves a significant recall response. Remarkably, oral application of probiotics before and during allergen sensitization led to a significant reduction of the allergen-induced proliferation of spleen MNC (Fig. 3). This effect however was not observed when mitogen-induced (ConA) proliferation of spleen MNCs was assessed (data not shown). These data suggest that probiotic microbiota-induced antigen-specific immune tolerance mechanisms without suppressing immune competence in the recipients.

Allergen-specific cytokine production by mesenteric mononuclear cells is modulated by oral probiotic treatment

Application of the probiotic strain LGG resulted in marked alterations in recall cytokine secretion by mesenteric lymph node (MLN) cells of sensitized mice upon stimulation with OVA. Th2 cytokine production of IL-4 and IL-5 (Table 1) were considerably reduced after probiotic feeding compared with cells from sham-fed and sensitized

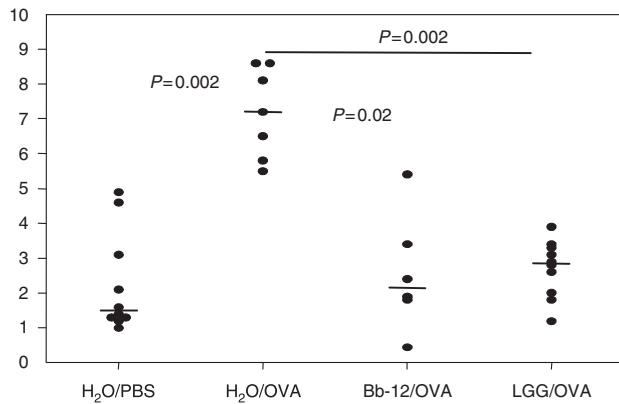


Fig. 3. Recall proliferation of spleen cells is reduced by the application of Bb-12 or LGG. Oral application of *Bifidobacterium lactis* Bb-12 (Bb-12)(Bb-12/OVA) or *Lactobacillus rhamnosus* GG (LGG/OVA) reduced OVA-induced recall proliferation (H₂O/OVA) to background levels (H₂O/PBS).

mice (H₂O/OVA), indicating a unique association between intestinal immune modulation and generalized effects on systemic sensitization, airway inflammation and airway reactivity presumably by alterations of these cytokines. Intriguingly, production of IL-10, which is known to exert regulatory properties and is reported to exert beneficial effects in immune modulation by probiotic bacteria [22, 23] was also down-regulated similarly to Th2 cytokines. Likewise, there was no parallel increase in Th1 responses, but rather a similar and marked decrease in IFN- γ production in response to probiotic feeding in this experimental group (LGG/OVA). This kind of immune modulation pointed towards an allergen-specific generalized immune suppressive effect in sensitized animals after application with LGG. The only cytokine measured not being suppressed in cell supernatants from animals treated with LLG and sensitized/challenged with OVA in comparison with positive controls was TGF- β .

Application of Lactobacillus rhamnosus GG increases numbers of transforming growth factor- β -secreting mesenteric T cells

In order to further elucidate possible mechanisms responsible for the strong effects of LGG on systemic sensitiza-

Table 1. Allergen-induced cytokine secretion by mesenteric lymph node (MLN) cells is markedly altered by the application of LGG

Cytokine (pg/mL)	1. H ₂ O/PBS	2. H ₂ O/OVA	3. LGG/OVA
IL-4	0 $\pm$ 0	279.2 $\pm$ 83.1	82.2 $\pm$ 21.3*
IL-5	30.0 $\pm$ 13.4	1952.0 $\pm$ 60.9	988.5 $\pm$ 139.5*
IL-10	0 $\pm$ 0	2368.1 $\pm$ 524.7	533.8 $\pm$ 165.6*
IFN- γ	5.1 $\pm$ 2.6	27.7 $\pm$ 10.7	12.4 $\pm$ 6.8*
TGF- β	57.8 $\pm$ 8.0	141.9 $\pm$ 20.6	128.9 $\pm$ 16.0 ^{NS}

Data are presented as mean $\pm$ SEM (median).

*Difference between H₂O/OVA and LGG/OVA groups; $P < 0.05$, NS, non-significant.

tion, airway inflammation and airway reactivity, we analysed the effects of oral LGG application on the cytokine production of individual T cells by assessing intracellular staining of CD4⁺MLN cells on the single-cell level. Interestingly, a ~ 3 -fold increase in numbers of TGF- β producing CD4⁺ T cells was present as compared with positive control mice ($6.95 \pm 0.89\%$ to $16.79 \pm 1.43\%$, $P = 0.05$, Mann-Whitney U -test, Fig. 4a). Numbers of IFN- γ , IL-4, IL-5 and IL-10 producing CD4⁺ MNCs were not significantly different between the groups (data not shown). These data suggest that LGG may inhibit allergen-specific Th2 responses via the induction of TGF- β producing T cells with regulatory capacity.

Lactobacillus rhamnosus GG and Bifidobacterium lactis strain bb-12 application results in up-regulation of Foxp3 in peribronchial lymph node cells

Since LGG induced an increase of TGF- β producing T cells with potential regulatory function in the intestine, we investigated whether oral probiotic administration also affected regulatory T cells in the lung compartment, the locus of allergen challenges. RT-PCR analysis was performed on PBLN's showing increased numbers of FoxP3 expressing CD4⁺ T cells in LGG-treated and OVA-sensitized animals (LGG/OVA group) by 1.7-fold, when compared with positive controls (H₂O/OVA). More striking results were observed in the Bb-12-treated group, where Foxp3 expression exhibited a 2.5-fold increase up to levels that were similar in negative control animals (H₂O/OVA), whereas β -actin expression remained constant (Fig. 4b). These results strongly suggest, that a generalized up-regulation of regulatory mechanisms is responsible for the suppression of the asthmatic phenotype in probiotics-treated animals.

Discussion

There is increasing evidence that the non-pathogenic flora of the gastrointestinal tract including probiotic microorganisms such as *Lactobacilli* or *Bifidobacteria* strongly influence the intestinal immune system [8, 24, 25]. These microbiota are intensively investigated as a novel alternative option for the management of allergic diseases including asthma and allergic rhinitis [4, 17, 26]. In order to dissect the immunological mechanisms governing this phenomenon we analysed the effects of oral probiotic administration on the development of allergen-induced sensitization and airway disease in a commonly used murine model of bronchial asthma. The results demonstrate that oral probiotic administration prior to and during allergen sensitization and airway challenges leads to a suppression of all features of the asthmatic phenotype, including specific IgE production, airway inflammation and development of AHR. Furthermore, this effect is

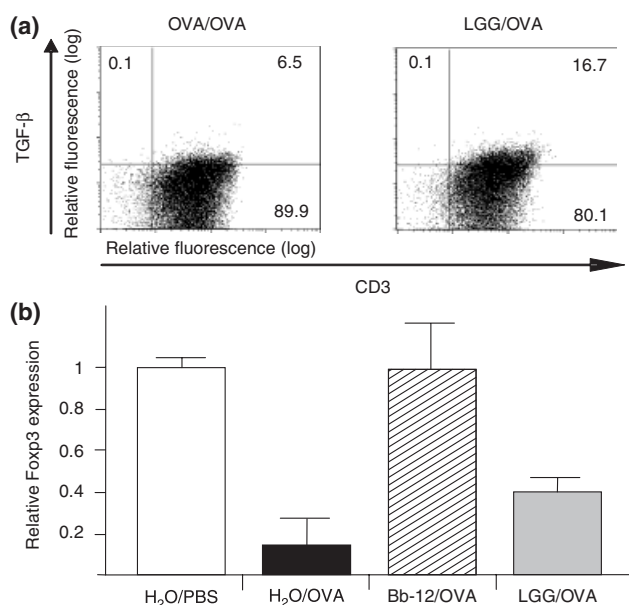


Fig. 4. Oral probiotic application up-regulates T regulatory cells. a. Oral application of or *Lactobacillus rhamnosus* (LGG) increases percentage of CD4/TGF- β -positive mesenteric lymph node cells among total mesenteric lymph node cells. The plots show cells which express CD3 and additionally intracellular TGF- β expression. Numbers represent % of gated cells expressing each antigen. Shown is one representative experiment of 4 animals. b. Oral application of *Bifidobacterium lactis* (Bb-12/OVA) or *Lactobacillus rhamnosus* GG (LGG/OVA) increased pulmonary Foxp3 transcript in peribronchial lymph node cells in comparison to cells obtained from OVA-sensitized and challenged animals (H₂O/OVA).

associated with the suppression of allergen-induced Th2 immune responses, paralleled by the induction of TGF- β -expressing T cells in the intestine, as well as with the enhanced expression of the Treg cell marker Foxp3 in the pulmonary compartment.

Interestingly, no preventive effects of probiotic administration on asthma were observed when the bacteria were applied to adult mice (preliminary experiments, data not shown), whereas the effects described here came to light only in experiments, in which probiotics were fed to newborn animals. These findings provide first functional evidence for the existence of a hypothetical 'time window of opportunity', as postulated by previous studies [3, 23, 27]. According to this hypothesis, probiotic intervention might be successful only in the initial stage of the colonization process, which is believed crucial for the maturation of the immune system to a balanced phenotype [8, 16, 17, 28].

The 'window of opportunity' has been postulated to be located in early infancy, since the first onset of allergy frequently occurs within the first months of life and this time is crucial for the maturation of the immune system including the gut defense mechanisms [8, 28]. This lies in line with our findings in which effects were observed in experiments where probiotic bacteria were administered

starting on the first post-natal day and continued for as long as 8 weeks, including the sensitization and challenge phases of the protocol. Own preliminary experiments in which probiotic bacteria were applied only prior to and not during the sensitization phase (data not shown) showed no significant effects in allergen sensitized and challenged animals. This also supports the notion that for successful immune modulation, application in the very early-stages of the development of the immune system and during the first 'hit' with allergen is required. These findings may also explain why all approaches using probiotic microorganisms for treatment of already established allergic diseases but not prevention failed to demonstrate significant or clinically meaningful effects [29–32]. Only those studies using probiotics that were conducted in early infancy showed somewhat acceptable success rates [9, 33–37] and further support this preventive strategy.

Our study clearly demonstrates that probiotic bacteria suppress Th2-driven, allergen-induced immune responses, supporting previous clinical studies suggesting that probiotic microorganisms suppress Th2-type effector mechanisms [38]. Recently, this effect was attributed to the strong anti-inflammatory effects of gut microbiota, demonstrated both, in allergic diseases [24] and inflammatory bowel disease [39–41]. This effect was associated with an increased expression of immunosuppressive markers, such as IL-10 and TGF- β [11, 41]. In this respect it was surprising that IL-10 – which is considered to be a main regulatory cytokine and a functional marker for Tregs – was down-regulated correspondingly to the reputed Th2 cytokines, IL-4 and IL-5, in probiotic-fed animals in response to allergen stimulation (Table 1). This is a somewhat controversial finding, since in a clinical study ingestion of LGG was associated with high serum IL-10 levels [22] and recent reports identify *in vitro* IL-10-producing regulatory cells as main lineage after *Lactobacilli*-induced stimulation of dendritic cells [23, 42].

In the present study, probiotic treatment was also associated with a parallel suppression of the classical Th1 cytokine-IFN- γ and a reduction of recall proliferation of spleen cells (Fig. 4). These findings pointed to a possible role of regulatory T cells inducing allergen-specific tolerance or immune suppression. In contrast to all cytokines measured including IL-10, TGF- β was presently the only cytokine that was not suppressed in probiotic-treated animals after allergen stimulation. This observation provides additional evidence for a role of TGF- β as leading candidate for the anti-inflammatory effects of probiotic microorganisms. Several studies already suggested TGF- β as the key initiator of regulatory mechanisms after probiotic treatment [9, 11, 12, 43] and pointed towards a potential new strategy for asthma prevention [44, 45]. Although a causal relationship between oral probiotic application and enhanced production of intestinal TGF- β

leading to suppression of the asthmatic phenotype was not demonstrated directly, it may be suggested that the increase of TGF-producing T cells is related to the suppressive effects observed after LGG application. Furthermore, these observations are supported by subsequent experiments, demonstrating that down-regulation of the FoxP3 expression (a critical marker of Treg cell activity) after sensitization was successfully reversed following application of probiotic bacteria. This effect can be attributed only to the increased activity of Treg in the pulmonary compartment following oral probiotic application.

Therefore, our results indicate that probiotic microorganisms can exert a major modulatory activity which is not confined to diseases affecting the digestive system but to other organs such as the respiratory tract. Hence, diverse microbiota may also be regarded as suitable tools to prevent allergic airway disease when administered in early infancy [46]. It may also be suggested that probiotic-induced immune regulatory mechanisms might offer a promising novel approach to target other harmful immune responses such as autoimmune or other atopic diseases. However, further clinical and experimental studies are required to delineate these preventive effects. These studies should also assess if priming with antigens other than OVA leads to the same preventive effects.

In summary, the present study in a murine asthma model provided functional data showing that inhibition of allergen-induced sensitization and airway disease after oral probiotic bacteria application is linked to increased T regulatory activity causing allergen-specific immunosuppressive effects and tolerance. Apart from revealing the underlying mechanisms of immune modulation by probiotics our findings may also help to interpret previous data from successful clinical trials with probiotics [9, 11, 33, 35–37].

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