

Neonatal colonization of germ-free mice with *Bifidobacterium longum* prevents allergic sensitization to major birch pollen allergen Bet v 1



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ABSTRACT

The main goal in reversing the allergy epidemic is the development of effective prophylactic strategies. We investigated the prophylactic effect of neonatal mother-to-offspring mono-colonization with *Bifidobacterium longum* ssp. *longum* CCM 7952 on subsequent allergic sensitization. Adult male and female germ-free (GF) mice were mono-colonized with *B. longum*, mated and their offspring, as well as age-matched GF controls, were sensitized with the major birch pollen allergen Bet v 1. Furthermore, signaling pathways involved in the recognition of *B. longum* were investigated *in vitro*. Neonatal mono-colonization of GF mice with *B. longum* suppressed Bet v 1-specific IgE-dependent β -hexosaminidase release as well as levels of total IgE and allergen-specific IgG2a in serum compared to sensitized GF controls. Accordingly, Bet v 1-induced production of both Th1- and Th2-associated cytokines in spleen cell cultures was significantly reduced in these mice. The general suppression of Bet v 1-specific immune responses in *B. longum*-colonized mice was associated with increased levels of regulatory cytokines IL-10 and TGF- β in serum. *In vitro*, *B. longum* induced low maturation status of bone marrow-derived dendritic cells and production of IL-10 in TLR2-, MyD88-, and MAPK-dependent manner. Our data demonstrate that neonatal mono-colonization with *B. longum* reduces allergic sensitization, likely by activation of regulatory responses via TLR2, MyD88, and MAPK signaling pathways. Thus, *B. longum* might be a promising candidate for perinatal intervention strategies against the onset of allergic diseases in humans.

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1. Introduction

There has been a remarkable increase in allergic diseases over the past few decades, especially in Western countries [1]. Accumulating evidence suggests that environmental changes, rather than genetic factors, are driving the epidemic character of allergy, and events acting within a narrow window of opportunity, either prenatally or early in life, might have major physiological effects [2,3].

The germ-free status of fetus changes rapidly after birth and the composition of colonizing microbiota can be influenced by multiple factors such as the mode of delivery, dietary changes, high hygiene or over-use of antibiotics [4]. The exposure to microbial stimuli is crucial for the development, maturation and function of the immune system and association between intestinal dysbiosis and allergic disease has been proposed [5]. Several prospective

studies have shown lower neonatal colonization by bifidobacteria and lactobacilli species accompanied by higher counts of *Clostridium difficile* in neonates who developed allergy later in life [6–8].

Due to the ability of bifidobacteria and lactobacilli to interact with the host immune system and to modulate host immune responses, they have been used with some success in prevention or treatment of allergic disease in infants at risk [9]. Several randomized clinical trials have shown that combined pre-natal/postnatal probiotic interventions reduced the cumulative incidence of eczema while less beneficial effects were found in trials using exclusively postnatal treatment approaches (recently reviewed in [10]). These data highlight the importance of the correct timing of probiotic interventions, for which the prenatal period seems to be a significant component.

Dendritic cells (DC) are pivotal in early bacterial recognition through engagement of TLR and C-type lectins, which leads to induction of distinct innate responses that shape the type of T helper cell responses [11,12]. In this respect, Konieczna et al. recently showed that preconditioning of DC with probiotic *Bifidobacterium infantis* led to induction of Foxp3 positive regulatory T (Treg) cells with enhanced IL-10 production [13]. Due to the fact

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that allergic diseases have been associated with a deficiency in Treg cells numbers and/or function [14], specific probiotic strains inducing regulatory immune responses might be beneficial in the prevention of allergic disorders. Indeed, we have previously shown that perinatal administration of *Lactobacillus paracasei* to pregnant/lactating mice protected against the development of airway inflammation in offspring by activating regulatory pathways [15]. Similarly, neonatal application of *Lactobacillus rhamnosus* GG or *Bifidobacterium lactis* Bb-12 suppressed allergic sensitization and airway inflammation in mice by induction of Treg cells associated with increased levels of TGF- β [16].

Germ-free (GF) animals represent a powerful model to study the interaction of a single bacterial strain or a defined mixture of microbial strains with the host immune system [17]. Taking the advantage of this model we have previously shown that neonatal mono-colonization of GF mice with the recombinant bacterial strain *Lactobacillus plantarum* producing major birch pollen allergen Bet v 1 reduced the development of allergic responses upon systemic sensitization with the same allergen [18].

Bifidobacterium longum subspecies *longum* CCM 7952 (*B. longum*) is a commensal bacterial strain originally isolated from the feces of a healthy breast fed infant. This strain has been shown to induce regulatory responses *in vitro* and to suppress the inflammatory responses in a mouse model of experimental colitis (Srutkova, unpublished results). In the present study we investigated whether neonatal mother-to-offspring mono-colonization of GF mice with *B. longum* protects the offspring against allergic sensitization to allergen Bet v 1 in a model of type I allergy.

2. Materials and methods

2.1. Animals

Germ-free BALB/c mice were kept under sterile conditions and were supplied with water and sterile pellet diet ST1 (Bergman, Czech Republic) *ad libitum*. Fecal samples were weekly controlled for microbial contamination as previously described [19]. Wild-type (WT) and TLR2^{-/-}, TLR4^{-/-}, and MyD88^{-/-} mice on a C57BL/6 background, obtained from Prof. M. Müller (Vienna, Austria), were kept under SPF conditions. All animal experiments were approved by the local ethics committee.

2.2. Bacterial strain

Probiotic strain *B. longum* ssp. *longum* CCM 7952 (*B. longum*), originally isolated from the feces of a breast-fed healthy child, was provided by Culture Collection of Dairy Microorganisms (Milcom, Czech Republic). *B. longum* cultures were grown anaerobically in MRS medium (Oxoid, UK) supplemented with L-cysteine hydrochloride (0.5 g/l) for 48 h at 37 °C. For *in vitro* experiments *B. longum* was inactivated with 1% formaldehyde-PBS as described before [20].

2.3. Colonization and experimental design

Eight-week-old GF male and female mice were colonized with a single dose (2×10^8 CFU) of freshly grown *B. longum* in 200 μ l of sterile PBS by intragastric administration and mated 20 days later. Stability of colonization was checked by plating of feces on MRS agar supplemented with L-cysteine hydrochloride (0.5 g/l) and CFU were counted after anaerobic cultivation for 48 h at 37 °C. Female offspring, neonatally colonized with *B. longum*, and control GF mice were subcutaneously (s.c.) sensitized on days 1, 14 and 28 with 1 μ g of recombinant Bet v 1 (Biomay, Austria) emulsified in 100 μ l of Al(OH)₃ (Serva, Germany). Seven days after the last

immunization, mice were killed by CO₂ asphyxia and samples were taken for further analysis (Fig. 1A).

2.4. Humoral immune responses

Blood samples were taken at sacrifice and serum levels of anti-Bet v 1 IgE, IgG1 and IgG2a were measured by ELISA as previously described [18]. The activity of Bet v 1-specific IgE in serum was measured by rat basophile leukemia cells degranulation assay as described previously [20]. Degranulation was tested by the release of β -hexosaminidase. Levels of total IgE and IgA in serum (dilution 1:10 and 1:400, respectively) were measured by a commercial ELISA kit as recommended by the manufacturers (Bethyl, USA). Levels of IL-10 and TGF- β in serum (final dilution 1:1000) were measured by ELISA Ready-Set-Go! kits (eBioscience, USA) according to manufacturer's instructions. Small intestine was excised and gut lavage was prepared as described previously [15]. Levels of total IgA and TGF- β in gut lavage were measured as described above with final dilution 1:2500 and 1:1000, respectively and reported as absolute units.

2.5. Cellular immune responses

Spleen and mesenteric lymph node (MLN) single cell suspensions from *B. longum*-colonized and control mice were prepared and cultured as previously described [15]. Mononuclear cells (3×10^6 cells/ml) were stimulated with Bet v 1 (20 μ g/ml) or media alone in 96-well plates at 37 °C for 60 h in culture medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin). Levels of cytokines in culture supernatants were measured by the MILLIPLEX MAP Mouse Cytokine/Chemokine Panel (Millipore, USA) according to manufacturer's instructions and analyzed with the Bio-Plex System (Bio-Rad Laboratories, USA). Values are expressed as pg/ml after subtraction of baseline levels of unstimulated cultures. Proliferative responses of spleen cell cultures with/without Bet v 1 restimulation were determined by scintillation counting after addition of ³H-Thymidine (0.5 μ Ci/well; Lacomed, Czech Republic) for the last 16 h of 76 h cultivation. Values are shown as stimulation index.

2.6. Preparation and activation of bone marrow-derived DC

Mouse bone marrow-derived DC (BM-DC) were prepared as previously described [15]. Briefly, the bone marrow precursors were isolated from femurs and tibias of mice. Cells were cultured at 4×10^5 /ml in bacteriological Petri dishes in 10 ml culture medium with GM-CSF (20 ng/ml; Sigma-Aldrich, USA). Fresh medium was added at days 3 and 6 and BM-DC were used on day 8 of culture. BM-DC (10^6 cells/ml) were stimulated with 10^6 or 10^7 CFU of inactivated *B. longum* for 18 h. As controls, BM-DC were incubated with Pam3Cys (Pam3CSK4, 1 μ g/ml, InvivoGen, USA) or ultrapure LPS (LPS-EB, 1 μ g/ml, InvivoGen, USA). Levels of IL-10, TGF- β , and IL-6 in culture supernatants were determined by ELISA Ready-Set-Go! kits (eBioscience, USA) according to manufacturer's instructions. Levels of IL-12p70 were measured with matched antibody pairs (BD Pharmingen, USA). Where indicated, BM-DC were pretreated with mitogen-activated protein kinase (MAPK) specific inhibitors (MEK: PD98059; p38: SB203580; JNK: SP600125) or NF- κ B (BAY 11-7082) at 10 μ M dissolved in DMSO or DMSO only for 1 h at 37 °C.

2.7. Flow-cytometry analysis

Single cell suspensions of spleens or MLN were stained for regulatory T cells with Foxp3 Staining Buffer Set (eBioscience, USA) as

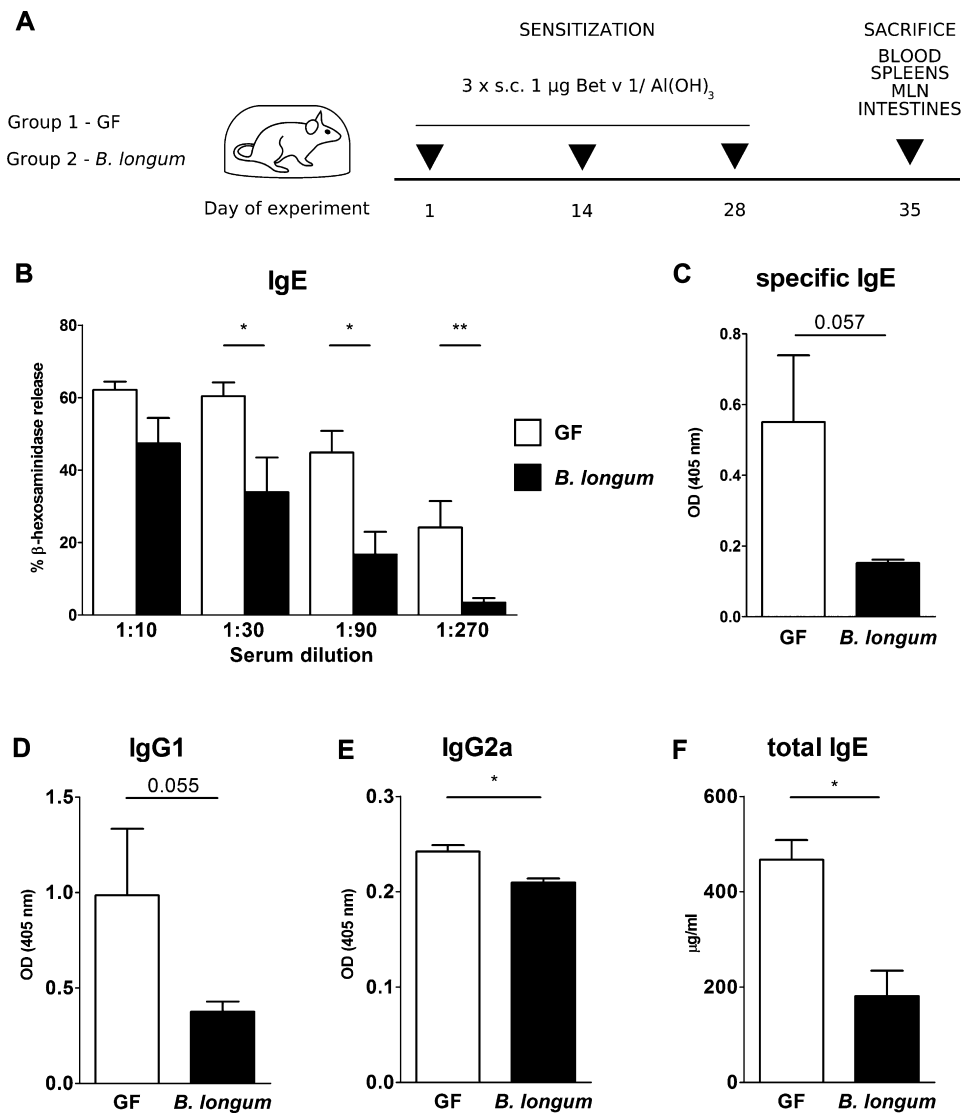


Fig. 1. Systemic sensitization of GF and *B. longum* neonatally colonized mice to Bet v 1. Experimental design: eight-week old germ-free mice (GF; white bars) and age-matched mice neonatally colonized with *B. longum* (*B. longum*; black bars) were subcutaneously (s.c.) sensitized with 1 μ g of recombinant Bet v 1 in Alum (Bet v 1/Al(OH)₃) on days 1, 14 and 28. Mice were sacrificed one week after the last immunization and blood, spleens, mesenteric lymph nodes and small intestines were collected for further analysis (A). Functional IgE in diluted serum (1:10, 1:30, 1:90, 1:270) was measured by Bet v 1-mediated β -hexosaminidase release from rat basophil leukemia cells (B). Levels of Bet v 1-specific IgE (C), IgG1 (D) and IgG2a (E), as well as levels of total IgE (F) in serum were measured by ELISA. Results are representative of two repeat experiments, each with four to six mice per group, and are expressed as mean $\pm$ SEM. * p < 0.05; ** p < 0.01.

described previously [21]. DC were labeled with monoclonal antibodies for CD11c (FITC), MHCII (APC), CD40, CD80 or CD86 (PE) (eBioscience, USA). Appropriate isotype antibodies were used as controls to determine non-specific binding. Cells were analyzed using FACSCalibur flow cytometer (Becton-Dickinson, USA) and obtained data were analyzed with FlowJo 7.6.2 software (TreeStar, USA).

2.8. Stimulation of Human embryonic kidney (HEK) 293 cells stably transfected with TLR

HEK293 cells stably transfected with plasmid carrying human (h)TLR2 gene were kindly provided by M. Yazdanbakhsh (Leiden, Netherlands) and cells transfected with hTLR4/MD2/CD14 were a gift of B. Bohle (Vienna, Austria). Cells were stimulated with formalin-inactivated *B. longum* (10⁶, 10⁷, or 10⁸ CFU/ml). TLR2 ligand Pam3Cys (1 μ g/ml) and TLR4 ligand LPS (1 μ g/ml) were used as positive controls. After the 20-h incubation period,

culture supernatants were harvested and concentration of human IL-8 was analyzed by ELISA (Thermo Scientific, USA) according to the manufacturer's instructions.

2.9. Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical analysis was performed by non-parametric Mann-Whitney *U*-test using GraphPad Software (GraphPad Prism 5.04, San Diego, USA); p values < 0.05 were considered significant.

3. Results

3.1. Neonatal mono-colonization with *B. longum* prevents systemic sensitization to Bet v 1

Colonization of mice with *B. longum* remained stable throughout the experiments and reached the level of approximately 5×10^9 CFU/g of feces (data not shown). Sensitization with Bet v

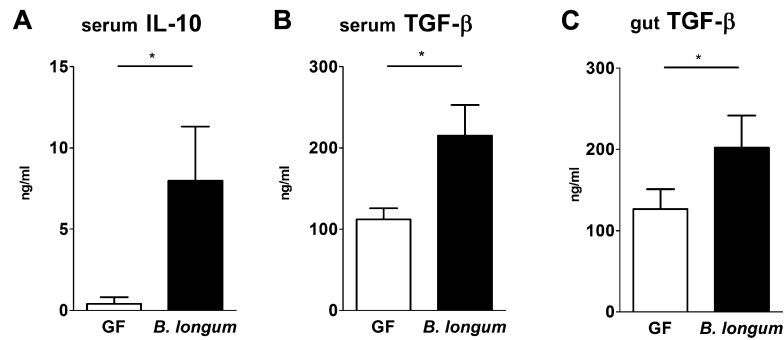


Fig. 2. Influence of *B. longum* neonatal colonization on levels of IL-10 and TGF- β in sera and gut lavage. Levels of IL-10 (A) and TGF- β (B) in sera and TGF- β (C) in gut lavage were measured by ELISA. Germ-free mice (GF, white bars) and *B. longum*-colonized mice (*B. longum*, black bars). Pooled values of two repeat experiments, each with four to six mice per group, are expressed as mean $\pm$ SEM. * p < 0.05.

1 induced high levels of allergen-specific antibodies in serum of GF mice. Neonatal mono-colonization with *B. longum* prevented the development of allergic sensitization as the production of Bet v 1-specific Th2-related IgE and IgG1 as well as Th1-related IgG2a antibodies in serum were reduced in these mice compared to GF controls (Fig. 1C, D, and E). Furthermore, *B. longum* reduced the IgE-dependent and Bet v 1-driven basophil degranulation (Fig. 1B) as well as levels of total IgE in serum (Fig. 1F). Concomitantly, *B. longum* colonization triggered increased production of IL-10 and TGF- β in serum (Fig. 2A and B). Increased levels of TGF- β were also measured in gut lavage of *B. longum*-colonized mice (Fig. 2C). Surprisingly, *B. longum* did not increase the production of total IgA, as levels of these antibodies in serum and gut lavage (data not shown) were comparable between *B. longum*-colonized mice and GF controls.

3.2. Neonatal mono-colonization with *B. longum* prevents the development of cellular allergic responses

Neonatal mono-colonization with *B. longum* reduced allergen-specific recall responses in spleen cell cultures *ex vivo*. Production of both Th2-related cytokines IL-5, IL-13 and IL-4 (Fig. 3A–C) and pro-inflammatory cytokines IFN- γ , IL-17 and TNF- α (Fig. 3E–G) as well as proliferative responses (Fig. 3H) were suppressed in *B. longum* mono-colonized mice compared with control GF mice. Interestingly, Bet v 1-induced levels of the regulatory cytokine IL-10 were also decreased by *B. longum* (Fig. 3D). In addition, *B. longum* suppressed Bet v 1-specific responses in MLN cell cultures (data not shown). This was accompanied by increase in population of CD4⁺Foxp3⁺ cells in spleen and MLN (Fig. 4A and B).

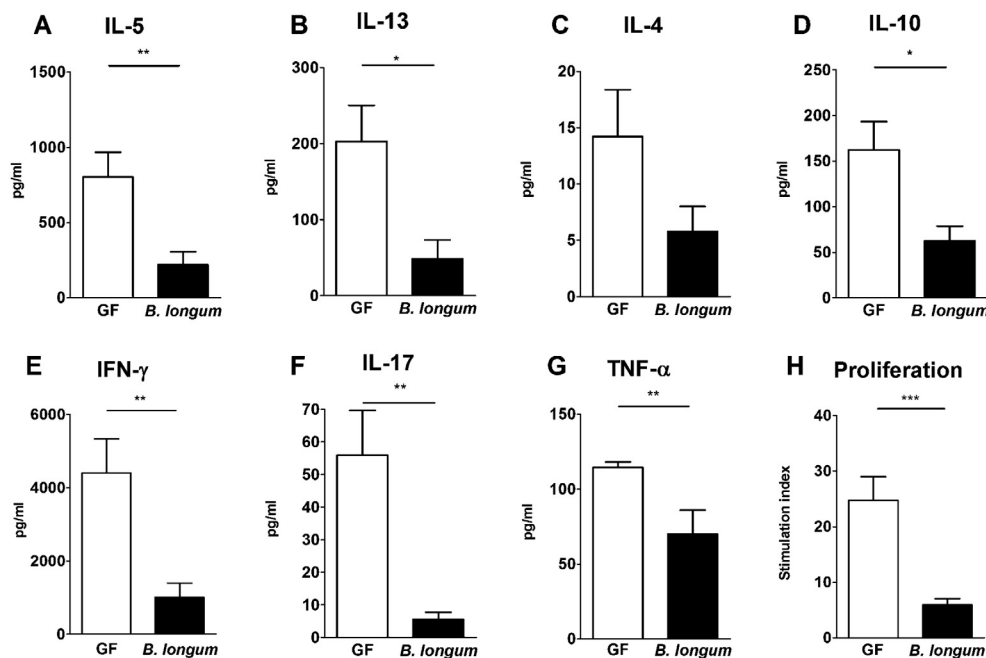


Fig. 3. Influence of *B. longum* neonatal colonization on Bet v 1-induced cytokine production and proliferative response in splenocytes. Spleen cells from germ-free mice (GF, white bars) and *B. longum*-colonized mice (*B. longum*, black bars) were cultured with 20 μ g/ml of Bet v 1 for 60 h. Levels of IL-5 (A), IL-13 (B), IL-4 (C), IL-10 (D), IFN- γ (E), IL-17 (F) and TNF- α (G) in culture supernatants were measured by MILLIPLEX Cytokine panel. Cytokine levels are expressed after subtraction of base line levels of unstimulated splenocytes. Proliferative responses of splenic cells were assessed by incorporation of ³H-Thymidine for the last 16 h of 76-h cultivation (H). Values are shown as stimulation index. Pooled values of two repeat experiments, each with four to six mice per group, are expressed as mean $\pm$ SEM. * p < 0.05; ** p < 0.01, *** p < 0.001.

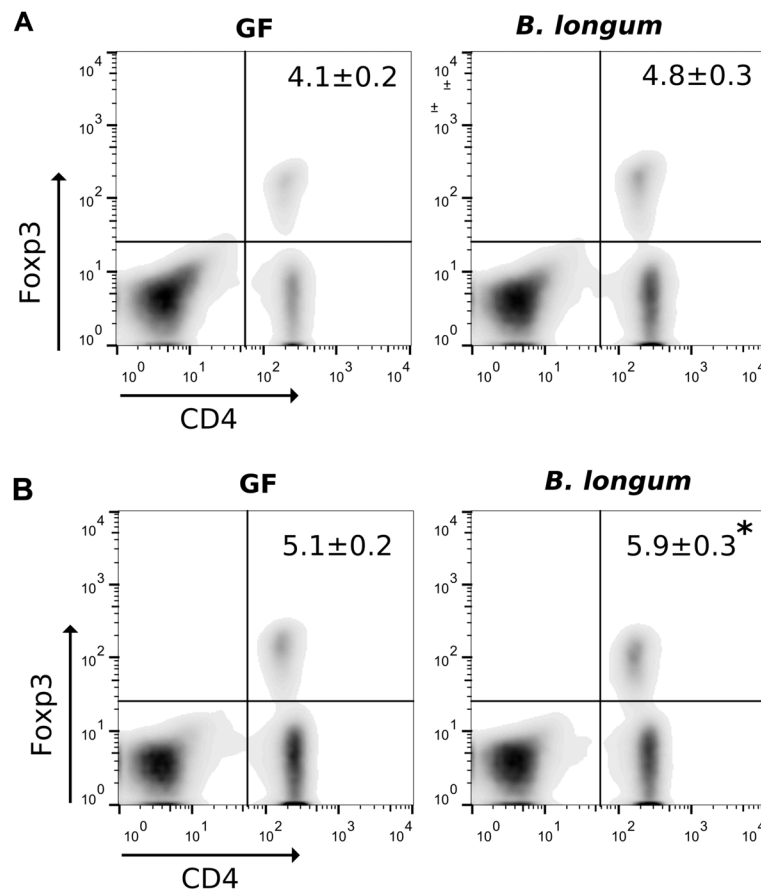


Fig. 4. Numbers of CD4⁺Foxp3⁺ cells in spleenocytes and mesenteric lymph nodes cells. Typical plots depicting numbers of Treg cells in suspensions isolated from spleens (A) and mesenteric lymph nodes (B) of germ-free mice (GF) and *B. longum*-colonized mice (*B. longum*). Pooled values of two repeat experiments are shown, each with four to six mice per group. Percentage of CD4⁺Foxp3⁺ cells from all gated lymphocytes are expressed as mean ± SEM. **p* < 0.05.

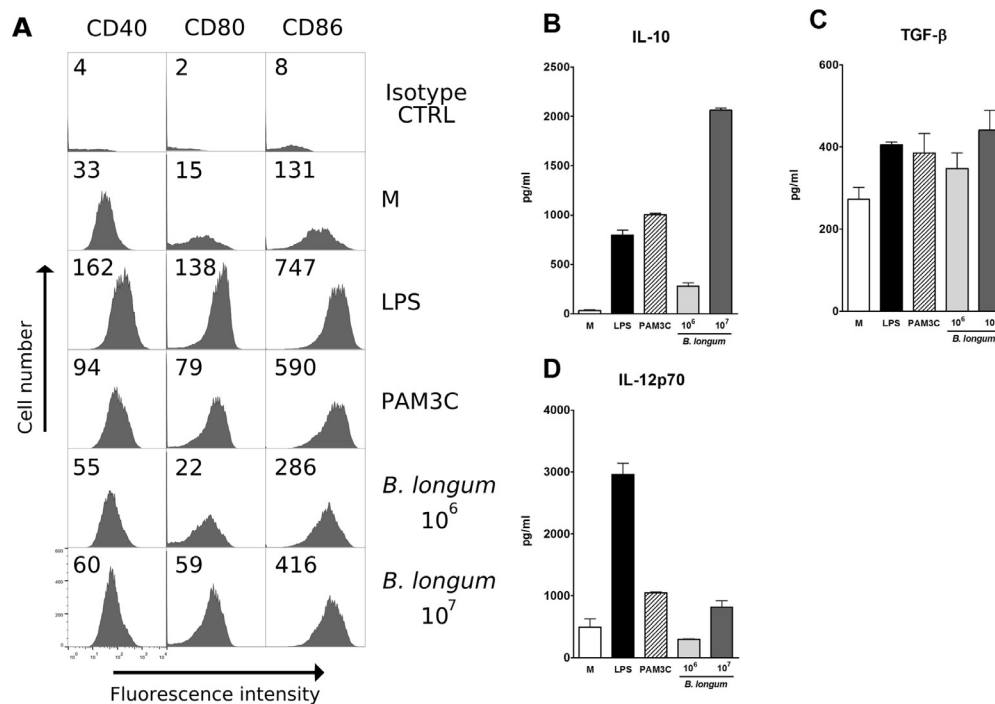


Fig. 5. Maturation of DC and cytokine production induced by *B. longum*. BM-DC cells from naive BALB/c mice were cultured with media alone (M), ultra-pure lipopolysaccharide from *E. coli* (LPS, 1 µg/ml), Pam3Cys (PAM3C, 1 µg/ml) or two different concentrations of formalin-inactivated *B. longum* (10⁶ or 10⁷ CFU/ml) for 18 h. BM-DC were gated as MHCII⁺CD11c⁺ and analyzed by flow cytometry for CD40, CD80 and CD86 expression. Isotype controls are shown in the first row (A). Numbers shown are median fluorescence units. One representative out of three experiments is shown. Production of IL-10 (B), TGF-β (C) and IL-12p70 (D) in culture supernatant was determined by ELISA. Mean ± SEM are shown. One representative out of three experiments is shown.

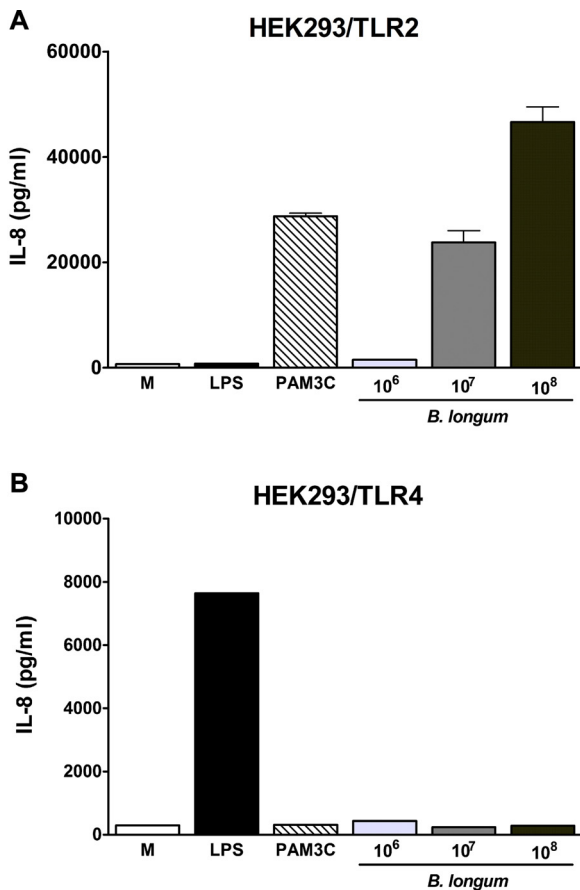


Fig. 6. Activation of TLR receptors by *B. longum*. HEK293 stably transfected with an expression vector for human TLR2 (293-hTLR2) (A) and TLR4 (293-hTLR4/MD2/CD14) (B) were cultured for 20 h with different concentrations of formalin-inactivated *B. longum* (10⁶, 10⁷, 10⁸ CFU/ml) or left unstimulated. Ultrapure lipopolysaccharide from *E. coli* (LPS; 1 µg/ml) and Pam3Cys (PAM3C, 1 µg/ml) were used as positive controls for TLR4 and TLR2, respectively. Unstimulated cells (M) were used as control. Stimulation was evaluated by measurement of IL-8 production; results are expressed as mean ± SEM. One representative experiment out of three is shown.

3.3. *B. longum* induces low maturation status of dendritic cells and production of regulatory cytokines

Stimulation of BM-DC derived from WT BALB/c mice with *B. longum* induced dose dependent but only moderate maturation status (increased levels of CD40, CD80 and CD86) when compared with LPS (TLR4 ligand) or Pam3Cys (TLR2 ligand) (Fig. 5A), but high levels of IL-10 and TGF-β (Fig. 5B and C). High levels of IL-12p70 were detected in cultures stimulated with LPS but only low levels were measured after *B. longum* stimulation (Fig. 5D).

3.4. TLR2 but not TLR4 is involved in recognition of *B. longum*

In order to investigate the role of TLR in *B. longum* recognition, HEK293 stably transfected with TLR2 or TLR4 were stimulated with different concentrations of inactivated *B. longum*. Pam3Cys was used as positive control for TLR2 and LPS for TLR4. Stimulation of HEK293/TLR2 with *B. longum* led to production of IL-8 in dose dependent manner (Fig. 6A). There was no production of IL-8 after stimulation of HEK293/TLR4 with *B. longum* (Fig. 6B).

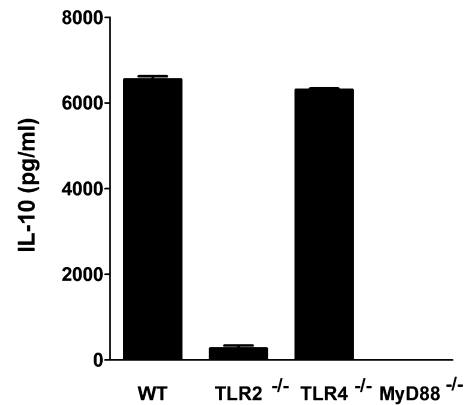


Fig. 7. Involvement of TLR in BM-DC IL-10 cytokine production induced by *B. longum*. BM-DC generated from wild-type (WT), TLR2-, TLR4- and MyD88-deficient mice with C57BL/6 background were cultured with formalin-inactivated *B. longum* (10⁷ CFU/ml) for 18 h. Production of IL-10 was measured by ELISA. Mean ± SEM after subtractions of baseline levels from unstimulated cells are shown. One representative out of three experiments yielding similar results is shown.

3.5. *B. longum* drives production of IL-10 in DC through TLR2 and MyD88

Similarly as observed for BM-DC derived from WT BALB/c mice (Fig. 5B), stimulation of BM-DC derived from WT C57BL/6 mice with *B. longum* induced up-regulation of IL-10 production (Fig. 7). This effect was significantly reduced in BM-DC derived from TLR2- or MyD88-deficient mice. The lack of TLR4 had no impact on IL-10 production by *B. longum* (Fig. 7).

3.6. Activation of MAPK and NF-κB in DC is critical for the production of IL-10 by *B. longum*

In order to investigate the functional role of MEK 1, p38, JNK or NF-κB pathways in *B. longum*-triggered production of IL-10, specific inhibitors of the MEK 1 (PD98059), p38 (SB203580), JNK (SP600125) or NF-κB (BAY 11-7082) signaling pathways were used. As shown in Fig. 8, inhibitors of the MEK 1, p38, or NF-κB markedly decreased the production of IL-10 induced by *B. longum* or by TLR2 ligand Pam3Cys in BM-DC derived from WT BALB/c mice. Interestingly, involvement of JNK signaling pathway was important for induction of IL-10 by *B. longum* but not by Pam3Cys (Fig. 8).

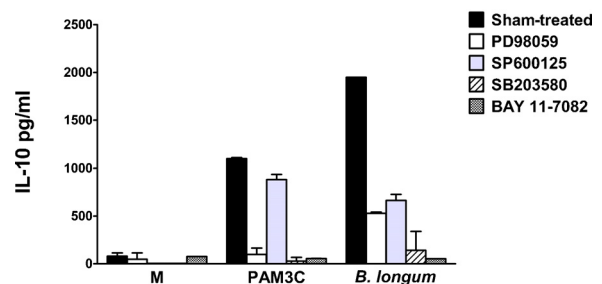


Fig. 8. Involvement of MAPK pathways and NF-κB in *B. longum*-induced production of IL-10. BM-DC from naive BALB/c mice were incubated with DMSO only (Sham-treated, black bars) or with MEK 1 (PD98059, white bars), JNK (SP600125, gray bars), p38 (SB203580, dashed bars) and NF-κB (BAY 11-7082, dotted bars) pathway inhibitors for 1 h prior to stimulation with formalin-inactivated *B. longum* (10⁷ CFU/ml) or Pam3Cys (PAM3C, 1 µg/ml). Unstimulated cells (M) were used as controls. Levels of IL-10 were determined by ELISA. Results are expressed as mean ± SEM, one representative experiment out of two is shown.

4. Discussion

In the current study, we demonstrate that neonatal mother-to-offspring mono-colonization with *B. longum* CCM 7952 significantly reduced the development of allergen-specific immune responses in a gnotobiotic mouse model of type I allergy, which was associated with induction of regulatory milieu. Furthermore we show that TLR2, MyD88 and MAPK signaling pathways are crucial for the recognition of *B. longum* and induction of IL-10.

Clinical and experimental studies differ greatly regarding the outcome of probiotic interventions in prevention/treatment of allergic diseases (reviewed in [22]). However, most of them claim that the probiotic application in prenatal/perinatal period appears to be crucial for manifestation of beneficial effects, confirming the existence of a 'window of opportunity' in the programming of the well balanced immune system in the neonatal period [15,16,23].

In order to investigate the interaction between a single bacterial strain and the host immune system, as well as the impact of the specific strain on the 'reprogramming' and maturation of the immune system, we established a mouse model of neonatal mother-to-offspring mono-colonization using GF mice. By that we previously showed that constitutive delivery of Bet v 1 by recombinant *L. plantarum*, but not the wild-type bacteria alone, was able to modulate subsequent systemic sensitization to Bet v 1 [18]. Here we show that mother-to-offspring mono-colonization of GF mice with *B. longum* decreased levels of Th2- as well as Th1-associated Bet v 1-specific antibodies in serum. These data suggest that the inhibition of sensitization was not achieved simply by immune shift toward the Th1 mode, but rather that general reduction of immune responses took place.

Consistent with the results observed in serum, *B. longum* also inhibited antigen-specific recall responses in spleen cells. The Bet v 1-induced production of both Th2 and Th1 cytokines, as well as antigen specific proliferation was dramatically reduced in mice colonized with *B. longum* in comparison to controls. These data are in agreement with our previous study where prophylactic mucosal application of *B. longum* NCC 3001 induced general suppression of allergen-specific cytokine production [24]. Similarly, *Bifidobacterium* has been shown to inhibit T-cell proliferation [25] and antigen-specific cytokine production [26] in OVA food allergy model. It is necessary to stress that although the reduction of humoral and cellular immune responses observed with *B. longum* is feasible in the context of sensitization to allergens, it remains important to evaluate whether the perinatal application of *B. longum* or other strains with immunosuppressive properties could interfere with immune responses against concomitant infections, vaccines or anti-tumor immunity.

We have shown that *B. longum* neonatally-colonized mice exhibited increased levels of Foxp3⁺ cells in MLN and high levels of regulatory cytokines IL-10 and TGF- β in serum. This is in agreement with recent studies both in animals and in humans [13,25], where probiotic bacteria have been shown to drive the generation of Treg cells. These Foxp3⁺ Treg cells have been shown to be protective in mouse allergy models [26] and they are indispensable for successful immunotherapy in humans [14]. Interestingly, it has been recently shown that bifidobacteria are able to induce another subset of Foxp3⁺ type 1 regulatory cells (Tr1) that exert their immunoregulatory properties by production of IL-10 [27]. However, IL-10 and TGF- β are produced by a broad variety of cells [11], thus the precise source and role of these cytokines as well as Tregs in prevention of allergic sensitization by *B. longum* in our model remains to be investigated.

Next we have shown that stimulation of BM-DC with *B. longum* triggered production of IL-10 and TGF- β ; however, the expression of surface markers was rather low. DC are viewed as the key players in routing the immune responses and inducing different T cell

subsets [13,27]. In this respect immature DC and/or IL-10-producing DC have been regarded as tolerogenic [28] and they were shown to be able to induce Treg cells [13,25].

Our data demonstrate that TLR2 is important for recognition of *B. longum* and signaling, through this receptor leads to induction of IL-10. Interestingly, stimulation of antigen-presenting cells with TLR2 ligand inhibited allergen-specific Th2 responses in sensitized patients [29] and stimulation of TLR2 on DC was shown to enhance their ability to induce Treg cells [30]. These data suggest that TLR2 ligands derived from *B. longum* might have beneficial effects on allergen-specific responses, providing an alternative to viable probiotics. Along these lines, IL-10 inducing properties have been suggested for *B. longum* BCRC 14634 derived exopolysaccharides (EPS) [31]. Recently, we have shown that *B. longum* CCM 7952 also produces EPS which lead to production of IL-10 in TLR2-dependent manner (Górska-Frączek, Schwarzer, unpublished results) and we are currently investigating the role of *B. longum*-derived EPS in allergy prevention/therapy.

Since TLR2 has been shown to activate MAPK and NF- κ B [32], we further investigated the role of these signaling pathways in IL-10 production induced by *B. longum*. Similar to the synthetic TLR2 ligand Pam3Cys, *B. longum*-induced IL-10 is critically dependent on NF- κ B activation and involves p38 and MEK pathways. In contrast, JNK signaling pathway was indispensable only for *B. longum*-induced, but not for Pam3Cys-induced IL-10 production.

Taken together, our data clearly show that mono-colonization with *B. longum* prevents the development of allergic sensitization to Bet v 1 in a mouse model of clinically relevant birch pollen allergy. This probiotic strain likely induces regulatory responses through TLR2/MyD88 signaling pathway. Identification and characterization of *B. longum* derived TLR2 ligands may provide a tool to investigate the molecular interaction between components of innate immunity and allergic-effector cells, leading to novel strategies to prevent/treat allergic diseases in humans.

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