

***Bifidobacterium bifidum* NCC 453 Promotes Tolerance Induction in Murine Models of Sublingual Immunotherapy**

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Key Words

Probiotics • Regulatory T cells • Sublingual immunotherapy • Bet v 1

Abstract

Background: Enhancing clinical efficacy remains a major goal in allergen-specific immunotherapy. In this study, we tested three strains of bifidobacteria as candidate adjuvants for sublingual allergy vaccines. **Methods:** Probiotic candidates were evaluated in human monocyte-derived dendritic cell (h-DC) maturation and CD4⁺ T-cell polarization in vitro models and further tested in murine models of sublingual immunotherapy in BALB/c mice sensitized to either ovalbumin or birch pollen. **Results:** *Bifidobacterium adolescentis*, *B. bifidum* and *B. longum* induced h-DC maturation and polarized naïve CD4⁺ T cells toward interferon- γ and interleukin-10 production. *B. bifidum* increased CD25^{high}, Foxp3⁺ cells within CD4⁺ T lymphocytes and was the most potent inducer of interferon- γ in Th2-skewed peripheral blood mononuclear cells and h-DC T-cell cocultures. It also induced a significant decrease in airway hyperresponsiveness in BALB/c mice sensitized to ovalbumin. Sublingual administration of *B. bifidum* together with recombinant Bet v 1 enhanced tolerance induction in BALB/c mice sensitized to

birch pollen, with a downregulation of both airway hyperresponsiveness, lung inflammation and Bet v 1-specific Th2 responses. **Conclusions:** Due to its capacity to reorient established Th2 responses toward Th1/regulatory T-cell profiles, *B. bifidum* represents a valid candidate adjuvant for specific immunotherapy of type I allergies.

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Introduction

As of today, allergen-specific sublingual immunotherapy (SLIT) has proven to be safe and effective in inducing allergen-specific tolerance in humans [1–12], as demonstrated by large double-blind placebo-controlled phase III clinical studies performed in patients allergic to grass pollen or house dust mites [6–12]. Regarding underlying immune mechanisms, the induction of Th1 and/or regulatory T-cell (Treg) responses is considered to be critical for the success of SLIT [13–17]. SLIT requires high doses of allergen administered over months and even years [11]. Thus, the coadministration of allergen(s) with adjuvants might help reducing the allergen dose and simplifying the immunization scheme. Specifically, adjuvants favoring Th1/Treg responses while downregulating Th2 and

possibly Th17 immune responses appear desirable for the development of new allergy vaccines.

Among adjuvants with such properties, selected probiotic strains have been considered, based on their capacity to modulate allergic reactions in animal models [18, 19] or in humans [20–23]. Most particularly, probiotics such as lactobacilli have been suggested to induce strong Th1 and Treg responses [18, 19]. In contrast to lactobacilli, the effect of bifidobacteria as adjuvants during SLIT has never been reported.

In this study, we tested the impact of three types of bifidobacteria, i.e. *Bifidobacterium adolescentis* NCC 251, *B. bifidum* NCC 453 and *B. longum* NCC 2705 using (1) human in vitro systems allowing to analyze human monocyte-derived dendritic cell (h-DC) maturation as well as polarization of naïve CD4⁺ T cells or Th2-skewed peripheral blood mononuclear cells (PBMCs), and (2) models of SLIT in BALB/c mice sensitized to either ovalbumin (OVA) or birch pollen. We demonstrate that *B. bifidum* NCC 453 can reorient established Th2 responses toward Th1/Treg profiles, and as such, enhances tolerance in SLIT murine models.

Material and Methods

Animals, Culture Media, Reagents and Bacterial Strains

Six-week-old female BALB/c mice were purchased from Charles River (L'Arbresle, France). International standards for the care and use of animals were applied in facilities certified by French authorities (No. A92-002-01). Birch pollen aqueous extract and recombinant Bet v 1 were produced at Stallergènes (Antony, France). The characterization of recombinant Bet v 1 was reported elsewhere [24]. Phosphate-buffered saline (PBS) was purchased from PAA (Pasching, Austria). Lipopolysaccharide (LPS) was purchased from Sigma Chemicals Aldrich (St. Louis, Mo., USA) and from InvivoGen (San Diego, Calif., USA) for in vitro and in vivo experiments, respectively. Probiotic bacteria including *B. adolescentis* (NCC 251), *B. bifidum* (NCC 453) and *B. longum* (NCC 2705) were obtained from the Nestlé Culture Collection (NCC). These strains were grown in MRS supplemented with 0.05% cysteine and collected 3 h after entering the stationary growth phase. Bacterial cells were washed twice with PBS and kept frozen in 20% glycerol at –80°C until use.

Lactic Acid Bacteria Testing in Human Immune Cells

h-DCs and allogeneic CD4⁺ naïve T cells were generated from PBMCs isolated from healthy volunteers as previously described [19]. Candidate probiotic bacteria were tested on h-DC maturation by assessing CD40, CD80, CD83, CD86, HLA-DR surface expression using specific antibodies and FACS analysis. Cytokine production [interleukin (IL)-1 β , IL-6, IL-8, IL-10, IL-12p70, IL-23 and tumor necrosis factor- α] was performed as previously described [19]. To assess the impact on T-cell polarization, bacteria-pretreated h-DCs were cultured with allogeneic CD4⁺ naïve T

cells for 24 h. The CD4⁺ naïve T-cell purification method as well as RNA isolation and quantitative real-time PCR analyses were performed as previously described [19]. The expression of the following genes was assessed in T cells: interferon (IFN)- γ (HS00174143_m1), IL-10 (HS00174086_m1) and IL-17A (HS00174383_m1). Data were interpreted for each gene in comparison with endogenous β -actin (HS99999903_m1). To assess Foxp3 expression in CD4⁺ T cells, cocultures between h-DCs and T cells were performed as above. After 4 days of culture, cells were stained with anti-CD4 ECD immunoglobulin G1, anti-CD25 PC5 immunoglobulin G2a (Beckman Coulter, Roissy, France) and anti-Foxp3 FITC using the FITC anti-human Foxp3 staining set kit (eBiosciences), according to the manufacturer's instructions. Samples were analyzed using a FC500 flow cytometer (Beckman Coulter). To analyze the impact of probiotic strains on committed Th2 cells, a Th2 cytokine phenotype was induced in PBMCs cultured in 48-well plates (Milan, Mayrin, Switzerland) at 1.5×10^6 cells/ml in the presence of 50 ng/ml of IL-4 (Bioconcept, Basel, Switzerland) and 1 μ g/ml anti-CD40 antibody (R&D Systems, Abington, England). After 3 days of culture, lactic acid bacteria were added at 10^7 colony-forming units (CFU)/ml for 2 additional days. The experiments were performed with cells from 12, 9 and 17 donors for *B. adolescentis*, *B. bifidum* and *B. longum*, respectively. To adjust for donor variation, data were standardized according to an arbitrary internal standard. Cytokine concentrations were normalized to 100% using control stimuli, corresponding to either LPS for IFN- γ , *B. lactis* (NCC 2818) for IL-10 or medium for IL-5.

Therapeutic SLIT Models in BALB/c Mice Sensitized to OVA or Birch Pollen

The OVA murine asthma model has been described elsewhere [25], both with respect to sensitization and SLIT protocols. In the present study, a sublingual dose of 2×10^8 CFU per mouse was used for each strain in the tolerance induction phase. The birch pollen allergy model is described herein. Briefly, BALB/c mice were sensitized with a birch pollen aqueous extract, using doses equivalent to 10 μ g Bet v 1 with 2 mg alum by two intraperitoneal injections at days 0 and 7. At day 14 and for 4 consecutive days, a 20-min aerosol challenge was performed with the birch pollen extract, using a dose equivalent to 1 mg Bet v 1, using an aerosol delivery system (Buxco Europe Ltd., Winchester, UK). For tolerance induction, 50 μ g Bet v 1 with or without *B. bifidum* (2×10^8 CFU per mouse) was applied sublingually to groups of 7 mice, twice a week during 2 months in 20 μ l PBS. Control mice were treated sublingually with PBS or bacteria alone. After SLIT, mice were challenged by aerosol during 2 consecutive days with the birch pollen extract (using a dose equivalent to 1 mg Bet v 1). After 24 h, airway hyperresponsiveness (AHR) was evaluated by measuring enhanced pause (Penh) by whole body plethysmography (Buxco Europe Ltd.), using increasing concentrations of methacholine (i.e. from 12.5 to 25 to 50 to 100 mg/ml). The Penh index was obtained as a ratio between Penh values measured after exposure to either inhaled methacholine (100 mg/ml) or nebulized PBS [25].

Cell counts were performed in bronchoalveolar lavage fluid as previously described [19]. To assess T-cell responses in vivo, lung (0.5×10^6 per well) and spleen cells (1×10^6 per well) were recovered and cultured with or without birch pollen extract using a dose equivalent to 100 μ g/ml Bet v 1 in RPMI-1640 plus glutamine

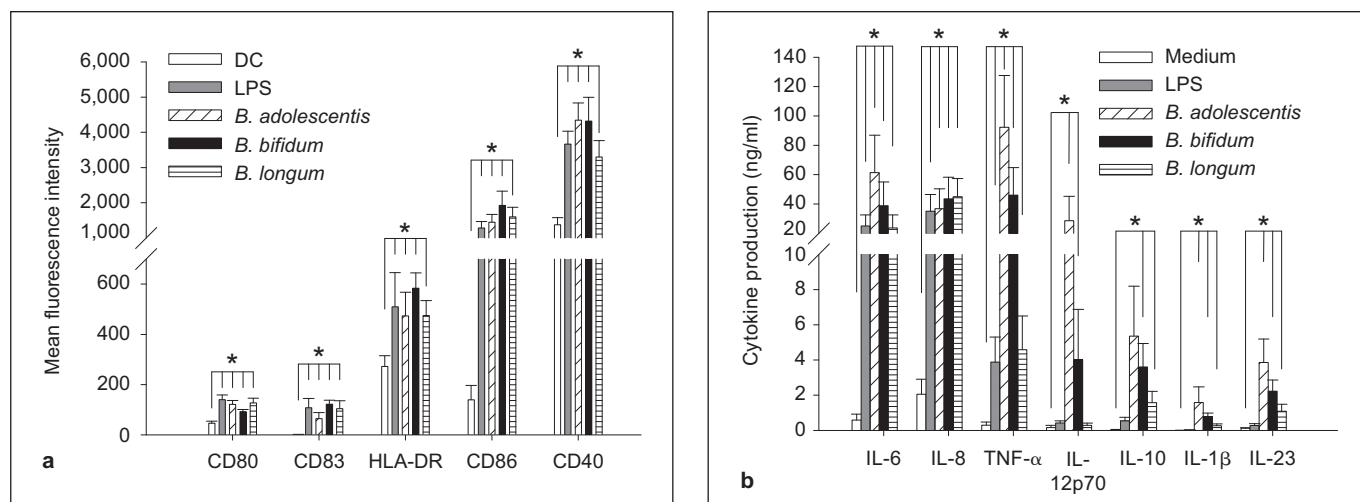


Fig. 1. Impact of probiotic candidates on h-DC phenotype and cytokine production. h-DCs were incubated for 24 h with either medium alone, LPS or live bifidobacteria strains (h-DC:bacteria ratio 1:10). Data were compared to medium using the Wilcoxon test (* $p < 0.05$). **a** The expression of various surface molecules was assessed by flow cytometry. Results are expressed as mean fluorescence intensity $\pm$ SEM ($n = 7$). **b** Cytokine levels in supernatants were determined by CBA or ELISA. Results are expressed as mean $\pm$ SEM ($n = 7$).

(PAA) and penicillin/streptomycin (Fisher Bioblock, Illkirch, France) supplemented with 10% fetal bovine serum (Gentaur, Brussels, Belgium) and 50 μ M β -mercaptoethanol (Fisher Bioblock). Cells were cultured in 96-well U bottomed plates (BD Biosciences, Le Pont de Claix, France) at 37°C, in 5% CO₂/95% air. After 4 days, IL-5, IL-10 and IL-13 were measured in supernatants by Cytometric Bead Array (CBA; BD Biosciences).

Statistical Analysis

Data from in vitro and in vivo experiments were analyzed using the nonparametric Wilcoxon and Mann-Whitney tests, respectively. Results with a p value < 0.05 were considered significant.

Results

Lactic Acid Bacteria Induce Human Dendritic Cell Maturation

We first investigated the capacity of *B. adolescentis* NCC 251, *B. bifidum* NCC 453 and *B. longum* NCC 2705 to induce maturation as well as cytokine production in h-DCs. Monocyte-derived DCs cultured in medium alone or in the presence of LPS were used as negative and positive controls, respectively. As shown in figure 1, when compared to medium alone, the three bifidobacteria strains tested significantly increased surface expression of costimulatory molecules (CD40, CD80 and CD86) to a level comparable to LPS ($p < 0.05$). Also, they induced

high amounts of cytokines (IL-6, IL-8, IL-10 and TNF- α ; $p < 0.05$). Interestingly, only *B. adolescentis* and *B. bifidum* strongly stimulated both IL-12p70 and IL-23 secretion by h-DCs (fig. 1).

Lactic Acid Bacteria Induce a Th1/Treg Polarization of Naïve CD4⁺ T Cells and IFN- γ Production in Th2-Skewed PBMCs

We next analyzed the polarization of allogeneic naïve CD4⁺ T cells incubated with h-DCs treated with either LPS, *B. adolescentis*, *B. bifidum* or *B. longum* for 24 h. Non-activated h-DCs were used as a control. The expression of genes encoding cytokines was subsequently monitored by quantitative RT-PCR after 1 day of coculture. As expected, LPS induced a significant IFN- γ gene expression in naïve CD4⁺ T cells in comparison to medium alone ($p < 0.05$; fig. 2a). All bifidobacteria stimulated statistically significant IFN- γ gene expression to a level comparable to LPS ($p < 0.05$; fig. 2a). Interestingly, all three bifidobacteria induced a 3- to 4-fold increase in IL-10 mRNA when compared to LPS. This IL-10 gene induction was only statistically significant with *B. bifidum* ($p < 0.05$), whereas IL-17A was induced at similar levels by the three strains (fig. 2a). Noteworthy, no significant expression of IL-4 was detected with the three bifidobacteria strains (data not shown). Those observations were confirmed at the protein level in culture supernatants

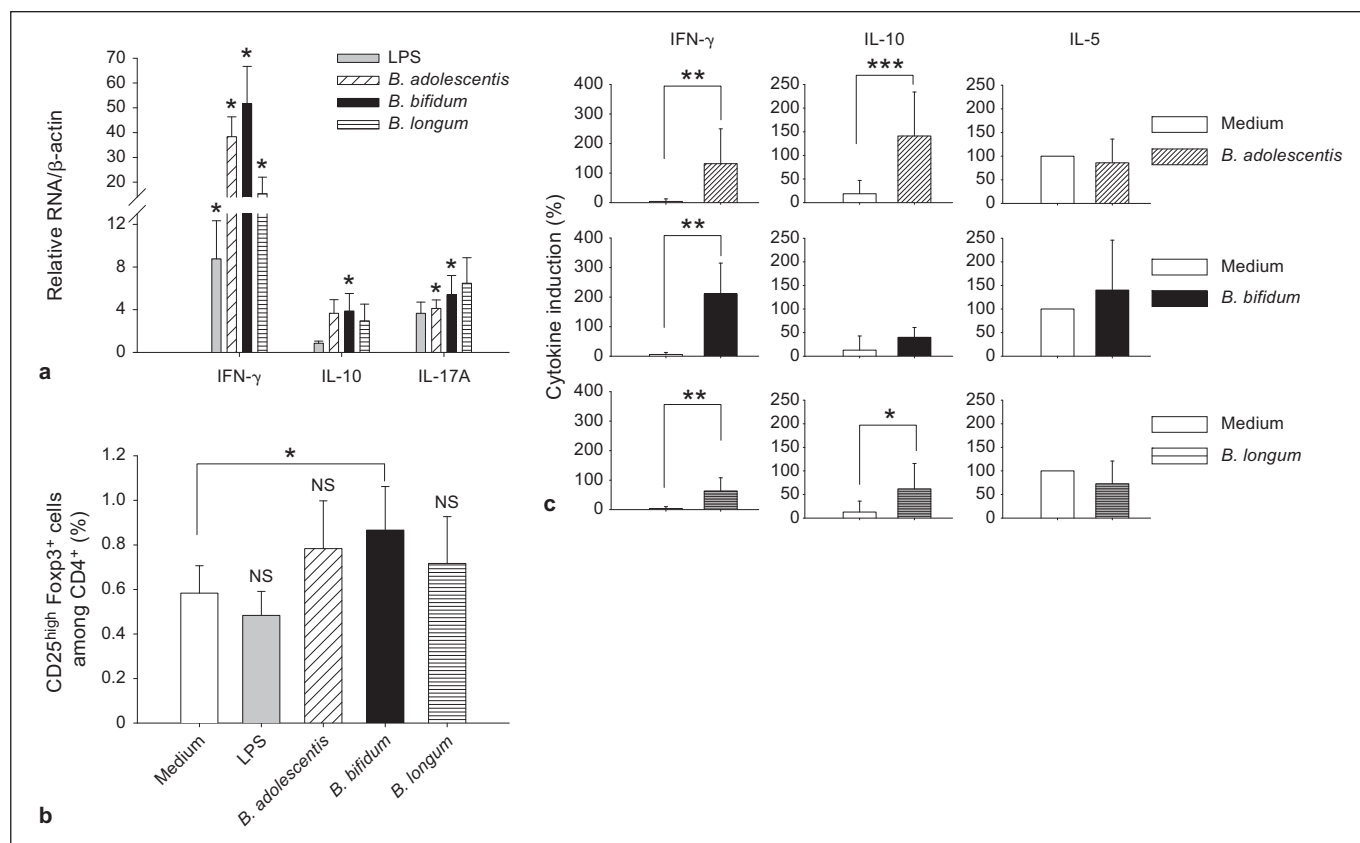


Fig. 2. Impact of probiotic candidates on CD4⁺ T cells. Naïve CD4⁺ T cells were cocultured for 1 or 4 days with allogeneic h-DCs from healthy donors pretreated with either medium alone, LPS or live probiotic candidates (h-DC:T-cell ratio 1:10). Data are expressed as the mean $\pm$ SEM and were compared to medium using the Wilcoxon test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **a** Gene expression was analyzed by quantitative PCR. Relative amounts of mRNA are shown compared with medium alone, ar-

bitrarily set to 1 ($n = 7$). **b** Foxp3 expression was analyzed by intracellular staining. The percentage of CD4⁺ CD25⁺ Foxp3⁺ T cells is shown compared with medium alone ($n = 6$). NS = Not significant. **c** Cytokine induction percentages obtained in Th2-skewed PBMCs are the mean values of 12, 9 and 17 donors for *B. adolescentis*, *B. bifidum* and *B. longum*, respectively. Results are normalized to 100% with LPS as a stimulus for IFN- γ , *B. lactis* for IL-10, medium for IL-5.

(data not shown). The T-bet transcription factor expression was also measured and revealed to be the most potently induced with *B. bifidum* (data not shown).

In parallel experiments, the expression of the Treg marker Foxp3 was assessed in CD4⁺ T cells, after 4 days of coculture. As shown in figure 2b, no significant enhancement of percentages of CD25^{high} Foxp3⁺ cells was found over LPS stimulation within CD4⁺ T lymphocytes cocultured with h-DCs treated with *B. adolescentis* or *B. longum*. In contrast, CD4⁺ T cells cocultured with *B. bifidum* treated h-DCs contain significantly more CD25^{high} Foxp3⁺ T cells ($p < 0.05$).

Subsequently, lactic acid bacteria were tested in Th2-skewed human PBMCs (fig. 2c) driven to secrete IL-5 and IL-10, but not IFN- γ , prior to incubation with bacteria.

Although Th2 cytokine (i.e. IL-5/IL-10) production was still detected in those cultures, *B. adolescentis*, *B. bifidum* and *B. longum* were able to reorient Th2-polarized cells toward a higher production of IFN- γ ($p < 0.01$). In agreement with the hDC T-cell coculture model, *B. bifidum* was the most potent IFN- γ inducer in Th2-skewed CD4⁺ T cells.

Sublingual Administration of *B. bifidum* Decreases AHR in Mice Sensitized to OVA

The three strains were first tested in a murine model of OVA-induced asthma [18]. OVA-sensitized mice were sublingually treated with either PBS, OVA or bifidobacteria strains alone. As shown in figure 3a, healthy mice used as a control (i.e. non-sensitized) exhibited low Penh

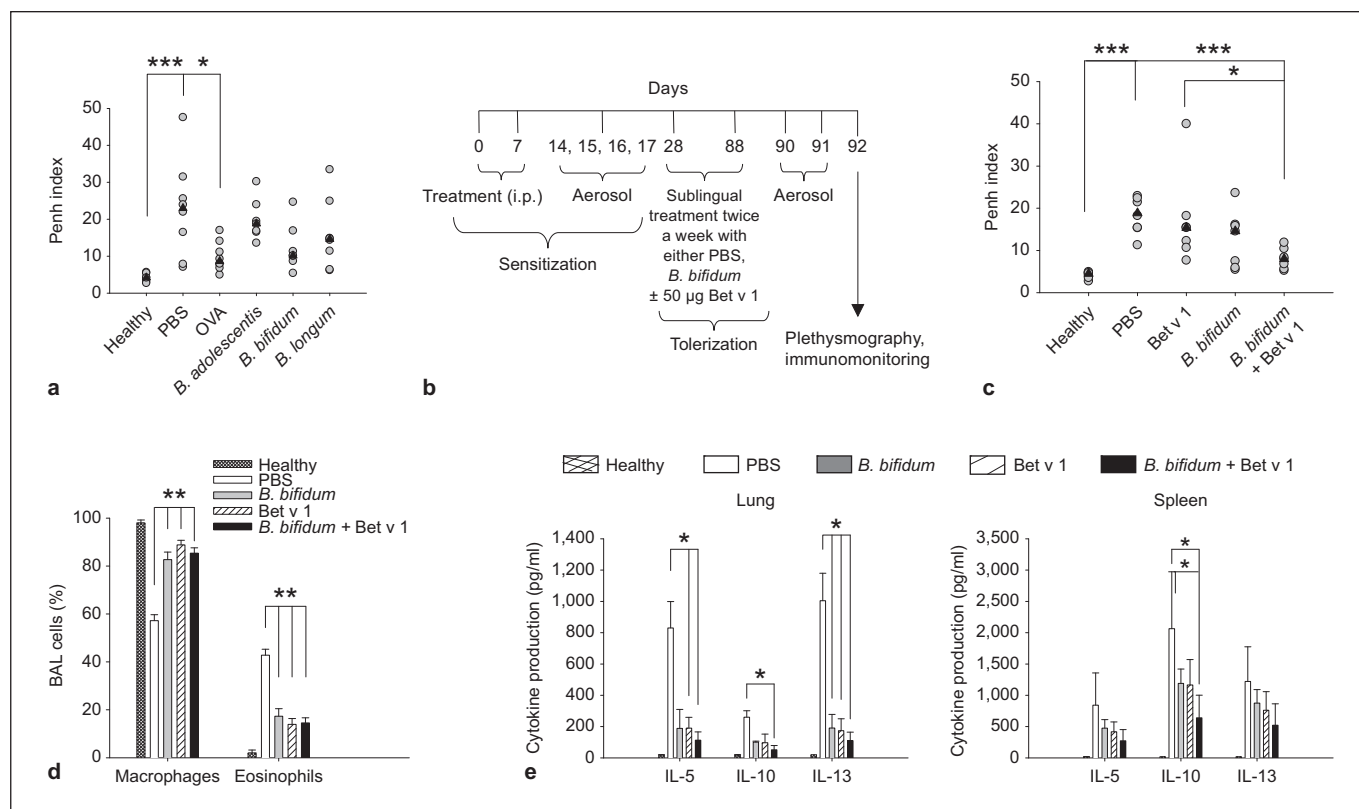


Fig. 3. Sublingual administration of *B. bifidum* decreases established AHR and Th2 responses in mice allergy models. Data were compared to OVA or Bet v 1-sensitized mice sublingually treated with PBS using the Mann-Whitney test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$). **a** AHR was determined by measuring the Penh index in OVA-sensitized mice sublingually treated with either PBS, OVA or live bacteria alone. Values are expressed as individual mice (grey circles) with median (black triangles; $n = 8$). **b** Experimental design of the birch pollen allergy model. **c** AHR was de-

termined by measuring the Penh index in birch-sensitized mice sublingually treated with either PBS, Bet v 1, live bacteria alone or Bet v 1 plus live bacteria. Values are expressed as individual mice (grey circles) with median (black triangles; $n = 7$). **d** Inflammatory cell infiltration into the lung was determined by counting 100 cells in BAL fluid. Values are expressed as mean percentages $\pm$ SEM ($n = 7$). **e** Th2 cytokines in the lung and spleen were measured by CBA. Values are expressed as mean $\pm$ SEM ($n = 7$).

values, whereas OVA-sensitized mice treated sublingually with PBS alone displayed a high statistically significant AHR ($p < 0.001$; with median $\pm$ SEM Penh values of 4.2 ± 0.5 and 23.0 ± 4.6 , respectively; $n = 8$). In addition, OVA treatment significantly reduced AHR to Penh values of 8.6 ± 1.4 ($n = 8$), close to the ones obtained in healthy mice ($p < 0.05$). *B. adolescentis* and *B. longum* had only a moderate impact on AHR (Penh values of 18.8 ± 2.1 and 14.6 ± 3.3 , respectively; $n = 8$), whereas *B. bifidum* induced an important reduction in AHR (Penh values of 10.1 ± 2.4 ; $n = 8$). The association of the OVA antigen with probiotics did not have a superior efficacy in those experiments (data not shown).

Altogether, these data established *B. bifidum* as the best candidate Th1/Treg adjuvant among the three strains

tested since (1) it increases Foxp3 expression in naïve CD4⁺ T cells, (2) it is the most potent inducer of IFN- γ in h-DC T-cell cocultures and in Th2-skewed PBMCs, and (3) it elicits tolerance independently of the antigen following sublingual administration.

Sublingual Administration of *B. bifidum* Induces Tolerance in Mice Sensitized to Birch Pollen

Based on the previous findings, *B. bifidum* was selected for further experiments in a murine model of respiratory birch pollen allergy, as summarized in figure 3b. After sensitization to birch pollen, mice were treated sublingually twice a week for 2 months with either PBS or *B. bifidum* with or without recombinant Bet v 1 (50 µg per dose). As expected, healthy mice in those experiments

exhibited low Penh values, whereas birch pollen-sensitized animals treated sublingually with PBS alone displayed a significantly higher AHR ($p < 0.001$; with median $\pm$ SEM Penh values of 4.5 ± 0.2 and 18.8 ± 1.1 , respectively; $n = 7$) (fig. 3c). Sublingual treatment with soluble Bet v 1 or with *B. bifidum* alone had a moderate impact on AHR (fig. 3c). In contrast, sublingual treatment with Bet v 1 administered together with *B. bifidum* resulted in a statistically significant ($p < 0.05$) reduction in AHR (Penh value of 8.0 ± 0.9) when compared with those treated with Bet v 1 alone ($p < 0.05$) or PBS ($p < 0.001$; fig. 3c).

We subsequently investigated immunological changes associated with symptomatic improvement in this therapeutic SLIT model. The statistically significant decrease in AHR observed after treatment with *B. bifidum* plus Bet v 1 correlated with a reduction in eosinophils and an increase in macrophages in bronchoalveolar lavage, when compared to PBS ($p < 0.01$; fig. 3d). Surprisingly, a similar impact on eosinophil counts was observed with *B. bifidum* or Bet v 1 alone when compared to the mix, despite their lower effect on AHR. Furthermore, the observed reduction in AHR and inflammation following treatment with *B. bifidum* plus Bet v 1 was associated with a statistically significant decrease in IL-5, IL-10 and IL-13 production by birch-specific Th2 cells in the lung and IL-10-secreting cells in the spleen ($p < 0.05$; fig. 3e). A clear-cut decrease was also induced by the strain alone for IL-13 production in the lung and IL-10 secretion in the spleen, and by Bet v 1 alone for IL-5 and IL-13 secretion in the lung ($p < 0.05$). Nonetheless, no IFN- γ production was detected in the spleen or in the lung (data not shown). Finally, we assessed bronchial inflammation by lung histology and observed that the decrease in AHR following treatment with *B. bifidum* + Bet v 1 was associated with a reduction in specific cellular infiltration (data not shown).

Discussion

SLIT has raised a lot of interest as a treatment for type I respiratory allergies in humans [1–12]. Nonetheless, SLIT efficacy could be further enhanced with the use of adjuvants potentiating regulatory immune responses. A better understanding of immune mechanisms underlying SLIT efficacy revealed that oral tissues contain tolerogenic antigen-presenting cells encompassing both Langerhans cells as well as myeloid and plasmacytoid DCs supporting the differentiation of Th1/Treg cells with a

suppressive capacity [25–31]. In this context, probiotics represent valid candidates in order to promote allergen-specific tolerance during SLIT, since they have the capacity to interact with mucosal antigen-presenting cells via pathogen recognition receptors (i.e. Toll-like receptors or mannose receptors) or C-type lectins (such as DC-SIGN3) [32–34], thus modulating allergic immune responses [35–37]. The latter hypothesis is reinforced by the observation that specific probiotic strains have been successfully used with allergies in therapeutic [18–20, 25, 26] or prophylactic approaches [21–23], both in animal models and humans.

Herein, we more specifically focused on bifidobacteria in the context of previous murine and human studies demonstrating their tolerogenic properties in allergic disorders following oral administration [38–43]. We tested three strains (i.e. *B. adolescentis* NCC 251, *B. bifidum* NCC 453 and *B. longum* NCC 2705) with respect to their capacity to modulate established Th2 responses. We observed that these strains tested induce h-DC maturation and polarize responses toward a Th1/Treg pattern (i.e. with IFN- γ and IL-10 secretion). Moreover, *B. bifidum* promotes the development of CD4⁺ CD25^{high} Foxp3⁺ T cells and is a strong inducer of IFN- γ in Th2-skewed PBMCs and in h-DC T-cell cocultures. This observation is in accordance with other studies demonstrating in human and mouse models that bifidobacteria activate regulatory T cells [44–47]. The enhancement of both IFN- γ production together with Foxp3⁺ expression in T cells cocultured with *B. bifidum*-treated h-DCs is in agreement with the observation that IFN- γ may contribute to Foxp3 induction and conversion of CD4⁺ CD25[−] naïve T cells into CD4⁺ Treg cells with an enhanced suppressive activity [48].

Noticeably, all bifidobacteria tested herein induce similar Th17 responses with only a minor difference observed between the three strains regarding IL-23 production by DCs. *B. bifidum* also decreased AHR in an OVA allergy model independently of the antigen. Lastly, when used as an adjuvant, *B. bifidum* dramatically enhanced SLIT efficacy in BALB/c mice sensitized to birch pollen, with a decrease in both AHR and associated Th2 responses (i.e. IL-5, IL-10 and IL-13). Although IL-10 has been described as a potent immunosuppressive cytokine [49], it can be produced by both Th1, Th2 and Th17 cells. As such, it is rather indicative of a negative feedback loop precluding uncontrolled inflammation. Thus, in our experimental allergy model, the observed decrease in IL-10 may be linked to the downregulation of OVA-specific Th2 responses.

We believe that differences observed between the various *Bifidobacterium* strains in terms of their tolerogenic properties are likely related to their capacity to induce Th1/Treg cells. Biological mechanisms underlying such differences in their immunomodulatory properties remain to be fully clarified but may involve the diversity of cell-wall glycomers observed in probiotics [50]. Our results are in agreement with previous experiments using lactobacilli, demonstrating that *Lactobacillus plantarum* and *L. helveticus* strains selected for their capacity to induce high levels of IFN- γ and IL-10 were the best adju-

vants for in vivo sublingual desensitization of asthmatic mice [18, 19]. We conclude that sublingual vaccines may benefit from the use of adjuvants capable to reorient established Th2 responses toward a Th1/Treg profile.

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