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The combination of *Bifidobacterium breve* with non-digestible oligosaccharides suppresses airway inflammation in a murine model for chronic asthma

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ABSTRACT

Over the last decade, there has been a growing interest in the use of interventions that target the intestinal microbiota as a treatment approach for asthma. This study is aimed at exploring the therapeutic effects of long-term treatment with a combination of *Bifidobacterium breve* with non-digestible oligosaccharides on airway inflammation and remodeling. A murine ovalbumin-induced chronic asthma model was used. Pulmonary airway inflammation; mRNA expression of pattern recognition receptors, Th-specific cytokines and transcription factors in lung tissue; expression of Foxp3 in blood Th cells; *in vitro* T cell activation; mast cell degranulation; and airway remodeling were examined. The combination of *B. breve* with non-digestible oligosaccharides suppressed pulmonary airway inflammation; reduced T cell activation and mast cell degranulation; modulated expression of pattern recognition receptors, cytokines and transcription factors; and reduced airway remodeling. The treatment induced regulatory T cell responses, as shown by increased *Il10* and *Foxp3* transcription in lung tissue, and augmented Foxp3 protein expression in blood CD4+CD25+Foxp3+ T cells. This specific combination of beneficial bacteria with non-digestible oligosaccharides has strong anti-inflammatory properties, possibly *via* the induction of a regulatory T cell response, resulting in reduced airway remodeling and, therefore, may be beneficial in the treatment of chronic inflammation in allergic asthma.

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1. Introduction

Allergic asthma is a T helper type-2 (Th2) cell-mediated chronic inflammatory disorder of the airways characterized by airway inflammation and hyper-responsiveness [1]. Persistent chronic inflammation and subsequent inadequate repair of injured tissue can result in structural changes in the airway walls of asthmatic patients, referred to as "airway remodeling" [2]. Airway remodeling is thought to be caused by an imbalance in regeneration and repair mechanisms leading to a disequilibrium between the synthesis and degradation of the extra-cellular matrix components [1] and characteristic thickening of the airway wall, increased smooth muscle mass, and vascularity and epithelial abnormalities [3]. The combination of these processes causes airway narrowing and reduced lung function [1,3]. Activation of mast cells induces degranulation and release of various inflammatory substances, including proteases [4]. Proteases are important regulators of mast cell function and mouse mast cell protease 1 (mMCP-1) has been shown to enhance airway narrowing in mice [5,6]. Additionally, eosinophils and neutrophils play key roles in the cellular airway inflammation [7].

The incidence and prevalence of allergic asthma are rapidly increasing throughout the world, especially in children and within developing countries. To date, the underlying immunological processes of asthma are still not fully understood as it is a complex multifactorial disorder in which both innate and adaptive immune responses are involved [8].

Although the majority of asthma patients is well controlled with inhaled corticosteroids with or without long-acting β_2-agonists, over 50% of asthmatics are poorly controlled largely due to poor adherence [9]. In addition, β_2-agonists are aimed at relieving the symptoms rather than treatment of the disease, and long-term corticosteroid therapy has undesirable side effects [10]. These limitations of the current treatments for asthma highlight the need for novel therapeutics targeting underlying allergic and immune responses with greater disease control, increased efficacy and a major clinical effect. Animal models for allergic asthma are important for understanding of the pathophysiology underlying the disease. Recently, there has been a focus on developing chronic allergen exposure models, especially in mice, to reproduce more of the clinical features of asthmatic patients, such as airway remodeling [1,11].

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Animal studies have demonstrated a substantial influence of the gut microbiota on immune function beyond the gut. There is now increasing evidence that changes in the gut microbiota contribute to the development of allergies and asthma [12–16].

Non-digestible oligosaccharides are oligosaccharides that resist digestion but are fermentable by intestinal microbiota, such as galacto-oligosaccharides (GOS), fructo-oligosaccharides (FOS) and pectin-derived acidic oligosaccharides (AOS), and are thought to be metabolized by or increase the survival of probiotics, "live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host", and specific bacterial strains in the intestinal tract [17]. A combination of *Bifidobacterium breve* M-16V with a specific mixture of short-chain GOS (sc-GOS) and long-chain FOS (lc-FOS) reduced allergic responses in mice [18]. The same combination also proved to be useful in patients, reducing the allergen-specific Th2-response and improving peak expiratory flow in allergic asthmatic adults, and preventing asthma-like symptoms in infants with atopic dermatitis [19,20]. Additionally, combinations of GOS/FOS and AOS were more effective in improving T helper-type 1 (Th1) immune responses and in reducing disease parameters of allergic asthma in mice than either of the oligosaccharides alone [21–23].

Although the mechanism of action of these beneficial bacteria still needs to be elucidated, probiotics have been shown to contain Toll-like receptor (TLR) ligands and can thereby modulate TLR-driven responses and skew the immune balance towards a Th1 response [24]. TLRs and NOD-like receptors (NLRs) are key pattern recognition receptor (PRR) families in the innate immune response, which are also involved in the activation and shaping of adaptive immunity [25]. In the human airways, the PRRs are expressed by dendritic cells (DCs), epithelial cells, eosinophils, macrophages and mast cells [26,27]. PRRs are especially crucial for modulating DC function and, ultimately, shaping the resulting adaptive immune response [28,29]. TLR and NLR activation in the lung also influences DCs, eosinophils and mast cells, encouraging them to produce various chemokines and cytokines that attract activated B-lymphocytes and Th lymphocytes to the lung [30].

The function and expression of PRRs are associated with susceptibility for the development of allergic asthma in humans [31,32]. Functional genetic variations in *TLR1*, *TLR10* and *TLR6* genes leading to increased mRNA expression have been shown to protect against atopic asthma in humans [32]. Genetic variations in *TLR2*, *NOD1* and *NOD2* genes that led to either decreased mRNA expression (*TLR2*) or affected microbial recognition function (*NOD1* and *NOD2*) were positively associated with disease susceptibility and pathogenesis [33–37]. Low *TLR2*, *TLR4*, and *TLR9* expression levels were detected in cord blood CD34(+) cells from high-atopic-risk infants. *TLR9* has been demonstrated to exert protective immunomodulatory effects on asthma [38–40]. Viral activation of *Tlr3* contributed to asthma exacerbations in mice [41] and a study in a murine macrophage cell line suggested a pro-inflammatory role of *Tlr4* and *5* in the disease [42].

In asthma, over 50 cytokines have now been identified that affect disease outcome. Interleukin-1β (IL-1β), IL-4, IL-5, IL-6, IL-9, IL-13, IL-17, IL-25 and tumor necrosis factor α (TNF-α) were reported to enhance the disease. On the other hand, IL-12, IL-18 and interferon-γ (IFNγ) were reported to reduce the symptoms of asthma in asthmatic patients [30]. Reduced levels of the anti-inflammatory cytokine IL-10 were found in the sputum of asthma patients. IL-10 is produced by alternatively activated macrophages and by a subset of regulatory T cells (Tregs). This cytokine exerts its effect by inhibiting the synthesis of inflammatory cytokines (including asthma-associated cytokines such as TNF-α and IL-5) and gene transcription [43]. Th2 cells play a key role in asthma, and Th1/Th2 imbalances as well as disturbed T helper type-17 (Th17)/Treg balances have been reported in asthmatics [44]. Treg cells are important in balancing immune responses and maintaining immunological tolerance to foreign and self-antigens, including allergens [43]. Imbalances in Th responses can also be detected using Th-specific transcription factors: T-bet for Th1 cells, GATA-3 for Th2 cells, retinoic acid orphan receptor-γt (RORγt) for Th17 cells and Foxp3 for Tregs [45]. Alterations in the expression and/or function of these transcription factors are also associated with asthma pathogenesis [46,47].

Although the potential of beneficial bacteria interventions in allergy and related inflammatory conditions is becoming more evident, more research in mice and asthma patients is needed to explore the immunomodulatory properties of these interventions. The aim of this current study is to explore the therapeutic effects of long-term administration of a combination of *B. breve* with non-digestible oligosaccharides on chronic airway inflammation and remodeling in mice. To mimic the repeated allergen exposure in asthmatic patients, we used the murine ovalbumin-induced asthma model combined with prolonged allergen exposure. Findings from this study will contribute to a better understanding of the immunomodulatory and therapeutic effects of beneficial bacteria in chronic allergic asthma.

2. Material and methods

2.1. Animals

Male BALB/c mice (6–8 weeks; Charles River Laboratories, France) were obtained and acclimated to their new environment for at least 1 week before the start of the experiment. Mice were housed under standard conditions and had free access to food and water. All *in vivo* experiments were approved by and were in accordance with the guidelines of the local Dutch Committee of Animal Experimentation.

2.2. Chronic asthma model

2.2.1. OVA sensitization

Sensitizations were performed on days 0 and 12. Mice were sensitized to OVA (chicken egg albumin, grade V, Sigma, St. Louis, MO, USA) by intraperitoneal injections of 0.1 mL alum-precipitated antigen, comprising 10 μg OVA absorbed into 2.25 mg alum (Imject Alum; Pierce, Rockford, IL, USA). Control animals received 0.1 mL saline alone (NaCl 0.9%; B. Braun Medical B.V., Oss, The Netherlands) (Fig. 1).

2.2.2. OVA challenge

A chronic model of asthma was established according to a modification of a model of prolonged allergen-induced airway inflammation described in [1]. Mice (OVA/OVA) were exposed daily to 5% OVA aerosol in saline using a PARI LC Star nebulizer (PARI GmbH, Starnberg, Germany) in an aerosol cabin for 20 min between days 17 and 23. Control animals (OVA/Sal) were exposed to nebulized saline aerosol alone. From day 24 until day 55, the frequency of challenge was reduced to three times a week and mice were exposed to aerosolized OVA (5%) or saline alone for 20 min (Fig. 1).

2.3. Bb/scFOS/lsFOS/AOS treatment

The combination consisted of 10^9 colony forming units (CFUs) of lyophilized *B. breve* M-16V powder with maltodextrin as carrier material (Bb, Morinaga Milk Industry, Tokyo, Japan) combined with a specific mixture of scFOS, lcFOS and pectin-derived acidic-oligosaccharides (AOS). Mice (OVA/Sal–Bb/scFOS/lcFOS/AOS and OVA/OVA–Bb/scFOS/lcFOS/AOS) received 0.2 mL of the mixture Bb/scFOS/lsFOS/AOS in phosphate buffered saline (PBS, Lonza, Leusden, The Netherlands) by oral gavage, 1 h prior to challenge, three times a week from day 22 until day 55. As a negative control treatment, mice in the OVA/Sal–PBS and OVA/OVA–PBS groups received 50 μL of PBS per day. PBS was administered to mice by oropharyngeal aspiration after induction of light isoflurane anesthesia as described previously in [48], 1 h prior to challenge, three times a week from day 22 until day 55. Mice were rendered asthmatic following the schema presented in Fig. 1.

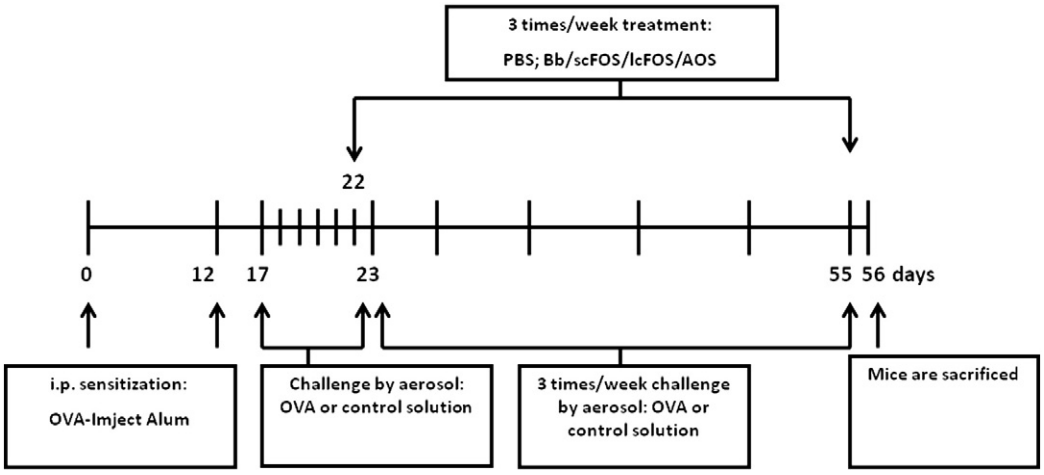


Fig. 1. Time schedule of the chronic asthma mouse model. Male BALB/c mice were sensitized intraperitoneally to OVA-Imject Alum on days 0 and 12 and mice were challenged from day 17 until day 23 daily with aerosolized OVA or saline. From day 22 until day 55, mice were treated 3 times a week with either PBS by oropharyngeal aspiration or Bb/scFOS/lcFOS/AOS by oral gavage. One hour after treatment, from day 24 until day 55, mice were challenged 3 times/week with aerosolized OVA or saline. Mice were sacrificed on day 56.

2.4. Bronchoalveolar lavage

After sacrifice, on day 56, lungs were first lavaged through a tracheal cannula with 1 mL saline containing protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Mannheim, Germany), pre-warmed at 37 °C. This was followed by 3 additional lavages with 1 mL saline only. Cytospin cell preparations were made by cytospinning the cells onto glass for 5 min (400 *g*, 4 °C) and cytospins were stained by DiffQuick (Merz & Dade AG, Düdingen, Switzerland). Numbers of eosinophils, macrophages, neutrophils and lymphocytes were scored by light microscopy. The bronchoalveolar lavage (BAL) fluid pellets were normalized in unit volumes to account for differences in recovery of lavage fluid from individual mice.

2.5. RNA isolation and quantitative real-time PCR

After mice were sacrificed on day 56, the lungs were dissected and mRNA was isolated from whole lung tissue. Messenger RNA isolation ($n = 6$ mice per group) was carried out according to the Qiagen RNeasy Mini Kit protocol (Qiagen Benelux B.V., Venlo, The Netherlands). Reverse transcriptase PCR was performed using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). The reactions were performed in a PTC-100TM Programmable Thermal Controller (M.J. Research, Inc., Waltham, Massachusetts, USA) according to manufacturer's protocol.

cDNA was amplified using iQ SYBR Green supermix in a 96-well PCR plate and run in a CFX96 Real-Time PCR Detection System (Bio-Rad). Primers for TLRs, NLRs, ribosomal protein S13 (RPS13, reference gene) and T cell transcription factors were purchased by Isogen (Isogen Life Science, De Meern, The Netherlands). The sequences are listed in Supplementary Table 1. For mouse T cell cytokines, RT^2 qPCR Primer Assays (SABiosciences, Venlo, The Netherlands) were used. The protocol used for amplification was 94 °C for 3 min, 94 °C for 10 s, specific melt temperature for 45 s, followed by 39 cycles at 94 °C for 10 s and 95 °C for 10 s.

Normalized gene expression ($\Delta\Delta C_T$) was calculated using the built-in gene expression analysis module in CFX Manager™ software (CFX Manager™ software version 1.6).

2.6. Foxp3 staining and flow cytometry

On days 0 and 56, blood samples were collected from mice by cardiac puncture in tubes containing lithium heparin to prevent coagulation. The blood was suspended in PBS and, after centrifugation, the pellet was subjected to red cell lysis using a buffer containing NH_4Cl (Merck, Darmstadt, Germany), $KHCO_3$ (Sigma), and ethylenediaminetetraacetic acid (EDTA, Merck) in demineralized water for 5 min on ice. After several washes with PBA (PBS containing 1% bovine serum albumin (BSA, Roche Diagnostics, Almere, The Netherlands)) cells were resuspended in PBA and kept on ice until Foxp3 staining.

The expression of Foxp3 was measured using the Foxp3 Staining Buffer Set (eBioscience, San Diego, CA, USA) using the following protocol: cells were incubated in Fixation/Permeabilization buffer for 30 min on ice. Cells were then washed once with PBA followed by two washes with permeabilization buffer. After a 15 min preincubation in total mouse serum blocking reagent on ice, cells were washed once with permeabilization buffer and then stained with anti-CD4 (FITC, eBioscience), anti-CD25 (PE, eBioscience) and anti-Foxp3 (APC, eBioscience) for 30 min on ice. Cells were washed twice with permeabilization buffer and resuspended in PBA for flow cytometry analysis.

Tregs were defined as CD4+Foxp3+CD25+ T cells. The stained cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences, USA). Data analysis was performed using BD FACSDiva™ software (BD Biosciences).

2.7. Measurement of cytokine production by T cells in thoracic lymph nodes after restimulation with anti-CD3 antibody in vitro

In order to examine specific T cell responses, after mice were sacrificed on day 56, lung-draining lymph nodes were collected from the thorax and transferred to cold sterile PBS. Single cell suspensions of the thoracic lymph nodes (TLNs) were made using a 70 μm nylon cell strainer (BD Biosciences) and rinsed with 15 mL of PBS. The cells were washed and resuspended in RPMI 1640 culture medium without L-glutamine and phenol red (Lonza) supplemented with 10% heat-inactivated fetal calf serum (FCS, HyClone Laboratories, USA) and 0.1% penicillin–streptomycin solution (pen–strep, Sigma). The total number of cells isolated from whole suspension of TLNs was determined using a Beckman Z1 coulter® Particle Counter (Beckman, USA). TLN cells (4×10^6 cells/mL; 100 μL/well) were cultured in a Greiner bio-one CellSTAR 96-well U-bottom plate (Greiner Bio-One B.V., Alphen a/d Rijn, The Netherlands) in a medium with or without O/N precoating of the wells with 50 μg/mL of anti-CD3 antibody (Bioceros B.V., Utrecht, The Netherlands). The supernatant was harvested after 5 days of culture at 37 °C in 5% CO_2 and stored at −20 °C until further analysis.

The levels of cytokines in the supernatant were measured by flow cytometry using a BD™ Cytometric Bead Array (CBA) Mouse Th1/Th2/Th17 Cytokine kit (BD Biosciences) according to manufacturer's

protocol on a FACSCanto II flow cytometer (BD Biosciences). Data analysis was performed using BD FCAP Array™ v3.0.1 Software (BD Biosciences).

2.8. Measurement of mouse mast cell protease 1 levels in serum and BAL fluid

To assess mast cell activation, after mice were sacrificed on day 56, blood samples were collected from mice by cardiac puncture. The blood was coagulated for 1 h at room temperature and subsequently centrifuged for 5 min at 17,500 *g*. BAL fluid samples were collected as described previously in this section. Serum and BAL fluid samples were stored at −80 °C until further analysis.

Mouse mast cell protease 1 (mMCP-1) protein expression levels in serum and BAL fluid were determined by enzyme-linked immunosorbent assay (ELISA) using the Mouse MCPT-1 (mMCP-1) ELISA Ready-SET-Go!® kit (eBioscience) according to manufacturer's protocol.

2.9. Histology and immunohistochemistry

After mice were sacrificed on day 56, lungs were fixed with 10% formalin infusion through a tracheal cannula at a constant pressure of 25 cm H_2O. The lungs were immersed in fixative for at least 24 h, after which the left lung was embedded in paraffin. After paraffin embedding, 5 μm sections were cut and deparaffin sections were first deparaffinized. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 (Merck) in methanol for 30 min at room temperature and rehydrated in a graded ethanol series to PBS and paraffin section were stained with hematoxylin/eosin (H&E) for inflammation, periodic acid-schiff (PAS) for goblet cells, Masson's trichrome for connective tissue, rabbit polyclonal anti-α-smooth muscle actin antibody (Abcam, Cambridge, UK) for smooth muscle cells and rabbit polyclonal anti-Ki76 antibody (Abcam) for proliferating cells according to standard methods.

Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital Camera.

Slides were reviewed in blinded fashion by two independent observers and were scored as *0* to *5* (no to abundant staining).

2.10. Statistical analysis

Data analysis was performed using a 1-way analysis of variance (one-way ANOVA) with the Bonferroni's post-hoc test. All statistical analyses were performed using GraphPad Prism software program (GraphPad Prism software version 5.03).

3. Results

3.1. Bb/scFOS/lcFOS/AOS treatment reduces pulmonary airway inflammation in chronic asthmatic mice

To study the extent of airway inflammation in the asthmatic mice after treatment with Bb/scFOS/lcFOS/AOS, BAL fluid was examined for leukocyte accumulation (Fig. 2). The total inflammatory cell number in the BAL fluid of OVA/OVA–PBS mice was significantly increased (Fig. 2A), which was due to a relative increase in the number

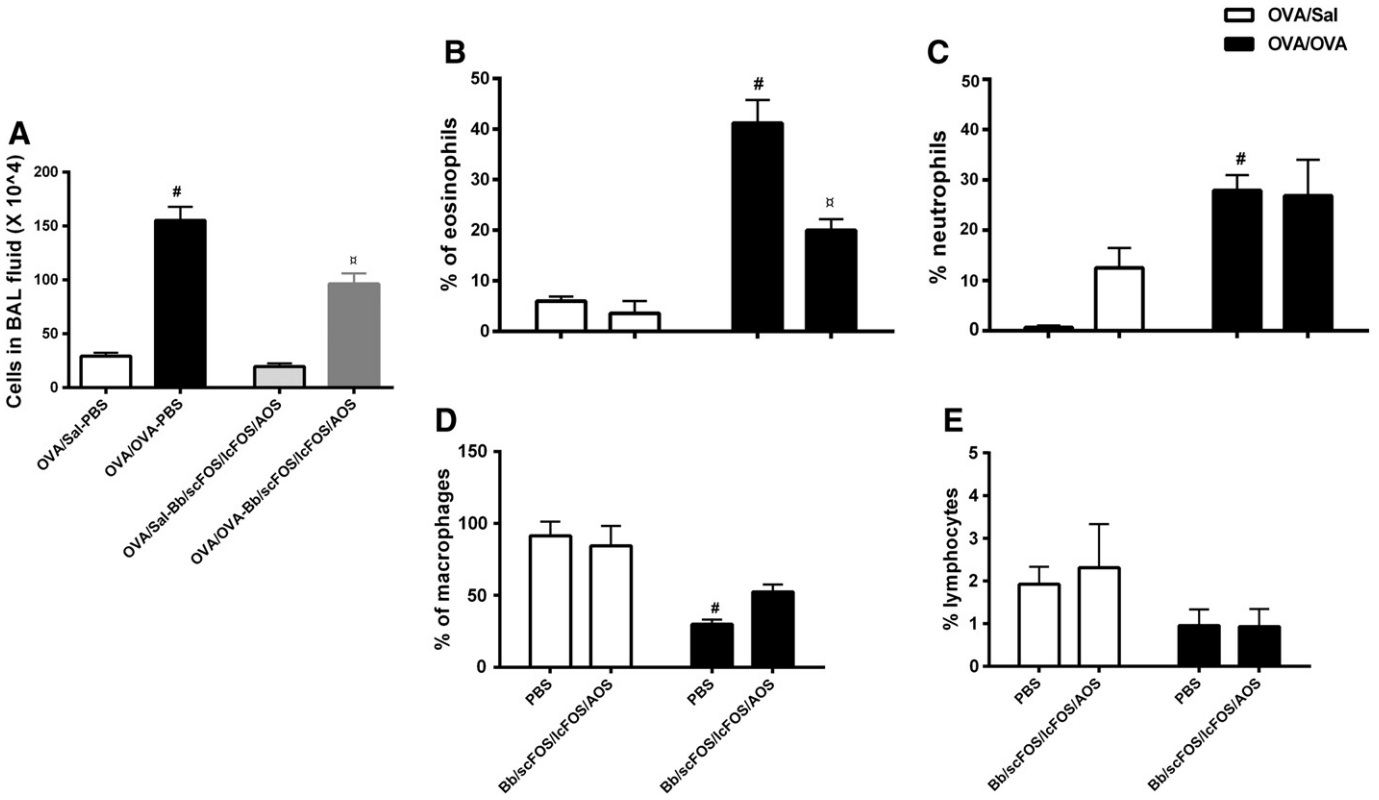


Fig. 2. Total and differential BAL fluid cell counts. Pulmonary inflammation represented by the influx of specific leukocytes in BAL fluid (B–E). Differential cell counts are shown as percentages of the total cell count for each group (A). Mice in all groups were sensitized with OVA. OVA/Sal mice were challenged with saline. OVA/OVA mice were challenged with OVA. Mice in the OVA/Sal–PBS and OVA/OVA–PBS groups received PBS as treatment, mice in the OVA/Sal–Bb/scFOS/lcFOS/AOS and OVA/OVA–Bb/scFOS/lcFOS/AOS were treated with Bb/scFOS/lcFOS/AOS. Results are shown as mean ± SEM, n = 6 mice/group, for one independent experiment. Statistical significance of differences was tested using post-hoc Bonferroni's multiple comparison test after one-way ANOVA. #Statistically significant difference ($p < 0.05$) compared to OVA/Sal–PBS mice. ¤Statistically significant difference ($p < 0.05$) compared to OVA/OVA–PBS mice.

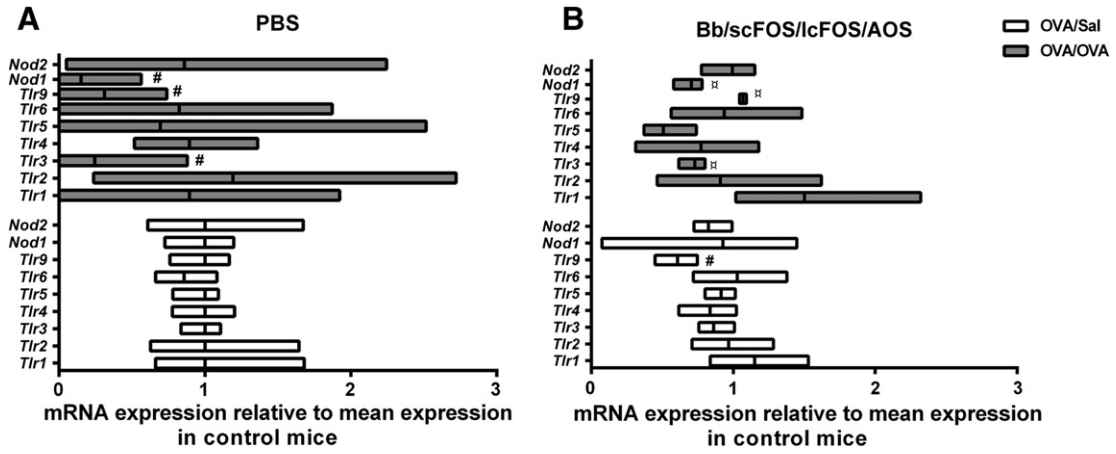


Fig. 3. Relative TLR and NLR mRNA expression in mouse whole lung tissue during chronic asthma. The bars indicate the min to max values and the line represents the mean, n = 6 mice/group, of the TLR and NLR mRNA expression levels in OVA-sensitized, saline-challenged (OVA/Sal, white bars) mice and OVA-sensitized, OVA-challenged (OVA/OVA, gray bars) mice treated with PBS or Bb/scFOS/lcFOS/AOS. Data is shown for one independent experiment. The results are presented as mRNA expression levels relative to levels found in the OVA/Sal–PBS mice. Statistical significance of differences was tested using post-hoc Bonferroni's multiple comparison test after one-way ANOVA. #Statistically significant difference ($p < 0.05$) compared to OVA/Sal–PBS mice. ¤Statistically significant difference ($p < 0.05$) compared to OVA/OVA–PBS mice.

of eosinophils and neutrophils (Fig. 2B and C) as compared to OVA/Sal–PBS mice. The relative number of macrophages was significantly decreased in OVA/OVA–PBS mice as compared to the OVA/Sal–PBS group (Fig. 2D). Bb/scFOS/lcFOS/AOS treatment, however, significantly reduced the total number of inflammatory cells (Fig. 2A) and relative number of eosinophils (Fig. 2B) as compared to OVA/OVA–PBS mice. The relative numbers of neutrophils, macrophages and lymphocytes (Fig. 2C, D and E) remained unchanged.

3.2. Bb/scFOS/lcFOS/AOS treatment modulates PRR mRNA expression in lung tissue of chronic asthmatic mice

To investigate whether PRRs are involved in the ongoing chronic inflammation during asthma, the mRNA expression of *Tlr1–9* and *Nod1–2* in lung tissue was measured (Fig. 3). The mRNA expression of *Tlr3*, *Tlr9* and *Nod1* was significantly decreased in OVA/OVA–PBS mice as compared to the OVA/Sal–PBS group (Fig. 3A). Interestingly, Bb/scFOS/lcFOS/AOS treatment prevented the decrease in*Tlr3*, *Tlr9* and *Nod1* expression (Fig. 3B). In non-asthmatic controls, the expression of *Tlr9* was significantly decreased in the OVA/Sal–Bb/scFOS/lcFOS/AOS group as compared to OVA/Sal–PBS mice; yet, the expression of the other PRRs in non-asthmatic controls remained unchanged (Fig. 3B).

3.3. Bb/scFOS/lcFOS/AOS treatment modulates cytokine mRNA expression in lung tissue of chronic asthmatic mice

To gauge the extent of inflammation and the Th response in the lung, the mRNA expression of various cytokines was measured (Fig. 4). The mRNA expression of *Il1β*, *Il6* and *Il17* was significantly increased and *Il5* was significantly decreased in OVA/OVA–PBS mice as compared to the OVA/Sal–PBS group (Fig. 4A). Bb/scFOS/lcFOS/AOS treatment, however, significantly decreased *Il1β*, *Il6*, *Il12* and *Tnfα* and significantly increased *Il10* and *Il23* expression in OVA/OVA mice as compared to the OVA/OVA–PBS group (Fig. 4B). Additionally, the expression of *Il17* tended to be decreased in OVA/OVA–Bb/scFOS/lcFOS/AOS mice when compared to the OVA/OVA–PBS group.

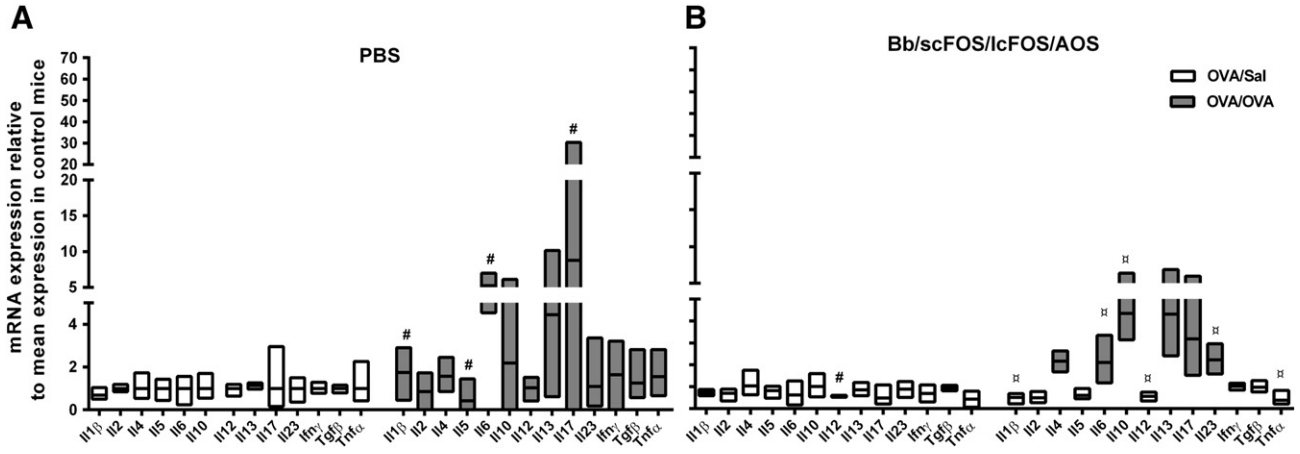


Fig. 4. Relative cytokine mRNA expression in mouse whole lung tissue during chronic asthma. The bars indicate the min to max values and the line represents the mean, n = 6 mice/group, of the cytokine mRNA expression levels in OVA-sensitized, saline-challenged (OVA/Sal, white bars) mice and OVA-sensitized, OVA-challenged (OVA/OVA, gray bars) mice treated with PBS or Bb/scFOS/lcFOS/AOS. Data is shown for one independent experiment. The results are presented as mRNA expression levels relative to OVA/Sal–PBS mice. Statistical significance of differences was tested using post-hoc Bonferroni's multiple comparison test after one-way ANOVA. #Statistically significant difference ($p < 0.05$) compared to OVA/Sal–PBS mice. ¤Statistically significant difference ($p < 0.05$) compared to OVA/OVA–PBS mice.

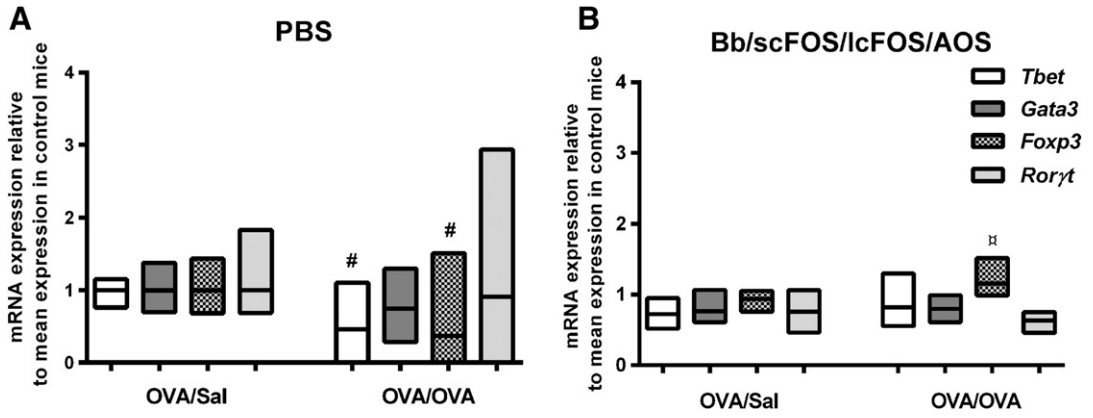


Fig. 5. Relative T cell transcription factor mRNA expression in mouse whole lung tissue during chronic asthma. The bars indicate the min to max values and the line represents the mean, n = 6 mice/group, of the transcription factor mRNA expression levels in OVA-sensitized, saline-challenged (OVA/Sal, white bars) mice and OVA-sensitized, OVA-challenged (OVA/OVA, gray bars) mice treated with PBS or Bb/scFOS/lcFOS/AOS. Data is shown for one independent experiment. The results are presented as mRNA expression levels relative to OVA/Sal–PBS mice. Statistical significance of differences was tested using post-hoc Bonferroni's multiple comparison test after one-way ANOVA. #Statistically significant difference ($p < 0.05$) compared to OVA/Sal–PBS mice. ¤Statistically significant difference ($p < 0.05$) compared to OVA/OVA–PBS mice.

3.4. Bb/scFOS/lcFOS/AOS treatment upregulates mRNA for Foxp3 and skews the immune response away from Th2 towards Treg in the lungs of chronic asthmatic mice

To further explore the effects of Bb/scFOS/lcFOS/AOS on Th and Treg responses and skewing in the lung, the mRNA expression of Th- and Treg-specific transcription factors was measured (Fig. 5). Ratios for *Gata3*/*Tbet* (Th2/Th1), *Foxp3*/*Rorγt* (Treg/Th17), *Foxp3*/*Gata3* (Treg/Th2) and *Foxp3*/*Tbet* (Treg/Th1) mRNA expression were also calculated (Table 1). The mRNA expression of Th1-(*Tbet*) and Treg-(*Foxp3*) transcription factors was significantly decreased in OVA/OVA–PBS mice as compared to the OVA/Sal–PBS group (Fig. 5A). The expression of Th2-(*Gata3*), and Th17-(*Rorγt*) transcription factors remained unchanged (Fig. 5A). The *Gata3*/*Tbet* ratio, however, was significantly increased and *Foxp3*/*Rorγt* and *Foxp3*/*Gata3* ratios were significantly decreased in OVA/OVA–PBS mice as compared to the OVA/Sal–PBS group (Table 1). After treatment with the Bb/scFOS/lcFOS/AOS, OVA/OVA mice showed a significant increase in the expression of *Foxp3* as compared to the OVA/OVA–PBS group (Fig. 5B) which led to significant increases in the *Foxp3*/*Rorγt* and *Foxp3*/*Gata3* ratios (Table 1).

Table 1
Effect of Bb/scFOS/lcFOS/AOS treatment on T helper cell balance in whole lung tissue.

Treatment	Ratio	OVA/Sal		OVA/OVA	
		Mean ratio	SEM	Mean ratio	SEM
PBS	*Gata3*/*Tbet*	1.03	0.10	1.66*	0.37
	Foxp3/*Roryt*	0.82	0.09	0.29*	0.20
	Foxp3/*Gata3*	0.83	0.09	0.41*	0.28
	Foxp3/*Tbet*	0.86	0.09	0.69	0.47
Bb/scFOS/IsFOS/AOS	*Gata3*/*Tbet*	1.09	0.12	1.11	0.09
	Foxp3/*Roryt*	1.01	0.05	1.49**	0.10
	Foxp3/*Gata3*	1.01	0.05	1.20**	0.08
	Foxp3/*Tbet*	1.10	0.06	1.21	0.08

Ratios for *Gata3*/*Tbet* (Th2/Th1), *Foxp3*/*Rorγt* (Treg/Th17), *Foxp3*/*Gata3* (Treg/Th2) and *Foxp3*/*Tbet* (Treg/Th1) mRNA expression in whole lung tissue are shown for OVA/Sal and OVA/OVA mice for each treatment. Data is represented as mean ratio ± SEM, n = 6 mice/group. The mean ratio was calculated by dividing the individual expression values for the first transcription factor (numerator) by the mean expression value for the second transcription factor (denominator). Statistical significance of differences was tested using post-hoc Bonferroni's multiple comparison test after one-way ANOVA.

* Statistically significant difference ($p < 0.05$) compared to the OVA/Sal–PBS group.

** Statistically significant difference ($p < 0.05$) compared to the OVA/OVA–PBS group.

3.5. Bb/scFOS/lcFOS/AOS treatment increases Foxp3 protein expression in blood CD4+CD25+Foxp3 + T cells of chronic asthmatic mice

In order to determine if the transcriptional changes induced in *Foxp3* expression could be found at a protein expression level, flow cytometry was used to examine Foxp3 protein expression in Treg cells in the blood of mice (Fig. 6). Treg cells were identified as CD4+CD25+Foxp3+ cells (Fig. 6A) and the magnitude of Foxp3 protein expression on each cell was determined by examining the percent of cells that are CD4+CD25+Foxp3+ (Fig. 6B) and the mean fluorescence intensity (MFI) of the Foxp3 staining (Fig. 6C). The percentage of CD4+CD25+Foxp3+ regulatory T cells was not significantly different between the groups. However, Bb/scFOS/lcFOS/AOS treatment increased Foxp3 protein expression in the blood Treg cells. The staining intensity of Foxp3 on day 56 was significantly higher in OVA/OVA–Bb/scFOS/lcFOS/AOS mice as compared to the OVA/OVA–PBS group. The staining intensity of Foxp3 on day 0 (before sensitization, challenge and treatment) did not differ among the different groups (data not shown).

3.6. Bb/scFOS/lcFOS/AOS treatment reduces T cell activity in chronic asthmatic mice

To investigate the effect of Bb/scFOS/lcFOS/AOS treatment on cytokine production by T cells in lung draining lymph nodes, TLN-cell cultures were stimulated with anti-CD3, a pan T cell stimulator, and the cytokine levels in the supernatant were measured (Fig. 7). The expression of IL-2, IL-4, IL-6, TNF-α and IFN-γ was significantly increased in anti-CD3-stimulated TLN cells of OVA/OVA–PBS mice as compared to samples from the OVA/Sal–PBS group. Bb/scFOS/lcFOS/AOS treatment, however, significantly decreased the expression of IL-2, IL-6, TNF-α and IFN-γ in anti-CD3-stimulated TLNs of OVA/OVA mice as compared to the OVA/OVA–PBS group. No differences in cytokine expression were observed in OVA-stimulated TLN cells in the different groups (data not shown).

3.7. Bb/scFOS/lcFOS/AOS treatment inhibits mucosal mast cell degranulation in chronic asthmatic mice

To examine the effect of Bb/scFOS/lcFOS/AOS treatment on mast cell activity, enzyme-linked immunosorbent assay was employed to measure mMCP-1 protein levels in serum and BAL fluid. The expression of mMCP-1 was more than doubled in serum of OVA/OVA–PBS

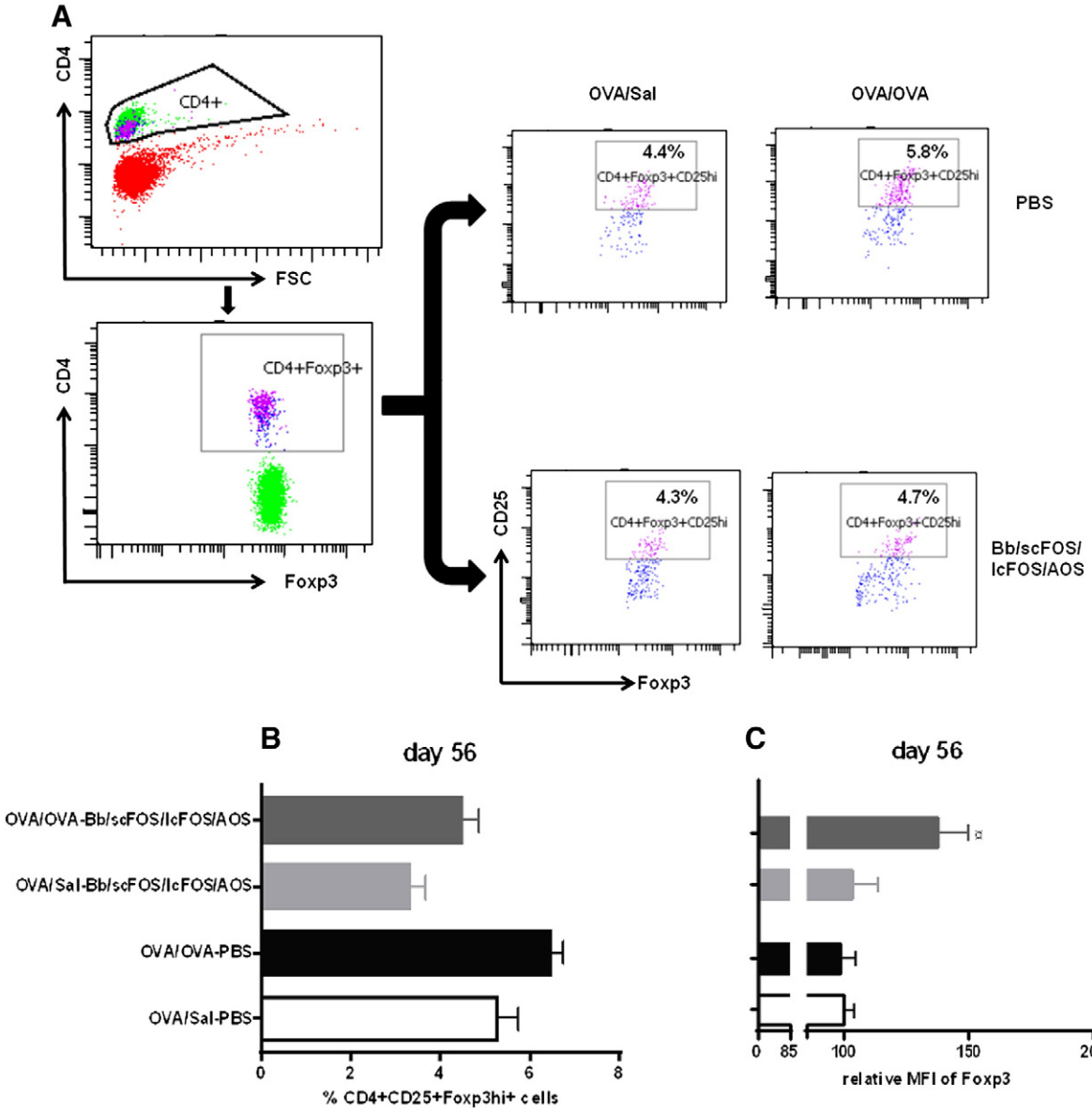


Fig. 6. Effect of Bb/scFOS/lcFOS/AOS treatment on Foxp3 protein expression levels in Treg cells during chronic asthma. T cells were isolated on day 56 from whole blood of OVA/Sal and OVA/OVA mice, treated with PBS or Bb/scFOS/lcFOS/AOS. (A) Representative dot plots of T cells analysis in the blood. T cells were gated based on FSC–SSC pattern, followed by analysis of expression of CD4. Then co-expression of Foxp3 and CD25 (regulatory T cells; Treg) was analyzed. (B) Percentage of CD4+CD25+Foxp3hi cells. (C) Mean fluorescence intensity (MFI) of Foxp3 on Treg cells relative to the OVA/Sal–PBS group (white bar). Data is shown as mean ± SEM, n = 6 mice/group, for one independent experiment. Statistical significance of differences was tested using post-hoc Bonferroni's multiple comparison test after one-way ANOVA. ¤Statistically significant difference ($p < 0.05$) compared to the OVA/OVA–PBS group.

(1.0 μg/mL ± 0.11; n = 5; $p < 0.0001$) mice as compared to the OVA/Sal–PBS group (0.5 μg/mL ± 0.20; n = 5). Interestingly, Bb/scFOS/lcFOS/AOS treatment reduced mMCP-1 levels by more than 80% in OVA/OVA mice (0.08 μg/mL ± 0.02; n = 5; $p < 0.0001$). Similar results were observed in the BAL fluid; mMCP-1 levels were more than 50% reduced in OVA/OVA–Bb/scFOS/lcFOS/AOS mice (402.3 pg/mL ± 141.5; n = 6; $p < 0.05$) as compared to the OVA/OVA–PBS group (980.9 pg/mL ± 208.5; n = 6).

3.8. Bb/scFOS/lcFOS/AOS treatment reduces airway remodeling features in chronic asthmatic mice

In order to examine the effect of Bb/scFOS/lcFOS/AOS treatment on airway remodeling, semi-quantitative histological and immunohistochemical analyses of lung tissue were performed (Fig. 8). In the lung sections of OVA/OVA–PBS mice, increased inflammation score and number of goblet cells, collagenous connective tissue fibers, airway smooth muscle cells and proliferating cells was observed as compared to the OVA/Sal–PBS group. Bb/scFOS/lcFOS/AOS treatment, however, reduced inflammation in the lungs of OVA/OVA–Bb/scFOS/lcFOS/AOS mice and decreased the number of collagenous connective tissue fibers and proliferating cells as compared to the OVA/OVA–PBS group.

4. Discussion and conclusions

Non-digestible oligosaccharides were shown to directly interact with the epithelium and to modulate the intestinal mucosal and systemic immune system from an allergic tuning towards a Treg and Th1 setting [49]. Additionally, previous studies have demonstrated that combination of *B. breve* M-16V with a mixture of short- and long-chain non-digestible oligosaccharides is more effective at reducing the allergic response in mice than non-digestible oligosaccharides alone [18].

The aim of this study was to investigate the therapeutic effects of long-term treatment with a combination of *B. breve* with a specific mixture of non-digestible oligosaccharides (Bb/scFOS/lcFOS/AOS) on

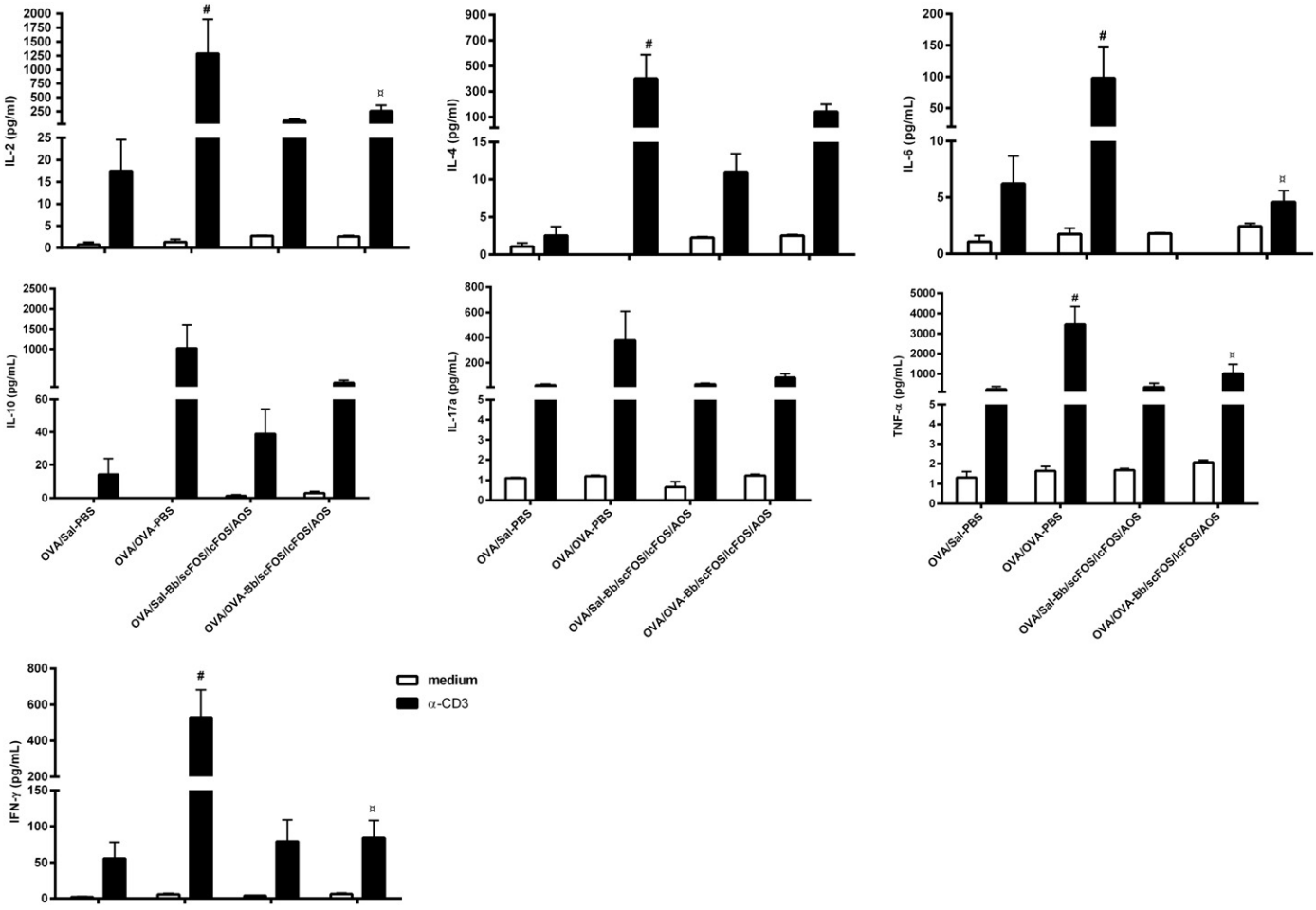


Fig. 7. Cytokine levels after TLN T cell stimulation. Thoracic lymph nodes (TLNs) were isolated from mice on day 56 and restimulated *in vitro* with plate-bound anti-CD3 antibody or medium only for 5 days (37 °C, 5% CO_2). Data is shown as mean of cytokine concentration (pg/mL) ± SEM, n = 6 mice/group, for one independent experiment. Statistical significance of differences was tested using post-hoc Bonferroni's multiple comparison test after one-way ANOVA. #Statistically significant difference ($p < 0.05$) compared to the OVA/Sal–PBS group. ¤Statistically significant difference ($p < 0.05$) compared to the OVA/OVA–PBS group.

features of the inflammatory response and airway remodeling in a murine model of chronic asthma. The total inflammatory cell number and BAL fluid eosinophil and neutrophil counts were significantly increased in OVA/OVA–PBS mice as compared to the OVA/Sal–PBS group. Additionally, increased number of inflammatory cells and goblet cells, collagenous connective tissue fibers, airway smooth muscle cells and proliferating cells was observed in lung sections of OVA–OVA–PBS mice. Hence, our model mimics the airway inflammation and remodeling found in chronic asthma [1]. Bb/scFOS/lcFOS/AOS treatment significantly reduced the total number of inflammatory cells and BAL fluid eosinophil counts; reduced the inflammation; and decreased the number of collagenous connective tissue fibers and proliferating cells in the lungs of sensitized and challenged mice. To our knowledge, we demonstrate here for the first time that Bb/scFOS/lcFOS/AOS treatment is effective at reducing chronic allergic inflammation by attenuating the total inflammatory cell number, particularly eosinophil counts in the BAL fluid; and airway remodeling.

PRRs are key components of the innate immunity of which the function and expression are associated with susceptibility towards allergic asthma. The mRNA expression of *Tlr3*, *Tlr9* and *Nod1* was significantly decreased in the lungs of OVA/OVA–PBS mice. Viral activation of *Tlr3* was reported to play a critical role in exacerbation of respiratory diseases in mice, including asthma [41]. However, no direct associations between *Tlr3* expression and function with asthma have been reported yet, and whether the decreased mRNA expression of *Tlr3* caused by the chronic inflammatory status of the animals is pro-inflammatory or anti-inflammatory is unknown. Single nucleotide polymorphisms in the *Nod1* gene that affected microbial recognition are positively associated with susceptibility towards asthma in farming children [34–36]; yet, the link between the decreased mRNA expression of *Nod1* observed in this current study, and the inflammatory status of the animals remains to be elucidated. TLR9 has been shown to exert its immunomodulatory effects on asthma by skewing the increased Th2/Th1 cell balance towards Th1 cell [38–40]. Bb/scFOS/lcFOS/AOS treatment normalized the expression levels of *Tlr3* and *Nod1*. Importantly, *Tlr9* expression was significantly increased in OVA/OVA mice treated with Bb/scFOS/lcFOS/AOS.

Th2 cells play a major role in allergic asthma, and Th1/Th2 imbalances as well as disturbed Th17/Treg balances were found in asthmatic patients [44]. Th2 dominance was observed in the OVA/OVA–PBS group represented by high *Gata3*/*Tbet* and low *Foxp3*/*Gata3* ratios. Additionally, a significant decrease in Th1 and Treg transcription factor mRNA expression, and low *Foxp3*/*Rorγt* ratio were found. Hence, our model mimics the Th2-responses found in chronic asthma [1]. Interestingly, Bb/scFOS/lcFOS/AOS treatment shifted the immune balance towards Treg, with significantly increased *Foxp3*/*Rorγt* and *Foxp3*/*Gata3* ratios compared to the OVA/OVA–PBS group. More importantly, the above observed effects of the Bb/scFOS/lcFOS/AOS on the Th and Treg responses in the lung are confirmed by the detection of high Foxp3-expressing Tregs in the blood of treated animals. Treg cells play a key role in

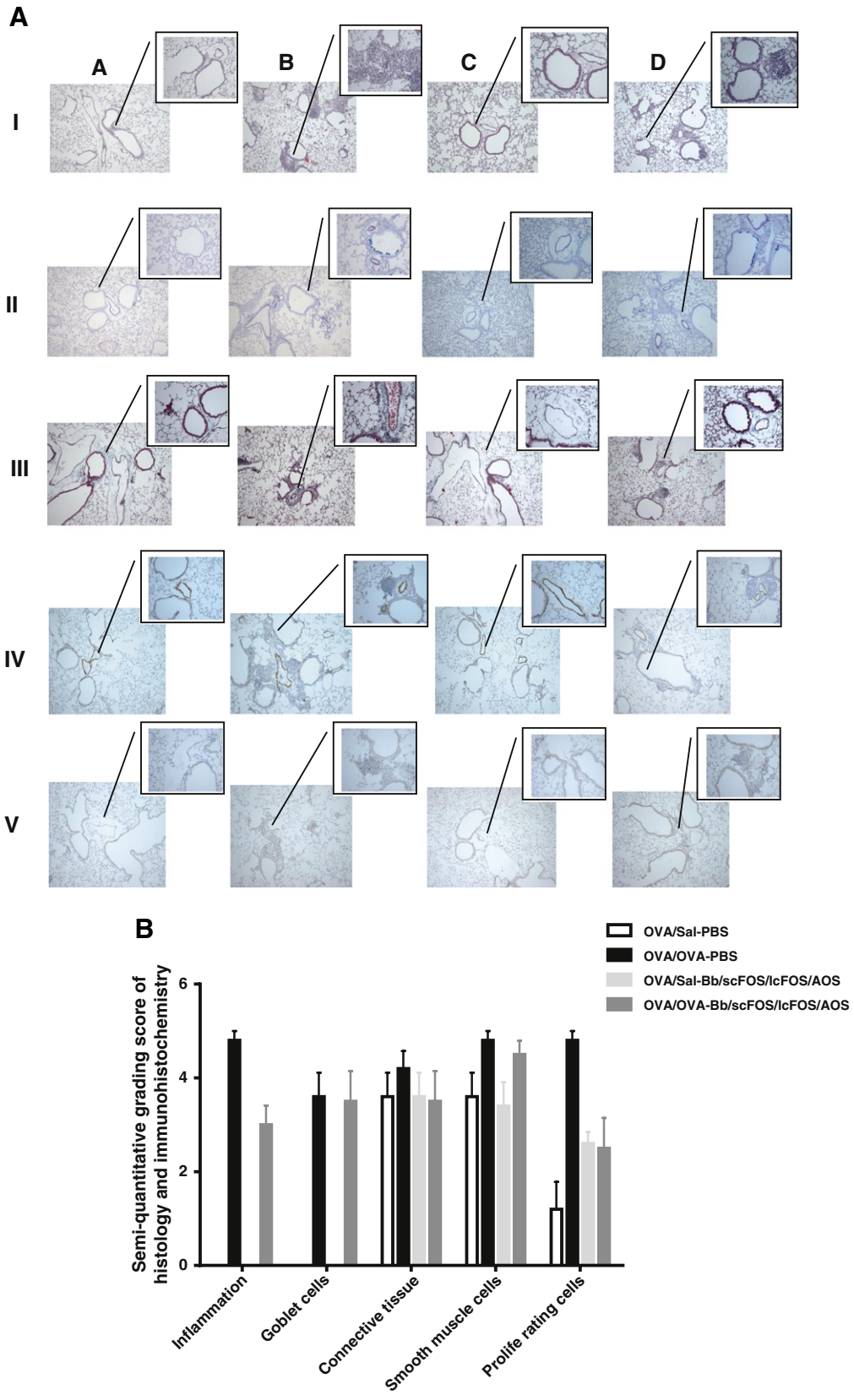


Fig. 8. Effect of Bb/scFOS/lcFOS/AOS treatment on airway remodeling during chronic asthma. (A) Representative photomicrographs for 5 mice/group, for one independent experiment, of histological and immunohistochemical staining for inflammation (hematoxylin–eosin, HE, **I**); goblet cells (periodic acid Schiff, PAS, **II**); connective tissue (Masson trichrome, Masson, **III**); anti-α-smooth muscle actin (α-smooth muscle, **IV**) and proliferating cells (anti-Ki67, **V**) in lung tissue of OVA/Sal–PBS mice (A), OVA/OVA–PBS mice (B), OVA/Sal–Bb/scFOS/lcFOS/AOS mice (C) and OVA/OVA–Bb/scFOS/lcFOS/AOS (D). Original magnification key figures 100×, original magnification boxes 200×. (B) Compiled data of semi-quantitatively graded histology and immunohistochemistry. Slides were reviewed in blinded fashion by two observers independently and slides were scored as 0 to 5 (no to abundant staining). Results are shown as mean score ± SEM of n = 5 mice/group.

balancing immune responses and it has been demonstrated that an increased expression of Foxp3 in Tregs is directly associated with increased suppressive function of these cells [43,50].

Expression of the cytokines *Il1β*, *Il6* and *Il17* was significantly increased in OVA/OVA–PBS mice and expression of the Th2 cytokine *Il5* was significantly decreased. These four cytokines have been reported to enhance asthma and increased levels of *Il1β*, *Il6* and *Il17* are found in the sputum and BAL fluid of individuals with asthma [30]. A reasonable explanation for the decrease in *Il5* mRNA expression remains to be elucidated as mRNA levels do not always reflect protein synthesis. Accordingly, no increase in *Gata3* mRNA expression in lung tissue was observed. Bb/scFOS/lcFOS/AOS treatment, however, significantly decreased the expression of the pro-inflammatory cytokines *Il1β*, *Il6* and *Tnfα*, restoring them to normal levels and also decreased *Il12*. Additionally, the expression of *Il10* and *Il23* was significantly increased. It has been reported that IL-12 is involved in the differentiation of Th1 cells. The decrease in *Il12* might be due to the increased expression of the anti-inflammatory cytokine *Il10*, as IL-10 is known to inhibit Il12 p40 transcription [30,51]. Interestingly, the observed effects of the Bb/scFOS/lcFOS/AOS treatment on cytokine expression in the lung are confirmed by cytokine levels in the TLNs. Bb/scFOS/lcFOS/AOS treatment significantly decreased the production of IL-2, IL-6 and TNF-α, restoring them to normal levels, and IFN-γ by T cells in the TLNs of OVA/OVA mice after a specific activation with an anti-CD3 antibody *in vitro*. In summary, although the expression of *Rorγt* was not increased, a predominant Th17 immunity in chronically asthmatic mice is observed with a less prominent role of Th2 immunity. These findings are in accordance with other animal and human studies in which the number of Th17 cells and the concentration of related cytokines were associated with asthma severity [52]. Bb/scFOS/lcFOS/AOS treatment, however, induced a strong anti-inflammatory response represented by the upregulation of Foxp3 and *Il10*, and suppression of pro-inflammatory cytokine expression.

Mast cells play a central role in both the early phase and late phase of allergen-induced airway inflammation [6]. Intriguingly, Bb/scFOS/lcFOS/AOS treatment was effective at inhibiting mucosal mast cell degranulation, as shown by the significant decrease in the protein levels of mMCP-1 systemically, in serum, as well as in the BAL fluid of treated mice. The mechanism by which Bb/scFOS/lcFOS/AOS treatment reduced mast cell degranulation still needs further investigation. Possibly, these findings might be explained by the induction of galectins by the Bb/scFOS/lcFOS/AOS mixture. Recently, a combination of *B. breve* M-16V with non-digestible oligosaccharides, although with a different oligosaccharide composition, was shown to induce galectin-9. The latter in turn suppressed allergic symptoms, including mast cell degranulation, in mice and humans [49]. Additionally, immune challenge in the airways might promote the trafficking of Treg cells, activated in the intestine, to the respiratory mucosa where they induce protective and anti-inflammatory responses as described in [53].

To our knowledge, this is the first report in which the therapeutic effects of long-term treatment with Bb/scFOS/lcFOS/AOS combination, on inflammatory response and airway remodeling are investigated in a murine OVA-induced chronic asthma model. We show here that Bb/scFOS/lcFOS/AOS treatment was effective at reducing pulmonary airway inflammation; modulating the mRNA expression of PRRs, T helper-specific cytokines and transcription factors; as well as reducing airway remodeling. More importantly, Bb/scFOS/lcFOS/AOS treatment was a potent inducer of regulatory responses; increasing *Il10* and *Foxp3* transcription in lung tissue and augmenting protein expression of Foxp3 in blood Treg cells. Additionally, Bb/scFOS/lcFOS/AOS treatment reduced T cell cytokine production and inhibited mast cell degranulation. Our current findings show that this specific combination of *B. breve* with non-digestible oligosaccharides may be beneficial in the management of chronic inflammation in allergic asthma.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.01.005.

Conflict of interest

Seil Sagar, Arjan P Vos, Mary E Morgan, Johan Garssen, Niki A Georgiou, Louis Boon, Aletta D Kraneveld, and Gert Folkerts have no conflicts of interest.

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