Beneficial bacteria and non-digestible oligosaccharides for the treatment of chronic allergic asthma: modulation of immune responses

Studies in murine models

Seil Sagar

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Gezondheidsbevorderende bacteriën en niet-verteerbare oligosacchariden voor het behandelen van chronisch allergisch astma: modulatie van immuunresponsen

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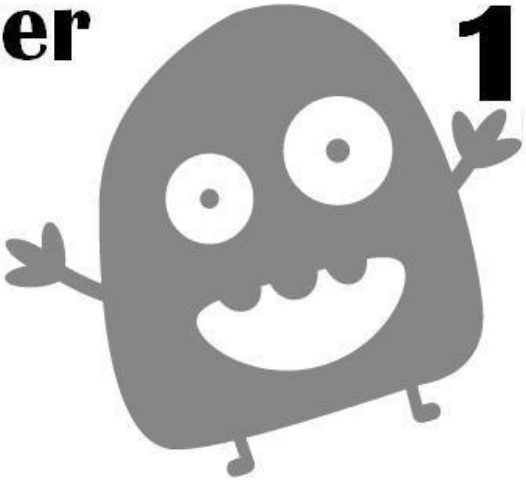
Contents

Chapter 1



General introduction

Part of this chapter is published in the book 'Toll-Like Receptors in Diseases of the Lung', 2012

eISBN: 978-1-60805-362-9, Chapter 3: Role of Toll-Like Receptors in Asthma

Seil Sagar[1,2], Niki A Georgiou[1,2], Gert Folkerts[1], Johan Garssen[1,2], Aletta D Kraneveld[1]

and in

Pharmacological Reviews, 2012, 64(2):337-58

Dual role of Toll-like receptors in asthma and chronic obstructive pulmonary disease

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Allergic asthma is a T helper type-2 (Th2) cell-mediated chronic inflammatory disorder of the airways characterized by episodes of reversible airway narrowing, bronchial hyper-responsiveness and chronic pulmonary inflammation and airway remodeling [1]. Asthma can be clinically classified according to the frequency of symptoms as intermittent, mild, moderate persistent or severe persistent [2, 3]. According to triggers causing the airway symptoms, asthma can also be classified as atopic, where symptoms are induced by allergens or non-atopic where symptoms are induced by non-specific triggers. Workplace exposures are the world's most common cause of so-called occupational asthma. 15-23% of new-onset asthma cases in adults are work-related [4]. The prevalence of asthma has been increasing since the 1980's, particularly in children and young adults [5]. To date, asthma is the most common chronic disease in children. More than 150 million people worldwide are diagnosed with asthma. Developed and westernized countries have higher asthma prevalence. More than 50% of the cases of asthma are of the atopic/allergic form [6, 7]. It is believed that this increase of prevalence of asthma is associated with western life style due to increased exposure to house dust mite, decreased exposure to various microorganisms, increased prevalence of obesity and changes in the western diet [8]. The pathophysiology of asthma is complex and involves both innate and adaptive immune responses, various inflammatory cells, cytokines and pattern recognition receptors. In this chapter the pathology of allergic asthma; current therapy of the disease; the role, expression, function and activation of the toll-like and nod-like receptors in asthma; the role of the gut microbiota in asthma and new preventive and therapeutic approaches for allergic asthma will be discussed. Following on the various aspects of allergic asthma presented in this chapter, a brief overview of this thesis is outlined in the final paragraph.

Asthma pathology

Asthma is an inflammatory disorder of the airways characterized by the presence of several inflammatory cells such as mast cells, eosinophils, neutrophils, B-lymphocytes and T-lymphocytes. Upon inhalation of a specific allergen, antigen-presenting cells (APCs), such as dendritic cells (DCs), in the airway migrate to the draining lymph nodes where they present the antigen to precursor T-helper (Th) cells in the lymph structures. These precursor Th cells then differentiate and mature into antigen-specific T-helper 2 (Th2) lymphocytes ($CD4^+$ Th2). Once the allergen is encountered by these antigen-specific Th2 cells, cytokines such interleukin (IL) 4 and IL13 are released which in turn leads to the activation of B cells and subsequent development of IgE-producing plasma cells (Figure 1) [9-16].

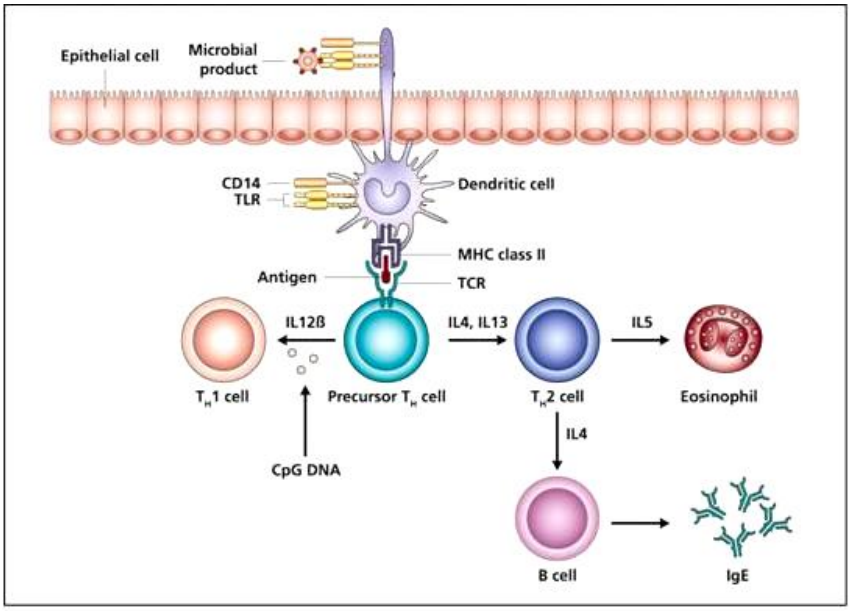

Figure 1: **Airway inflammation.** Upon inhalation of an allergen, DCs migrate to the draining lymph nodes where they present the antigen to precursor Th cells. Precursor Th cells then maturate and differentiate into Th2 cells leading to the production and activation of eosinophils, the release of IL4, IL13 and the development of antigen-specific B cells. The B cells become plasma cells which produce antigen-specific IgE. Possibly, toll-like receptor 9 (TLR9) ligands (CpG DNA) restore the immune balance by shifting the balance from a Th2 response to a Th1 response. The figure is adapted from [9, 131].

Additionally, mast cells play a crucial role in the induction of allergic airway inflammation [9, 17]. Mast cells originate from bone marrow-derived mast cell progenitors and mature into mast cells upon entry to the peripheral organs [18]. Upon binding of extravascularly IgE to its high-affinity receptors (FcεRI) on the surface of mast cells and re-exposure to the same antigen these cells become activated. Mast cell activation can be divided into three different phases. In the first phase, degranulation, preformed mediators such as histamine, serotonin, proteoglycans and tumor necrosis factor α (TNFα) stored in granules are rapidly released in a matter of seconds resulting in bronchoconstriction [17, 19, 20]. In the second phase, arachidonic acid metabolites (leukotrienes) are formed, followed by the *de novo* production of chemokines and cytokines, such as IL4, IL5, IL9 and IL13, in the third phase. These chemokines and cytokines conjugate with arachidonic acid metabolites resulting in the induction of chronic inflammation (Figure 2) [17, 19, 20]. In addition to these inflammatory features of allergic asthma, structural changes are observed in the airway walls of asthmatic patients [21]. These changes are referred to as "airway remodeling" and include subepithelial fibrosis, increased deposition of extracellular matrix protein, mucus gland hypertrophy and goblet cell hyperplasia, epithelial damage and smooth muscle hypertrophy and hyperplasia [22]. The combination of these processes causes airway narrowing, and subsequent reduced lung function [23]. Although, the mechanism by which these structural changes are initiated and maintained is still incompletely understood, it is thought that these features are caused by persistent inflammation, due to the involvement of Th2 and eosinophils, and subsequent inadequate repair of damaged airway epithelium [1].

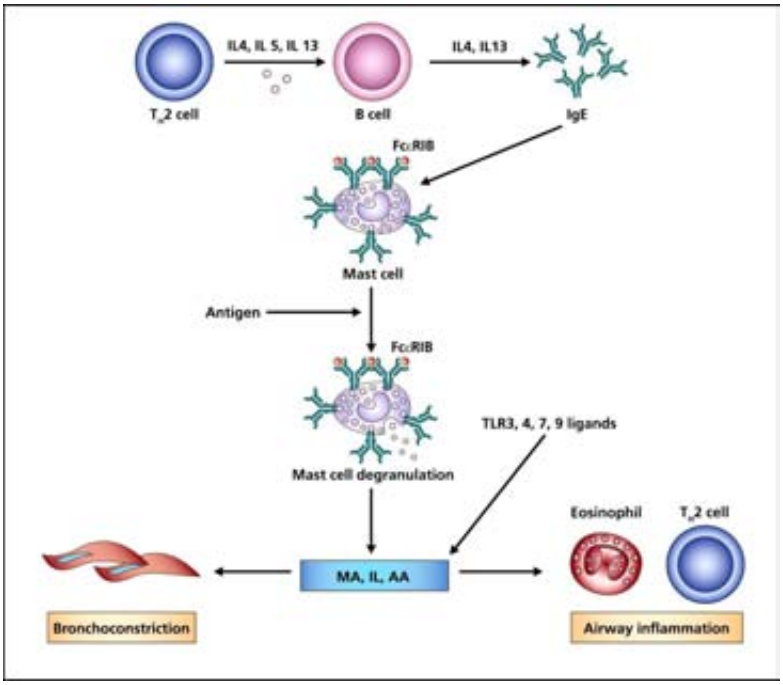

Figure 2: Role of mast cells in airway inflammation. Th2 cells release IL4, IL5 and IL13 leading to the development of antigen-specific B cells which differentiate into IgE-producing plasma cells. Mast cells express receptors for IgE on their surface. Binding of IgE to mast cells leads to activation of these cells followed by degranulation. During degranulation of mast cells monoamines (MA; histamine, serotonin, prostaglandins), interleukins (IL; IL4, IL5, IL13) and arachidonic acid metabolites (AA) are released resulting in increased flow of inflammatory mediators and cells into the antigen-encountered site leading to bronchoconstriction. The released chemokines and cytokines recruit eosinophils and Th2 cells to the lung leading to chronic inflammation. Toll-like receptor (TLR) 3, 4, 7 and 9 ligands seem to induce mast cells activation and the release of cytokines and chemokines without the induction of degranulation and arachidonic acid metabolism. The figure is adapted from [9, 131].

Current therapy of allergic asthma

Classically, asthma has been regarded as a bronchoconstrictive disease and is predominantly treated with bronchodilators, such as β2 agonists [14]. Currently, the chronic inflammatory

process is target of treatment with inhaled corticosteroids with or without long-acting β_2-agonists. Corticosteroids are by far the most effective anti-inflammatory treatment for asthma. However, over 50% of asthmatics are poorly controlled and there is still a need for new therapies. Corticosteroids have long-term side effects and often show poor compliance [24]. Furthermore, the long-term safety of long acting β2 agonists, which has been linked to increased risk of mortality and desensitization of the β2 receptor, is of concern [25, 26].

Blocking the synthesis or receptor for a single mediator (such as vasoactive amines or lipid mediators) involved in asthma seems unlikely to be very effective. Anti-leukotrienes that block (cysteinyl leukotriene receptor 1 (CysLt1), are currently used in therapy; yet, these drugs are less effective than inhaled corticosteroids and leukotriene B4 (LTB4) receptor antagonists had shown no effect in mild asthma. Compounds targeting another arachidonic metabolite and its receptor, prostaglandin D2, are now in clinical development for asthma [14]. Since cytokines play a crucial role in orchestrating chronic inflammation, they have become important targets for asthma treatments. However, over 50 cytokines have been implicated in asthma and several cytokines and chemokines (IL4, IL5, IL13, TNFα, C-C chemokine receptor type 3(CCR3)) blocking antibodies are now in clinical development but clinical studies in asthmatic patients have been disappointing [14]. Antibodies blocking IgE are only used in the treatment of patients with severe asthma, due to the high costs of the treatment and the unclear mechanism of clinical efficacy [27].

Phosphodiesterase (PDE) 4 inhibitors, targeting T cells, eosinophils, smooth muscle cells and epithelial cells are promising anti-inflammatory therapy. PDE4 inhibitor, an oral PDE4 inhibitor, proved to be as effective as low doses of inhaled corticosteroids at inhibiting allergen-induced responses in asthmatic subjects [28]. Undesirable side effects, such as nausea, headaches and

diarrhea, are the major limitations for using PDE4 inhibitor. Mammalian p38 mitogen-activated protein kinases (MAPKs) are another promising target for anti-inflammatory therapy as these proteins play a crucial role in regulating the expression of inflammatory genes involved in asthma. Several tyrosine kinase inhibitors are currently in clinical development [29]. A different approach is specific immune therapy, whereby asthmatic patients are exposed in a controlled way to allergens or allergen-peptides, (either subcutaneously or sublingually) to induce tolerance and desensitization. This approach has shown some efficacy; yet, long-term studies and

comparison with inhaled corticosteroids are needed to determine efficacy [14]. To date, the number of clinical studies that target the innate immune system is still limited.

The role of TLRs in health and disease

The toll-like receptors (TLRs) is a key pattern recognition receptor (PRR) family in the innate immune response, and these PRRs have an important role in the activation of epithelial cells, monocytes, DCs, mast cells and neutrophils. Additionally, these receptors are also involved in the activation and shaping of adaptive immunity [30]. TLRs exhibit their protective role against microbial infections by both the detection of and response to pathogens in infective as well as non-inflammatory responses in the lung [10, 31]. It is also becoming clear that these receptors have homeostatic roles in the lung. Hence, breakdown products of the extracellular matrix component hyaluranon were suggested to signal via TLRs and this signaling via TLRs seems to be required for epithelial integrity maintenance in health and for epithelial survival and proliferation after injury [32]. The latter is in turn necessary for restoration of normal tissue architecture, [15, 33, 34]. In the context of airway inflammation, TLRs expressed on the surface of immature pulmonary DCs activate these cells during infection or inflammation leading to up-regulation of co-stimulatory molecules. TLR-primed DCs present antigen to antigen-specific T cells leading to proliferation of these cells and the release of Th2 cytokines, such as IL4, IL5 and IL13, which in turn leads to the activation of B cells and subsequent development of IgE-producing plasma cells. Additionally, the release of IL5 results in eosinophil proliferation and infiltration leading to enhanced mucus production and subsequent chronic airway inflammation (Figure 1 & 2) [9-13]. Moreover, ligands of TLR3, TLR4, TLR7 and TLR9 were reported to induce mast cell activation and subsequent secretion of pro-inflammatory cytokines and chemokines through a degranulation- and arachidonic acid metabolism-independent mechanism (Figure 3) [17, 19, 35-40].

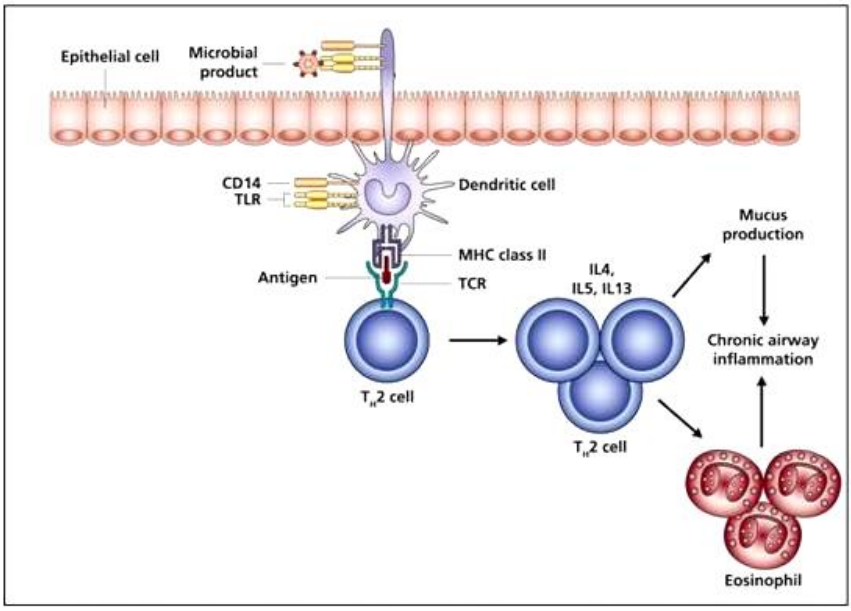

Figure 3: **Toll-like receptor-mediated role of dendritic cells in airway inflammation.** Immature DCs express TLRs on their surface. These TLRs become activated during infection or inflammation. TLR-primed DCs also present the antigen to antigen-specific Th2 cells leading to proliferation of these cells, release of IL4, IL5 and IL13 and eosinophil proliferation. All these events lead to enhanced mucus production and eventually airway inflammation. The figure is adapted from [9, 131].

Expression, function and activation of TLRs in the asthmatic/allergic lung

TLRs exhibit different cell- and stimulus specific patterns of expression in various tissues. The ***TLR1*** gene is ubiquitously expressed and at a higher level than the other TLRs [41, 42]. Single nucleotide polymorphisms (SNPs) in the *TLR1* gene were demonstrated to protect against atopic asthma in humans by skewing the Th1/Th2 balance towards Th1 through increasing the levels of interferon γ (IFNγ) and reducing IL4 production [43]. ***TLR2*** has a restricted pattern of expression and it is expressed in human monocytes, polymorphonuclear (PMN) and DCs [41, 44]. As this PRR acts as a heterodimer with TLR1, TLR6 and TLR10, different structures of lipopeptides and lipoproteins are recognized by the different TLR2 heterodimers [45-49].

Stimulation of airway smooth muscle cells (ASMCs), which express *TLR2*, from asthmatic subjects with the TLR2 ligand peptidoglycan (PGN) induced the release of the chemokine IL8 (CXCL8) in ASMCs of patients [50]. Animal studies, on the other hand, have shown that TLR2 agonists have the potency to both inhibit and promote the development of allergic immune responses [51]. This dual role of TLR2 in asthma seems to depend partly on which TLR2 heterodimer, TLR2/TLR1 or TLR2/TLR6, is addressed. A synthetic TLR2/TLR1 ligand reversed established ovalbumin (OVA)-induced airways inflammation in mice [51, 52]. In contrast, another study showed asthma aggravation in mice immunized with OVA allergen in combination with a TLR2/TLR1 agonist [51, 53]. On the other hand, treatment of asthmatic mice with a TLR2/TLR6 agonist in combination with IFNγ reduced airway hyper-responsiveness, eosinophilia and IL5 and IL13 levels in the bronchoalveolar lavage (BAL) fluid [51, 54]. Consistent with this, a TLR2/6 agonist was shown to reduce eosinophilic infiltration in a murine model of chronic allergic airway inflammation [55]. ***TLR6*** is highly expressed on human B cells and at lower levels on monocytes and natural killer (NK) cells [41, 56]. Additionally, *TLR6* was also demonstrated to be expressed on human cord blood-derived mast cells mast [57]. TLR6 signals as a heterodimer with TLR2 and binds specifically to di-acetylated lipopeptides [45, 56]. SNPs in the *TLR6* gene were demonstrated to protect against atopic asthma in asthmatic children [43]. Moreover, one non-synonymous SNP in the *TLR6* gene was significantly associated with protection from asthma in African Americans [58]. Conflicting effect of the same SNP on asthma was reported in another study, and this SNP was significantly associated with childhood asthma [59]. ***TLR3*** has a selective pattern of expression and is exclusively expressed by human immature DCs [41, 44]. The exact role of TLR3 in the pathogenesis of allergic asthma still needs to be elucidated. Polyinosine-polycytidylic acid poly (I:C), a known ligand of TLR3 and a synthetic analog of double-stranded RNA (dsRNA), was demonstrated to induce upregulation of *TLR2*, *TLR3*, *TLR7* and *TLR9* gene expression as well as the upregulation of chemokines, cytokines and signaling molecules in the lung of wild-type mice. These findings were accompanied by increased total cell number, especially neutrophil counts, in the BAL fluid, bronchial epithelial cell hypertrophy and subsequent impaired lung function in these mice [60]. On the other hand, stimulation of ASMCs from asthmatic subjects with dsRNA, TLR3 ligand, induced *TLR3* mRNA expression, as ASMCs only express TLR3 protein under basal conditions, and the release of the chemokine CXCL8 in ASMCs of patients [50]. There is also rising

evidence for functional role of TLR3 in the pathogenesis of severe asthma. TLR3 ligands in combination with TSLP, a human thymic stromal lymphopoietin expressed in the lungs of asthmatic patients, were shown to activate human DCs and thereby promote the differentiation of T helper-17 (Th17) cells [61-64]. ***TLR4*** is expressed in a variety of human cell types including macrophages, DCs, epithelial and endothelial cells but not in lymphocytes [41, 44]. This receptor is activated by lipopolysaccharide (LPS), an outer cell wall component of gram negative bacteria [50]. Asthma patients were shown to have lower *TLR4* expression on their monocytes, lymphocytes and DCs as compared to control subjects. Additionally, LPS-stimulated peripheral blood mononuclear cell (PBMC) from asthmatic subjects were shown to produce low TNFα, IL10 and IL1β levels *ex vivo* as compared to PBMC from control subjects suggesting that reduced TLR4 activation leads to reduced release of IL1β and IL10 and thereby this receptor contributes to the immunological mechanisms of asthma [65]. In addition, *TLR4* expression in irradiated chimeric mice was shown to be necessary for DCs activation and the priming of T helper responses to house dust mite in the asthmatic lung. Inhalation of a TLR4 antagonist at the same time of house dust mite injection reduced the features of asthma in mice [66]. It is proposed that de dose of LPS determines which type of immune response (Th1 or Th2) will be induced. The combination of low dose of LPS with OVA was shown to enhance antigen-induced inflammation in lung tissue of asthmatic mice. A high dose of LPS, however, induced Th1 response, histologically characterized by neutrophil recruitment and increased IFNγ levels in the BAL fluid. In another study, the expression of *TLR4* mRNA in alveolar macrophages showed no correlation with the dose of LPS [67]. ***TLR5*** is expressed on human monocytes, immature DCs, epithelial cells, NK cells and T cells [41, 44, 56]. To date, very little is known about the function of TLR5 in allergic asthma. Decreased functional response of TLR5 was demonstrated in asthmatic patients. Compared with healthy subjects, the *ex vivo* flagellin, a structural component of flagellated bacteria and the only known TLR5 ligand so far, stimulated production of TNFα, IL10 and IL1β by PBMCs was shown to be significantly lower in asthmatic patients. Additionally, the expression of *TLR5* was also found to be significantly decreased in monocytes, lymphocytes and DCs of asthmatic patients [65]. ***TLR7*** is expressed on human B cells and plasmacytoid precursor DCs [41, 56]. A non-synonymous SNP in the *TLR7* gene was significantly associated with allergic phenotypes, including asthma [68]. Adolescents with asthma were shown to have reduced TLR7 function in PBMCs compared to healthy individuals

[69]. Furthermore, the synthetic TLR7 ligand R-837 was shown to activate human eosinophils from allergic patients and healthy subjects. The TLR7 responses of eosinophils were higher in allergic patients and activation of TLR7 by R-837 resulted in the activation of eosinophils at several levels, suggesting that during viral respiratory infections TLR7-mediated activation of eosinpohils may contribute to allergic exacerbations [70]. Yet, a TLR7/TLR8 ligand was shown to prevent chronic asthma-induced airway remodeling and the enhanced protein expression of both Th1 and Th2 cytokines in the lungs of rats [71]. Activation of TLR7 in early life, however, seems to promote the development of Th2 cells resulting in allergic airway inflammation upon allergen challenge in later life [72]. ***TLR8*** is highly expressed on human monocytes and at lower levels on NK and T cells [41, 56]. The exact role of TLR8 in allergic asthma still needs to be elucidated. However, the TLR8 gene was identified as a novel risk gene in asthma [68]. ***TLR9*** is one of the most extensively studied TLRs in asthma. This receptor is expressed on human plasmacytoid precursor DCs, B cells, macrophages, polymorphonuclear leukocytes (PMLs) and microglial cells [41, 73, 74]. TLR9 recognizes unmethylated bacterial cytosine-guanine repeat (CpG) dideoxynucleotides, which are almost methylated and less common in vertebrates [75, 76]. TLR9 agonists proved to be highly effective immune modulators with applications as vaccine adjuvants, as stand-alone therapy or in combination with other therapies for the treatment of cancer, infectious diseases, allergy and asthma. In the specific context of asthma, CpG ODN demonstrated benefit in various rodent and primate models of asthma and they have shown positive results in a number of early human clinical trials [77, 78]. These CpG DNA motifs were shown to activate NK cells via stimulation of DCs leading to the induction of a Th1 innate response and subsequent production of IFNγ, as well as IL10 and IL12 to counterbalance the allergic Th2 dominated phenotype [79-81]. ***TLR10*** is the latest discovered human TLR and it is highly expressed on B cells and at lower levels on plasmacytoid precursor DCs [41, 56]. The ligand of TLR10 has not been identified yet, it is an orphan receptor, and it has no rodent homologue. As mentioned previously, TLR10 signals in homodimers and heterodimers with TLR1 and TLR2 [45, 46]. SNPs in the *TLR10* gene were demonstrated to protect against atopic asthma by increasing Th1 cytokine expression and reducing Th2 cytokine production [43]. Other two SNPs in the *TLR10* gene, however, showed significant association with asthma in European American subjects [82].

In summary, the various TLRs exhibit different cell- and stimulus specific pattern of expression. According to their cellular expression pattern, the TLRs can be categorized as either ubiquitous (*TLR1*), restricted (*TLR2, 4, 5, 6, 7, 8, 9* and *10*) or selective (*TLR3*). Besides their protective role against microbial infections, the TLRs also exhibit homeostatic roles. Functional genetic variations in *TLR1*, *TLR6* and *TLR10* genes were shown to have protective effects on atopic asthma in human. However, TLR2 and TLR4 have the potency to both inhibit and promote the development of allergic immune responses. Activation of TLR4 and TLR5 was shown to be reduced in asthmatic patients and this was suggested to contribute to the immunopathological mechanisms of asthma by reducing the release of Th1 and anti-inflammatory cytokines. TLR3 was shown to contribute to exacerbations of asthma and TLR3 agonists seem to play a pro-inflammatory role in respiratory diseases, including asthma. *TLR7* and *TLR8* were identified as novel risk genes in asthma. However, the function of TLR7 was shown to be reduced in adolescents with asthma and consistently a TLR7 ligand was described to inhibit airway remodeling features in a rodent allergic asthma model. TLR9 was reported to modulate allergic responses by skewing the balance from a Th2 towards a Th1 response and CpG ODN, ligands of TLR9, demonstrated benefit in various rodent and primate models of asthma and they have shown positive results in a number of early human clinical trials. The exact role of TLR10 in asthma still needs to be elucidated. The expression, function and role of the different TLRs are summarized in Table 1.

Table 1: Role of TLRs and NLRs in allergic asthma

Receptor	Cell type	Human studies	Animal studies	Role in allergic asthma	References
TLR1	Ubiquitous	↑ Th1 ↓ Th2		Protective role of SNPs	[41-43]
TLR2	Monocytes, PMN , DCs	↑ CXCL8 in ASMCs by PGN	↑↓ airway inflammation by TLR2/TLR1 ↓ AHR and Th2 by TLR2/TLR6	Protective effect of TLR2/TLR6 and Dual role of TLR1/TLR2	[41, 44-57, 122]
TLR3	Immature DCs	↑ CXCL8 in ASMCs by poly (I:C)	↑ airway inflammation by poly (I:C)	Exacerbation of the disease	[41, 44, 50, 60-64, 123]
TLR4	Macrophages, DCs, endothelial cells	↓ Th1 in PBMCs ↑ CXCL8 in ASMCs by LPS	↑ Th2 by low dose LPS with OVA ↑ Th1 by high dose LPS with OVA	Induction of airway inflammation	[13, 41, 44, 50, 65, 67]
TLR5	Monocytes, immature DCs, epithelial cells, NK cells, T cells	↓ Th1 in PBMCs		Promotion of the disease	[41, 44, 45, 56, 65, 124-126]
TLR6	High expression in B cells, lower expression in monocytes and NK cells	↑ Th1 ↓ Th2	↓ AHR and Th2 by TLR2/TLR6	Protective role	[43, 45, 54-56, 58, 59]
TLR7	B cells, plasmacytoid precursor DCs	Risk gene ↑ CXCL8 in eosinophils by R-837	↑ Th2 by ssRNA in later life ↓ Th1 and Th2 expression by R-848	Exacerbation of the disease	[41, 56, 68-72]
TLR8	High expression in monocytes, low expression in NK cells, T cells	Risk gene	↓ Th1 and Th2 expression by R-848	Not known yet	[41, 56, 68, 71]

Table 1: *continued……*

Receptor	Cell type	Human studies	Animal studies	Role in allergic asthma	References
TLR9	plasmacytoid precursor dendritic cells, B cells, macrophages, PMN, micoglial cells	Positive disease modulation in early clinical trials	↑ Th1 and ↓ Th2 by CpG DNA Prenatal protection	Modulation of the disease	[41, 70, 73-81, 127-130]
TLR10	High expression in B cells, low expression in plasmacytoid precursor DC	↑ Th1 and ↓ Th2 by SNPs Association of SNPs with asthma		Not known yet	[41, 43, 45, 46, 56, 82]
NOD1	Expression in various tissues	Lower frequency of allergies in farming children by SNPs		Protective role of SNPs	[83, 85, 87]
NOD2	monocytes, granulocytes, DCs, epithelial cells	Association of SNPs with asthma		Development of allergic asthma	[84, 86, 88-94]

Expression, function and activation of other pattern recognition receptors in the asthmatic/allergic lung

The nod-like receptors (NLRs) are another family of PRRs. Nod-like receptor 1 (NOD1) and 2 (NOD2), also known as nucleotide-binding oligomerization domain protein 4 (CARD4) and 15 (CARD15) respectively, are cytosolic PRRs [83, 84]. The expression and function of these NLRs is linked with susceptibility towards development of allergic diseases, including asthma [85, 86]. ***NOD1*** is expressed in various types of tissues and it interacts with muropeptides which are a component of Gram-negative bacteria [83, 87]. Polymorphisms in the *NOD1* gene were strongly associated with the presence of asthma [85]. A specific *NOD1* allele was reported to protect children, which live on farms and are heavily exposed to microbial products, against asthma and other atopic diseases [83]. ***NOD2*** is expressed in monocytes, granulocytes, DCs and epithelial cells [88, 89]. This receptor functions as a cytosolic receptor for LPS and peptidoglycan [84, 90]. Three different relevant polymorphisms in the *NOD2* gene were associated with atopic diseases

including asthma [91-94]. To date, no studies have been published showing the exact mechanism on cellular or molecular level of the polymorphisms of *NOD1* or *NOD2* in relation to allergic asthma. The expression, function and role of NOD1 and NOD2 are summarized in Table 1.

The gut microbiota and asthma

In the past years, the role of the microbiota in regulating the (systemic) immune system has gained attention. Changes in the gut microbiota have been thought to contribute to or at least to be correlated with the development of various inflammatory diseases, including asthma. Additionally, animal studies have demonstrated a substantial influence of the gut microbiotica on immune function beyond the gut [95, 96]. On the other hand, animal studies indicated a link between bronchial asthma and the gastrointestinal tract (GIT), and it has been hypothesized that, besides its effects on the airways and lungs, asthma can also affect the GIT in the same pathophysiological manner [97]. Recently, beneficial bacterial strains with or without non-digestible oligosaccharides, as potential modulators of the intestinal microbiota and mucosal and systemic immune responses, have gained a lot of attention [98-100].

Regarding the microbiome–immune system interaction, TLRs, especially TLR2 and TLR9, were reported to be involved in the induction of regulatory T cell (Tregs) responses by the intestinal microbiota and this might be responsible for the protective effects in allergy [101].

Beneficial bacterial strains and non-digestible oligosaccharides in asthma

The United Nations Food and Agricultural Organization and the World Health Organization define probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit to the host" [102]. *Bifidobacterium* and *Lactobacillus* are the most frequently used bacterial genera since these occur naturally as part of the gut microbiota [103]. Recently an international expert group from the International Life Sciences Institute (ILSI) evaluated the published evidence of the functionality of these beneficial bacterial strains in allergy, chronic intestinal inflammatory and functional disorders, infections and metabolic processes [104]. It was stated that there is substantial evidence from clinical studies to suggest a role of these bacterial strains in the prevention and management of allergy [105]. Clinical studies

with children suffering from atopic disease have shown that the composition of the gut microbiota differs between healthy and allergic infants and in countries of high and low prevalence of allergy [106-110]. Additionally, specific bacterial strains proved to be beneficial in reducing symptom severity and medication use in allergic rhinitis patients [111]. In the context of allergic asthma, different species of *Lactobacillus* have been shown to inhibit the allergic airway hyper-responsiveness, modulate Th1/Th2 immunobalance and prevent asthma in mice [103, 112-114]. Administration of *Bifidobacterium* during lactation suppressed both allergic and autoimmune responses in the progeny [115], and these bacteria reduced allergic symptoms in ovalbumin (OVA)-sensitized mice [116]. The rationale for a potential effect stems from the fact that these strains, as Gram positive bacteria, contain TLR ligands and thus can potentiate a TLR driven response as well as shifting the balance from a Th2 to a Th1 and/or Treg response.

Non-digestible oligosaccharides are oligosaccharides that resist digestion but are fermentable by intestinal microbiota, such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), and are thought to be metabolized by or increase the survival of beneficial bacteria in the intestinal tract [99]. 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 [117, 118]. 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 [119, 120]. Additionally, a specific mixture of oligosaccharides consisting of scGOS/lcFOS and scGOS/lcFOS/pectin-derived acidic oligosaccharides (pAOS) decreased several parameters of allergic asthma in mice [121].

Aim and outline of this thesis

The prevalence of allergic diseases, such as asthma, worldwide is rising dramatically in developed as well as developing countries. Despite the effectiveness of the current therapies for asthma, a high percentage of asthmatics are poorly controlled. Novel therapeutic strategies for asthma management are strongly needed. Understanding the immune responses and the different inflammatory markers involved in asthma is crucial for the development of effective, safe and potential therapeutic treatment approaches. This thesis aims at understanding the immune responses underlying allergic asthma and the modulation of these responses and asthma symptoms by using beneficial bacterial strains with or without specific non-digestible oligosaccharides.

In this chapter, a general introduction about asthma pathology; current therapy; the role, expression, function and activation of the different toll-like receptors and nod-like receptors in asthma; the role of the gut microbiota in asthma and new preventive and therapeutic approaches for allergic asthma is given. In addition, current knowledge on the possible immunomodulatory effect of specific beneficial bacterial strains with or without non-digestible oligosaccharides is also discussed. In **chapter 2**, the differences in inflammatory and immune responses between mild and severe experimental asthma are described. Different mRNA expression profiles of TLRs, NLRs, T cell cytokines and transcription factors are observed in mild and severe experimental asthma. The therapeutic effects of long-term treatment with *Bifidobacterium breve* and *Lactobacillus rhamnosus* treatment, on the inflammatory response in a murine model for chronic allergic asthma are discussed in **chapter 3.** *Bifidobacterium breve* has strong anti-inflammatory properties, possibly via the induction of a regulatory T cell response. In **chapter 4**, the therapeutic effects of long-term treatment with a combination of *Bifidobacterium breve* with non-digestible oligosaccharides on airway inflammation and remodeling in a murine ovalbumin-induced chronic asthma model are described. This specific combination of *Bifidobacterium breve* with non-digestible oligosaccharides has strong anti-inflammatory properties and is effective at inducing a regulatory T cell response and reducing airway remodeling. The therapeutic effects of long-term treatment with *Bifidobacterium breve* and *Lactobacillus rhamnosus* on asthma symptoms in a murine model for chronic allergic asthma are further

explored in **chapter 5**. An overview of the dual role of mast cells in allergic asthma is given in **chapter 6**. Preliminary data regarding the protective effects of *Bifidobacterium breve* on mast cell progenitors in the bone marrow and mast cells degranulation *in vitro* is described in **chapter 7**. A general overview and discussion of the findings demonstrated in this thesis is given in **chapter 8**.

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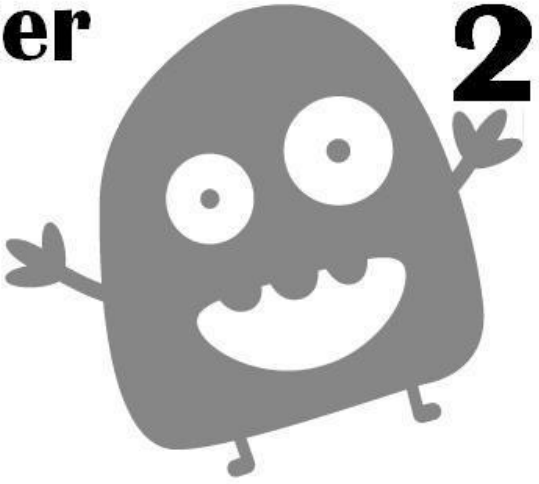
Chapter 2



Differential regulation of inflammation and immunity in mild and severe experimental asthma

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Abstract

Background: This study aimed at exploring innate and adaptive immunity in allergic asthma by investigation of mRNA expression of pattern recognition receptors, T cell-specific cytokines and transcription factors. Mouse models for mild and severe asthma, with similar pathological characteristics observed in humans, were used to study the involved inflammatory markers as a first step in the development of phenotype-directed treatment approaches.

Methods: In the mild model, mice were sensitized to ovalbumin-Imject Alum and challenged with ovalbumin. In the severe model, mice were sensitized to trinitrophenyl conjugated-ovalbumin and challenged with trinitrophenyl-ovalbumin/IgE immune complex. Pulmonary airway inflammation and mRNA expression of toll-like receptors (TLRs), Nod-like receptors (NLRs), T cell cytokines and transcription factors in lung tissue were examined.

Results: Different mRNA expression profiles of TLRs, NLRs, T cell cytokines and transcription factors were observed. In the mild model, *Il10* showed the largest increase in expression, whereas in the severe model it was *Infγ* with the largest increase. Expression of *Tbet* was also significantly increased in the severe model.

Conclusion: Inflammation and immunity are differentially regulated in mild and severe experimental asthma. This preclinical data may help in directing clinical research towards a better understanding and therapy in mild and severe asthmatic patients.

Introduction

Asthma is a chronic inflammatory disease of the airways affecting over 300 million people worldwide and its prevalence is rising, especially in children and within developing countries [1]. New studies indicate that in allergic disorders, including asthma, innate immunity is deregulated by allergens that promote sensitization [2]. However, the underlying immunological processes are still not fully understood as asthma is a complex multifactorial disease in which both innate and adaptive immune responses are involved [3]. In addition, asthma is considered a complex syndrome with different clinical phenotypes in children and adults. Eosinophils and neutrophils play a key role in the cellular airway inflammation [4,5]. The different phenotypes of asthma have distinct immunological and pathological features. Mild asthma is characterized by chronic inflammation of the airways that is mostly eosinophilic in nature and allergic sensitization. The resulting airway inflammation is thought to be caused by a breakdown of immune tolerance toward environmental antigens and leads to a Th2-biased immune response [6]. On the contrary, neutrophil accumulation in the bronchial mucosa is an important feature of severe asthma and frequently includes a Th1 component as well as a Th2 immune response [4, 6]. This heterogeneity highlights the importance of more specific treatment approaches based on asthma phenotypes.

Pattern recognition receptors (PRRs), like the toll-like (TLRs) and nod-like (NLRs) families of receptors, are key components of the innate immune system. These PRRs exhibit different cell and stimulus specific patterns of expression [7]. In the human airways, *TLRs* are expressed in and on dendritic cells (DCs), epithelial cells, eosinophils, macrophages and mast cells [8]. *NOD1* and *NOD2* are intracellular pattern recognition molecules (PRMs) expressed in various human epithelial cells including lung cells [9]. Multiple DC functions are controlled by PRRs and, ultimately, modulate the resulting adaptive immune response [8, 10]. Upon PRR activation in the lung, various chemokines and cytokines are produced by mast cells and eosinophils that recruit activated B-lymphocytes and Th lymphocytes to the lung, starting the inflammation process in the airways [11].

Mast cells express the high-affinity receptors (FcεRI) for immunoglobulin E (IgE) on their surface and animal studies have demonstrated that mast cells play an important role in the induction of allergic airway inflammation [12]. In addition, asthmatic patients have been shown

to have an increased number of lung mast cells and allergen specific IgE, a phenomenon of allergic asthma shared with mouse models for this disease [13]. In order to reproduce more of the clinical features of severe asthmatic patients, there has been a focus on using IgE immune complexes as inducers of immune responses in the murine lung. Trinitrophenyl (TNP)-ovalbumin(OVA)-IgE immune complexes have been shown to be more potent inducers of immune responses than antigens alone, since challenge of sensitized mice with these complexes resulted in increased migration of mast cell progenitors to the lung [12, 13].

The expression and function of PRRs have been linked to susceptibility towards allergic asthma [2, 14]. Functional genetic variations in *TLR1*, *TLR6* and *TLR10* genes affecting gene and protein expression have been shown to be associated with increased mRNA expression of these TLRs and to protect against atopic asthma in humans [14]. Single nucleotide polymorphisms (SNPs) in the *TLR2* gene that led to decreased mRNA expression were positively associated with asthma susceptibility [15]. Cord blood CD34 (+) cells from high-atopic-risk infants exhibited low *TLR2, TLR4 and TLR9* expression [16]. Additionally, amino acid changes in the *TLR2* gene have been linked to reduced *TLR2* receptor function and to an increase in atopy risk in humans [17]. *Tlr3* contributes to asthma exacerbation in mice [18]. A study in a murine macrophage cell line suggested a pro-inflammatory role of *Tlr4* and *5* in the disease [19]. Animal studies have demonstrated that the dose of the *Tlr4* ligand, lipopolysaccharide (LPS), determines the type of inflammatory response generated and that lung epithelial cell activation by *Tlr4* is crucial for induction of airway inflammation via activation of mucosal DCs [20-22]. *TLR7* and *8* were identified as novel risk genes for asthma [23]. TLR9 is one of the most extensively studied TLRs in asthma and it is currently thought to modulate allergic responses by skewing the balance from a T helper type 2 (Th2) towards a Th1 response [24]. In addition, SNPs in the *TLR9* gene were associated with increased risk of asthma [25]. *TLR11*, *12* and *13* are not encoded in the human genome and there are currently no data on associations with asthma in mice [14]. Insertion-deletion polymorphisms in the *NOD1* gene has been associated with increased risk of developing asthma, and genetic variations in *NOD1* that affected microbial recognition were positively associated with disease susceptibility and pathogenesis [26, 27]. Polymorphisms in *NOD2,* that affected LPS recognition and TLR4 function, were associated with atopic diseases and were suggested to indirectly increase the severity of asthma [28].

In asthma, over 50 cytokines have now been identified to determine disease outcome. Pro-inflammatory and Th2-associated cytokines; including interleukin 4 (IL4), IL5, IL6, IL13 and tumor necrosis factor α (TNFα), are reported to enhance the disease. On the other hand, interferon γ (IFNγ), a Th1-associated cytokine was reported to reduce the symptoms of asthma in asthmatic patients [11]. In addition, asthmatic patients have been shown to have reduced levels of the anti-inflammatory cytokine IL10 in the sputum. IL10 is produced by macrophages and by a subset of regulatory T cells (Tregs) and exerts its effects by inhibiting the synthesis of inflammatory cytokines (including asthma-associated cytokines such as TNFα and IL5) and gene presentation [29]. Th2 cells play a key role in asthma and asthmatic subjects have been reported to have Th1/Th2 imbalances as well as disturbed T helper type-17 (Th17)/Treg imbalances [30]. Each type of Th cell is regulated by a specific transcription factor: T-bet for Th1 cells, GATA3 for Th2 cells, retinoic acid orphan receptor γt (RORγt) for Th17 cells and forkhead box P3 (FOXP3) for Tregs [31]. Animal and human studies demonstrated that alterations in expression and/or of functions of these transcription factors can contribute to asthma pathogenesis [32, 33].

The aim of this current study is to explore the innate and adaptive immune responses and inflammation in allergic asthma by investigation of the mRNA expression profiles of the different PRRs, T cell-related cytokines and transcription factors. To this end, we have used a mouse model for both mild allergy and severe asthma with similar pathological characteristics seen in humans. Findings from this observational study may contribute to elucidating underlying mechanisms of mild and severe asthma and the involved inflammatory markers, a first step in the development of phenotype-directed treatment approaches.

Material and Methods

Animals

Male BALB/c mice (6-8 weeks; Charles River Laboratories, France) were 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.

Mild asthma model

OVA sensitization

Sensitizations were performed on days 0 and 7. Mice were sensitized to ovalbumin (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 (AlumImject; Pierce, Rockford, IL, USA). Control animals received 0.1 mL saline only (NaCl 0.9%; B. Braun Medical B.V., Oss, The Netherlands) (Figure 1).

OVA challenge

Mice were exposed to 10 mg/mL OVA aerosol in saline using Pari LC Star nebulizer (PARI GmbH, Starnberg, Germany) in an aerosol cabin for 30 min on days 35, 38 and 41. Control animals were exposed to nebulized saline aerosol only (Figure 1).

Severe asthma model

TNP-OVA sensitization

Sensitizations were performed on days 0 and 7. Mice were sensitized with trinitrophenyl (TNP) conjugated-OVA by intraperitoneal injections of 0.1 mL alum-precipitated antigen, comprising 10 μg TNP-OVA absorbed into 2.25 mg alum. Control animals received 0.1 mL saline only (Figure 1).

TNP-OVA-IgE challenge

From day 14 up to and including day 20, mice were challenged daily by intranasal administration of a TNP-ovalbumin/IgE immune complex (2 μg TNP-OVA plus 20 μg DNP-specific IgE (clone H1 26.82)), as described previously [34]. Control animals received 50 μL of saline only (Figure 1).

Bronchoalveolar lavage

After sacrifice, on day 42 (mild model) or 21 (severe model), lungs were first washed 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 repeated 3 times 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.

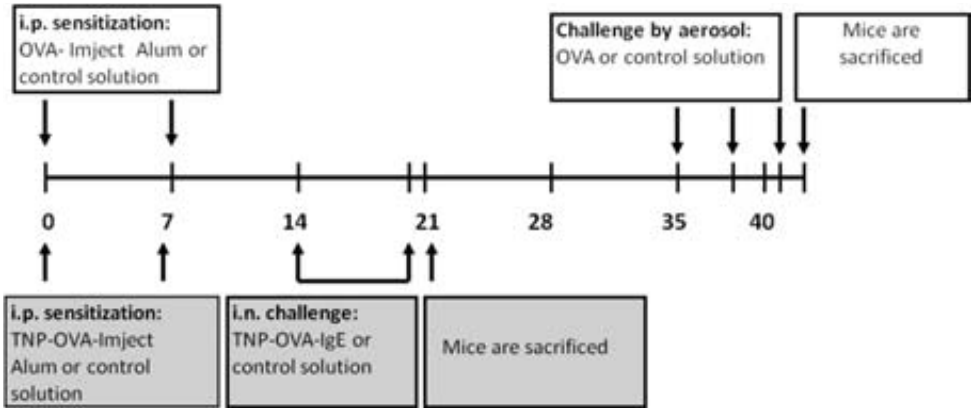


Figure 1: Time schedule of the mild and severe allergic asthma mouse models. Mild model (white): male BALB/c mice were sensitized intraperitonealy with alum-precipitated OVA or saline only on days 0 and 7 and challenged with OVA or saline aerosol on days 35, 38 and 41; mice were sacrificed on day 42. Severe model (grey): male BALB/c mice were sensitized intraperitonealy with alum-precipitated TNP-OVA or saline only on days 0 and 7 and challenged intranasally with TNP-OVA/IgE immune complex or saline from day 14 up to and including day 20; mice were sacrificed on day 21.

RNA isolation and quantitative real-time PCR

After mice were sacrificed, on day 42 or 21, the lungs were dissected and mRNA was isolated from whole lung tissue. Messenger RNA isolation (n= 3 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 94ºC for 3 min, 94ºC for 10 sec, specific melt temperature for 45 sec, followed by 39 cycles of 94ºC for 10 sec and 95ºC for 10 sec.

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

Statistical analysis

Data analysis was performed using a 1-way analysis of variance (one-way ANOVA) with the Bonferroni's post-hoc test. In some studies, the Student's t-test was used. Linear regression analysis was used to calculate correlations. All statistical analyses were performed using the GraphPad Prism software program (GraphPad Prism software version 5.03).

Results

Allergen-sensitized and challenged mice in the severe asthma model show higher total inflammatory cell number

Mice were rendered asthmatic following the scheme presented in Figure 1. To examine the extent of pulmonary inflammation in the asthmatic mice, bronchoalveolar lavage (BAL) fluid was examined for leukocyte accumulation (Figure 2). In mild asthma, allergen-sensitized and challenged mice showed a significant increase in the total inflammatory cell number (Figure 2A) which was due to a relative increase in the number of lymphocytes and eosinophils (Figure 2C) in BAL fluid compared to challenged only mice. In severe asthma, a significantly higher total inflammatory cell number (Figure 2B) was observed in allergen-sensitized and challenged mice compared to challenged only mice and this was due to a relative increase in the number of eosinophils (Figure 2D).

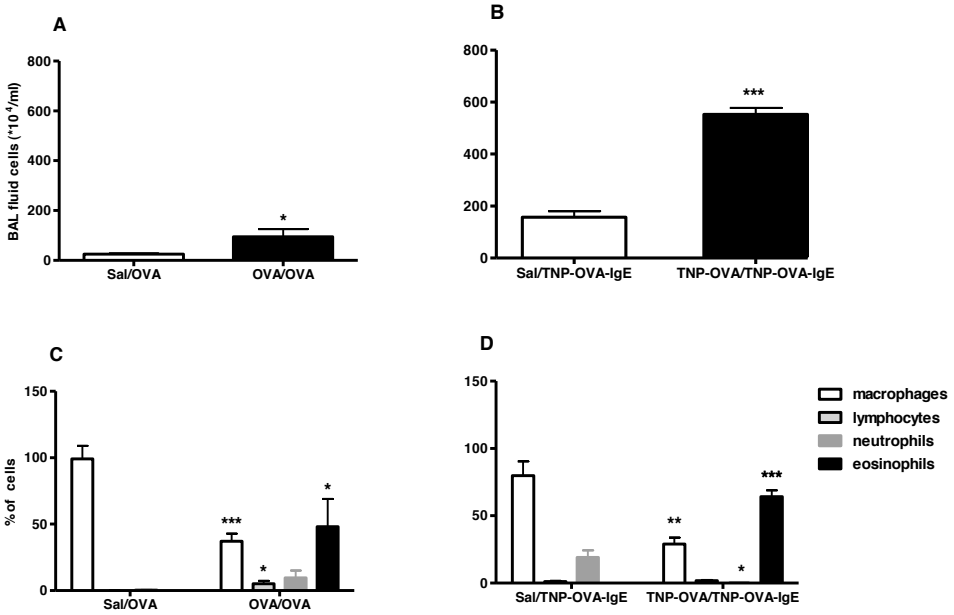
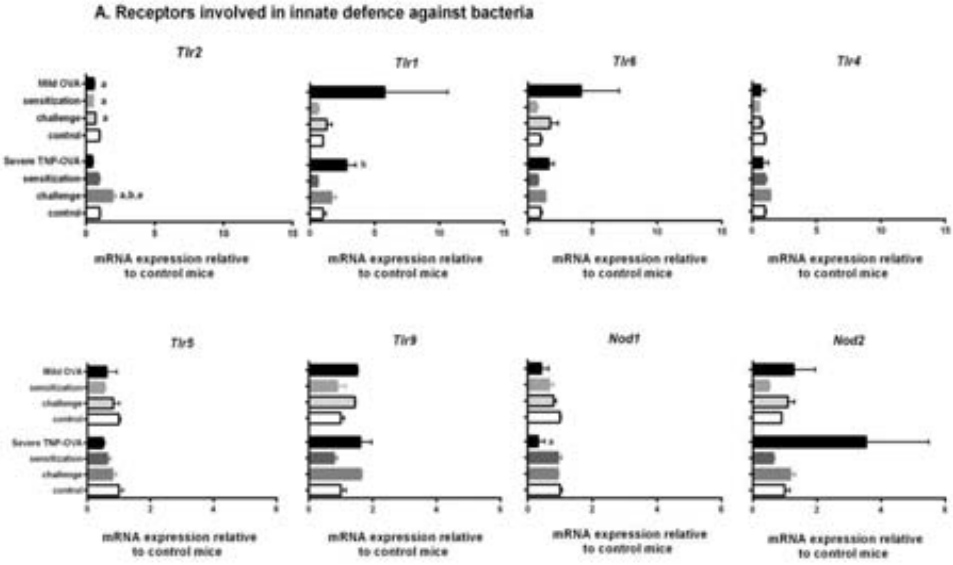


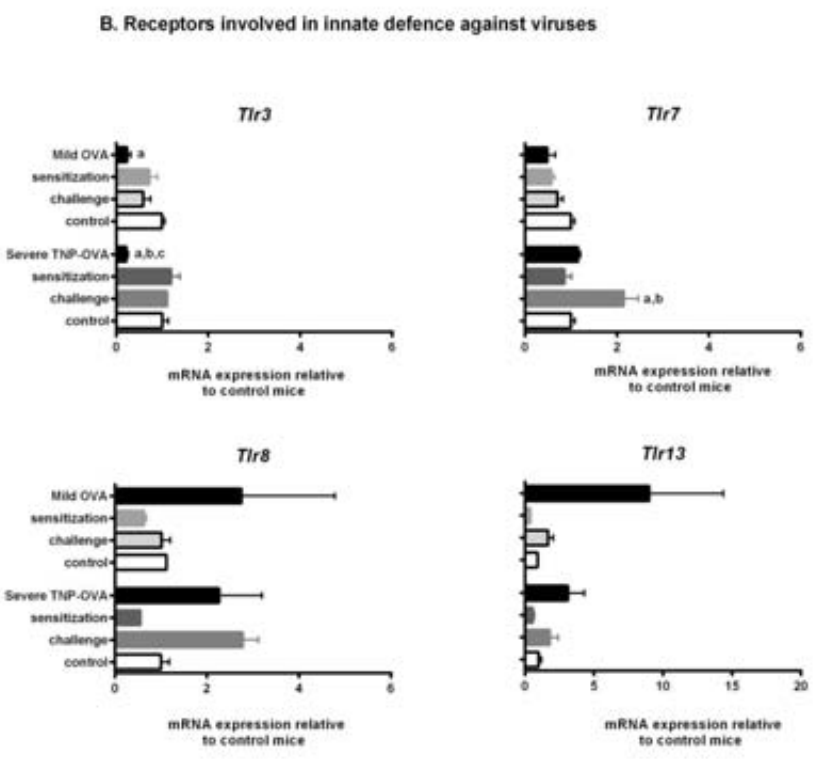
Figure 2: Total and differential BAL fluid cell counts in mild and severe allergic asthma. The total inflammatory cell number in BAL fluid is shown in A (mild model) and B (severe model). The differential cell

counts are shown as percentages of the total cell count in C (mild model) and D (severe model). Results are presented as mean ± SEM, n=9 mice/group. The Student's t-test was used to compare the means of the OVA-sensitized and challenged mice with the means of only challenged mice for each cell type, *, $p<0.05$; **, $p<0.01$; ***, $p<0.0001$.

TLR and NLR mRNA expression in lung tissue is differentially modulated in mild and severe asthma

As PRRs in the lung can modulate ongoing chronic inflammation during asthma, the mRNA expression of *Tlr1-13* and *Nod1 & 2* were measured (Figure 3). In the mild model, *Tlr2* expression was significantly increased in control mice when compared with sensitized only, challenged only and sensitized and challenged mice (Figure 3A). In the severe model, *Tlr2* expression was significantly increased in challenged only mice compared to control, sensitized only and allergen-sensitized and challenged mice (figure 3A). In addition, sensitized and challenged mice showed significantly higher *Tlr1* expression compared to sensitized only mice and significantly lower *Nod1* expression in comparison to control mice (Figure 3A). In mild asthma, *Tlr3* expression was significantly decreased in allergen-sensitized and challenged mice when compared with control mice (Figure 3B). In severe asthma, *Tlr3* expression was significantly lower in sensitized and challenged mice but not in control, sensitized only and challenged only mice (Figure 3B). In addition, *Tlr7* expression was significantly higher in challenged only mice compared to control and sensitized only mice (Figure 3B). The expression of *Tlr11* and *Tlr12* remained unchanged in both models (Figure 3C, 3D). Interestingly, in the severe model, the mRNA expression of *Tlr1*, *Tlr3*, *Tlr6*, *Tlr9*, *Tlr11*, *Tlr13*, *Nod1*, and *Nod2* was significantly correlated with the total inflammatory cell number in BAL fluid (Table 1).


A. Receptors involved in innate defence against bacteria
Tlr2
Tlr1
Tlr6
Tlr4
Tlr5
Tlr9
Nod1
Nod2
Mild OVA
sensitization
challenge
control
Severe TNP-OVA
mRNA expression relative to control mice


B. Receptors involved in innate defence against viruses
Tlr3
Tlr7
Tlr8
Tlr13
Mild OVA
sensitization
challenge
control
Severe TNP-OVA
mRNA expression relative to control mice

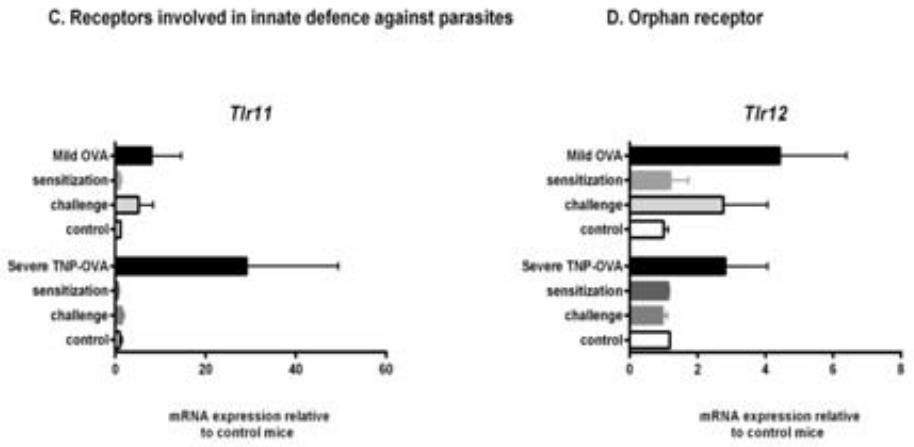


Figure 3: Relative TLR and NLR mRNA expression in mouse whole lung tissue during mild and severe allergic asthma. The results are presented as mRNA expression levels relative to control mice. Data is shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparison test after one-way ANOVA. [a] $p<0.05$ compared to control mice; [b] $p<0.05$ compared to sensitized only mice; [c] $p<0.05$ compared to challenged only mice; [e] $p< 0.05$ compared to OVA-sensitized and challenged mice.

Table 1: Correlations between TLR and NLR mRNA expression in whole lung tissue and the total cell number in BAL fluid during mild and severe allergic asthma

TLR/NLR	Model	Correlation
Tlr1	**mild**	p>0.05
Tlr1	**severe**	r^2= 0.74
Tlr3	**mild**	p>0.05
Tlr3	**severe**	r^2=0.61
Tlr6	**mild**	p>0.05
Tlr6	**severe**	r^2=0.62
Tlr9	**mild**	p>0.05
Tlr9	**severe**	r^2=0.36
Tlr11	**mild**	p>0.05
Tlr11	**severe**	r^2=0.95
Tlr13	**mild**	p>0.05
Tlr13	**severe**	r^2=0.68
Nod1	**mild**	p>0.05
Nod1	**severe**	r^2=0.43
Nod2	**mild**	p>0.05
Nod2	**severe**	r^2=0.39

Correlation is calculated using linear regression analysis**.** R square values are shown for the statistically significant correlations, P>0.05 represents not significant correlations.

Allergen-sensitization and challenge in the severe asthma model enhances the mRNA expression of *Il5*, *Il6* and *Il10* in lung tissue

To determine the extent of inflammation in the lung, the mRNA expression of various cytokines was measured (Table 2). In the severe model (white rows), the expression of almost all cytokines tends to be increased in TNP-OVA-sensitized and TNP-OVA/IgE challenged mice compared to

OVA-sensitized and challenged mice in the mild model (grey rows). *Il10* expression was significantly increased in both models. In severe asthma, an 8 and 33-times higher *Il5* and *IL6* expression respectively, was observed in allergen-sensitized and challenged mice but not in control, sensitized only and challenged only mice.

Table 2: Relative T cell cytokine mRNA expression in mouse whole lung tissue during mild and severe allergic asthma

Cytokine	Model	Control a	Sensitized only b	Challenged only c	Sensitized and challenged
Il2	mild	1.00 ± 0.02	2.24 ± 1.41	12.96 ± 9.17	11.63 ± 11.32
Il2	severe	1.00 ± 0.14	1.21 ± 0.20	1.53 ± 0.55	90.66 ± 37.70
Il4	mild	1.00 ± 0.21	1.95 ± 0.49	12.29 ± 8.58	27.71 ± 27.25
Il4	severe	1.00 ± 0.05	1.00 ± 0.11	0.82 ± 0.31	43.38 ± 38.41
Il5	mild	1.00 ± 0.01	2.91 ± 2.08	8.09 ± 4.63	30.60 ± 0.00[d]
Il5	severe	1.00 ± 0.22	0.62 ± 0.11	0.56 ± 0.02	8.02 ± 0.89[a,b,c]
Il6	mild	1.00 ± 0.34	0.48 ± 0.09	2.94 ± 1.01	5.80 ± 3.49
Il6	severe	1.00 ± 0.25	0.90 ± 0.46	1.45 ± 0.83	33.44 ± 4.31[a,b,c]
Il10	mild	1.00 ± 0.28	2.22 ± 0.37	1.55 ± 0.49	18.93 ± 0.67[a,b,c]
Il10	severe	1.00 ± 0.33	1.24 ± 0.14	0.89 ± 0.43	25.94 ± 8.76[a,b,c]
Il13	mild	1.00 ± 0.32	2.75 ± 2.63	11.44 ± 7.40	9.58 ± 2.20
Il13	severe	1.00 ± 0.28	0.98 ± 0.21	0.87 ± 0.37	64.21 ± 34.25
Tnfα	mild	1.00 ± 0.11	1.01 ± 0.11	2.39 ± 0.87	4.83 ± 4.02
Tnfα	severe	1.00 ± 0.29	0.86 ± 0.14	1.17 ± 0.25	8.78 ± 4.87
Ifnγ	mild	1.00 ± 0.31	3.51 ± 2.77	25.26 ± 21.10	8.78 ± 1.16
Ifnγ	severe	1.00 ± 0.00	0.75 ± 0.14	0.74 ± 0.26	82.32 ± 60.37

The values shown in the table are relative to the cytokine mRNA expression levels in control mice. Data is shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparison test after one-way ANOVA. [a] $p<0.05$ compared to control mice; [b] $p<0.05$ compared to sensitized only mice; [c] $p<0.05$ compared to challenged only mice; [d] a single value.

Cytokine mRNA expression in lung tissue is differentially modulated in mild and severe asthma

In mild asthma, *Il10* showed the largest change in expression followed by *Il5, Il4*, *Tnfα*, *Il6*, *Il2*, *Il13* and *Ifnγ* respectively (Figure 4A). In severe asthma, the largest change (110-fold) is observed in *Ifnγ* expression followed by *Il13*, *Il2*, *Il4*, *Il10*, *Il6*, *Il5*, and *Tnfα* respectively (Figure 4B).

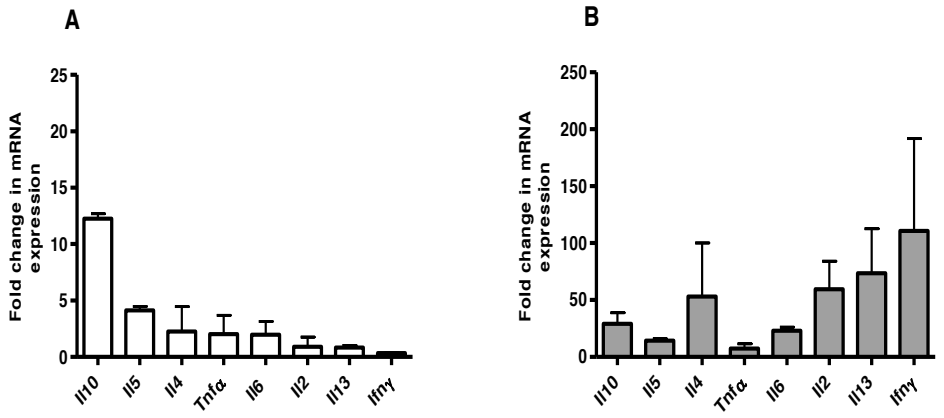


Figure 4: Change in T cell cytokine mRNA expression in mouse whole lung tissue during mild and severe allergic asthma. A: mild model, B: severe model. Data is shown as mean ± SEM. Fold change is calculated by dividing the cytokine mRNA expression in OVA-sensitized and challenged mice by cytokine mRNA expression in challenged only mice.

Allergen-sensitization and challenge in the severe asthma model results in a strong upregulation of mRNA for *Tbet* and *Foxp3* in the lungs and skews the immune response away from Th2 and towards Treg

To examine the T cell responses in the lung, the mRNA expression of T cell-specific transcription factors was measured and to determine the extent of Th response skewing in the lung, ratios for *Gata3*/*Tbet* (Th2/Th1), *Foxp3/Rorγt* (Treg/Th17), *Foxp3*/*Gata3* (Treg/Th2) and *Foxp3*/*Tbet* (Treg/Th1) mRNA expression were calculated (Table 3A). The expression of *Tbet* and *Foxp3* was 7 and 2-times higher respectively, in allergen-sensitized and challenged mice in the severe model (grey rows) compared to allergen-sensitized and challenged mice in the mild model (white rows). In severe asthma, sensitized and challenged mice showed 16 and 17-times

higher *Tbet* and *Foxp3* expression respectively, when compared to control, sensitized only and challenged only mice. Most interestingly, allergen-sensitization and challenge resulted in a significant decrease in *Gata3*/*Tbet* ratio as compared to control and sensitized only mice in both models. The ratio of *Foxp3*/*Rorγt* was 12-times higher in the severe model in comparison to the mild model and this ratio was significantly increased in allergen-sensitized and challenged mice compared to control, sensitized only and challenged only mice in both models. In the mild model, allergen-sensitization and challenge significantly increased the *Foxp3*/*Gata3* ratio compared to control mice. The ratio of *Foxp3*/*Gata3* in allergen-sensitized and challenged mice was significantly increased compared to control, sensitized only and challenged only mice, and this ratio was almost 4-times higher than the mild model.

Table 3A: Relative T cell transcription factor mRNA expression in mouse whole lung tissue during mild and severe allergic asthma

Transcription Factor	Model	Control a	Sensitized only b	Challenged only c	Sensitized and challenged
Tbet	**mild**	1.00 ± 0.40	1.06 ± 0.35	4.56 ± 2.23	2.32± 0.51
	severe	1.00 ± 0.19	1.05 ± 0.31	1.45 ± 0.02	15.94 ± 5.09[a,b,c]
Gata3	**mild**	1.00 ± 0.01	0.68 ± 0.16	1.10 ± 0.14	1.44 ± 0.45
	severe	1.00 ± 0.17	0.67 ± 0.08	0.57 ± 0.06	0.84 ± 0.21
Rorγt	**mild**	1.00 ± 0.11	0.67 ± 0.33	0.72 ± 0.15	0.35 ± 0.02
	severe	1.00 ± 0.02	0.71 ± 0.19	1.02 ± 0.14	0.117[d]
Foxp3	**mild**	1.00 ± 0.31	1.87 ± 0.50	0.95 ± 0.34	8.55 ± 2.82[a]
	severe	1.00 ± 0.11	0.92 ± 0.21	2.03 ± 0.39	17.11 ± 1.06[a,b,c]
Ratio					
Gata3/Tbet	**mild**	1.00±0.10	0.64± 0.26	0.24±0.03	0.10±0.03[a,b]
	severe	1.00±0.17	0.63±0.07	0.30 ±0.03	0.05±0.01[a,b]
Foxp3/Rorγt	**mild**	1.00±0.31	2.78±0.63	4.33±3.05	37.12±12.24[a,b,c]
	severe	1.00±0.11	1.29±0.29	2.00± 0.38	439.08±27.31[a,b,c]
Foxp3/Gata3	**mild**	1.00±0.31	2.77±0.63	2.83±1.98	5.95±1.96[a]
	severe	1.00±0.11	1.37±0.31	3.55±0.67	20.40±1.27[a,b,c]
Foxp3/Tbet	**mild**	1.00±0.31	1.77±0.50	0.69±0.48	0.61±0.20
	severe	1.00±0.11	0.87±0.20	1.07±0.20	1.07±0.07

Tbet, *Gata3*, *Rorγt*, and *Foxp3* represent Th1,Th2, Th17 and Treg cells, respectively. The values shown in the table are relative to transcription factor mRNA expression levels in control mice. Data is shown as mean ± SEM. Ratio's for Th2/Th1 (*Gata3*/*Tbet*), Treg/Th17 (*Foxp3*/*Rorγt*), Treg/Th2 (*Foxp3*/*Gata3*) and Treg/Th1 (*Foxp3*/*Tbet*) mRNA expression are also shown. 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. [a] $p<0.05$ compared to control mice; [b] $p<0.05$ compared to sensitized only mice; [c] $p<0.05$ compared to challenged only mice; [d] a single value.

T cell transcription factor mRNA expression in lung tissue is differentially modulated in mild and severe asthma

In mild asthma, the largest change (9-fold) was observed in *Foxp3* expression followed by *Tbet*, *Gata3* and *Rorγt* (Figure 5A). *Tbet* showed the largest change in expression in severe asthma followed by *Foxp3*, *Gata3* and *Rorγt* (Figure 5B).

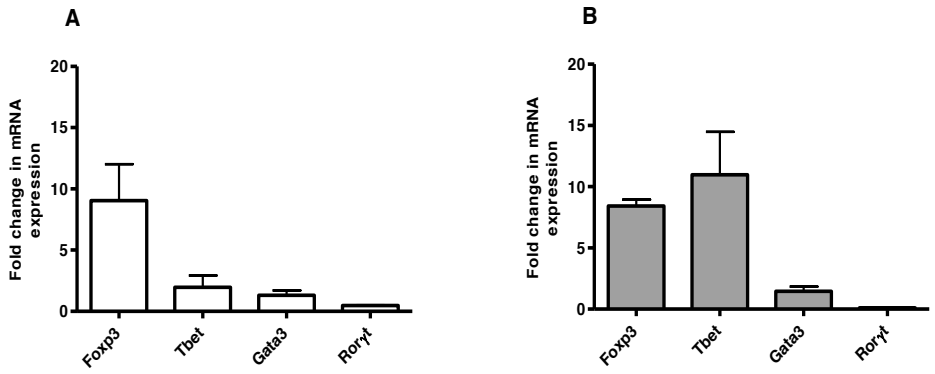


Figure 5: Change in T cell transcription factor mRNA expression in mouse whole lung tissue during mild and severe allergic asthma. A: mild model, B: severe model. Data is shown as mean ± SEM. Fold change is calculated by dividing the transcription factor mRNA expression in OVA-sensitized and challenged mice by cytokine mRNA expression in challenged only mice.

Different correlations between T cell transcription factor mRNA expression and T cell cytokine mRNA expression are found in mild and severe asthma

As imbalances in T cell responses can also be detected using T cell-specific transcription factors, the correlations between the mRNA expression of T cell transcription factors and T cell-specific cytokines were calculated (Table 3B). In mild asthma, *Tbet* expression was significantly correlated with *Il2*, *Il4*, *Il5*, *Il6*, *Il13* and *Tnfα* expression. *Rorγt* expression showed a significant correlation with *Il10* expression and *Foxp3* expression was significantly correlated with and *Il2*, *Il4*, *Il5*, *Il6*, *Il10* and *Tnfα* expression. In severe asthma, *Tbet* expression was strongly correlated with *Il2*, *Il6* and *Il10* expression. *Rorγt* expression was significantly correlated with *Il2*, *Il4*, *Il5*,

Il6, *Il10* and *Il13* expression. Interestingly, *Foxp3* expression was strongly correlated with *Il2*, *Il4*, *Il5*, *Il6* and *Il13* and significantly correlated with *Il10* and *Ifnγ* expression.

Table 3B: Correlation between T cell transcription factor mRNA expression and T cell cytokine mRNA expression in whole lung tissue during mild and severe allergic asthma

Transcription Factor	*Il2*	*Il4*	*Il5*	*Il6*	*Il10*	*Il13*	*Tnfα*	*Ifnγ*
***Tbet*/mild**	r^2=0.60	r^2=0.91	r^2=0.93	r^2=0.82	p>0.05	r^2=0.86	r^2=0.88	p>0.05
***Tbet*/severe**	r^2=0.92	p>0.05	p>0.05	r^2=0.97	r^2=0.96	p>0.05	p>0.05	p>0.05
***Gata3*/mild**	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
***Gata3*/severe**	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05	p>0.05
***Rorγt*/mild**	p>0.05	p>0.05	p>0.05	p>0.05	r^2=0.53	p>0.05	p>0.05	p>0.05
***Rorγt*/severe**	r^2=0.50	r^2=0.51	r^2=0.42	r^2=0.46	r^2=0.54	r^2=0.44	p>0.05	p>0.05
***Foxp3*/mild**	r^2=0.63	r^2=0.52	r^2=0.88	r^2=0.58	r^2=0.83	p>0.05	r^2=0.46	p>0.05
***Foxp3*/severe**	r^2=0.98	r^2=0.92	r^2=0.98	r^2=0.99	r^2=0.58	r^2=0.98	p>0.05	r^2=0.80

Correlation is calculated using linear regression analysis**.** R square values are shown for the statistically significant correlations, P>0.05 represents not significant correlations

Discussion

The aim of this study was to explore the innate and adaptive immune responses and inflammation in a mouse models for mild and severe allergic asthma. Our results clearly show that pulmonary inflammation is differentially regulated in mild and severe experimental asthma. In the severe asthma model, a higher (3-fold) cell influx in BAL fluid was seen compared to the mild model. As expected, TNP-OVA-sensitized and TNP-OVA/IgE-challenged mice showed significantly higher total inflammatory cell number and a relative increase in the number of eosinophils, lymphocytes and neutrophils as compared to challenged only mice. These findings are in accordance with other animal studies in which IgE immune complexes have been used as inducers of airway inflammation [12, 13, 34].

Besides being key components of the innate immunity, PRRs are also involved in the activation and shaping of adaptive immunity. The function and expression of PRRs has been linked to susceptibility towards allergic asthma. In mild asthma, *Tlr2* expression was lower in sensitized only, challenged only and OVA-sensitized and challenged mice compared with control mice. Previous human studies have demonstrated that decreased *TLR2* mRNA expression and receptor function due to SNPs in the *TLR2* gene are positively associated with asthma susceptibility and high-atopic-risk infants have been reported to have low *TLR2* expression on their cord blood CD34 (+) cells [15-17]. In severe asthma, allergen challenge only increased the expression of *Tlr2* and allergen-sensitization, and challenge significantly increased *Tlr1* expression compared to sensitized only mice. These results are supported by data from animal and human studies which have shown that *Tlr2/Tlr1* heterodimers can play both pro- and anti-inflammatory roles in allergic asthma [16, 35]. Interestingly, allergen-sensitization and challenge decreased *Tlr3* expression in both models. Previous *in vitro* studies have demonstrated that upon the activation of *Tlr3* by its ligand, this PRR induces upregulation of its own expression as well as expression of other TLRs, various cytokines and chemokines and thereby contributes to exacerbation of inflammation [18]. However, no direct associations between *Tlr3* expression and function and 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 also unknown. In severe asthma, allergen-challenge increased *Tlr7* expression. This could be due to a response of plasmacytoid DCs to the inhaled antigen as these cells strongly express *Tlr7* [36]. In addition, allergen-sensitization and challenge decreased *Nod1* expression compared to control

mice. NOD1 is an intracellular sensor of pathogenic bacteria. Single nucleotide polymorphisms in the *NOD1* gene were positively associated with susceptibility towards asthma in children living on farms, and this PRR has been reported to be necessary for neutrophil function in mice [26, 27, 37]. However, no direct associations between *Nod1* expression and function and asthma have been reported yet.

Interestingly, *Tlr1*, *Tlr3*, *Tlr6*, *Tlr9*, *Tlr11*, *Tlr13*, *Nod1* and *Nod2* expression was significantly correlated with the total inflammatory cell number in BAL fluid in the severe model. These correlations might be explained by the increase in the number of inflammatory cells which express these receptors, as is the case for *Tlr1* and *Nod1*. Additionally, these receptors could also contribute to the increased sensitivity to inflammation/exacerbation since a small trigger can lead to an inflammatory cascade. However, the link between these correlations and asthma pathogenesis remains to be investigated.

Different mRNA expression profiles of T cell-related cytokines are observed in the mild and severe models for allergic asthma. To our knowledge, this is the first report in which cytokine mRNA expression is measured in mouse whole lung tissue. In severe asthma, allergen-sensitization and challenge increased *Il6*, *Il5* and *Il10* expression. These results are in line with findings in human asthmatics [11]. Of particular interest, *Il10* showed the largest change in mRNA expression in mild asthma. This profound, 13-fold change in *Il10* expression might be necessary to limit the inflammation in the airways and to counter the effects of the other cytokines on disease progression as previously described [38]. This hypothesis is supported by the findings in the severe model, in which *Ifnγ* showed the largest change in expression suggesting a Th1-skewed response.

Th2 cells play a key role in the pathogenesis of allergic asthma, and asthmatic patients have been reported to have Th1/Th2 imbalances as well as disturbed Th17/Treg imbalances. In mild asthma, expression of *Foxp3* was the most prominent. In severe asthma, *Foxp3* and *Tbet* showed the highest expression in lungs of allergen-sensitized and challenged mice. These findings are in line with our cytokine expression data (described above) and are supported by the proposed role of Tregs and *Il10* in the airways [38]. Intriguingly, allergen-sensitization and challenge resulted in a strong Treg response in both models. Accordingly, *Foxp3* showed the largest change in expression in the mild model. In severe asthma, the largest change in expression was found in the Th1-related transcription factor, *Tbet*, and this result is in contrast with data obtained from

asthmatic patients [30, 32, 33], possibly due to measurement of mRNA expression in mouse whole lung tissue instead of in PBMCs of asthmatics and/or measurement of mRNA expression instead of protein expression. It has been reported that both *in vivo* and *in vitro Gata3* can inhibit *Foxp3* gene induction by directly binding to the *Foxp3* promoter in mice [39]. An opposite action of *Foxp3* might also be possible and our results could be explained by a counter-regulatory mechanism of Treg/Th1 to suppress the Th2 immune response. Interestingly, the mRNA expression of *Tbet* was strongly correlated with the expression of innate cytokines (*Il2*, *Il6* and *Tnfα*) and Th2 cytokines (*Il4*, *Il5* and *Il13*), but not with Th1 cytokine (*Ifnγ*). In severe asthma, *Rorγt* expression was significantly correlated with *Il6* expression, but not in mild asthma. Expression of *Foxp3* was significantly correlated with *Il10* expression in both models. IL2, IL4, IL5, IL6, IL13 and TNFα have been reported to enhance asthma in humans and Th1 cells have been shown to suppress Th2 cells through the release of IFNγ. Additionally, Tregs suppress other Th cell effector functions through the release of IL10 [11]. No correlations were found between *Gata3* and Th2 cytokines expression suggesting a counter-regulatory mechanism of Treg/Th1 to suppress the Th2 immune response as described previously.

To our knowledge, our results demonstrated for the first time that in mild and severe models for experimental asthma immune and inflammatory responses are regulated differently. We showed that in mild and severe allergic asthma different mRNA expression of TLRs, NLRs and T cell-specific cytokines and transcription factors is observed. How this determines asthma severity remains to be investigated. This study adds to our understanding of the allergic characteristics of mild and severe allergic asthma which can contribute to the identification of phenotype-specific therapeutic targets.

Aknowledgments

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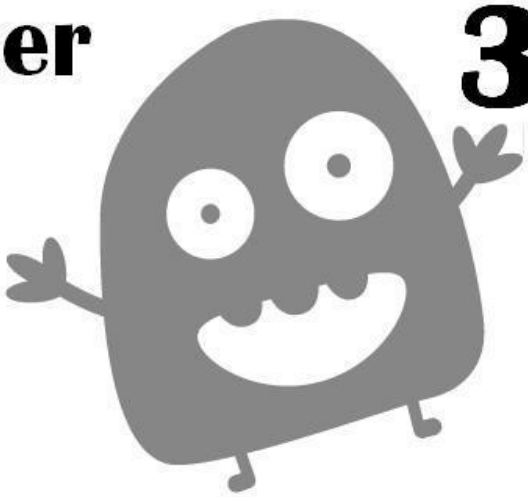
Supplementary Table 1: Forward and reverse primers used for quantitative real-time PCR analysis

Molecule	Forward primer	Reverse primer
Tlr1	GGTGTTAGGAGATGCTTATGGGG	GATGTTAGACAGTTCCAAACCGA
Tlr2	CCAGACACTGGGGGTAACATC	CGGATCGACTTTAGACTTTGGG
Tlr3	GGGGTCCAACTGGAGAACCT	CCGGGGAGAACTCTTTAAGTGG
Tlr4	GCCTTTCAGGGAATTAAGCTCC	AGATCAACCGATGGACGTGTAA
Tlr5	TCAGACGGCAGGATAGCCTT	AATGGTCAAGTTAGCATACTGGG
Tlr6	GACTCTCCCACAACAGGATACG	TCAGGTTGCCAAATTCCTTACAC
Tlr7	TCTTACCCTTACCATCAACCACA	CCCCAGTAGAACAGGTACACA
Tlr8	GGCACAACTCCCTTGTGATT	CATTTGGGTGCTGTTGTTTG
Tlr9	ACTCCGACTTCGTCCACCT	GGCTCAATGGTCATGTGGCA
Tlr11	AAAACCAGACAACATCACAA	GCATCCCAAATAGATAGAGG
Tlr12	GAACTCTTGGATCCCCTAC	GGCAGAAGTTCCTCTATCAC
Tlr13	ATCTCAGGAACAAAAGATGG	TGTTCCCATAGACATCAAAA
Nod1	GAAGGCACCCCATTGGGTT	AATCTCTGCATCTTCGGCTGA
Nod2	CCGCTTTCTACTTGGCTGTC	GTGATTTGCAGGTTGTGTGG
Tbet	GCCAGCCAAACAGAGAAGAC	AAATGTGCACCCTTCAAACC
Gata3	GCGGTACCTGTCTTTTTCGT	CACACAGGGGCTAACAGTCA
Foxp3	CACTGGGCTTCTGGGTATGT	AGACAGGCCAGGGGATAGTT
Roryt	TGCAAGACTCATCGACAAGG	AGGGGATTCAACATCAGTGC
RPS13	GTCCGAAAGCACCTTGAGAG	AGCAGAGGCTGTGGATGACT

Chapter 3



Bifidobacterium breve treatment is as effective as budesonide at reducing inflammation in a murine model of chronic asthma

Manuscript submitted

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Abstract

Background: Glucocorticoids are by far the most effective treatment for asthma. Yet, despite their effectiveness at resolving airway inflammation, glucocorticoids do not produce long-term benefits. Over the last decade, there has been a growing interest in the use of beneficial bacteria for allergic diseases. This study is aimed at exploring the therapeutic effects of long-term treatment with two different beneficial bacterial strains (*Bifidobacterium breve* and *Lactobacillus rhamnosus*) and a glucocorticoid (budesonide), as a reference treatment, on inflammatory response in allergic asthma.

Methods: To mimic the chronic disease in asthmatic patients, we used the murine ovalbumin-induced asthma model combined with prolonged allergen exposure. Pulmonary airway inflammation; mRNA expression of pattern recognition receptors, Th-specific cytokines and transcription factors in lung tissue; and expression of Foxp3 in blood Th cells were examined.

Results: Pulmonary airway inflammation was suppressed by all treatments. Beneficial bacteria and budesonide differentially modulated the expression of toll-like receptors (TLRs), nod-like receptors (NLRs), cytokines and T cell transcription factors. *Bifidobacterium breve* was the most potent inducer of regulatory T cell responses, increasing *Il10* and *Foxp3* transcription in lung tissue and augmenting the mean fluorescence intensity of Foxp3 in blood CD4+ T cells. These observations suggest that *Bifidobacterium breve* is an inducer of regulatory T cell responses in the airways as well as systemic.

Conclusion: These and previous findings show that *Bifidobacterium breve* has strong anti-inflammatory properties that are comparable to budesonide and therefore may be beneficial in the treatment of chronic asthma.

Introduction

Allergic asthma is a T helper type-2 (Th2) cell-mediated chronic inflammatory disorder of the airways with rapidly increasing incidence and prevalence throughout the world, especially in children and within developing countries [1]. The underlying immunological processes are still not fully understood as asthma is a complex multifactorial disease in which both innate and adaptive immune responses are involved [2]. In addition, asthma is a heterogeneous disease with different phenotypes in which eosinophils and neutrophils play key roles in the cellular airway inflammation [3]. Observations from animal models of allergic asthma have contributed to a better 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 [4, 5].

Glucocorticoids (GCs) are by far the most effective treatments for asthma. It is suggested that GCs exert their anti-inflammatory actions by suppression of pro-inflammatory genes or by the activation of anti-inflammatory gene expression [6, 7] and these drugs enhanced toll-like receptor 2 (*Tlr2*) expression in murine primary bronchial epithelial cells, activated *in vitro,* and in human bronchial epithelial cell line [8, 9]. Moreover, the mRNA expression of forkhead box P3 (*FOXP3),* regulatory T cells (Tregs)-specific transcription factor, was increased in asthmatic patients receiving GC treatment and *FOXP3* was tightly correlated with the anti-inflammatory cytokine interleukin10 (*IL10)* mRNA expression [10]. Despite the effectiveness of GCs at resolving airway inflammation, the treatment does not produce long-term benefits and the therapy has undesirable side effects. Additionally, a significant number of asthmatics is steroid resistant and fails to respond to GC therapy [7, 11]. These limitations of GCs therapy highlight the need for novel therapeutics with long-term benefits, greater disease control and increased efficacy.

In the past years, the role of the microbiota in regulating the immune system has gained attention. Changes in gut microbiota were suggested to contribute to the development of various inflammatory diseases, including asthma [12-14]. Interestingly, animal studies indicated a link between bronchial asthma and the gastrointestinal tract (GIT), and it has been hypothesised that, besides its effects on the airways and lungs, asthma can also affect the GIT in the same pathophysiological manner [15]. The potential role of beneficial bacteria as modulators of the

intestinal microbiota and mucosal immune responses has been extensively investigated and discussed in the last few years [16-18].

Probiotics are"live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host" [19, 20]. In the context of allergic asthma, different strains of *Lactobacillus* have been shown to inhibit the allergic airway response, modulate Th1/Th2 immunobalance and prevent asthma in mice [21-24]. Administration of *Bifidobacteria* during lactation suppressed both allergic and autoimmune responses in the progeny [25], and these bacteria reduced allergic symptoms in ovalbumin (OVA)-sensitised mice [26].

Because they contain TLR ligands, it is believed that beneficial bacteria can modulate TLR-driven responses and also skew the immune balance towards a Th1-associated response [27]. The TLRs and nod-like receptors (NLRs) are key pattern recognition receptor (PRR) families in the innate immune response. In the human airways, the RRRs are expressed in or on dendritic cells (DCs), epithelial cells, eosinophils, macrophages and mast cells [28, 29]. Multiple DC functions are controlled by PRRs and, ultimately, modulate the resulting adaptive immune response [30]. Following TLR and NLR activation in the lung, various chemokines and cytokines are produced by eosinophils and mast cells that attract activated B-lymphocytes and Th lymphocytes to the lung to orchestrate the inflammation in the airways [31].

The function and expression of PRRs were linked to susceptibility towards allergic asthma [32, 33]. Functional genetic variations in *TLR1*, *TLR10* and *TLR6* genes affecting gene and protein expression were associated with increased mRNA expression of these TLRs and protected against atopic asthma in humans [33]. Genetic variations in *TLR2, NOD1 and NOD2* genes that led to either decreased mRNA expression and affected microbial recognition, respectively, were positively associated with disease susceptibility and pathogenesis [34-38]. Cord blood CD34 (+) cells from high-atopic-risk infants have been reported to have low *TLR2*, *TLR4*, and *TLR9* expression and the latter was demonstrated to exert protective immunomodulatory effects on asthma [39-41]. *Tlr3* contributes to asthma exacerbations in mice [42] and a study in a murine macrophage cell line suggested a pro-inflammatory role of *Tlr4* and *5* in the disease [43].

In asthma, over 50 cytokines have now been identified to affect disease outcome. Strong pro-inflammatory and Th2-associated cytokines; including interleukin1β (IL1β), IL4, IL5, IL6, IL9, IL13, IL17, IL25 and tumor necrosis factor α (TNFα) were reported to enhance the disease. On the other hand, Th1-associated cytokines; IL12, IL18 and interferon γ (IFNγ) were reported to

reduce the symptoms of asthma in asthmatic patients (31). Additionally, subjects with asthma were reported to have reduced levels of IL10 in the sputum. IL10 is an anti-inflammatory cytokine produced by macrophages and by a subset of Tregs and exerts its effect by inhibiting the synthesis of inflammatory cytokines (including asthma-associated cytokines such as TNFα and IL5) and gene presentation [44]. Th2 cells play a key role in the etiology of asthma, and Th1/Th2 imbalances as well as disturbed T helper type-17(Th17)/Treg balances were reported in asthmatic patients [45]. Imbalances in Th responses can also be detected using Th-specific transcription factors: T-bet for Th1 cells, GATA3 for Th2 cells, retinoic acid orphan receptor γt (RORγt) for Th17 cells and FOXP3 for Tregs [46]. Alterations in the expression and/or function of these transcription factors were associated with asthma pathogenesis [47, 48].

Interestingly, a gut-lung axis of probiotic action has been proposed, and it was suggested that probiotics might induce Tregs in the gut-associated lymphoid tissue (GALT) that can, subsequently, spread to the airways upon allergen challenge and inflammation [49]. This idea was supported by the findings that the bacterial extracts, Broncho-Vaxom, were able to prevent asthma in mice via the recruitment of Tregs to the airways [1] and that *Lactobacillus* species regulated the immune response in mice by Treg-mediated mechanisms [50]. Tregs are important in balancing immune responses and maintaining immunological tolerance to foreign and self-antigens, including allergens [44].

Despite the increasing number of animal studies, beneficial bacteria research in asthma patients is still in its infacy. To dat, more research has been conducted to investigate the preventive effects of beneficial bacteria rather than the therapeutic effects. The aim of this current study is to explore the therapeutic effects of long-term administration of *Bifidobacterium breve* and *Lactobacillus rhamnosus* on the inflammatory response in mice by investigation of the mRNA expression profiles of the different PRRs, T cell-related cytokines and transcription factors; and by exploring Foxp3 expression in blood Th cells. To mimic the continued exposure in asthmatic patients, we used the murine ovalbumin-induced asthma model combined with prolonged allergen exposure. A glucocorticoid (budesonide) was used as a reference treatment. Findings from this study could contribute to a better understanding of the immunomodulatory and therapeutic effects of beneficial bacteria in allergic asthma.

Material and Methods

Animals

Male BALB/c mice (6-8 weeks; Charles River Laboratories, France) were 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.

Chronic asthma model

OVA sensitisation

Sensitisations were performed on days 0 and 12. Mice were sensitised 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 (AlumImject; Pierce, Rockford, IL, USA). Control animals received 0.1 mL saline only (NaCl 0.9%; B. Braun Medical B.V., Oss, The Netherlands) (Figure 1).

OVA challenge

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

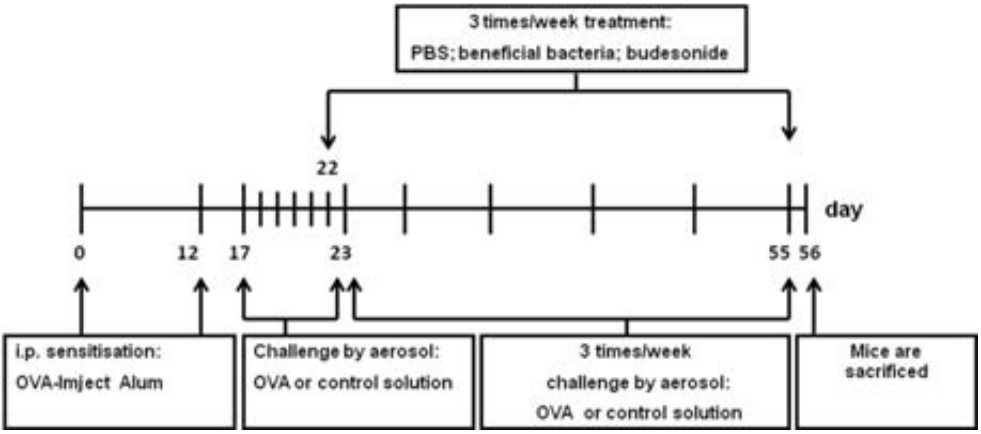

Figure 1: Time schedule of the chronic asthma mouse model. Male BALB/c mice were sensitised intraperitoneally with alum-precipitated OVA on days 0 and 12 and mice were challenged from day 17 until day 23 daily with aerosolised OVA or saline. From day 22 until day 55, mice were treated 3 times a week with either PBS or budesonide by oropharyngeal aspiration or beneficial bacteria (*B. breve or L. rhamnosus*) by oral gavage. 1h after treatment, from day 24 until day 55, mice were challenged 3 times/week with aerosolised OVA or saline. Mice were sacrificed on day 56 after pulmonary function measurement.

Beneficial bacteria treatment

Bifidobacterium breve M-16V (*B. breve*, Morinaga Milk Industry, Tokyo, Japan) and *Lactobacillus rhamnosus* NutRes1 (*L. rhamnosus*, Danone Research, Wageningen, the Netherlands) were grown in MRS (Oxoid, Basingstoke, UK), supplemented with 0.5 g/L L-cysteine for *Bifidobacteria*, at pH 6.5 and under anaerobic conditions. Bacteria were harvested in the early stationary phase, washed with phosphate buffered saline (PBS, Lonza Leusden, The Netherlands) and stored with glycerol 20% (w/v), in aliquots at -80°C. Cell counts were determined by plating serial dilutions and fluorescent microscopy by staining with DAPI. The bacteria were resuspended in PBS prior to use.

After development of airway inflammation, mice received 10^9 colony forming units (CFUs) of *B. breve* or $1.1x10^9$ CFUs of *L. rhamnosus* per animal per day. Bacterial strains were suspended in 0.2 mL of PBS and given by oral gavage, 1 h prior to challenge, three times a week from day 22 until day 55 (Figure 1).

Budesonide treatment

As a reference treatment, mice received 0.5 μg/g of mouse/day of budesonide (Sigma) in PBS. Budesonide was administered to mice by oropharyngeal aspiration after induction of light isoflurane anesthesia as described previously in [51], 1 h prior challenge, three times a week from day 22 until day 55. Control animals received 50 uL of PBS by the same administration route. Mice were rendered asthmatic following the schema presented in Figure 1.

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 (400g, 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.

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 94ºC for 3 min, 94ºC for 10

sec, specific melt temperature for 45 sec, followed by 39 cycles of 94ºC for 10 sec and 95ºC for 10 sec.

Normalised 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).

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 then washed 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), ethylenediaminetetraacetic acid (EDTA, MERCK) in demineralised 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+CD25+Foxp3+ T cells. The stained cells were analysed on a FACSCanto II flow cytometer (BD Biosciences, USA). Data analysis was performed using BD FACSDiva™ software (BD Biosciences).

Statistical analysis

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

Results

B. breve and *L. rhamnosus* are as effective as budesonide in reducing pulmonary inflammation in chronically asthmatic mice

To gauge the extent of inflammation in the asthmatic mice after treatments, bronchoalveolar lavage (BAL) fluid was examined for leukocyte accumulation (Table 1). OVA/OVA-PBS mice showed a significant increase in the total inflammatory cell number (155.25 $x10^4$ ± 12.58; n=6; $p<0.0001$) in the BAL fluid, which was due to a relative increase in the number of macrophages, eosinophils and neutrophils as compared to OVA/Sal-PBS mice (29.25 $x10^4$ ± 3.12; n=6). All treatments significantly reduced the total numbers of inflammatory cells in the BAL fluid of OVA/OVA mice. Budesonide treatment significantly decreased the total inflammatory cell number (83.70 $x10^4$ ± 13.66; n=6; $p<0.0001$) and relative number of eosinophils and neutrophils in OVA/OVA-BUD mice. *B. breve* treatment, however, further decreased the total inflammatory cell number (73.50 $x10^4$ ± 6.42, n=6; $p<0.0001$) and significantly decreased the relative number of eosinophils and neutrophils. *L. rhamnosus* treatment significantly decreased the total inflammatory cell number (79.75 $x10^4$ ± 17.49; n=6; $p<0.0001$) and relative number of neutrophils.

Table 1: Differential BAL fluid cell counts

Group	% Macrophages		% Lymphocytes		% Eosinophils		% Neutrophils	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM
OVA/Sal- PBS	91.40	9.87	1.92	0.41	5.99	0.92	0.68	0.39
OVA/OVA-PBS	29.96$^{\#}$	3.24	0.96	0.38	41.18$^{\#}$	4.57	27.90$^{\#}$	3.09
OVA/Sal- *B. breve*	95.34	24.83	4.50	1.26	0.16	0.10	0.00	0.00
OVA/OVA- *B. breve*	53.47	6.18	4.90	1.46	22.63$^{¤,\ddagger}$	3.23	19.0$^{¤,\ddagger}$	1.64
OVA/Sal-BUD	97.00	25.54	2.84	0.53	0.16	0.16	0.00	0.00
OVA/OVA-BUD	67.0	13.00	1.12	0.59	20.39$^{¤,\ddagger}$	1.92	9.50$^{¤,\ddagger}$	2.32
OVA/Sal- *L. rhamnosus*	93.85	29.84	4.08	2.33	0.53	0.29	0.00	0.00
OVA/OVA- *L. rhamnosus*	43.65	11.04	3.71	2.11	37.88‡	8.52	14.76$^{¤,\ddagger}$	5.30

Pulmonary inflammation represented by the influx of specific leukocytes in bronchoalveolar lavage (BAL) fluid. Differential cell counts are shown as percentages of the total cell count for each group. Mice in all groups were sensitised 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, and the different test groups were treated with *B. breve* (OVA/Sal-*B. breve*; OVA/OVA-*B. breve*), *L. rhamnosus* (OVA/OVA-*L. rhamnosus*; OVA/OVA-*L. rhamnosus*) or budesonide (BUD, OVA/Sal-BUD; OVA/OVA-BUD). The table indicates the mean ± SEM, n=6 mice/group. Statistical significance of differences was tested using post hoc Bonferronis' 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. ‡ Statistically significant difference ($p<0.05$) compared to OVA/Sal mice in each treatment group.

B. breve and *L. rhamnosus* modulate TLR and NLR mRNA expression in lung tissue of chronically asthmatic mice

As PRRs in the lung can modulate ongoing chronic inflammation during asthma, the mRNA expression of *Tlr1-9* and *Nod1-2* were measured (Figure 2). The mRNA expression of *Tlr3* and *Nod1* was significantly decreased and *Tlr9* was also decreased ($p>0.05$) in OVA/OVA-PBS mice as compared to the OVA/Sal-PBS group (Figure 2A). After *B. breve* treatment, the OVA/OVA-*B. breve* mice showed a significant increase in *Tlr9* expression compared to the OVA/OVA-PBS group. In non-asthmatic controls, *Tlr1 and Tlr2* expression was significantly increased in the OVA-Sal-*B. breve* group as compared to OVA/Sal-PBS mice, yet, the expression of the other PRRs in non-asthmatic controls remained unchanged. *B.breve* treatment in asthmatic mice significantly increased *Tlr9* expression and decreased *Tlr2, Tlr3, Tlr5* and *Nod1* expression in the OVA/OVA-*B. breve* group as compared to the OVA/Sal-*B. breve* group (Figure 2B). Mice in the OVA/OVA-BUD group showed significant increases in the expression of *Tlr3* and *Nod1* and *Tlr9* expression was higher ($p>0.05$) as compared to the OVA/OVA-PBS group. The expression of *Tlr3* and *Tlr5* was significantly decreased in OVA/Sal-BUD mice as compared to the OVA/Sal-PBS group (Figure 2C). The OVA/OVA-*L. rhamnosus* group showed a significant decrease in *Tlr4*, an increase in *Tlr3* and higher *Tlr9* expression compared to OVA/OVA-PBS mice. The mRNA expression of *Tlr1*, *Tlr2*, *Tlr4*, *Tlr6, Nod1* and *Nod2* was significantly decreased in OVA/OVA- *L. rhamnosus* mice as compared to the OVA/Sal-*L. rhamnosus* group (Figure 2D).

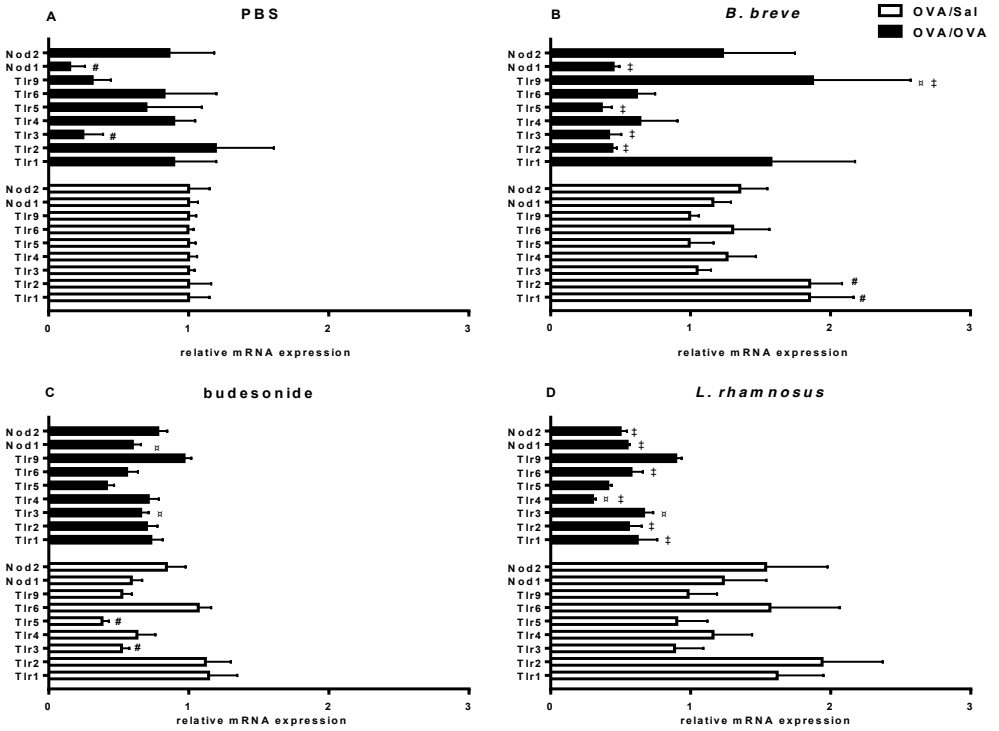

Figure 2: Beneficial bacteria modulate TLR and NLR mRNA expression in lung tissue of chronically asthmatic mice. Data is shown as mean ± SEM, n=6 mice/group**,** of the TLR and NLR mRNA expression levels in OVA-sensitised, Sal-challenged (OVA/Sal; white bars) mice and OVA-sensitised, OVA-challenged (OVA/OVA; black bars) mice treated with PBS (A), *B. breve* (B), budesonide (C) or *L. rhamnosus* (D). The results are presented as mRNA expression levels relative to levels found in the OVA/Sal-PBS mice (white bars in A). Statistical significance of differences was tested using the 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. ‡ Statistically significant difference ($p<0.05$) compared to the OVA/Sal group in each treatment group.

Beneficial bacteria and budesonide modulate cytokine mRNA expression in lung tissue of chronically asthmatic mice

To determine the extent of inflammation and the Th response in the lung, the mRNA expression of various cytokines was measured (Figure 3). The mRNA expression of *Il1β* and *Il6* was significantly increased and *Il13* and *Il17* expression was also increased ($p>0.05$) in OVA/OVA-

PBS mice as compared to the OVA/Sal-PBS group (Figure 3A). The OVA/OVA-*B. breve* group showed a significant increase in *Il4* and *Il10* expression as compared to the OVA/OVA-PBS group. Expression of *Il1β* and *Il6* was significantly increased in OVA/Sal-*B. breve* mice as compared to the OVA/Sal-PBS group. The mRNA expression of these two cytokines was significantly decreased in OVA/OVA-*B. breve* mice as compared to the OVA/Sal-*B. breve* group (Figure 3B). The OVA/OVA-BUD group showed a significant decrease in the expression of *Tnfα* as compared to OVA/OVA-PBS mice. Expression of *Il13* was significantly increased and *Tgfβ* expression was significantly decreased in OVA/Sal-BUD mice as compared to the OVA/Sal-PBS group. The mRNA expression of *Il23* and *Tgfβ* was significantly increased in OVA/OVA-BUD mice as compared to the OVA/Sal-BUD group (Figure 3C). *L. rhamnosus* treatment significantly decreased the expression of *Il6* in OVA/OVA-*L. rhamnosus* mice as compared to the OVA/OVA-PBS group. The expression of *Il1β, Il6* and *Ifnγ was* significantly increased and *Tgfβ* expression was significantly decreased in OVA/Sal-*L. rhamnosus* mice compared to the OVA/Sal-PBS group. The mRNA expression of *Il1β* and *Il6* was significantly decreased in OVA/OVA- *L. rhamnosus* mice as compared to the OVA/Sal-*L. rhamnosus* group (Figure 3D).

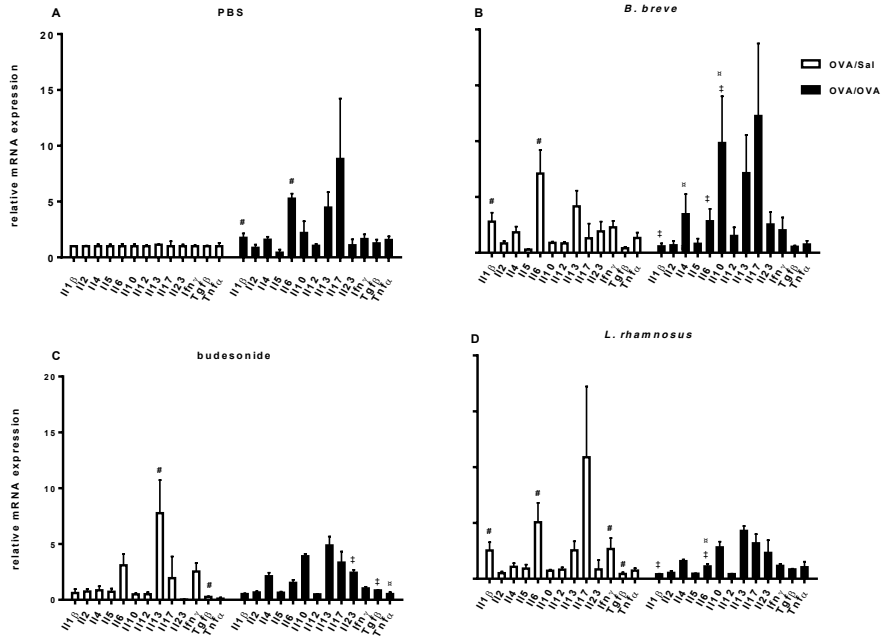

Figure 3: Beneficial bacteria and budesonide modulate cytokine mRNA expression in lung tissue of chronically asthmatic mice. Data is shown as mean ± SEM, n=6 mice/group**,** of the cytokine mRNA expression levels in OVA-sensitised, Sal-challenged (OVA/Sal; white bars) mice and OVA-sensitised, OVA-challenged (OVA/OVA; black bars) mice treated with PBS (A), *B. breve* (B), budesonide (C) or *L. rhamnosus* (D). The results are presented as mRNA expression levels relative levels found in the OVA/Sal-PBS mice (white bars in Figure A). Statistical significance of differences was tested using the 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. ‡ Statistically significant difference ($p<0.05$) compared to the OVA/Sal group in each treatment group.

B. breve treatment results in a strong up-regulation of mRNA for *Tbet* and *Foxp3* and relevant Th cytokines mRNA expression in the lungs of chronically asthmatic mice

To further explore the effects of the different treatments on Th responses in the lung, the mRNA expression of Th-specific transcription factors was measured (Figure 4). The expression of Th1-(*Tbet*) and Treg-(*Foxp3*) transcription factors was significantly decreased in the OVA/OVA-PBS group as compared to the OVA/Sal-PBS group. No correlations were found between the expression of these transcription factors and relevant Th cytokines. The expression of Th2-(*Gata3*), and Th17-(*Rorγt*) transcription factors remained unchanged (Figure 4A). However, *Tbet* and *Foxp3* expression was significantly increased in OVA/OVA-B. *breve* mice as compared to the OVA/OVA-PBS group. Interestingly, expression of *Tbet* was tightly correlated with *Ifnγ* (R^2=0.903; p=0.013) and *Il12* (R^2=0.994; p=0.003) and expression of *Foxp3* was tightly correlated with *Il10* (R^2=0.860; p=0.024) expression in whole lung tissue of OVA/OVA-*B. breve* mice. Compared to the OVA/Sal-PBS group, *Foxp3* expression was almost two-fold increased (P>0.05) in the OVA/Sal-*B. breve* group. Expression of *Tbet* was significantly increased and expression of *Gata3* was significantly decreased in OVA/OVA-B. *breve* mice as compared to the OVA/Sal-*B. breve* group (Figure 4B). A significant increase in the expression of *Foxp3* was observed in the OVA/OVA-BUD group as compared to OVA/OVA-PBS mice (Figure 4C). The expression of all Th-specific transcription factors remained unchanged in the OVA/Sal-*L. rhamnosus* and OVA/OVA-*L. rhamnosus* groups (Figure 4D). Expression of *Foxp3,* however, was tightly correlated with *Il10* expression (R^2=0.926; p=0.009) in OVA/OVA-*L. rhamnosus* mice. None of the test groups showed correlations between the expression of *Gata3* and Th2-related cytokines (*Il4, Il5, Il13*) and *Rorγt* and Th17-related cytokines (*Il17, Il23*).

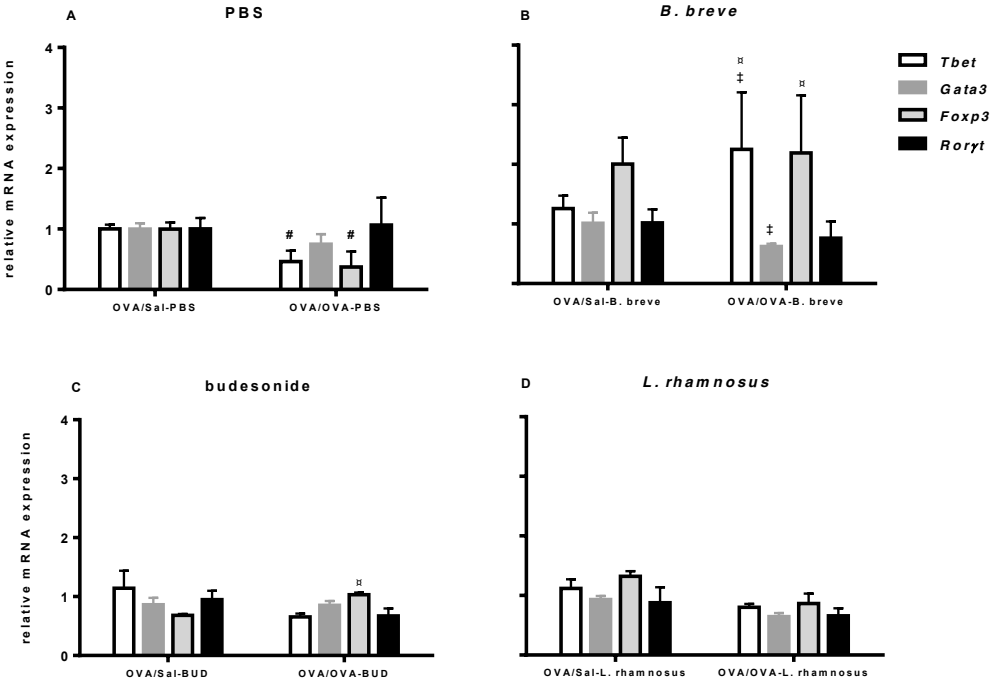

Figure 4: *B. breve* treatment up-regulates *Tbet* and *Foxp3* mRNA expression in lungs of chronically asthmatic mice. Data is shown as mean ± SEM, n=6 mice/group**,** of the transcription factor mRNA expression levels in OVA-sensitised, Sal-challenged (OVA/Sal) mice and OVA-sensitised, OVA-challenged (OVA/OVA) mice treated with PBS (A), *B. breve* (B), budesonide (C) or *L. rhamnosus* (D). The results are presented as mRNA expression levels relative to levels found in the OVA/Sal-PBS mice (white bars in A). Statistical significance of differences was tested using the 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. ‡ Statistically significant difference ($p<0.05$) compared to the OVA/Sal group in each treatment group.

B. breve skews the immune response away from Th2 and towards Treg in the lungs of chronically asthmatic mice

To determine the extent of Th response skewing in the lung, ratios for *Gata3/Tbet* (Th2/Th1), *Foxp3/Rorγt* (Treg/Th17), *Foxp3/Gata3* (Treg/Th2) and *Foxp3/Tbet* (Treg/Th1) mRNA

expression were calculated (Table 2). The OVA/OVA-PBS groups showed a Th2-skewed immune response represented by a significant increase in *Gata3/Tbet* ratio as compared to OVA/Sal-PBS mice. This ratio was significantly decreased in the OVA/OVA-*L. rhamnosus* and OVA/OVA-*B. breve* groups, but not the OVA/OVA-BUD group. The Th2-reducing effect of OVA-OVA-*B. breve* was even more effective in challenged mice than healthy mice as the *Gata3/Tbet* ratio was decreased in OVA/OVA-*B. breve* mice as compared to the OVA/Sal-*B. breve* group. This was not the case for budesonide. Compared to the OVA/Sal-BUD group, there was a significant increase in the *Gata3/Tbet* ratio in the OVA/OVA-BUD group. Interestingly, the *Foxp3/Rorγt* and *Foxp3/Gata3* ratios were significantly increased in OVA/OVA- *B. breve* mice as compared to the OVA/OVA-PBS group indicating an increase in Treg-associated responses. The ratio of *Foxp3/Tbet* did not differ significantly among the different treatment groups.

Beneficial bacteria and budesonide increase Foxp3 expression in blood Treg cells of chronically 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 employed to examine Foxp3 expression on Treg cells in the blood (Figure 5). Treg cells were identified as CD4+CD25+Foxp3+ cells and the magnitude of Foxp3 expression on each cell was determined by examining the mean fluorescence intensity (MFI) of the Foxp3 staining. All treatments increased the Foxp3 expression in the blood Treg cells. The staining intensity of Foxp3 on day 56 was significantly higher in the OVA/OVA-*B. breve*, OVA/OVA-BUD and OVA/OVA-*L. rhamnosus* groups as compared to both OVA/Sal-PBS and OVA/OVA-PBS groups. The staining intensity of Foxp3 on day 0 (before sensitisation, challenge and treatment) did not differ among the different groups (data not shown).

Table 2: *B. breve* skews the immune response away from Th2 and towards Treg in lungs of chronically asthmatic mice

Treatment	Ratio	OVA/Sal		OVA/OVA	
		mean ratio	SEM	mean ratio	SEM
PBS	*Gata3/Tbet*	1.03	0.10	1.66[#]	0.37
	Foxp3/Rorγt	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
B. breve	*Gata3/Tbet*	0.83	0.14	0.29[¤, ‡]	0.02
	Foxp3/Rorγt	1.62	0.36	2.36[¤]	1.04
	Foxp3/Gata3	1.65	0.36	2.92[¤]	1.29
	Foxp3/Tbet	1.36	0.30	0.83	0.37
budesonide	*Gata3/Tbet*	0.78	0.10	1.34[‡]	0.11
	Foxp3/Rorγt	0.59	0.02	1.26	0.04
	Foxp3/Gata3	0.66	0.02	1.00	0.03
	Foxp3/Tbet	0.51	0.02	1.35	0.04
L. rhamnosus	*Gata3/Tbet*	0.86	0.06	0.83[¤]	0.08
	Foxp3/Rorγt	1.02	0.22	1.07	0.21
	Foxp3/Gata3	0.98	0.21	1.11	0.20
	Foxp3/Tbet	0.84	0.18	0.92	0.18

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. [‡] Statistically significant difference ($p<0.05$) compared to OVA/Sal mice in each treatment group.

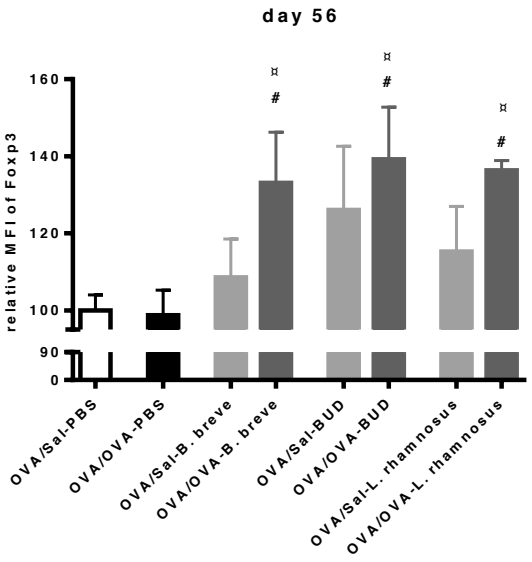

Figure 5: Beneficial bacteria and budesonide increase Foxp3 expression in blood Treg cells of chronically asthmatic mice. Data is shown as mean fluorescence intensity (MFI) of Foxp3 in Treg cells isolated from mouse whole blood on day 56 in OVA-sensitised, Sal-challenged (OVA/Sal) mice and OVA-sensitised, OVA-challenged (OVA/OVA) mice for each treatment. Data is shown as mean ± SEM, n=6 mice/group, and the results are presented as MFI levels relative to the OVA/Sal-PBS group (white bar). Statistical significance of differences was tested using the 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.

Discussion

The aim of this study was to investigate the effects of two different beneficial bacterial strains (*B. breve* and *L. rhamnosus)* and a reference treatment (budesonide) on features of the inflammatory response in a murine model of chronic asthma. The total inflammatory cell number and individual BAL fluid cell counts, except lymphocytes, were significantly increased in OVA/OVA-PBS mice as compared to the OVA/Sal-PBS group. Interestingly, all treatments were effective in suppressing cellular infiltration into the airways, significantly decreasing the total inflammatory cell number, particularly eosinophils and neutrophils in BAL fluid as compared to the OVA/OVA-PBS group. These findings are in accordance with other studies in which beneficial bacteria and budesonide have been individually investigated [1, 11, 21, 22, 24, 26, 50]. This study is the first, however, to examine the therapeutic effects of *B. breve*, *L. rhamnosus* and budesonide on various immune parameters in a murine model of chronic asthma. To our knowledge, we demonstrate here for the first time that beneficial bacteria, especially *B. breve*, are as effective as budesonide in reducing chronic allergic inflammation by attenuating the total inflammatory cell number as well as individual BAL fluid cell counts and skewing the immune response towards Treg after prolonged allergen exposure.

PRRs are key components of the innate immunity which are also involved in the activation and shaping of adaptive immunity. The function and expression of PRRs was linked to susceptibility towards allergic asthma. The mRNA expression of *Tlr3* and *Nod1* was significantly decreased in the lungs of OVA/OVA-PBS mice. Previous *in vitro* studies have demonstrated that upon activation by its natural or endogenous ligands, *Tlr3* induces up-regulation of other TLRs, various cytokines and chemokines as well as its own expression and thereby contributes to exacerbation of inflammation [42]. NOD1 is an intracellular sensor of pathogenic bacteria. Single nucleotide polymorphisms in *NOD1* gene were positively associated with susceptibility towards asthma in farming children and this PRR was reported to be necessary for neutrophil function in mice [34-37]. However, no direct associations between *Tlr3* and *Nod1* expression and function and asthma have been reported yet, and whether the decreased mRNA expression of *Tlr3* and *Nod1* caused by the chronic inflammatory status of the animals is pro-inflammatory or anti-inflammatory is also unknown. Remarkably, *B. breve* treatment significantly increased *Tlr9* expression, suggesting that this strain beneficially regulates the immune reponse via a TLR9-mediated mechanism. Since TLR9 was shown to exert its immunomodulatory effects on asthma

by skewing the increased Th2/Th1 balance towards Th1, this may very well be the situation [39-41]. Budesonide treatment significantly increased the expression of *Tlr3* and *Nod1,* restoring them to normal levels, and raised *Tlr9* (P>0.05) expression. This further supports a positive role for *Tlr9* and suggests that lowered *Tlr3* and *Nod1* measured in the asthmatic mice is pro-inflammatory in nature [35, 37, 42]. *L. rhamnosus*, besides significantly increasing *Tlr3* expression and increasing the expression of *Tlr9* expression (P>0.05), significantly decreased the expression of *Tlr4*. Asthma patients have been shown to have low expression of *TLR4* on their monocytes, lymphocytes and DCs suggesting that this reduction in TLR4 activation might contribute to the disease by reducing the release of Th1 and anti-inflammatory cytokines [52]. These findings might suggest that *L. rhamnosus* treatment in asthmatic mice could not restore the expression of *Tlr4* to its normal level. Taken together, we demonstrate here that the mRNA expression of PRRs in mouse lung tissue is differentially regulated by the different treatments. Yet, all treatments, especially *B. breve*, increased the expression of *Tlr9*.

Th2 cells play a key role in the pathogenesis of allergic asthma, and asthmatic patients were reported to have Th1/Th2 imbalances as well as disturbed T helper type-17(Th17)/Treg balances. Th2 dominance was observed in the OVA/OVA-PBS group represented by a significant decrease in Th1 and Treg transcription factors and high *Gata3*/*Tbet* ratio. Hence, our model mimics the Th2-responses found in chronic asthma [1]. Importantly, *B. breve* shifted the immune balance towards Th1 and Treg, with significantly increased *Foxp3*/*Rorγt* and *Foxp3*/*Gata3* ratios and a significantly decreased *Gata3*/*Tbet* ratio. A different strain of *Bifidobacterium*, *B. animalis*, has been already shown to skew the Th1/Th2 balance towards Th1 in a preventative, acute mouse model for respiratory allergy [25]. Budesonide showed only a moderate effect and significantly increased the expression of *Foxp3,* but did not affect the *Gata3/Tbet, Foxp3/Rorγt, Foxp3/Gata3* and *Foxp3*/*Tbet* ratios. These findings are consistent with results of other studies which have shown that GCs treatment of asthmatic subjects encourages regulatory responses [10]. *L. rhamnosus* did not influence the mRNA expression of the different Th-specific transcription factors, but significantly decreased the *Gata3*/*Tbet* ratio. More importantly, the above observed effects of beneficial bacteria and budesonide on the Th responses in the lung are mirrored by the detection of high Foxp3-expressing Treg cells in the blood of treated animals. Treg cells play a key role in balancing immune responses and it was demonstrated that an increased expression of Foxp3 in Treg cells is directly associated with increased function of these cells [44, 53].

Expression of the pro-inflammatory cytokines *Il1β* and *Il6* was significantly increased in the OVA/OVA-PBS group and expression of both *Il13* and *Il17* was also increased but not significantly. These four cytokines were reported to enhance asthma [31]. *B. breve* treatment, however, significantly increased the expression of the anti-inflammatory cytokine *Il10* and the Th2-associated cytokine *Il4,* yet, the mRNA expression of *Gata3* was decreased. Interestingly, the expression of *Tbet* was tightly correlated with *Ifnγ* and *Il12*, while *Foxp3* expression was tightly correlated with *Il10* expression. It has been reported that IL12 is involved in the differentiation of Th1 cells and that these cells suppress Th2 cells through the release of IFNγ. Additionally, Treg cells suppress other Th cell effector functions through the release of IL10 [31]. Budesonide treatment significantly decreased the expression of the pro-inflammatory cytokine *Tnfα* which is in agreement with previously published studies demonstrating the anti-inflammatory effects of budesonide [6]. *L. rhamnosus* treatment significantly decreased the expression of *Il6,* another highly pro-inflammatory cytokine. The mRNA expression of *Foxp3* was tightly correlated with *Il10* expression.

To our knowledge, this is the first report in which the therapeutic effects of long-term treatment with *B. breve* and *L. rhamnosus* and budesonide on inflammatory response are investigated in a murine OVA-induced chronic allergic asthma model. We show here that pulmonary airway inflammation and mRNA expression of PRRs, T helper-specific cytokines and transcription factors are differentially modulated by the different treatments. More importantly, *B. breve* was the most potent inducer of regulatory responses, increasing *Il10* and *Foxp3* transcription in lung tissue and augmenting the mean fluorescence intensity of Foxp3 in blood CD4+ T cells. Our current findings show that beneficial bacteria, especially *B. breve,* may be beneficial in the management of chronic asthma.

Aknowledgments

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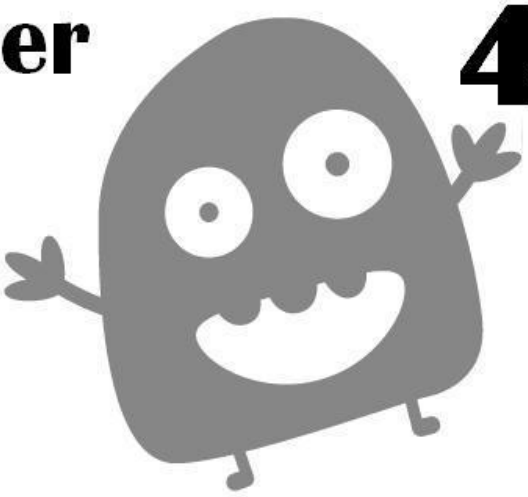
Supplementary Table 1: Forward and reverse primers used for quantitative real-time PCR analysis

Molecule	Forward primer	Reverse primer
Tlr1	GGTGTTAGGAGATGCTTATGGGG	GATGTTAGACAGTTCCAAACCGA
Tlr2	CCAGACACTGGGGGTAACATC	CGGATCGACTTTAGACTTTGGG
Tlr3	GGGGTCCAACTGGAGAACCT	CCGGGGAGAACTCTTTAAGTGG
Tlr4	GCCTTTCAGGGAATTAAGCTCC	AGATCAACCGATGGACGTGTAA
Tlr5	TCAGACGGCAGGATAGCCTT	AATGGTCAAGTTAGCATACTGGG
Tlr6	GACTCTCCCACAACAGGATACG	TCAGGTTGCCAAATTCCTTACAC
Tlr9	ACTCCGACTTCGTCCACCT	GGCTCAATGGTCATGTGGCA
Nod1	GAAGGCACCCCATTGGGTT	AATCTCTGCATCTTCGGCTGA
Nod2	CCGCTTTCTACTTGGCTGTC	GTGATTTGCAGGTTGTGTGG
Tbet	GCCAGCCAAACAGAGAAGAC	AAATGTGCACCCTTCAAACC
Gata3	GCGGTACCTGTCTTTTTCGT	CACACAGGGGCTAACAGTCA
Foxp3	CACTGGGCTTCTGGGTATGT	AGACAGGCCAGGGGATAGTT
Roryt	TGCAAGACTCATCGACAAGG	AGGGGATTCAACATCAGTGC
RPS13	GTCCGAAAGCACCTTGAGAG	AGCAGAGGCTGTGGATGACT

Chapter 4



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

Manuscript submitted

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Abstract

Background: 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.

Methods: 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 cells degranulation; and airway remodeling were examined.

Results: The combination of *Bifidobacterium 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.

Conclusion: 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.

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, subsequent, 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 cells 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 corticosteroids 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].

Animal studies have demonstrated a substantial influence of the gut microbiotica on immune function beyond the gut and 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 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 has 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)* and 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. Interleukin1β (IL1β), IL4, IL5, IL6, IL9, IL13, IL17, IL25 and tumor necrosis factor α (TNFα) were reported to enhance the disease. On the other hand, IL12, IL18 and interferonγ (IFNγ) were reported to reduce the symptoms of asthma in asthmatic patients [30]. Reduced levels of the anti-inflammatory cytokine IL10 were found in the sputum of asthma patients. IL10 is produced by alternatively activated macrophages and by a subset of regulatory T cells (Tregs) and exerts its effect by inhibiting the synthesis of inflammatory cytokines (including asthma-associated cytokines such as TNFα and IL5) 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, GATA3 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].

Despite the increasing number of animal studies analyzing the role of beneficial bacteria, with or without non-digestible oligosaccharides, interventions in allergy and related inflammatory conditions, 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 *Bifidobacterium 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.

Material and Methods

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.

Chronic asthma model

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 (AlumImject; Pierce, Rockford, IL, USA). Control animals received 0.1 mL saline only (NaCl 0.9%; B. Braun Medical B.V., Oss, The Netherlands) (Figure 1).

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 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 were exposed to nebulized saline aerosol only. 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 only for 20 min (Figure 1).

Bb/scFOS/lsFOS/AOS treatment

The combination consisted of 10^9 colony forming units (CFUs) of lyophilized *Bifidobacterium breve* M-16V powder with maltodextin 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 challenge, three times a week from day 22 until day 55. Mice were rendered asthmatic following the schema presented in Figure 1.

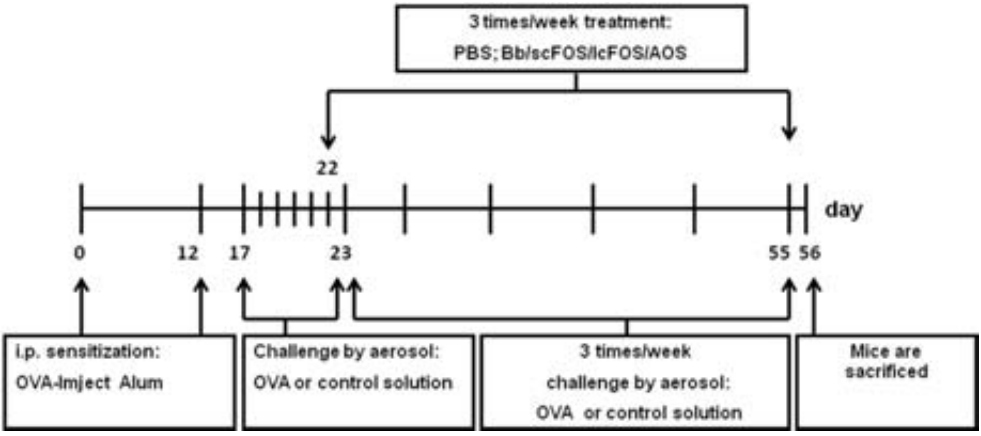

Figure 1: Time schedule of the chronic asthma mouse model. Male BALB/c mice were sensitized intraperitoneally with alum-precipitated OVA on days 0 and 12 and mice were challenged from day 17 until day 23 daily with aerosolized OVA or saline. From day 22 until day 55, mice were treated 3 times a week 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.

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.

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, RT² qPCR Primer Assays (SABiosciences, Venlo, The Netherlands) were used. The protocol used for amplification was 94ºC for 3 min, 94ºC for 10 sec, specific melt temperature for 45 sec, followed by 39 cycles of 94ºC for 10 sec and 95ºC for 10 sec.

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).

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), 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).

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 was determined using a Beckman Z1 coulter® Particle Counter (Beckman, USA). TLN cells (4 x 10^6 cells/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 medium with or without O/N pre-coating 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% CO2 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 Bisosciences).

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.

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 the paraffin 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 on the basis of the percentage of positive stained cells in the following way: -,no positive staining; +/-, less than 25% of cells stained positive; +,25 to 50% cells stained positive; ++, 50 to 75% cells stained positive.

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).

Results

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, bronchoalveolar lavage (BAL) fluid was examined for leukocyte accumulation (Figure 2). The total inflammatory cell number in the BAL fluid of OVA/OVA-PBS mice was significantly increased (Figure 2A), which was due to a relative increase in the number of eosinophils and neutrophils (Figure 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 (Figure 2D). Bb/scFOS/lcFOS/AOS treatment, however, significantly reduced the total number of inflammatory cells (Figure 2A) and relative number of eosinophils (Figure 2B) as compared to OVA/OVA-PBS mice. The relative numbers of neutrophils, macrophages and lymphocytes (Figure 2C, D and E) remained unchanged.

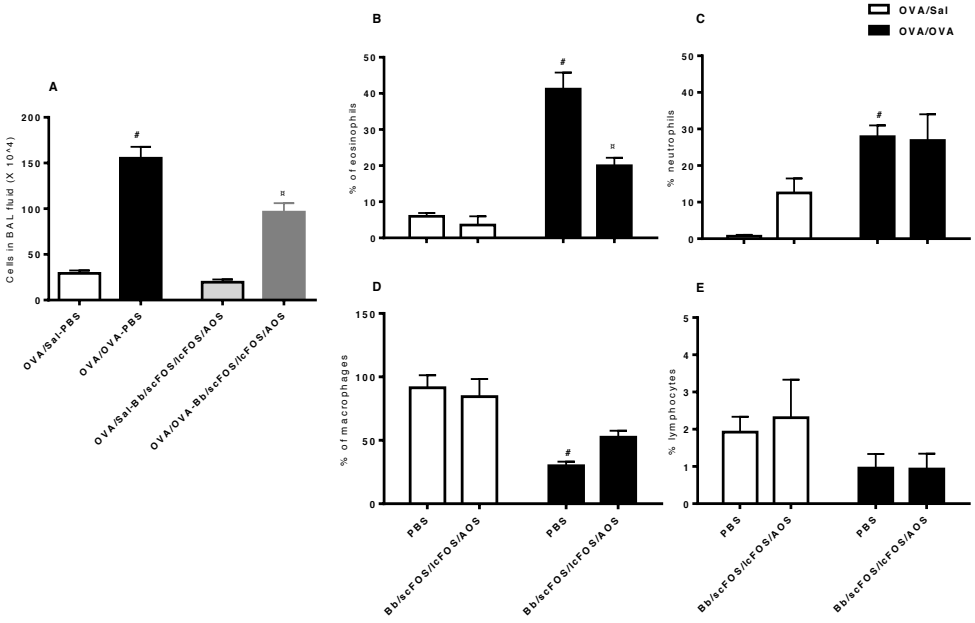

Figure 2: Total and Differential BAL fluid cell counts. Pulmonary inflammation represented by the influx of specific leukocytes in BAL fluid (B-E). Differential cell counts are shown as percentages of the total cell count for

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. 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.

Bb/scFOS/lcFOS/AOS treatment modulates PRRs 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 (Figure 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 (Figure 3A). Interestingly, Bb/scFOS/lcFOS/AOS treatment prevented the decrease in*Tlr3*, *Tlr9* and *Nod1* expression (Figure 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 (Figure 3B).

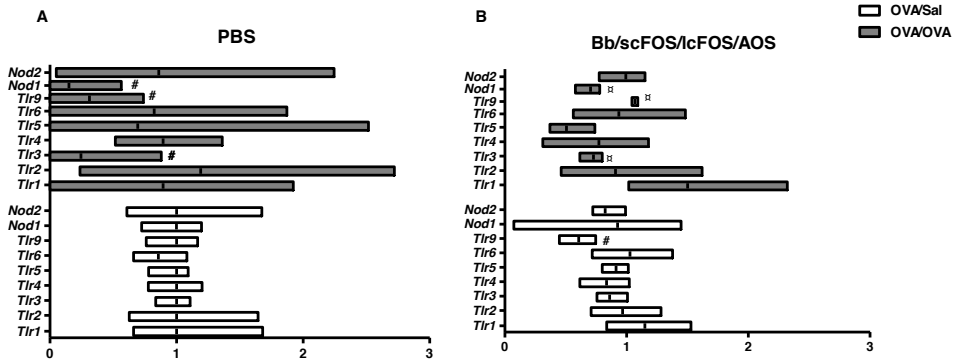

Figure 3: Relative TLR and NLR mRNA expression in mouse whole lung tissue during chronic asthma. The bars indicate the min to max values and the line represents the mean, n=6 mice/group**,** of the TLR and NLR mRNA expression levels in OVA-sensitized, saline-challenged (OVA/Sal, white bars) mice and OVA-sensitized, OVA-challenged (OVA/OVA, grey bars) mice treated with PBS or Bb/scFOS/lcFOS/AOS. 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.

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 (Figure 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 (Figure 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 (Figure 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.

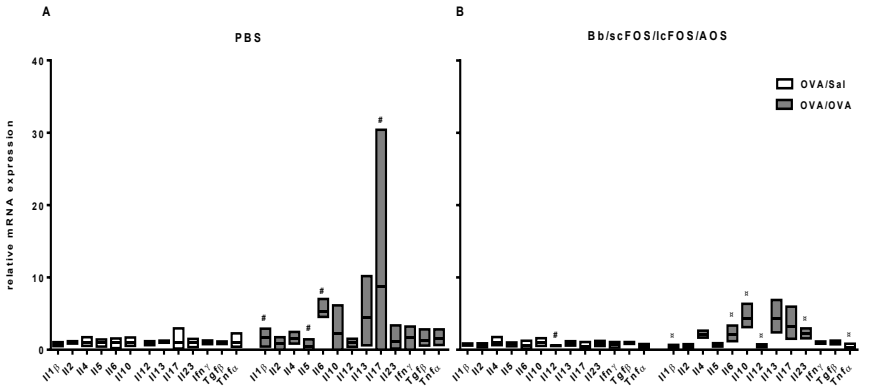

Figure 4: Relative cytokine mRNA expression in mouse whole lung tissue during chronic asthma. The bars indicate the min to max values and the line represents the mean, n=6 mice/group**,** of the cytokine mRNA expression levels in OVA-sensitized, saline-challenged (OVA/Sal, white bars) mice and OVA-sensitized, OVA-challenged (OVA/OVA, grey bars) mice treated with PBS or Bb/scFOS/lcFOS/AOS. 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.

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 (Figure 5) and ratios for *Gata3*/*Tbet*(Th2/Th1), Foxp3/Rorγt(Treg/Th17), *Foxp3*/*Gata3* (Treg/Th2) and *Foxp3*/*Tbet*(Treg/Th1) mRNA expression were 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 (Figure 5A). The expression of Th2-(*Gata3*), and Th17-(*Rorγt*) transcription factors remained unchanged (Figure 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 (Figure 5B) which led to significant increases in the *Foxp3*/*Rorγt* and *Foxp3*/*Gata3* ratios (Table 1).

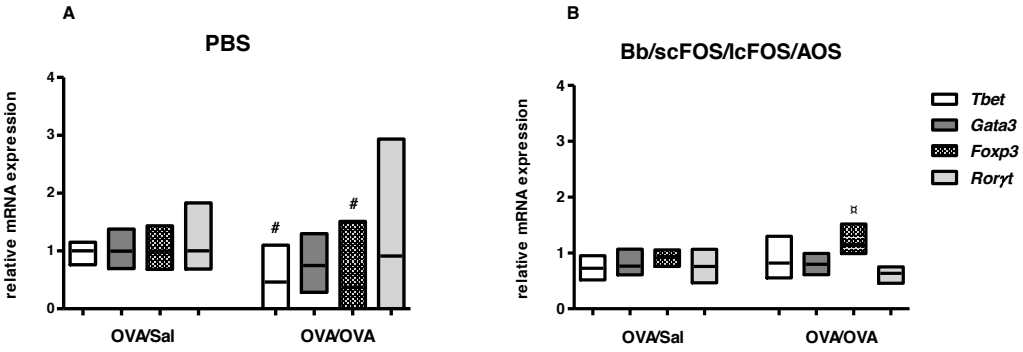

Figure 5: Relative T cell transcription factor mRNA expression in mouse whole lung tissue during chronic asthma. The bars indicate the min to max values and the line represents the mean, n=6 mice/group**,** of the transcription factor mRNA expression levels in OVA-sensitized, saline-challenged (OVA/Sal, white bars) mice and OVA-sensitized, OVA-challenged (OVA/OVA, grey bars) mice treated with PBS or Bb/scFOS/lcFOS/AOS. 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.

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/Rorγt	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/lsFOS/AOS	*Gata3/Tbet*	1.09	0.12	1.11	0.09
	Foxp3/Rorγt	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 presented 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.

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 (Figure 6). Treg cells were identified as CD4+CD25+Foxp3+ cells and the magnitude of Foxp3 protein expression on each cell was determined by examining the mean fluorescence intensity (MFI) of the Foxp3 staining. 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).

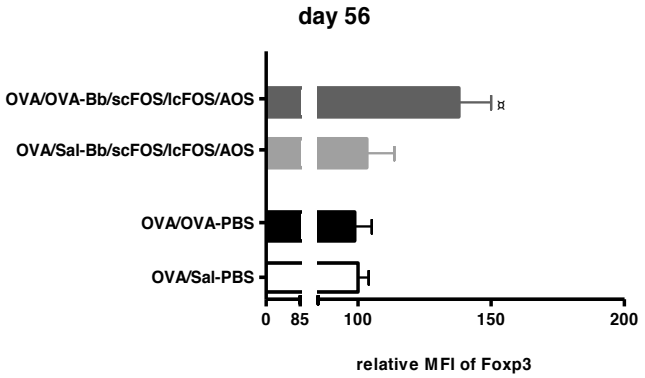

Figure 6: Effect of Bb/scFOS/lcFOS/AOS treatment on Foxp3 protein expression levels in Treg cells during chronic asthma. Mean fluorescence intensity (MFI) of Foxp3 on Treg cells isolated from mouse whole blood on day 56 in OVA/Sal and OVA/OVA mice, treated with PBS or Bb/scFOS/lcFOS/AOS. Data is shown as mean ± SEM, n=6 mice/group, and the results are presented as MFI levels relative to the OVA/Sal-PBS group (white bars). 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.

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 (Figure 7). The expression of IL2, IL4, IL6, TNFα and IFNγ 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 IL2, IL6, TNFα and IFNγ 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).

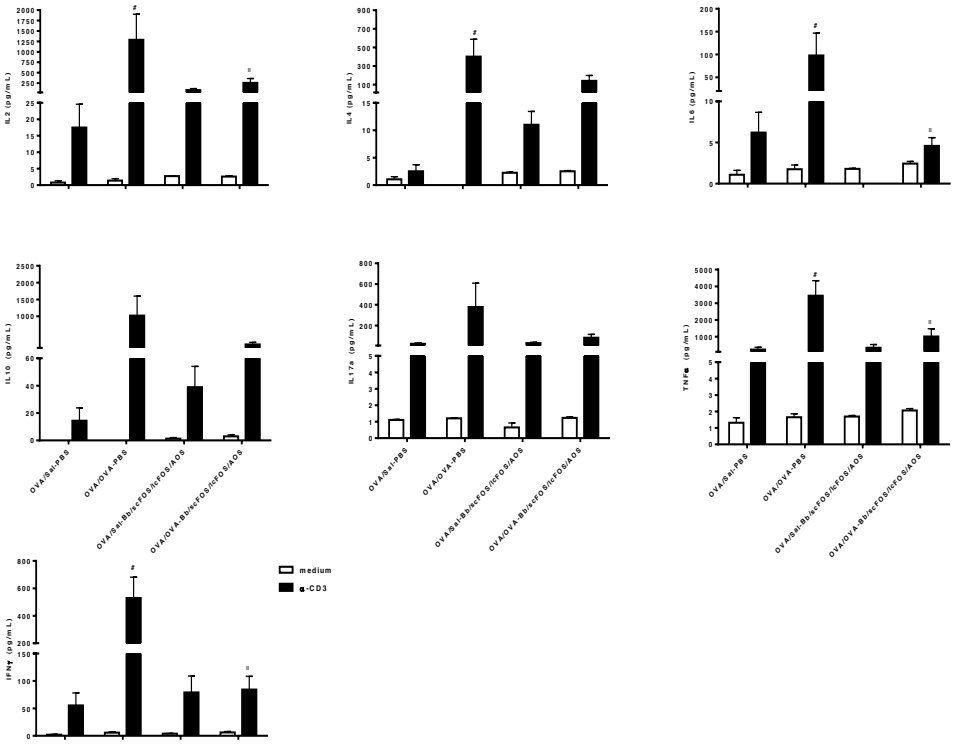

Figure 7: Cytokine levels after TLN T cell stimulation. Thoracic lymph nodes (TLNs) were isolated from mice on day 56 and restimulated in vitro with plate-bound anti-CD3 antibody or medium only for 5 days (37ºC, 5% CO_2). Data is shown as mean of cytokine concentration (pg/ml) ± SEM, n=6 mice/group. 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.

Bb/scFOS/lcFOS/AOS treatment inhibits mucosal mast cells 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 (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).

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-quantivitive histological and immunhistochemical analyses of lung tissue were performed (Figure 8 and Table 2). 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.

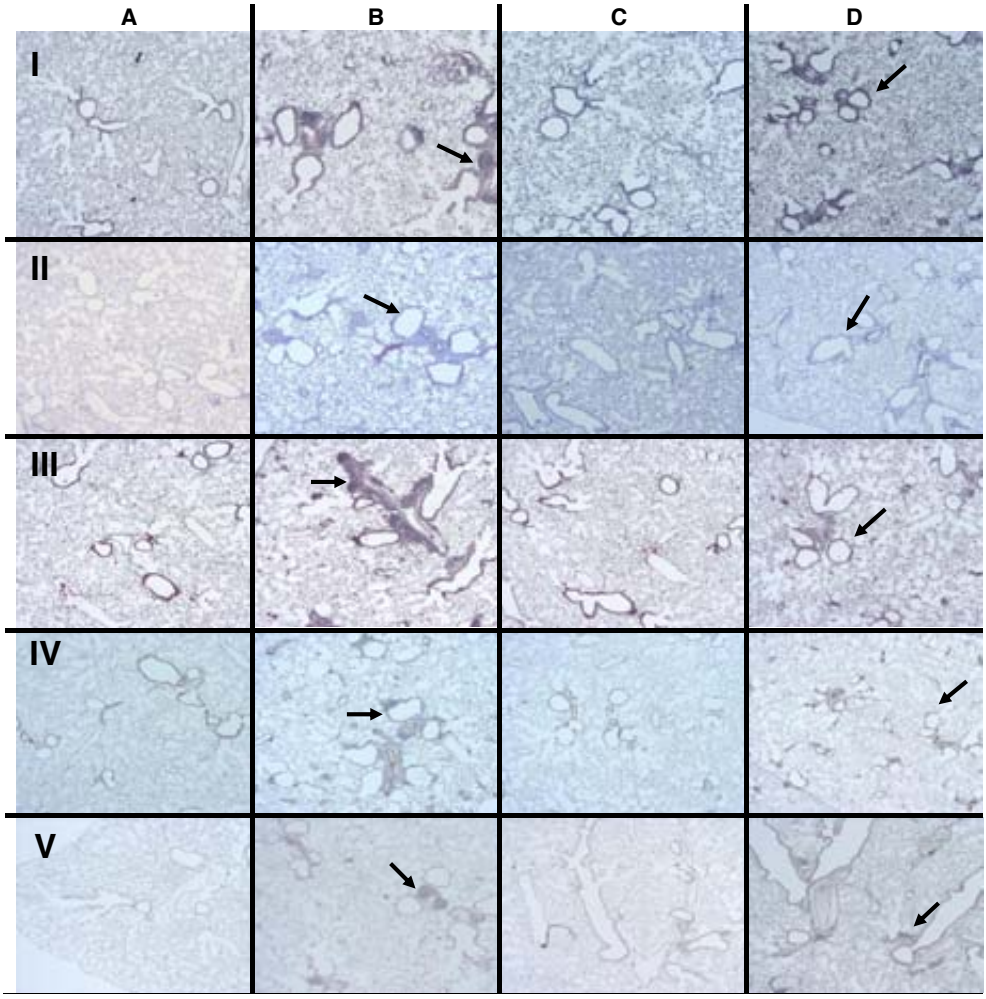

Figure 8: Effect of Bb/scFOS/lcFOS/AOS treatment on airway remodeling during chronic asthma. Representative photomicrographs for 5 mice/group of hitstological and immunohistochemical staining for inflammation (hematoxylin-eosin, HE, **I**); goblet cells (periodic acid Schiff, PAS, **II**); connective 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, 40X except for Anti-Ki67 100X.

Table 2: Overview of the hitstological and immunohistochemical score

Group	Inflammation (HE)	Goblet cells (PAS)	Connective tissue (Masson)	α-smooth muscle cells (anti-α-smooth muscle actin)	Proliferating cells (anti-Ki67)
OVA/Sal-PBS	-	-	-	+	-
OVA/OVA-PBS	++	+	++	++	++
OVA/Sal-Bb/scFOS/lcFOS/AOS	-	-	+	+	+/-
OVA/OVA-Bb/scFOS/lcFOS/AOS	+	+	+	++	+

Slides were reviewed in blinded fashion by two observers independently and slides were scored on the basis of the percentage of positive stained cells in the following way: -,no positive staining; +/-, less than 25% of cells stained positive; +, 25 to 50% cells stained positive; ++, 50 to 75% cells stained positive. Results are presented as average score of 5 animals/group.

Discussion

The aim of this study was to investigate the therapeutic effects of long-term treatment with a combination of *Bifidobacterium breve* with a specific mixture of non-digestible oligosaccharides (Bb/scFOS/lcFOS/AOS) on 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 were 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. 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 factors 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 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, 49].

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 lL1β, IL6 and IL17 are found in the sputum and BAL fluid of individuals with asthma [30]. The decrease in *Il5* mRNA expression might suggest that the number of Th2 cells in lung tissue was not increased as these cells release IL5 and other cytokines to orchestrate allergic inflammation [30]. Likewise, 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 IL12 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 IL10 is known to inhibit IL12 p40 transcription [30, 50]. Interestingly, the observed effects of the Bb/scFOS/lcFOS/AOS treatment on cytokine expression in the lung are confirmed by significant decreases in the production of IL2, IL6 and TNFα, restoring them to normal levels, and IFNγ by T cells in the TLNs of OVA/OVA mice after aspecific 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 [51]. 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 cytokines 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 futher investigation. Possibly, these findings might be explained by the induction of galectins by the Bb/scFOS/lcFOS/AOS mixture. Recently, a combination of *Bifidobacterium breve* M-16V with non-digestible oligosaccharides, although with a different oligosaccharide composition, was shown to induce galectin-9 which in turn suppressed allergic symptoms, including mast cell degranulation, in mice and humans [52].

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 at 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 *Bifidobacterium breve* with non-digestible oligosaccharides may be beneficial in the management of chronic inflammation in allergic asthma.

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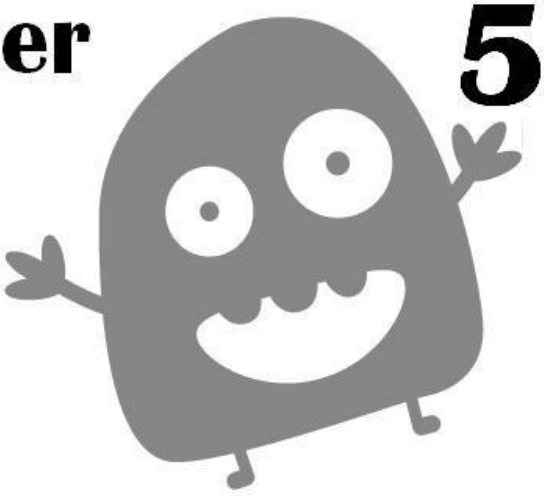
Supplementary Table 1: Forward and reverse primers used for quantitative real-time PCR analysis

Molecule	Forward primer	Reverse primer
Tlr1	GGTGTTAGGAGATGCTTATGGGG	GATGTTAGACAGTTCCAAACCGA
Tlr2	CCAGACACTGGGGGTAACATC	CGGATCGACTTTAGACTTTGGG
Tlr3	GGGGTCCAACTGGAGAACCT	CCGGGGAGAACTCTTTAAGTGG
Tlr4	GCCTTTCAGGGAATTAAGCTCC	AGATCAACCGATGGACGTGTAA
Tlr5	TCAGACGGCAGGATAGCCTT	AATGGTCAAGTTAGCATACTGGG
Tlr6	GACTCTCCCACAACAGGATACG	TCAGGTTGCCAAATTCCTTACAC
Tlr9	ACTCCGACTTCGTCCACCT	GGCTCAATGGTCATGTGGCA
Nod1	GAAGGCACCCCATTGGGTT	AATCTCTGCATCTTCGGCTGA
Nod2	CCGCTTTCTACTTGGCTGTC	GTGATTTGCAGGTTGTGTGG
Tbet	GCCAGCCAAACAGAGAAGAC	AAATGTGCACCCTTCAAACC
Gata3	GCGGTACCTGTCTTTTTCGT	CACACAGGGGCTAACAGTCA
Foxp3	CACTGGGCTTCTGGGTATGT	AGACAGGCCAGGGGATAGTT
Rorγt	TGCAAGACTCATCGACAAGG	AGGGGATTCAACATCAGTGC
RPS13	GTCCGAAAGCACCTTGAGAG	AGCAGAGGCTGTGGATGACT

Chapter 5



Bifidobacterium breve and *Lactobacillus rhamnosus* suppress chronic allergic asthma in mice

Manuscript submitted

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Abstract

Background: Asthma is estimated to affect as many as 300 million people worldwide and its incidence and prevalence are rapidly increasing throughout the world, especially in children and within developing countries. Recently, there has been a growing interest in the use of potentially beneficial bacteria for allergic diseases. This study is aimed at exploring the therapeutic effects of long-term treatment with *Bifidobacterium breve* and *Lactobacillus rhamnosus* on asthma symptoms in a murine model for chronic allergic asthma. A glucocorticoid (budesonide) was used as a reference treatment.

Methods: A murine ovalbumin-induced chronic asthma model was used. Airway function; pulmonary inflammation; mast cell degranulation; *in vitro* T cell activation; and airway remodelling were examined.

Results: *Lactobacillus rhamnosus* reduced lung resistance to a similar extent as budesonide treatment in chronic asthmatic mice. Pulmonary inflammation (infiltration of eosinophils and neutrophils into the airways; mast cell degranulation; T cell activation) and airway remodelling were suppressed by all treatments.

Conclusion: These findings show that *Bifidobacterium breve* and *Lactobacillus rhamnosus* are effective in suppressing asthma by modulating inflammation and disturbed immunity, with an overall efficacy that is similar to glucocorticoid treatment. Therefore these bacterial strains may be beneficial in the treatment of chronic allergic asthma.

Introduction

Asthma is a chronic inflammatory disorder of the airways characterised by airway inflammation and hyper-responsiveness (AHR) [1]. Structural changes in the airway walls of asthmatic patients, referred to as "airway remodelling", are caused by persistent inflammation and subsequent inadequate repair of damaged airway epithelium [2]. Subepithelial fibrosis, increased deposition of extracellular matrix protein, mucus gland hypertrophy and goblet cell hyperplasia, epithelial damage and smooth muscle hypertrophy and hyperplasia are hallmark features of airway remodelling in asthmatic patients [3]. The combination of these processes causes airway narrowing, and subsequent reduced lung function [1, 4].

Various cells are involved in cellular airway inflammation and subsequent airway remodelling, such as eosinophils, neutrophils, macrophages, epithelial cells, T lymphocytes, airway smooth muscle cells, fibroblasts and mast cells [3]. Infiltration of mast cells into the airway smooth muscle cell layer of allergic asthmatics is a key feature of asthma and is thought to be associated with AHR [5, 6]. Additionally, mast cells degranulation is detected in asthmatic lungs and various mast cell mediators are found in the BAL fluid of asthma patients [7]. Following capture and presentation of an allergen by antigen presenting cells, antigen-specific T helper 2 (Th2) cells are developed [8]. Once the allergen is encountered by these antigen-specific Th2 cells, cytokines such interleukine 4 (IL4) and IL13 are released which in turn leads to the activation of B cells and subsequent development of IgE-producing plasma cells. Allergen-specific immunoglobulin E (IgE) molecules produced by plasma cells bind to their high-affinity receptor (FcεRI) on surface of basophils and mast cells [8, 9]. Allergen-induced cross-linking of the IgE bound FcεRI results in the activation and degranulation of mast cells followed by the release of various inflammatory mediators, such as vasoactive amines and the cytokine tumor necrosis factor α (TNFα) [10]. Allergic inflammation and IgE production is further enhanced by IL4, IL9 and IL13. Additionally, IL5 is also produced by Th2 cells and this cytokine is important for growth and differentiation of eosinophils [8, 9]. In addition to the release of cytokines, proteases, such as tryptase, are also released after degranulation of mast cells. Proteases are involved in the pathogenesis of various inflammatory disorders in which mast cells play a role, such as asthma. Hence, mouse mast cell protease 1 (mMCP-1) was reported to enhance airway narrowing in mice through effects on the epithelium [7, 11].

Besides IL4, IL5, IL9, IL13 and TNFα, many other cytokines were reported to affect disease outcome in asthmatic patients. IL6, which is involved in the expansion of Th2 and T helper type-17 (Th17) cells, enhanced inflammation in patients. Additionally, IL17, produced by Th17 cells, indirectly increased the number of neutrophils and thereby enhanced asthma in patients. On the other hand, IL10 produced by regulatory T cells (Tregs), and interferon γ (IFNγ) decreased asthma symptoms by reducing the inflammation and the number of Th2 cells, respectively. Reduced levels of IL10 and IFNγ are found in individuals with asthma [12].

Asthma is estimated to affect as many as 300 million people worldwide and its incidence and prevalence are rapidly increasing throughout the world, especially in children and within developing countries [13]. Animal models for allergic asthma have contributed to a better understanding of the disease pathophysiology. Recently, there has been a focus on developing chronic allergen exposure models, especially in mice, to reproduce more of the clinical features of chronic asthmatic patients, such as airway remodelling [1, 14]. Glucocorticoids (GCs) are by far the most effective anti-inflammatory treatment for asthma. However, over 50% of asthmatics are poorly controlled with inhaled corticosteroids with or without long-acting β_2-agonists largely due to poor adherence [15]. β_2-agonists are aimed at relieving the symptoms rather than treatment of the disease [16]. Besides the undesirable side effects of long-term GC therapy, a significant number of asthmatics is steroid resistant and fails to respond to this therapy [17, 18]. These limitations of the current treatments for asthma highlight the need for novel therapeutics targeting underlying allergic and immune responses with a greater disease control, increased efficacy and a major clinical effect.

Animal studies indicated a substantial influence of the gut microbiota on immune function beyond the gut. Hence, changes in the microbiota were reported to contribute to the development of allergies and asthma [19, 20]. The potential role of beneficial bacteria, such as beneficial bacteria, as modulators of the intestinal microbiota and mucosal immune responses has been extensively investigated and discussed in the last few years [21-23]. Probiotics are"live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host" [24, 25]. *Bifidobacteria* and *Lactobacilli* are the most frequently used bacterial genera since these occur naturally as part of the gut microbiota [26]. *Bifidobacteria* and *Lactobacilli* were effective in suppressing both allergic and autoimmune responses, reducing allergic

symptoms and inhibiting allergic airway response in murine models of acute airway inflammation [26-30].

To date, more research has been conducted to investigate the preventive effects of beneficial bacteria rather than the therapeutic effects. The aim of this current study is to explore the therapeutic effects of long-term administration of *Bifidobacterium breve* and *Lactobacillus rhamnosus* on lung function, airway remodelling, mast cell degranulation and T cell activation in a mouse model for chronic asthma. Findings from this study will contribute to a better understanding of the immunomodulatory and therapeutic effects that may be induced using specific bacterial strains in chronic allergic asthma.

Material and Methods

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.

Chronic asthma model

OVA sensitisation

Sensitisations were performed on days 0 and 12. Mice were sensitised 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 (AlumImject; Pierce, Rockford, IL, USA). Control animals received 0.1 mL saline only (NaCl 0.9%; B. Braun Medical B.V., Oss, The Netherlands) (Figure 1).

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 were exposed daily to 5% OVA aerosol in saline using a Pari LC Star nebuliser (PARI GmbH, Starnberg, Germany) in an aerosol cabin for 20 min between days 17 and 23. Control animals were exposed to nebulised saline aerosol only. From day 24 until day 55, the frequency of challenge was reduced to three times a week and mice were exposed to aerosolised OVA (5%) or saline only for 20 min (Figure 1).

Beneficial bacteria treatment

Bifidobacterium breve M-16V (*B. breve*, Morinaga Milk Industry, Tokyo, Japan) and *Lactobacillus rhamnosus* NutRes1 (*L. rhamnosus*, Danone Research, Wageningen, The Netherlands) were grown in MRS (Oxoid, Basingstoke, UK), supplemented with 0.5 g/L L-cysteine for *Bifidobacteria*, at pH 6.5 and under anaerobic conditions. Bacteria were harvested in the early stationary phase, washed with phosphate buffered saline (PBS, Lonza Leusden, The Netherlands) and stored with glycerol 20% (w/v), in aliquots at -80°C. Cell counts were

determined by plating serial dilutions and fluorescent microscopy by staining with DAPI. Mice received 10^9 colony forming units (CFUs) of *Bifidobacterium breve* M-16V or $1.1x10^9$ CFUs of *Lactobacillus rhamnosus* NutRes1 per animal per day. The bacteria were resuspended in 0.2 mL of PBS and given therapeutically by oral gavage, 1 h prior to challenge, three times a week from day 22 until day 55 (Figure 1).

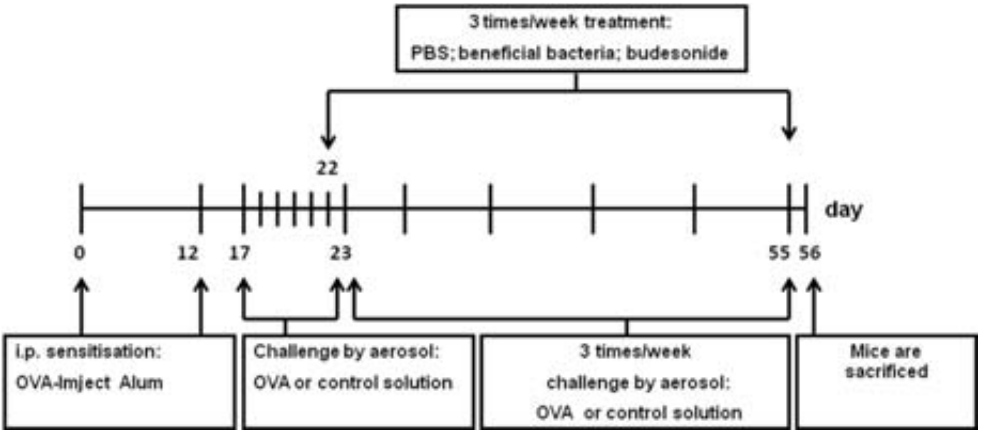

Figure 1: Time schedule of the chronic asthma mouse model. Male BALB/c mice were sensitised intraperitoneally with alum-precipitated OVA on days 0 and 12 and mice were challenged from day 17 until day 23 daily with aerosolised OVA or saline. From day 22 until day 55, mice were treated 3 times a week with either PBS or budesonide by oropharyngeal aspiration or probiotics (*B. breve* or *L. rhamnosus*) by oral gavage. 1h after treatment, from day 24 until day 55, mice were challenged 3 times/week with aerosolised OVA or saline. Mice were sacrificed on day 56 after pulmonary function measurement.

Budesonide treatment

As a reference treatment, mice received 0.5 μg/g of mouse/day of budesonide (Sigma) in PBS. Budesonide was administered to mice by oropharyngeal aspiration after induction of light isoflurane anesthesia as described previously in [31], 1 h prior challenge, three times a week from day 22 until day 55. Control animals received 50 uL of PBS by the same administration route. Mice were rendered asthmatic following the schema presented in Figure 1.

Airway response to methacholine

On day 56, 24 h after the last OVA aerosol challenge, the airway response to increasing doses of methacholine was analysed after insertion of a cannula in the trachea. Lung resistance in anesthetised, mechanically ventilated mice was measured directly using whole-body plethysmography (Emka technologies, Paris, France). Mice were exposed to saline (0 mg/mL methacholine) and increasing doses (0.38 to 25 mg/mL) of aerosolised methacholine (Sigma).

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.

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 was determined using a Beckman Z1 coulter® Particle Counter (Beckman, USA). TLN cells (4 x 10^6 cells/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 medium with or without O/N pre-coating of the wells with 50 μg/mL of anti-CD3 antibody (Bioceros BV, Utrecht, The Netherlands). The supernatant was harvested after 5 days of culture at 37°C in 5% CO2 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 Bisosciences).

Measurement of mouse mast cell protease 1 levels in serum

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*. Serum samples were stored at -80°C until further analysis. Mouse mast cell protease 1 (mMCP-1) protein expression levels in serum 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.

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 de paraffin sections were first deparaffinised. 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 observers independently and slides were scored on the basis of the percentage of positive stained cells in the following way: -,no positive staining; +/-, less than 25% of cells stained positive; +,25 to 50% cells stained positive; ++, 50 to 75% cells stained positive.

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).

Results

L. rhamnosus and budesonide reduce the increased lung resistance in chronically asthmatic mice

To investigate the lung function in the chronic asthmatic mice after treatments, lung resistance was measured (Figure 2 and Table 1). OVA-sensitised and challenged control mice (OVA/OVA-PBS) showed a significant increase in the basal lung resistance (Figure 2) and lung resistance at all concentrations of methacholine (Table 1) as compared to the sensitised only control group (OVA/Sal-PBS). Treatment of sensitised and challenged mice with *L. rhamnosus* (OVA/OVA-*L. rhamnosus*) resulted in a significant decrease in the basal lung resistance (Figure 2) and lung resistance at 0, 0.75 and 1.56 mg/mL methacholine (Table 1) as compared to the OVA/OVA-PBS group. Budesonide treatment significantly decreased the basal lung resistance (Figure 2) and lung resistance at 0, 0.38, 0.75 and 1.56 mg/mL methacholine (Table 1) in allergic mice (OVA/OVA-BUD) as compared to OVA/OVA-PBS mice. The basal lung resistance (Figure 2) and lung resistance at all concentrations of methacholine remained unchanged in *B. breve*-treated, sensitised and challenged mice (OVA-OVA-*B. breve*) as compared to the OVA/OVA-PBS group (Table 1).

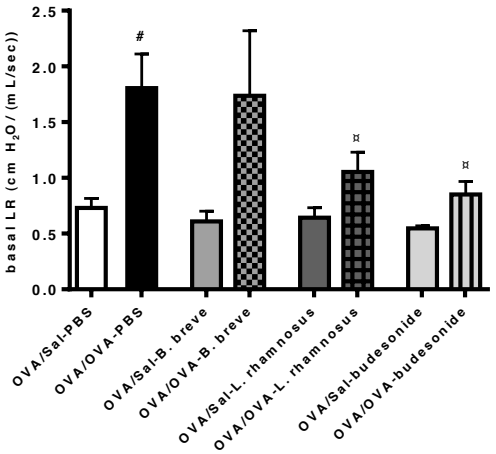

Figure 2: *L. rhamnosus* and budesonide reduce the basal lung resistance in chronically asthmatic mice. Basal airway response measured 24h after the last OVA or saline challenge as expressed by lung resistance (LR). Mice in all groups were sensitised with OVA. OVA/Sal mice were challenged with saline; OVA/OVA mice were challenged

with OVA. Mice were treated with PBS (OVA/Sal-PBS; OVA/OVA-PBS), *B. breve* (OVA/Sal-*B. breve*; OVA/OVA-*B. breve*), *L. rhamnosus* (OVA/Sal-*L. rhamnosus*; OVA/OVA-*L. rhamnosus*) or budesonide (OVA/Sal-budesonide; OVA/OVA-budesonide). The results are expressed as mean ± SEM, n=6 mice/group. 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.

Table 1: *L. rhamnosus* and budesonide treatment reduce the lung resistance in chronically asthmatic mice

Group	Methacholine (mg/mL)																	
	basal		0 (saline)		0.38		0.75		1.56		3.13		6.25		12.5		25	
	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM	mean	SEM
OVA/Sal-PBS	0.73	0.09	0.76	0.09	0.80	0.06	0.88	0.06	0.97	0.08	1.04	0.09	1.16	0.06	1.23	0.07	1.41	0.12
OVA/OVA-PBS	1.81[#]	0.31	1.79[#]	0.22	1.76[#]	0.19	1.85[#]	0.20	1.86[#]	0.21	1.82[#]	0.19	1.73[#]	0.15	1.96[#]	0.18	2.01[#]	0.21
OVA/Sal-*B. breve*	0.61	0.09	0.76	0.15	0.93	0.21	0.96	0.17	0.97	0.13	1.00	0.07	1.12	0.09	1.17	0.09	1.16	0.06
OVA/OVA-*B. breve*	1.74	0.58	1.66[#]	0.59	1.54	0.43	1.55	0.33	1.66	0.28	1.86	0.30	1.97	0.27	2.18	0.29	2.36	0.30
OVA/Sal-*L. rhamnosus*	0.64	0.09	0.73[#]	0.16	0.74	0.09	0.81	0.11	0.83	0.08	0.93	0.08	1.02	0.10	1.05	0.08	1.20	0.12
OVA/OVA-*L. rhamnosus*	1.05[¤]	0.18	1.15[¤]	0.14	1.13	0.13	1.18[¤]	0.13	1.30[¤]	0.12	1.48	0.13	1.55	0.14	1.69	0.16	1.80	0.16
OVA/Sal-BUD	0.55	0.02	0.56[#]	0.02	0.63	0.04	0.71	0.07	0.79	0.09	0.85	0.10	0.96	0.09	1.08	0.12	1.17	0.11
OVA/OVA-BUD	0.85[¤]	0.12	0.88[¤]	0.09	1.01[¤]	0.09	1.10[¤]	0.09	1.23[¤]	0.13	1.38	0.13	1.57	0.15	1.73	0.10	1.80	0.16

Airway response to saline and increasing doses of methacholine measured 24h after the last OVA or saline challenge as expressed by lung resistance (LR). Basal airway response is also shown. Results are shown as mean ± SEM. 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.

***B. breve* and *L. rhamnosus* are as effective as budesonide in reducing pulmonary inflammation in chronically asthmatic mice**

To gauge the extent of inflammation in the asthmatic mice after treatments, bronchoalveolar lavage (BAL) fluid was examined for leukocyte accumulation (Figure 3). OVA/OVA-PBS mice showed a significant increase in the total inflammatory cell number (155.25 $x10^4$ ± 12.58; n=6; $p<0.0001$; vs 29.25 $x10^4$ ± 3.12; n=6 $p<0.0001$) and the percentage of macrophages, eosinophils and neutrophils in the BAL fluid as compared to OVA/Sal-PBS mice. All treatments significantly reduced the total numbers of inflammatory cells in the BAL fluid of OVA/OVA mice. *B. breve* treatment, significantly decreased the total inflammatory cell number (73.50 $x10^4$ ± 6.42, n=6; $p<0.0001$) and the percentage of eosinophils and neutrophils, and tended to increase the percentage of macrophages and lymphocytes as compared to the OVA/OVA-PBS group. *L. rhamnosus* treatment significantly decreased the total inflammatory cell number (79.75 $x10^4$ ± 17.49; n=6; $p<0.0001$) and the percentage of neutrophils in OVA/OVA-*L. rhamnosus* mice as compared to the OVA/OVA-PBS group. Budesonide treatment significantly decreased the total inflammatory cell number (83.70 $x10^4$ ± 13.66; n=6; $p<0.0001$) and the percentage of eosinophils and neutrophils in OVA/OVA-BUD mice as compared to the OVA/OVA-PBS group.

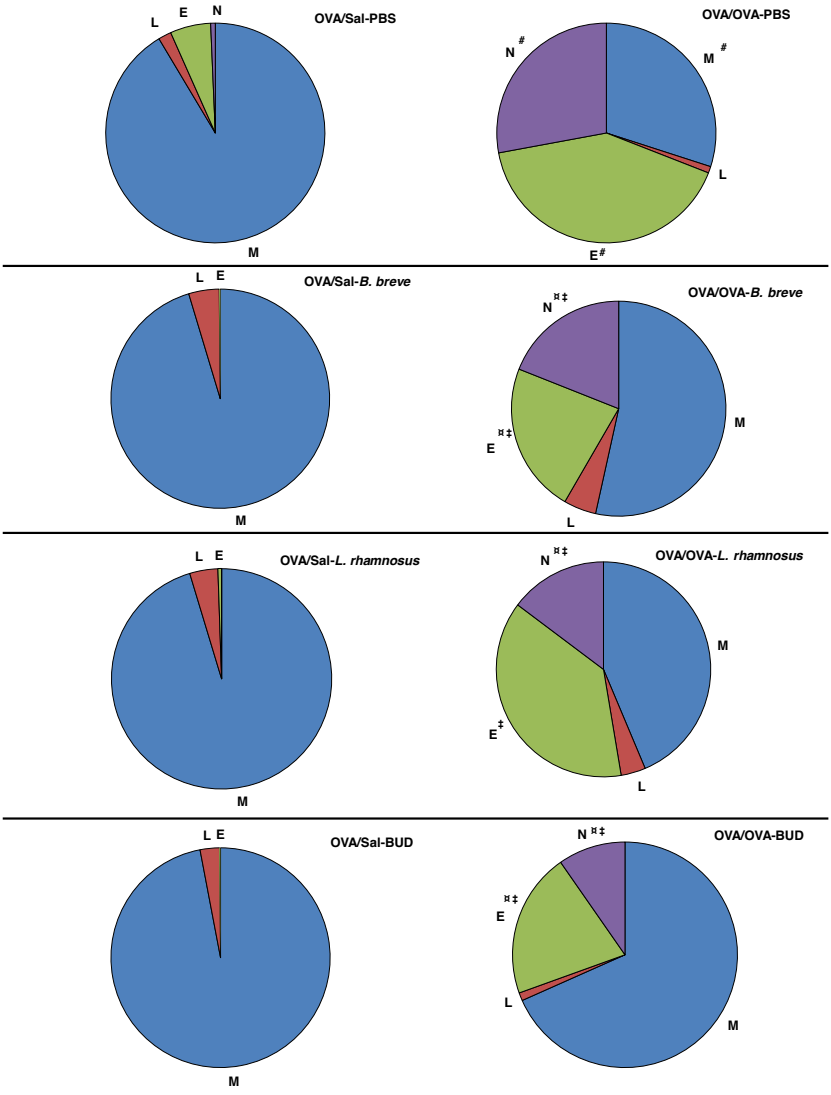

Figure 3: Beneficial bacteria and budesonide reduce the differential BAL fluid cell counts in chronic asthmatic mice. Pulmonary inflammation is represented by the influx of macrophages (M), lymphocytes (L), eosinophils (E) and neutrophils (N) in the BAL fluid. Differential cell counts are shown as percentages of the total cell count for each group. The charts show the mean, n=6 mice/group. 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. BUD=budesonide.

B. breve and *L. rhamnosus* are as effective as budesonide in suppressing mucosal mast cell degranulation in chronically asthmatic mice

To examine the effect of the different treatments on mast cells activity, enzyme-linked immunosorbent assay was employed to measure mMCP-1 protein levels in serum (Figure 4). The expression of mMCP-1 was significantly increased in OVA/OVA-PBS mice as compared to the OVA/Sal-PBS group. All treatments were effective in suppressing mucosal mast cell degranulation in OVA/OVA mice, as depicted by decreased protein levels of mMCP-1 in serum when compared to the OVA/OVA-PBS group.

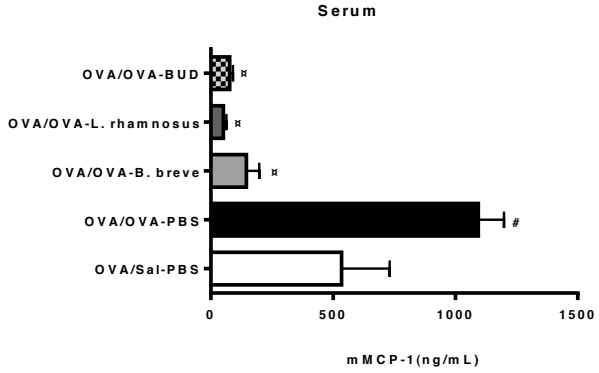

Figure 4: beneficial bacteria and budesonide reduce mouse mast cell protease-1 levels in serum of chronically asthmatic mice. Mouse serum was isolated on day 56 and mouse mast cell protease-1 (mMCP-1) levels were measured by ELISA. Data is shown as mean ± SEM, n=5-6mice/group. # Statistically significant difference ($p<0.05$) compared to the OVA/Sal-PBS group. ¤ Statistically significant difference ($p<0.05$) compared to the OVA/OVA-PBS group.

Beneficial bacteria and budesonide reduce T cell activity in chronically asthmatic mice

To investigate the effect of the treatments 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 (Figure 5). Cytokine expression levels in

medium-stimulated TLN cells did not differ among the different groups. The levels of IL2, IL4, IL6, IL17a, TNFα and IFNγ were significantly increased in anti-CD3-stimulated TLN cells of OVA/OVA-PBS mice as compared to samples from the OVA/Sal-PBS group. All treatments were effective in inhibiting T cell activity in OVA/OVA mice, significantly decreasing the levels of IL2, IL4, IL6, IL17a and TNFα as compared to the OVA/OVA-PBS group. Additionally, *L. rhamnosus* and budesonide treatment significantly decreased the levels of IFNγ in OVA/OVA mice as compared to the OVA/OVA-PBS group. IL10 levels were increased ($p>0.05$) in the OVA/OVA-PBS and in all treatment groups as compared to the OVA/Sal-PBS group. No differences in cytokine expression were observed in the TLNs of the different control groups (data not shown).

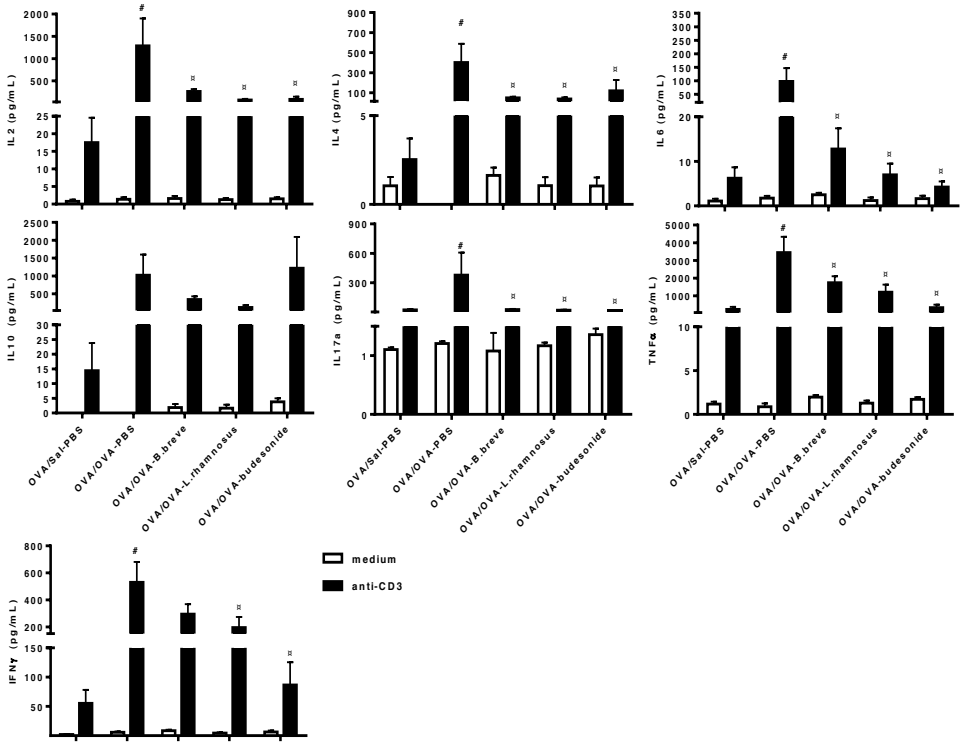

Figure 5: Beneficial bacteria and budesonide suppress cytokine production by T cells in TLNs of chronically asthmatic mice. Thoracic lymph nodes (TLNs) were isolated from mice on day 56 and restimulated in vitro with plate-bound anti-CD3 monoclonal antibody or medium only for 5 days (37ºC, 5% CO_2). Data is shown as mean of

cytokine concentration (ng/mL) ± SEM, n=6 mice/group. 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.

B. breve and *L. rhamnosus* are as effective as budesonide in suppressing airway remodelling features in chronically asthmatic mice

In order to examine the effect of the different treatments on airway remodelling features, semi-quantitative histological and immunhistochemical analyses of lung tissue were performed (Figure 6 and Table 2). 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. All treatments were effective in reducing the inflammation score and decreasing the number of collagenous connective tissue fibers and proliferating cells in sensitised and challenged mice as compared to the OVA/OVA-PBS group. Yet, the number of airway smooth muscle cells remained unchanged in the different treatment groups. Budesonide treatment was also effective in reducing the number of goblet cells in the lung sections of OVA/OVA-BUD mice as compared to the OVA/OVA-PBS group.

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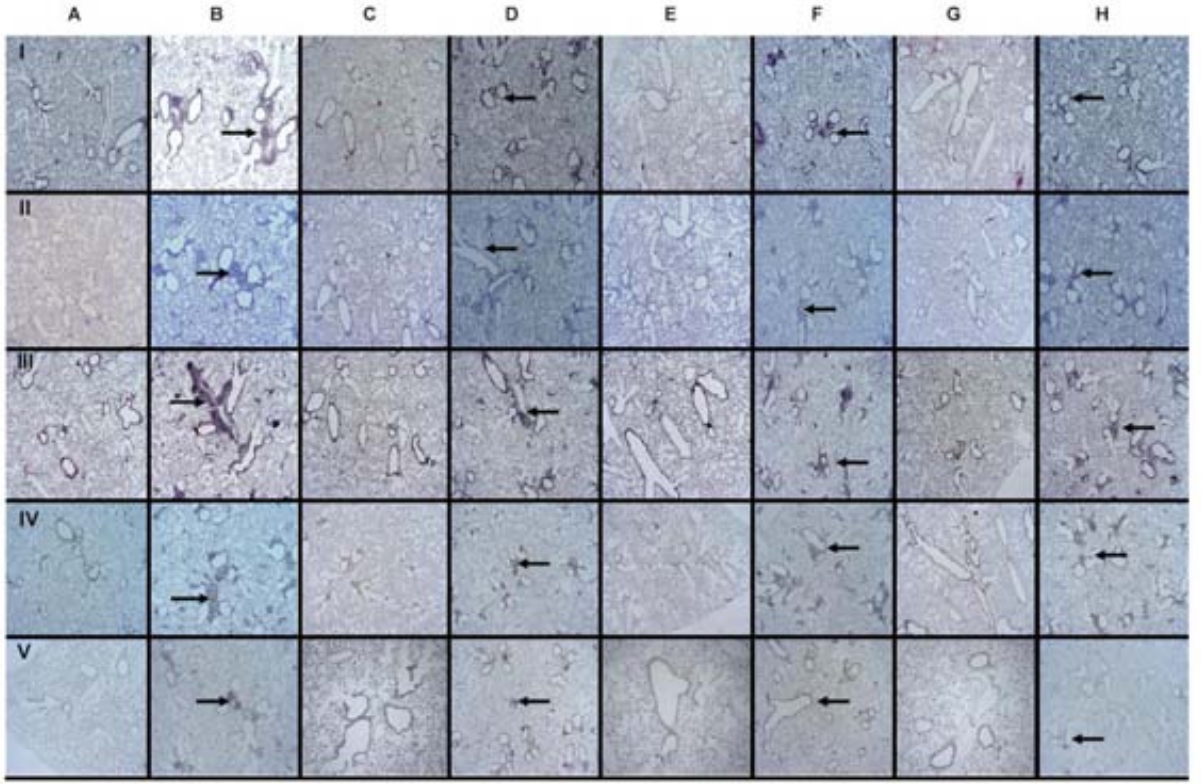

Figure 6. Beneficial bacteria and budesonide suppress airway remodelling features in chronically asthmatic mice. Representative photomicrographs for 5 mice/group of histological and immunohistochemical staining for inflammation (hematoxylin-eosin, HE, **I**); goblet cells (periodic acid Schiff, PAS, **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 and OVA/OVA-PBS mice (A and B), OVA/Sal-*B. breve* and OVA/OVA-*B. breve* mice (C and D), OVA/Sal-*L. rhamnosus* and OVA/OVA-*L. rhamnosus* mice (E and F), OVA/Sal-budesonide and OVA/OVA-budesonide mice (G and H) . Original magnification, 40X, except for Ki67 100X.

Table 2: Overview of the hitstological and immunohistochemical score

Group	Inflammation (HE)	Goblet cells (PAS)	Connective tissue (Masson)	α-smooth muscle cells (anti-α-smooth muscle actin)	Proliferating cells (anti-Ki67)
OVA/Sal-PBS	-	-	-	+	-
OVA/OVA-PBS	++	+	++	++	++
OVA/Sal-*B. breve*	-	-	+	+	+/-
OVA/OVA-*B. breve*	+	+	+	++	+
OVA/Sal-*L. rhamnosus*	-	-	+	+	+/-
OVA/OVA-*L. rhamnosus*	+	+	+	++	+/-
OVA/Sal-budesonide	-	-	+	+	+/-
OVA/OVA-budesonide	+	-	+	++	+

Slides were reviewed in blinded fashion by two observers independently and slides were scored on the basis of the percentage of positive stained cells in the following way: -, no positive staining; +/-, less than 25% of cells stained positive; +, 25 to 50% cells stained positive; ++, 50 to 75% cells stained positive. Results are presented as average score of 5 animals/group.

Discussion

The aim of this study was to explore the therapeutic effects of *B. breve* and *L. rhamnosus* on allergic asthma symptoms in a murine model of chronic asthma. The lung resistance was significantly increased in OVA/OVA-PBS mice at all concentrations of methacholine as compared to the OVA/Sal-PBS group. Additionally, the total inflammatory cell number and BAL fluid macrophage, eosinophil and neutrophil counts were significantly increased in OVA/OVA-PBS mice as compared to the OVA/Sal-PBS group. Increased inflammation score and number of 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 AHR, airway inflammation and remodelling in chronic asthma [1, 14, 32]. Interestingly, *L. rhamnosus* and budesonide treatments were effective in suppressing the lung resistance in OVA/OVA mice, significantly decreasing the response to methacholine as compared to the OVA/OVA-PBS group. *B. breve* treatment did not affect the airway hyper-response to methacholine. Yet, all treatments were effective in suppressing inflammatory cellular infiltration into the airways, significantly decreasing the total inflammatory cell number, particularly eosinophils and neutrophils in BAL fluid as compared to the OVA/OVA-PBS group. These findings are in line with other studies in which different strains of *Bifidobacteria* and *Lactobacilli,* and budesonide have been individually investigated in acute allergic asthma models [17, 26, 28, 29, 37]. Additionally, *B. breve*, *L. rhamnosus* and budesonide treatments reduced the inflammation score and decreased the number of collagenous connective tissue fibers and proliferating cells in ovalbumin-sensitised and challenged mice as compared to the OVA/OVA-PBS group. Budesonide treatment, but not *B. breve* or *L. rhamnosus,* was also effective in reducing the number of goblet cells in the lung sections of OVA/OVA-BUD mice. The number of airway smooth muscle cells remained unchanged in the different treatment groups as compared to OVA/OVA-mice.

Our study is the first to examine the therapeutic effects of *B. breve*, *L. rhamnosus* and budesonide (as a reference treatment) on allergic asthma symptoms in a murine model for chronic asthma. To our knowledge, we demonstrate here for the first time that *L.rhamnosus* is as effective as budesonide in suppressing lung resistance in chronic allergic mice. Additionally, we demonstrate here that both bacterial strains are as effective as budesonide in reducing chronic allergic inflammation and several airway remodelling features.

Mast cells are key effector cells in allergic inflammation and mast cell degranulation is detected in asthmatic lungs. The expression of serum mMCP-1 was significantly increased in OVA/OVA-PBS mice as compared to the OVA/Sal-PBS group indicating pulmonary mast cell degranulation. Hence, previous studies demonstrated that mice undergoing a hypersensitivity reaction in the airways have high serum levels of mMCP-1 of pulmonary origin [33]. All treatments were effective in inhibiting mucosal mast cell degranulation, as shown by the significant decrease in the protein levels of mMCP-1 systemically, in serum, in treated mice as compared to the OVA/OVA-PBS group. The mechanism by which these beneficial bacteria reduced mast cells degranulation still needs to be investigated; yet, these effects might be galectines-mediated. Recently, a combination of *Bifidobacterium breve* M-16V with galacto- and fructo-oligosaccharides was shown to induce galectin-9 release from intestinal epithelial cells which in turn systemically suppressed allergic symptoms, including mast cells degranulation, in mice and humans. The same study demonstrated a negative correlation between increased serum galectin-9 levels and serum mMCP-1 levels [34]. The mechanism by which budesonide affects mast cell degranulation still needs further investigation; yet, glucocorticoids were demonstrated to suppress mast cell activation *in vitro* through the upregulation of inhibitory regulators of these cells [35]. In addition to their effects on mast cells, *B. breve*, *L. rhamnosus* and budesonide were also effective at reducing cytokine production by T cells in the TLNs. The levels of IL2, IL4, IL6, IL17a, TNFα and IFNγ were significantly increased in anti-CD3-stimulated TLN cells from OVA/OVA-PBS mice as compared to the OVA/Sal-PBS group. IL2, IL4, IL6, IL17a and TNFα were reported to be involved in asthma. Increased levels of IFNγ are found in individuals with severe asthma and acute exacerbation [12, 36]. The levels of IL2, IL4, IL6, IL17a and TNFα were significantly decreased in OVA/OVA mice treated with *B. breve*, *L. rhamnosus* or budesonide as compared to the OVA/OVA-PBS group. Levels of IFNγ were significantly decreased in OVA/OVA-*L. rhamnosus* and OVA/OVA-budesonide mice. The level of IL10 did not differ among the different groups, suggesting that the different treatments could not normalise IL10 production in chronic allergic mice.

Taken together, our current findings show that *B. breve* and *L. rhamnosus*, are as effective as budesonide in suppressing airway remodelling and inhibiting mast cell degranulation. Additionally, *L. rhamnosus,* but not *B. breve,* reduced lung resistance and IFNγ production by T cells in the TLNs indicating that treatment effects differ between the bacterial strains. These

findings show that *B. breve* and *L. rhamnosus* may be beneficial in the management of chronic allergic asthma in a therapeutic way.

Acknowledgments

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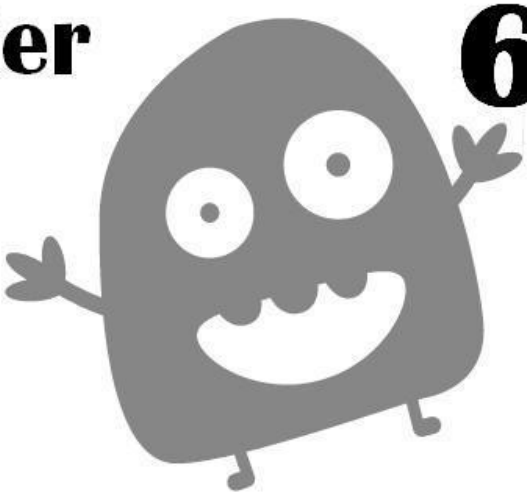
Chapter 6



The two faces of mast cells in food allergy and allergic asthma: the possible concept of Yin Yang

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Abstract

The purpose of this review is to discuss the role of mast cells in allergic inflammation. We have focused on inflammation associated with allergic asthma and food allergy. Mast cells are 'first line of defense' innate/adaptive immune cells and are widely distributed in tissues in surfaces exposed to the environment. Especially in allergic settings mast cells are extensively studied, as they can be activated to release a wide range of mediators by allergen-IgE specific triggers. In addition, in allergic inflammation mast cells can also be activated by non-allergic triggers. Recent studies revealed that mast cells, besides the classical role of pro-inflammatory effector cell, have also emerged as modulators of allergic sensitization and down-regulators of allergic inflammation. Therefore, mast cells can be regarded as 'Yin Yang' modulators in allergic responses in intestinal tract and airways.

1. Introduction

Over the past decades the prevalence of allergic diseases, such as asthma and food allergy, has increased dramatically. Allergic diseases arise as a result of unwanted and exaggerated immune responses to harmless proteins, also called allergens. A certain percentage of the population experiences adverse immunological reactions to these allergens that in non-allergic individuals only induce tolerance. This failure of tolerance is believed to result in the induction of an active immune response to allergens. One of the first events in the pathogenesis of allergy is capture and presentation of the allergen by antigen presenting cells at mucosal sites in the airways or intestinal tract and in draining lymph nodes, respectively. This will lead to the development of a large population of antigen-specific T helper 2 cells [1]. Activated allergen-specific T cells are found in the circulation of allergic patients. These T helper 2 cells, once encountering the allergen, will in turn activate B cells via the release of cytokines such as interleukin 4 (IL4) and IL13 critical for the development of plasma cells that will start the production of allergen specific immunoglobulin E (IgE) as well as IgG [1, 2, 3]. These IgEs will bind to their receptor FcεRI on basophils and extravascularly to mast cells. Allergen-induced cross linking of the IgE bound FcεRI results in the activation of mast cells. The release of mast cell derived pre-stored vasoactive amines, such as histamine and serotonin, and cytokines, such as tumor necrosis factor α (TNFα), as well as produced cytokines and arachidonic acid metabolites, such as leukotrienes and prostaglandins. IL4, IL9 and IL13 will further enhance the propagation of the production of IgE and allergic inflammation in general. IL5 and granulocyte-macrophage colony-stimulating factor (GM CSF) are also produced and are important for growth and differentiation of eosinophils. Eosinophils are immune effector cells that can leave the circulation through the interactions of adhesion molecules due to chemo-attractants and migrate to the site of inflammation (for review see [3]).

Mast cells play a critical role in allergy and this makes them attractive candidates for targeting allergic diseases. This review will focus on the central role of mast cells in inflammation associated with allergy, specifically in (allergic) asthma and food allergy.

2. Allergic Asthma

Asthma represents a chronic inflammatory airway disease characterized by episodes of reversible airway narrowing, bronchial hyper-responsiveness and chronic pulmonary inflammation. Asthma can be classified either according to frequency of symptoms (clinical) or according to triggers causing the airway symptoms. Clinical classification of asthma severity consists of four types [4]. Intermittent; patients suffer less than once a week from symptoms having a forced expiratory volume in 1 sec (FEV1) of more than 80% predicted. Mild: patients suffer more than once per week, but less than once per day from symptoms having a FEV1 of more than 80% predicted. Moderate persistent: patients suffer daily from symptoms having a FEV1 of 60-80% predicted. Severe persistent: patients suffer daily from symptoms associated with nighttime symptoms and having a FEV1 less than 60% predicted [5].

Asthma may also be classified as atopic, where symptoms are induced by allergens or non-atopic where symptoms are induced by non-specific triggers. Workplace exposures are the world's most common cause of this so-called occupational asthma and 15-23% of new-onset asthma cases in adults are work-related [6]. During remission, patients suffer from symptoms such as nighttime coughing and shortness of breath when exercising [7, 8]. Exacerbation of the disease consists of an acute asthma attack. During an asthma attack patients are short of breath, suffer from chest tightness and have a rapid heart rate (tachycardia) and wheezing can occur [7, 8]. In the pathology of asthma, the inflammation in the airways is characterized by several inflammatory cells such as eosinophils, B-lymphocytes and T-lymphocytes; however, the role of mast cells is undisputed (see paragraph 3.1).

More than 300 million people worldwide are diagnosed with asthma. Developed and westernized countries have higher asthma prevalence [9, 10]. The prevalence of asthma has been increasing since the 1980's, particularly in children and young adults. To date, asthma is the most common chronic disease in children. Asthma is associated with a western-life style and this has been shown clearly for example by the rapid increase in the prevalence of asthma in children who have been migrated from developing countries to developed countries [11]. Four reasons could be involved in the increase of prevalence of asthma associated with life style [12]. First, exposure to house dust mite (major allergen in asthma) has increased due to modern housing and more time indoor. Secondly, exposure to a wide range of microorganisms has changed because of improved hygiene and wide spread antibiotic used. Thirdly, the prevalence of obesity has

increased in children. Last but not least, changes of the western diet (decline of fresh vegetables, raw materials).

Classically, asthma has been regarded as a bronchoconstrictive disease and is predominantly treated with bronchodilators, such as β2 agonists [13]. Currently, the chronic inflammatory process is target of treatment with inhaled corticosteroids. For the management of asthma, patients take regularly inhaled corticosteroids with or without long acting β2 agonists. However, 50% of the patients are poorly controlled and there is still a need for new therapies. Blocking the synthesis or receptor for a single mediator (such as lipid mediators) involved in asthma seems unlikely to be very effective. Anti-leukotrienes that block cysteinyl leukotriene receptor 1 (CysLt1), are currently used in therapy, but these drugs are less effective than inhaled corticosteroids [13]. In addition, leukotriene B4 (LTB4) receptor antagonists had shown no effect in mild asthma [14]. Compounds targeting another arachidonic metabolite and its receptor, prostaglandin D2, are now in clinical development for asthma. Since cytokines play a crucial role in orchestrating chronic inflammation, they have become important targets for asthma treatments. However, over 50 cytokines have been implicated in asthma and several cytokines and chemokines (IL4, IL5, IL13, TNFα, C-C chemokine receptor type 3(CCR3)) blocking antibodies are now in clinical development but clinical studies in asthmatic patients have been disappointing [13]. Antibodies blocking IgE are only used in the treatment of patients with severe asthma, due to high cost of treatment and the unclear mechanism of clinical efficacy (for review see [15]). The most promising anti-inflammatory therapy is by the use of phosphodiesterase (PDE) 4 inhibitors targeting T cells, eosinophils, smooth muscle cells and epithelial cells. Roflumilast, an oral PDE4 inhibitor, demonstrated inhibitory effects on allergen-induced responses in asthma similar to low doses of inhaled corticosteroids [16]. A different approach is specific immune therapy, whereby asthmatic patients are exposed in a controlled way to allergens or allergen-peptides, (either subcutaneously or sublingually) to induce tolerance and desensitization. Some efficacy by this approach has been demonstrated, however, longer studies and comparison with inhaled corticosteroids are needed to determine efficacy [13].

Targeting mast cells in allergic asthma is directed towards mast cell sensitization, stabilization and their mediators released. Interestingly, besides acting as a smooth muscle relexants, β2 receptor antagonists have been shown to stabilize mast cells [17, 18]. Other mast cell stabilizers,

such as nedocromil and cromoglycate have only modest effects in allergic asthma [19, 20, for review see 18]. Today, mast cell stabilizers are not used in therapy. To targeting mast cell sensitization the above mentioned anti-IgE-antibody therapy can be regarded. The humanized anti-IgE-antibody, omalizumab, did not show expected results and are only recommended to use in difficult-to-treat asthma [21, 22, 23, for review see 24].

As described above, several compounds have been developed to target mast cell mediators such as leukotrienes, prostaglandins and cytokines, however, none of these compounds can beat the corticosteroids.

3. Food allergy

Food allergy is a growing problem in Western Europe and the USA. Intolerance or hypersensitivity reactions toward food or food additives can result in adverse reactions after ingestion of a particular food (for review see [25]). Food hypersensitivity reactions develop in genetically predisposed individuals associated with failure in the development or breaking down of tolerance. Clinical symptoms may involve dermatitis, respiratory distress and gastrointestinal symptoms such as nausea, diarrhea and stomachache and can even lead to a systemic anaphylactic reaction [26]. Food allergy, in contrast to food intolerance, is immunological hypersensitivity reaction mostly due to an inappropriate IgE- or non-IgE-mediated immune response. IgE-mediated food allergy accounts for the majority of food allergic reactions [26].
Typical foods accounting for food allergy are: cow milk, egg, peanut, tree nuts, soy, wheat, shell fish and fish proteins [27]. The prevalence of food allergy is the greatest during the first years of life where it affects around 8% of infants younger than 3 years of age [28]. Uncontrolled penetration of antigens through the not yet fully developed mucosal epithelial barrier of the intestinal tract might play a role in the increased prevalence during early life [27]. In this age group cow milk allergy is the most common type of food allergy. About 80% of these children develop clinical tolerance at the age of 5, however, this group is at risk to develop allergic asthma at later age [27, 28, 29]. In adults, peanut hypersensitivity is the most common, severe and dangerous food allergy [30, 31]. Peanut allergy remains for life and the prevalence rates exceed 1%.

Allergic reactions to food proteins can also occur independent of IgE. To date the mechanisms underlying these reactions are not clear. It has been suggested that T cells play a role in the immunopathogenesis as in cell-mediated or delayed type hypersensitivity reactions [32]. Studies performed in our group suggest the involvement of immunoglobulin free light chains (IgLC) in cow milk allergy [33, 34].

The only remedy in food allergy is strict avoidance of allergic food components determined after a skin prick test. In several clinical trails treatments with humanized recombinant anti-IgE antibodies have shown to increase the threshold of sensitivity to peanut allergens [35]. However, in order to cure food allergy, re-education of the intestinal immune system is necessary to establish oral tolerance. This can be realized by performing allergen-specific immunotherapy. Recent reports have demonstrated partial success with oral immunotherapy in the treatment of food allergy [36, 37, 38]. Other strategies with immuno-modulating dietary interventions, such as pre- and probiotics, are currently under research in preclinical and clinical studies [39, 40, 41].

4. Mast cells in allergic inflammation

The role of mast cells in allergic inflammation has extensively been studied [42, 43]. Triggering of mast cells and subsequent release of pre-stored mediators play an important role in the immediate phase of the allergic reaction resulting in vasodilatation, edema formation and/or bronchoconstriction. Through the production and release of chemotactic and other pro-inflammatory cytokines from mast cells, other non-specific (eosinophils) and allergen-specific (T cells) inflammatory cells infiltrate and get activated resulting in the late phase response.

Basophils share several characteristics with mast cells and represent less than 1% of peripheral blood leukocytes. They have been ignored for a long time and were often considered to play a minor role in allergic reactions. However, now it is widely acknowledged that basophils like mast cells respond to IgE-dependent stimuli and are involved in the onset, the effector phase and exacerbations of allergic (T helper 2 cell-mediated) responses. Basophils are often recruited to the site of allergic inflammation and release histamine, leukotrienes C4 and T helper 2 cytokines and play a role in T helper 2 cell differentiation. In addition, they contribute to immunoglobulin synthesis and can present antigens to T helper cells (for reviews see [44, 45]). The role of basophils in allergy is beyond the scope of this review.

As mentioned before, there is a prominent role for mast cells in allergic asthma. In the healthy human lung the major mast cell type is tryptase positive, whereas 20 % is double tryptase and chymase positive. [46] Tryptase positive mast cells are predominantly found at mucosal sites. In contrast, tryptase and chymase positive mast cells are located in the skin and submucosa, protected from external triggers. [47] It is generally believed that the microenvironment is important for the phenotype of mast cells and changes in this microenvironment (for example during allergic inflammation) can induce changes in mast cell type. The two mast cell phenotypes functionally differ: tryptase and chymase positive mast cells seem to be more responsive to allergic triggers when compared to only tryptase positive mast cells. Recently it was demonstrated that increased asthma severity is associated with an enhanced shift towards tryptase and chymase positive mast cells in airway mucosa [48]. In addition, it was suggested that these mast cells contribute to the increased level of PGD2 in bronchoalveolar lavage fluid of severe asthmatics.

The role of mast cells in food allergy is indicated by high levels of histamine, TNFα, IL5 and tryptase in serum, intestinal lavage fluid and stool samples of patients suffering from food allergy [49, 50]. Impairment of intestinal barrier function is of importance in food allergy and it has been shown that inflammation enhances intestinal mucosal permeability by mast cell-dependent mechanisms [51, 52]. As a result intestinal mast cells can contribute to an ongoing inflammation due to enhanced influx of potential allergens and harmful microbes into the intestinal tissue [50]. Indeed, in vitro studies using epithelial cells, have shown that human chymase can induce increased intestinal permeability [53]. Mast cell degranulation in the intestinal mucosa has been demonstrated in food allergic individuals [54]. Furthermore, it has been shown that IgE-stimulated mast cells trigger enteric nerves resulting in pain and diarrhoea [52, 55]. The development of oral antigen-induced diarrhoea in mice is associated with a marked intestinal mucosal mast cell degranulation.

As described for the human lung, tryptase and chymase positive mast cells are found in the submucosa of the intestinal tract, whereas tryptase positive mast cells are located in the mucosa [56]. To our knowledge, not much is known about the mast cell phenotype involved in food allergic patients. Based on the direct effects of human chymase on epithelial integrity, however, it is tempting to speculate that the tryptase and chymase positive mast cell population is responsible for the food allergy symptoms such as decreased barrier function and diarrhoea.

The role of mast cells in the pathogenesis of allergic asthma and food allergy has been studied in different animal models, especially using genetic mast cell deficient mice (WBB6F1-*Kit* $^{W/Wv}$ or C57BL/6-*Kit* $^{W\text{-}sh/W\text{-}sh}$) and mast cell-reconstituted mice [57, 58, 59, 60]. In these studies, mast cells have been shown to either directly or indirectly enhance the magnitude of several features of allergic asthma: airway hyperresponsiveness, infiltration of leukocytes, mucus production and tissue remodeling. However, in experimental setting where high doses of antigen and adjuvants were used to induce allergic asthma no contribution of mast cells were detected.

Mice deficient in mast cell or mast cell chymase show enhanced intestinal barrier function [53]. To our knowledge only one study in mast cell deficient and reconstituted mice is performed in a murine model for food allergy, where it was shown that mast cells are important in promoting the development of peanut-induced intestinal responses [57]. All studies in mast cell deficient mice do not exactly pinpoint where mast cells play a role in the pathogenesis of allergic inflammation. Therefore, below we will describe the current knowledge of the role of mast cells in the sensitization and effector phase of allergic inflammation as well as their possible role in suppression of the allergic inflammation.

4.1 Involvement of mast cells in sensitization to allergens

It has been hypothesized that mast cells can be involved in the process of sensitization to allergens because of their role as regulators of epithelial barrier integrity. In the gastrointestinal tract, it has been demonstrated that non-specific mast cell activation (for example chronic stress) can lead to increased epithelial permeability resulting in a higher exposure to allergens and thus induction of sensitization to food allergens [61, 62]. In addition, studies in mast cell deficient mice have shown that mast cells are important in the recruitment of dendritic cells, in the induction of major histocompatibility complex-I (MHC-I) dependent T cell priming and proliferation of CD4+ and CD8+ T cells [63]. Mast cell-derived TNFα induces the migration of T cells to draining lymph nodes at sites of inflammation demonstrating a role for mast cells in the processing of environmental allergens [64]. Furthermore, mast cells are proposed to become antigen-presenting cells for T cells due to factors from the micro-environment that induce the expression of MHC class II on mast cells [65]. For instance the TLR4 ligand, lipopolysaccharide, induces an increased number of MCHII-expressing mast cells in lymph nodes of mice [65]. Mast cells are able to produce and release IL4 and IL13. These cytokines promote Ig class switching and IgE production. Finally, it is shown that allergens can be stored in lysosomes of mast cells

after first being internalized by IgE-FcεRI-mediated phagocytosis [66]. Noted should be that these antigen-containing mast cells can activate antigen-specific T cells responses *in vitro* only when they undergo apoptosis and are then ingested by antigen-presenting cells [58]. Thus mast cells can drive further IgE production and epitope spreading in allergic diseases.

4.2 The role of mast cells in the onset of allergic inflammation

Allergen-IgE dependent mast cell activation is widely regarded to be the major initiator of the clinical signs and symptoms that are demonstrated rapidly after exposure of small amounts of allergen to an allergic individual. However, mast cells express many stimulatory and inhibitory receptors. Allergen-specific mast cell activation can occur via IgE, IgG1, IgG2a and IgG2b through the cross-linking of ther high affinity IgE receptor , FcεRI, FcγRI (human), FcγRIIa(human/mouse) or FcγRIIIa (mouse) receptors [67, 68]. In addition, antigen-specific mast cell degranulation can also be induced by cross-linking of immunoglobulin free light chains IgLC [32]. This mechanism might also be involved in asthma or food allergy, since blockage of IgLC was effective in down regulating airway and skin hypersensitivity reactions in murine models of asthma and cow milk allergy [33, 69].

Allergen-independent mast cell activation may also occur in an allergic setting. Activation of neuropeptide receptors, c-Kit, toll-like receptors (TLRs), complement receptors or direct interaction with G-proteins can also be involved in mast cell activation [6, 70, 71, 72, 73, 74]. Of interest are TLRs, since mast cells found in the submucosa and serosa express greater levels of TLR2, TLR3, TLR4, TLR7 and TLR9 compared to mucosal mast cells [75]. TLR2 and TLR4 ligands directly induce the generation and release of inflammatory mediators from mast cells [for review see 18]. In addition, in the context of allergic asthma, TLR ligands such as lipopolysaccharide (TLR4 ligand) are suggested to synergize with the crosslinking of allergen-IgE-FcεRI to enhance allergic inflammation [78]. Furthermore, it has been demonstrated that commensal bacteria in the intestinal tract via TLR signalling promote the migration of mast cells to the intestine [79]. Similar functions have been reported for chemokine receptors: CCR1 and CCR3 are involved in IgE-induced mast cell activation and epithelial derived CXCR2 ligands are important in the TLR-mediated mast cell recruitment to the intestinal tract [17, 79].

Activated mast cells release their pre-stored mediators (vasoactive amines, cytokines such as TNFα, proteases, peptides such as bradykinin) via exocytose of their granules or via differential

release of mediators without degranulation, so-called piece meal degranulation [80]. The pre-stored vasoactive amines, such as histamine and serotonin, mediate mainly vascular effects associated with the inflammatory response such as vasodilatation/constriction and changes in vascular permeability as well as acute bronchoconstriction [80]. The acutely released proteases, such as tryptase and chymase are involved in tissue damage and the peptides (bradykinin, substance P, vasoactive intestinal peptide and vascular endothelial growth factor (VEGF)) are also involved in the acute inflammation and pain perception [80].

Mast cell activation also leads to the *'de novo'* synthesis and release of lipid mediators and chemokines and cytokines. The newly synthesized arachidonic acid metabolites such as LTB4 and LTC4, platelet activating factor (PAF) and prostaglandin D2, cytokines, amongst which IL1, IL4, IL5, IL6, IL9, IL13, interferon γ (IFNγ) and TNFα, and chemokines are responsible for the late phase event during an allergic inflammatory response: leukocyte migration, infiltration and activation [73, 74, 80, 81].

4.3 Mast cells as suppressors of allergic inflammation

Recently, attention has been drawn to the potential role of mast cells in suppressing or breaking of allergic inflammation.

First, it was demonstrated that mast cell-derived proteases (such as β-tryptase) can cleave IgE. Treatment with protamine (an inhibitor of proteases) enhanced IgE-mediated allergic skin inflammation in man [82]. Although not yet demonstrated in an allergic setting, it has been shown that endogenous and exogenous peptides (endothelin-1, neurotensin and snake venom) that induce mast cell activation are also cleaved by mast cell proteases and thereby down-regulating the peptide damaging effects [83, 84, 85]. In addition, mast cells have been shown to have immunosuppressive functions following UVB radiation, mosquito bites or in peripheral tolerance to skin allocrafts [86, 87, 88]. How mast cells mediate these immunosuppressive effects remain to be elucidated, but naturally arising regulatory T cells seem to be involved [88]. IL9 derived from these regulatory T cells induces the recruitment and activation of mast cells. In turn, mast cells can mediate this immunomodulatory function *in vivo* by the production of IL10. Recently, the role of naturally arising regulatory T cells in the induction of oral tolerance to peanut allergens was challenged. Rezende and coworkers [89] have demonstrated that adoptive transfer of naturally arsing regulatory T cells from peanut tolerant mice did not induced tolerance

in recipient mice that were sensitized to peanut. They found evidence that TGFβ-secreting regulatory T cells may play an important role instead. However, in several clinical trails of allergen specific immunotherapy in allergic subjects, the induction of tolerance seems to be related to naturally arising regulatory T cells and IL10 producing type 1 regulatory T cells [for review see 90].

IL10 derived from mast cells is important for the limitation of chronic skin inflammation following contact hypersensitivity responses to the allergen-containing components of poison ivy [91]. The pathways that link mast cells-IL10 to the observed changes remain to be defined and to be shown in an allergic setting.

Lastly, mast cells are equipped to sense the environment to decide to become potentially suppressive in allergic inflammation. It has been suggested that such action could be realized via sialic acid-binding immunoglobulin-like lectin 8 (SIGLEC8) [92]. Siglecs are cell surface receptors and member of immunoglobulin super family (I-type lectins) that recognize sugars [93]. Siglec-8 is highly similar in structure to CD33 and has two conserved immunoreceptor tyrosine-based inhibitory motif (ITIMs)-like motifs in their cytoplasmic tails [94]. This suggests that SIGLEC8 function as inhibitory molecules. *In vitro*, SIGLEC8 promotes the apoptosis of eosinophils and inhibits IgE-FcεRI-induced mast cell mediator release [95, 96]. Recently, Gao and coworkers have described that polymorphisms of the *SIGLEC8* gene are associated with the susceptibility to asthma [97]. Further studies are required to validate SIGLEC8 as useful target to induce mast cell-dependent down regulation of allergic inflammation.

5. The two faces of mast cells in allergic inflammation: Yin Yang concept

Mast cells are typically regarded as troublesome cells due to their prominent role in IgE-dependent allergic hypersensitivity reactions such as allergic asthma and food allergy. Further, it seems that mast cells are also able to play an additional role in the ‘allergic’ sensitization-processes. Recent findings show that mast cell functionality is not only pro-inflammatory, but can on the contrary have suppressive or immunomodulatory effects in allergic inflammation (Figure 1).

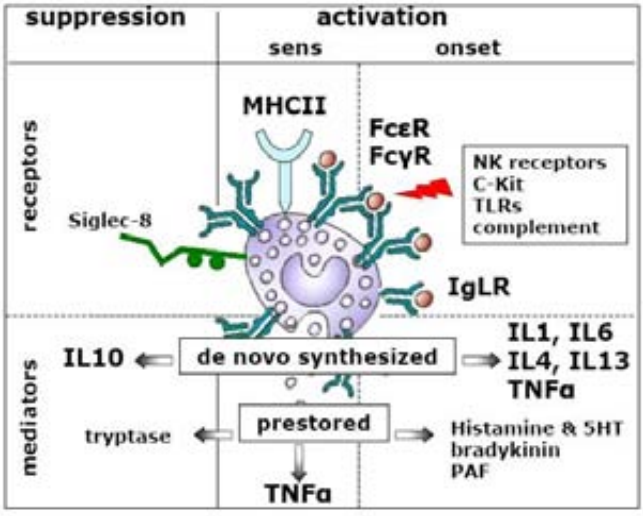

Figure1. The two faces of mast cells in allergic inflammation: inducer/activator and suppressor. MHC: major histocompatibility complex; IL: interleukin; PAF: platelet activating factor; FcεR: IgE receptor; FcγR: IgG receptor; IgLR: Ig light chain receptor; TNFα: tumor necrosis factor α; NK receptor: neurokinin receptor; 5HT: serotonin; TLR: toll like receptor; sens: sensitization phase. Figure adapted from [98].

The latter is mainly based on *in vitro*-studies and one should keep in mind that these circumstances do not reflect the true nature of mast cells in their micro-environment in intestinal wall or airways. More evidence needs to be obtained from *in vivo* and human studies to prove the immunomodulatory role of mast cells in allergic inflammation. In Chinese philosophy, the concept of yin yang is used to describe how seemingly contrary forces are interconnected and interdependent in the natural world, and how they give rise to each other in turn. Maybe we should regard the suppressor and activator roles of mast cells in allergic inflammation as such. Whilst inducing inflammation; Mother Nature has created a downregulating role for mast cells as well. Only during allergic exacerbations there is an imbalance of these mast cell functions resulting in the symptoms related to allergic asthma and food allergy. Opposite roles for mast cells in allergic inflammation thus only exist in relation to each other (Figure 2).

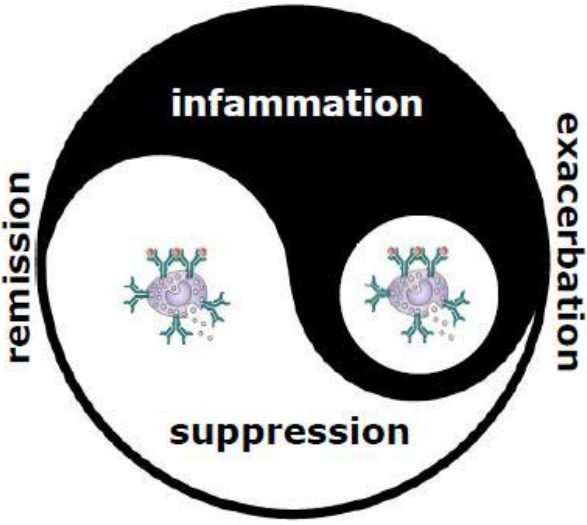

Figure 2. Yin Yang concept for mast cells. The suppressor and activator roles of mast cells in allergic inflammation are seemingly contrary. While mast cells play an important activator role in allergic inflammation; Mother Nature has created a downregulating role for mast cells as well. Only during allergic exacerbations there is an imbalance of these mast cell functions resulting in the symptoms related to allergic asthma and food allergy. During remission the balance is restored. Opposite roles for mast cells in allergic inflammation thus only exist in relation to each other. Thus mast cells follow the Yin Yang concept in allergy.

Still, the possible suppressor role of mast cell in allergic inflammation and/or the possible role of mast cells in the development of immunological tolerance, leads to the following questions:

1. How careful should we be in targeting mast cells in allergic inflammation?
2. How can we manipulate specific mast cell suppressor functions therapeutically?

A better understanding of mast cell biology will give us new insights for improving strategies to treat allergic inflammation associated with asthma and food allergy.

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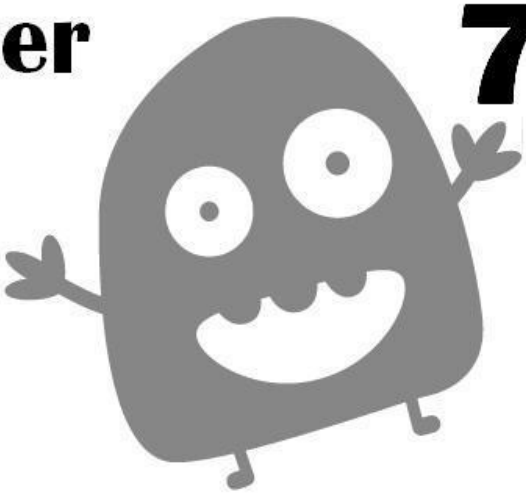
Chapter 7



Oral treatment of chronic allergic mice with *Bifidobacterium breve* or *Lactobacillus rhamnosus* prevents recruitment of bone marrow cells during inflammation and has long-term inhibitory effects on mast cell progenitors

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Abstract

Background: Asthma is a chronic inflammatory disorder of the airways in which different inflammatory/immune cells and mediators play a role. Mast cells are crucial in inducing both the early and late phase allergic responses and these cells are involved in chronic inflammation. We and others showed that potentially beneficial bacteria can suppress allergic inflammatory responses in acute allergic asthma models. This study is aimed at exploring the therapeutic effects of long-term treatment with two different beneficial bacterial strains (*Bifidobacterium breve* and *Lactobacillus rhamnosus*) on the recruitment and function of bone marrow cells in a murine model of chronic allergic asthma. A glucocorticoid (budesonide) was used as a reference treatment.

Methods: A murine ovalbumin-induced chronic asthma model was used. Bone marrow cells were isolated from animals that were exposed to aerosols of ovalbumin for five weeks and the cells were accordingly cultured in the presence of stem cell factor and interleukin-3 for four weeks to generate bone marrow-derived mast cells (BMMC). Development of mast cells was determined by expression of c-Kit and FcεRI receptors. IgE-mediated activation of BMMC was analyzed by the release of beta-hexosaminidase.

Results: *In vivo* treatment of allergic mice with *Bifidobacterium breve* and *Lactobacillus rhamnosus*, but not budesonide, resulted in decreased antigen-IgE-specific degranulation of BMMC cultured from bone marrow *in vitro*. *In vitro* co-culture of BMMC with *Bifidobacterium breve* reduced BMMC degranulation cultured from bone marrow of control and budesonide-treated allergic mice, while BMMC cultured from *Bifidobacterium breve-* and *Lactobacillus rhamnosus*-treated animals were not further inhibited by direct interaction with probiotics.

Conclusion: Our study demonstrates that the bacterial strains *Bifidobacterium breve* and *Lactobacillus rhamnosus* can prevent the recruitment of bone marrow cells during allergic inflammation. Oral treatment with these beneficial bacterial strains also causes long-term changes in the mast cell progenitor population in the bone marrow. Mast cells cultured from bone marrow of beneficial bacteria-treated animals show a greatly inhibited response to IgE-

mediated activation. These inhibitory effects may be caused by the induction of epigenetic changes following treatment with *Bifidobacterium breve* and *Lactobacillus rhamnosus*.

Introduction

Allergic diseases, such as asthma, are now estimated to affect approximately 30% of the population in western countries [1]. Asthma is a chronic inflammatory disorder of the airways characterized by airway inflammation and hyper-responsiveness (AHR), and structural changes in the airway walls, referred to as "airway remodeling" [2,3]. Different inflammatory/immune cells and mediators are involved in the pathogenesis of the disease.

Animal and human studies indicated an important role for mast cells in asthma [4, 5]. Mast cells originate from bone marrow-derived mast cell progenitors and mature into mast cells upon entry to the peripheral organs [6]. Infiltration of mast cells into the airway smooth muscle cell layer of allergic asthmatics is a key feature of asthma and is thought to be associated with AHR [7, 8]. Binding of the inhaled allergen to the allergen-specific immunoglobulin E (IgE) molecules that are already bound to the high affinity receptors (FcεRI) on surface of mast cells leads to cross-linking of the FcεRI receptors and subsequent degranulation and activation of mast cells and rapid release of mediators [9]. Additionally, in the late phase reaction, arachidonic acid metabolites are formed and various cytokines and chemokines are produced. The released pro-inflammatory pre-stored mediators such as the enzyme beta-hexosaminidase, as well as *de novo* sensitized substances induce smooth muscle contraction and mucus secretion, two key features of asthma [10]. Hence, mast cells degranulation and activation is detected in asthmatic lungs and various mast cell mediators are found in the bronchoalveolar lavage (BAL) fluid of asthma patients [11].

In addition to their expression of the FcεRI receptor, which they share with other cells, mast cells express exclusively the stem cell factor (SCF) receptor c-Kit [12]. Mast cell development and growth are regulated by SCF and a variety of growth factors and cytokines, such as interleukin 3 (IL3). Interleukin 3 directly stimulates proliferation of uncommitted progenitors in rodents as well as in humans (13). Culturing of mouse bone marrow cells in IL3-containing media results in the generation of more than 95% pure populations of immature mast cells termed mucosal bone marrow-derived mast cells (BMMC). BMMC have been extensively used in the investigation of mouse mast cell biology [13].

Despite the effectiveness of the current therapies for asthma, these treatments have many limitations and side effects [14-17] and there is still a need for novel therapeutics targeting

underlying allergic and immune responses with greater disease control, increased efficacy and a major clinical effect.

Over the past decades there has been an increasing awareness of the role played by the gut microbiotica in the development of allergies and asthma [18-21]. Currently, there is a great interest in the therapeutic potential of bacteria-based strategies, such as the use of probiotics, for immune disorders, allergies and asthma. Probiotics are defined as"live microorganisms which, when consumed in adequate amounts, confer a health benefit on the host" [22-24]. It is proposed that these beneficial bacteria exhibit their therapeutic effects at site of inflammation and infection by modulating both the local gut mucosal and systemic immune responses [25, 26]. Animal studies have demonstrated effectiveness of *Bifidobacteria* and *Lactobacilli* at suppressing both allergic and autoimmune responses, reducing allergic symptoms and inhibiting allergic airway response in mice [24, 27-31].

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 [2, 32]. Besides their well established role in the initiation of inflammation, mast cells and IgE play a potential role in chronic asthma [33].

Previously, we have demonstrated that *Bifidobacterium breve* and *Lactobacillus rhamnosus* inhibited mucosal mast cell degranulation by reducing mMCP-1 levels in serum of mice in a murine model of chronic asthma (chapter 5). In this current study, we further investigated the effect of long-term treatment with *B. breve* and *L. rhamnosus* on development and IgE-induced activation of mast cells cultured from bone marrow of allergic mice. Additionally, the effect of *in vitro* co-culture of BMMC with *Bifidobacterium breve* was investigated. Findings from this preliminary study could contribute to a better understanding of the anti-allergic and therapeutic effects of these specific bacterial strains in chronic allergic asthma.

Material and Methods

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.

Chronic asthma model

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 (AlumImject; Pierce, Rockford, IL, USA). Control animals received 0.1 mL saline only (NaCl 0.9%; B. Braun Medical B.V., Oss, The Netherlands) (Figure 1).

OVA challenge

A chronic model of asthma was established according to a modification of a model of prolonged allergen-induced airway inflammation described in [2]. Mice 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 were exposed to nebulized saline aerosol only. 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 only for 20 min (Figure 1).

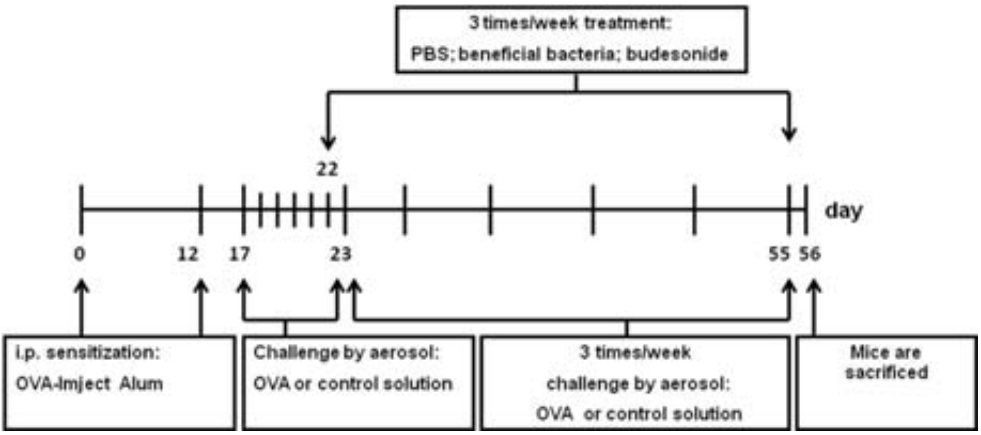

Figure 1: Time schedule of the chronic asthma mouse model. Male BALB/c mice were sensitized intraperitoneally with alum-precipitated OVA on days 0 and 12 and mice were challenged from day 17 until day 23 daily with aerosolized OVA or saline. From day 22 until day 55, mice were treated 3 times a week with budesonide or PBS by oropharyngeal aspiration; *B. breve* or *L. rhamnosus* by oral gavage. 1 h after treatment, from day 24 until day 55, mice were challenged 3 times/week with aerosolized OVA or saline. Animals were sacrificed on day 56 after pulmonary function measurement.

Beneficial bacteria treatment

Bifidobacterium breve M-16V (*B. breve*, Morinaga Milk Industry, Tokyo, Japan) and *Lactobacillus rhamnosus* NutRes1 (*L. rhamnosus*, Danone Research, Wageningen, The Netherlands) were grown in MRS (Oxoid, Basingstoke, UK), supplemented with 0.5 g/L L-cysteine for *Bifidobacteria*, at pH 6.5 and under anaerobic conditions. Bacteria were harvested in the early stationary phase, washed with phosphate buffered saline (PBS, Lonza Leusden, The Netherlands) and stored with glycerol 20% (w/v), in aliquots at -80°C. Cell counts were determined by plating serial dilutions and fluorescent microscopy by staining with DAPI. The bacteria were resuspended in PBS prior to use.

Mice received 10^9 colony forming units (CFUs) of *B. breve* or $1.1x10^9$ CFUs of *L. rhamnosus* per animal per day. The bacteria were suspended in 0.2 mL of PBS and given by oral gavage, 1 h prior to challenge, three times a week from day 22 until day 55 (Figure 1).

Budesonide treatment

As a reference treatment, mice received 0.5 μg/g of mouse/day of budesonide (Sigma) in PBS. Budesonide was administered to mice by oropharyngeal aspiration after induction of light isoflurane anesthesia as described previously in [34], 1 h prior challenge, three times a week from day 22 until day 55. Control animals received 50 uL of PBS by the same administration route. Mice were rendered asthmatic following the schema presented in Figure 1.

Mouse bone marrow isolation

After sacrifice, on day 56, intact femurs and tibia were removed and bone marrow cells were isolated from the bone marrow of male BALB/c mice according the following protocol: sterile PBS was repeatedly flushed through the bone shaft using a syringe with a needle. Single cell suspensions of the bone marrow were made using a 70 μm nylon cell strainer (BD Biosciences, USA). The cells were centrifuged at 1,300 rpm and 22°C for 4 min, resuspended in PBS and centrifuged again. The pellet was subjected to red cell lysis using a buffer containing NH_4Cl (MERCK, Darmstadt Germany), $KHCO_3$ (Sigma) and 5% ethylenediaminetetraacetic acid (EDTA, MERCK) in demineralized water and cells were centrifuged at 1,460 rpm and 22°C for 4 min. The pellet was resuspended in PBS and the total number of cells was determined using a haemocytometer (Assistent, Germany). After centrifugation, the supernatant was removed and the cells were resuspended (dropwise) in freezing medium containing 10% dimethyl sulfoxide (DMSO; Sigma, USA) and 90% heat-inactivated fetal calf serum (FCS, Hyclone Laboratories, USA) and frozen at -70°C for 24 hrs and then stored in liquid nitrogen until further analysis. An overview of the groups included in this study is given in the table below.

Table 1: mouse groups included in the study

Sensitization	Challenge	Treatment	Group abbreviation
OVA	Saline	PBS	**OVA/Sal-PBS**
OVA	OVA	*B. breve*	**OVA/OVA-*B. breve***
OVA	OVA	*L. rhamnosus*	**OVA/OVA-*L. rhamnosus***
OVA	OVA	budesonide	**OVA/OVA-budesonide**

Mouse bone marrow cultures

Bone marrow-derived mast cells (BMMC) were generated from the bone marrow of male BALB/c mice according the following protocol: after thawing the cells at room temperature, bone marrow cells were cultured in a 175 cm^2 tissue culture flask with filter (Cellstar, Germany) with 100 mL of complete medium (BMMC medium 1), consisting of RPMI 1640 with glutamine and phenol red (Lonza) supplemented with 10% FCS, 1% penicillin-streptomycin solution (pen-strep, Sigma), 8 mM L-Glutamine (Sigma, Germany), 2 μM sodium pyruvate (Sigma, USA), 6 μL 2-mercaptoethanol (Sigma, USA) and 20 mL MEM non-essential amino acid solution (GIBCO, USA) combined with 1 ng interleukin 3 (IL3 mouse; Prospec, USA) and 2 ng stem cell factor (SCF mouse; Prospec, USA), at 37°C in a humidified atmosphere with 5% CO_2. The amounts of mouse IL3 and SCF were reduced to 0.5 ng of IL3 and 1 ng of SCF in culture weeks 1, 2 and 3. Nonadherent cells were centrifuged and resuspended in fresh medium once a week. Bone marrow cells were cultured for 3 weeks to develop BMMC and cells were used in week 4 for the experiments.

Degranulation assay

Cells were resuspended and transferred to a Cellstar sterile 96-well V-bottom plate (GREINER BIO ONE, USA) and washed twice with BMMC medium 2 supplemented with gentamycine. Cells were transferred to a Costar sterile 96-well flat bottom plate (Corning Life Sciences, USA) and sensitized with 20% anti- 2,4-Dinitrophenol (DNP) IgE from hybridoma clone 26.82 at 37 °C and 5% CO_2, for 2 hrs. BMMC were transferred to a 96-well V-bottom plate and washed twice with BMMC medium 2 supplemented with gentamycine and then transferred to a 96-well flat bottom plate. Cells were activated with 20 ng/mL 2,4-Dinitrophenyl hapten conjugated to human serum albumin (HSA), DNP-HSA, at 37 °C and 5% CO_2, for 1 hr. For the dose response curve of DNP-HSA, BMMC generated from bone marrow cells of naïve male BALB/c mice were incubated with 1, 2.5, 5, 10, 25, 50, 100, 250 and 500 ng/mL of DNP-HSA. 10% Triton X-100 (TX-100; Merck, Germany) was used to obtain 100% release of beta-hexosaminidase. Cells were centrifuged at 1,200 rpm and 22°C, for 5 min and the supernatant was transferred to a new 96-well flat bottom plate. BMMC were incubated with 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUG; Sigma, USA) solution in 0.1M citrate buffer (Merck, Germany) (pH = 7.2), as a substrate for beta-hexosaminidase, at 37°C and 5% CO_2 for 1 h. The reaction was stopped with 0.2 M glycine buffer (MP, USA) (pH = 10.7). Beta-hexosaminidase release was

measured by fluorescence (excitation: 351 nm, emission: 462 nm).The percentage of degranulation was calculated as: [(a-b)/(t-b)] X 100 where a is the amount of beta-hexosaminidase released from stimulated cells, b is that released from unstimulated cells, and t is total cellular content.

Measurement of c-kit and FcεRI receptor expression on BMMC

After centrifugation at 1,500 rpm and 22°C for 10 min, the pellet was resupended in BMMC medium 1 and cells were counted. BMMC were incubated at a density of 6 x 10^5 cells with 1.5 μL of fluorescein isothiocyanate (FITC)-conjugated anti-FcεRI alpha (FcεRI, IgE receptor) antibody or 3 μL of r-phycoerythrin (PE) conjugated anti-CD117 (c-Kit, SCF receptor) antibody (eBioscience, San Diego, CA, USA) for 1 h at room temperature. After centrifugation, the pellet was washed twice with FACS buffer containing PBS (Lonza) supplemented with 1 % FCS (Hyclone Laboratories) and 0.1% Na-Azide (Sigma) and resuspended in FACS buffer for flow cytometry analysis.

The stained cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences, USA). Data analysis was performed using BD FACSDIVA^{TM} software (BD Biosciences).

In vitro co-culture of BMMC with *Bifidobacterium breve*

BMMC were cultured at a density of 1.5 x 10^6 cells/mL in BMMC medium 2 consisting of RPMI 1640 without L-glutamine and phenol red, and supplemented with all factors mentioned in the previous paragraph for BMMC medium 1 plus 10 μg/mL gentamycine (Invitrogen, USA). BMMC were overnight incubated with different concentrations (1:1; 10:1 and 50:1 bacteria:cell) of viable *B. breve* bacteria at 37°C and 5% CO_2.

Cell viability

To determine cell viability, BMMC were transferred to fresh BMMC medium 2 and incubated overnight at 37°C in a humidified atmosphere with 5% CO_2. On the next day, cells were centrifuged at 100 rcf and 22°C for 5 min, and the pellet was resuspended in PBS. Cells were diluted twice with 0.4% trypan blue solution (Sigma, Germany) and the unstained (viable) and stained (non-viable) cells were counted twice by two independent observers with the haemocytometer. The percentage of viable cells was calculated by dividing the total number of viable cells by the total number of cells and multiplying by 100.

Statistical analysis

Data analysis was performed using a 1-way or 2-way analysis of variance (one-way ANOVA or two-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).

Results

Treatment with *B. breve* and *L. rhamnosus* prevents depletion of bone marrow cells during allergic inflammation

In contrast to all other treatment groups, the bone marrow of OVA/OVA-PBS mice was deprived from mast cell progenitors as the number of the isolated bone marrow cells was very low, compared to the other groups, and no BMMC could be induced. Treatment with *B. breve*, *L. rhamnosus* and budesonide prevented the depletion of bone marrow cells in OVA/OVA mice (Figure 2A). Subsequently bone marrow cells were isolated and cultured in presence of IL3 and SCF to differentiate them into BMMC. The number of cultured cells observed in weeks 1, 2, 3 and 4 after start of the bone marrow culture did not differ significantly across the different treatment groups (Figure 2B).

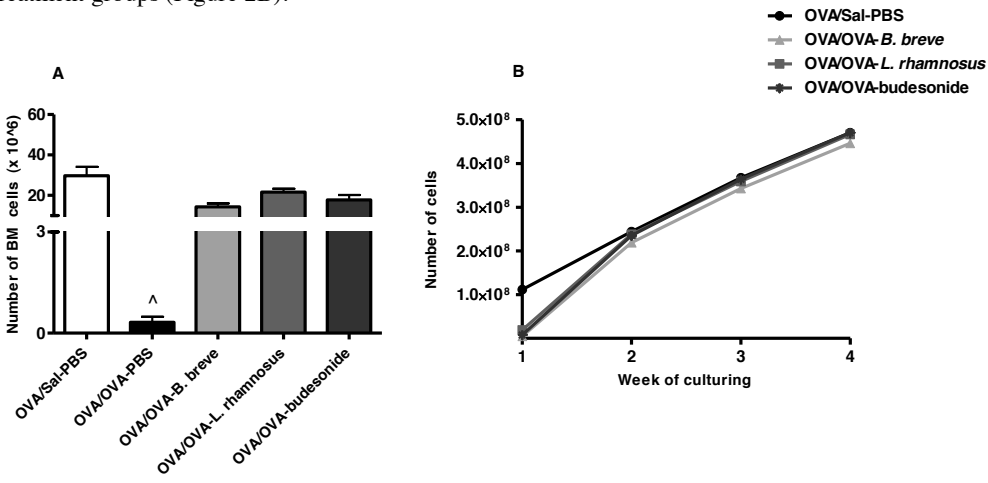

Figure 2: Number of bone marrow cells isolated from mouse bone marrow and the number of BMMC cultured. The number of bone marrow cells from mice from the different treatment groups is shown in A. The number of BMMC observed after 1, 2, 3 and 4 weeks of culturing is shown in B. Data are expressed as mean ± SEM, n=3-5 mice/group. BM= bone marrow cells. 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 all other groups.

Activation of BMMC from naive mice with 10 and 25 ng/mL of DNP-HSA induced the highest mast cell degranulation

To determine the optimal DNP-HSA concentration, bone marrow cells were isolated form naïve mice and BMMC were generated and cultured as described previously in material and methods. A dose response curve of DNP-HSA with a range from 1 to 500 ng/mL was made (Figure 3). Significantly higher beta-hexosaminidase release was observed with 25 ng/mL (top of the curve) followed by 10 ng/mL of DNP-HSA as compared to all other concentrations, therefore 20 ng/mL was chosen as the optimal concentration for further assays.

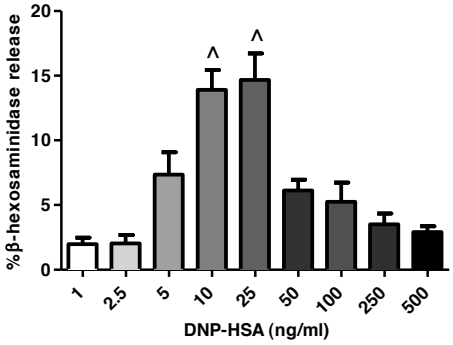

Figure 3: DNP-HSA dose response curve. BMMC ($1.5x10^6$ cells/mL) cultured from bone marrow cells of untreated mice were sensitized with anti-DNP IgE for 2 hrs and then incubated with different DNP-HSA concentrations for 1 h. Degranulation was measured by using beta-hexosaminidase release assay. Data are expressed as mean ± SEM, n=1 mouse. All concentrations were tested in sextuplicate. 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 1, 2.5, 5, 50, 100, 250, 500 ng/mL DNP-HSA.

BMMC cultured from bone marrow of mice long-term treated with *L. rhamnosus* show reduced expression of c-Kit and FcεRI

In order to explore the effect of the different treatments on the development of mast cell, the expression levels of c-Kit and FcεRI on the surface of BMMC was determined by flow cytometry (Figure 4). In the OVA/Sal-PBS group, 97.8% of BMMC exhibited surface receptor for SCF, c-Kit (Figure 4A). 96% of BMMC obtained from OVA/Sal-PBS mice exhibited surface

high affinity IgE FcεRI receptor (Figure 4B). BMMC derived from bone marrow of *L. rhamnosus*-treated OVA/OVA mice showed a small, but significant, decrease in the relative number of c-Kit-positive BMMC and tended to decrease the relative number of FcεRI-positive BMMC as compared to the OVA/Sal-PBS group. Likewise, BMMC cultured from bone marrow of budesonide-treated OVA/OVA mice showed reduced expression of c-Kit and FcεRI, as shown by the significant decrease in the relative numbers of c-Kit-positive BMMC and FcεRI-positive BMMC. *B. breve* treatment, did not affect the relative number of c-Kit-positive BMMC obtained from OVA/OVA mice, but significantly increased the relative number of FcεRI-positive BMMC.

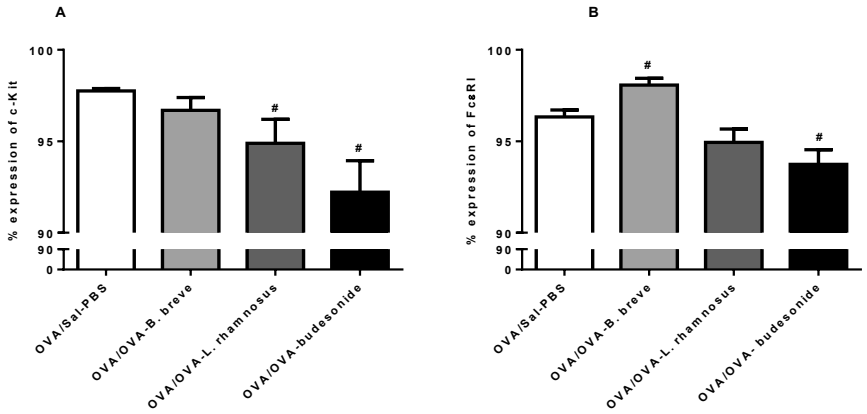

Figure 4: Long-term treatment of mice with *L. rhamnosus* or budesonide reduces c-Kit and FcεRI expression on surface of BMMC. c-Kit and FcεRI expression was measured by flow cytometry. Data are expressed as mean ± SEM, n=3 mice/group. All samples were tested in triplicate. Statistical significance of 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.

Treatment of mice with *B. breve* and *L. rhamnosus* induces long-term inhibitory effects on mast cell progenitors to IgE-mediated activation

To examine the effect of *in vivo B. breve* and *L. rhamnosus* treatment of allergic mice on *in vitro* mast cell degranulation, beta-hexosaminidase release was measured *in vitro* in BMMC generated from bone marrow cells of allergic mice from the different treatment groups (Table 1) after sensitization with DNP-specific IgE and activation with DNP-HSA (Figure 5). BMMC obtained from the bone marrow of OVA-sensitized, saline-challenged and PBS-treated (OVA/Sal-PBS) mice, showed a normal degranulation response. Long-term treatment with *B. breve* almost

completely suppressed the degranulation of BMMC obtained from OVA/OVA mice treated with *B. breve*. BMMC obtained from the OVA/OVA-*L. rhamnosus* group showed significantly lower degranulation response as compared to the OVA/Sal-PBS group. Budesonide treatment tended to increase the degranulation of BMMC obtained from OVA/OVA mice as compared to all other groups.

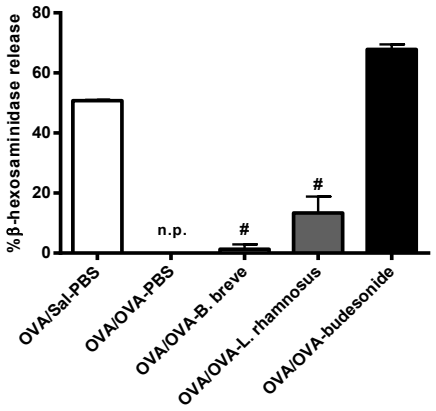

Figure 5: Long-term treatment of mice with *B. breve* and *L. rhamnosus* attenuates IgE-mediated BMMC degranulation. BMMC (1.5 x 10^6 cells/mL) were sensitized with anti-DNP IgE for 2 hrs and then incubated with 20 ng/mL of DNP-HSA for 1 h. Degranulation was measured by using beta-hexosaminidase release assay. Mice in all groups were sensitized with ovalbumin, OVA/OVA mice were challenged with ovalbumin and OVA/Sal mice were challenged with saline. Mice were treated with PBS (OVA/Sal-PBS; OVA/OVA-PBS), *B. breve* (OVA/OVA-*B. breve*), *L. rhamnosus* (OVA/OVA-*L. rhamnosus*) or budesonide (OVA/OVA-budesonide). Data are expressed as mean ± SEM, n=1 mouse/group. All samples were tested in triplicate. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparison test after two-way ANOVA. # Statistically significant difference ($p<0.05$) compared to the OVA/Sal-PBS group. n.p = not performed.

In vitro co-culture of BMMC with *B. breve* reduces IgE-mediated degranulation of BMMC

To determine whether *B. breve* could directly modulate mast cell degranulation, BMMC obtained from mice were co-cultured *in vitro* with *B. breve* at different bacteria:cell ratios prior to sensitization with DNP-specific IgE and activation with DNP-HSA (Figure 6). *In vitro* co-culture of BMMC obtained from bone marrow cells of OVA/Sal-PBS mice with *B. breve* reduced degranulation by almost 50% at all tested probiotic:cell ratios as compared to control cells in this group. Likewise, *in vitro* co-culture of BMMC obtained from bone marrow cells of

OVA/OVA-budesonide mice with *B. breve* reduced degranulation by almost 50% at all tested probiotic:cell ratios as compared to control cells in this group.

The *in vitro* exposure of BMMC obtained from bone marrow cells of OVA/OVA-*B. breve* and OVA/OVA-*L. rhamnosus* to *B. breve* did not alter subsequent degranulation in response to antigen exposure as compared to control cells in these groups.

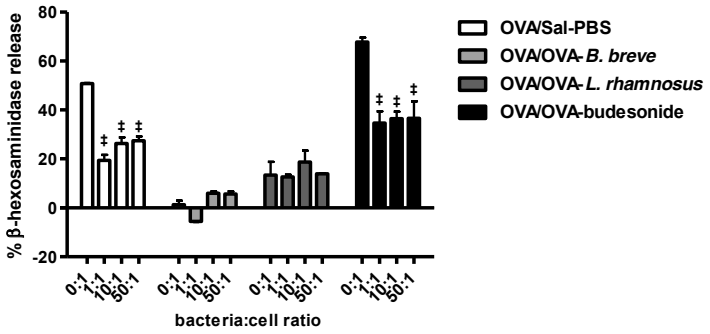

Figure 6: Effects of in vitro co-culture of BMMC with *B. breve* on mast cell degranulation. BMMC (1.5 x10^6 cells/mL) were overnight incubated with different concentrations of B. breve in the presence of 10 ug/mL gentamycine. BMMC degranulation was studied by measurement of beta-hexosaminidase release after sensitization with anti-DNP IgE and stimulation with 20 ng/mL DNP-HSA for 1 h. Data are expressed as mean ± SEM, n=1 mouse/group. All samples were tested in triplicate. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparison test after two-way ANOVA. ‡ Statistically significant difference ($p<0.05$) compared to control cells in each group.

In vitro co-culture of BMMC with *B. breve* does not affect cell viability

To examine the effect of *in vitro* co-culture of BMMC with *B. breve* on cell viability, a trypan blue viability assay was performed (Figure 7). *In vitro* exposure to *B. breve* did not affect BMMC viability and the percent viable cells did not differ across all groups.

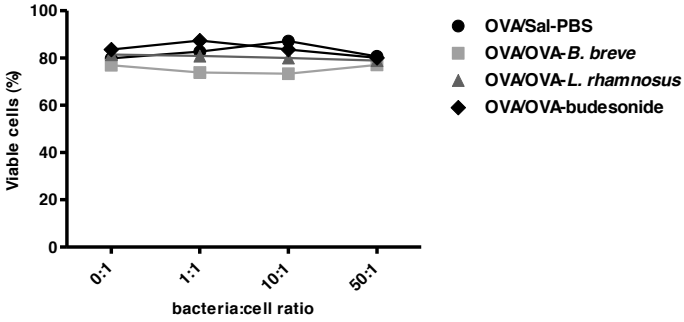

Figure 7: Cell viability test. The number of viable cells in the cell suspension after overnight incubation with *B. breve* was determined by using trypan blue exclusion test.

Discussion

The aim of this study was to explore further the anti-allergic effects of *B. breve* and *L. rhamnosus* in a murine model of chronic asthma. We have demonstrated that oral treatment of mice with *B. breve* and *L. rhamnosus,* previously demonstrated to inhibit mucosal mast cell degranulation *in vivo* by reducing mMCP-1 levels in serum of mice in a murine model of chronic asthma (chapter 5), prevent the depletion of bone marrow cells during the allergic inflammation elicited in the airways. These bacterial strains were equally active as treatment with the corticosteroid budesonide. The bone marrow of OVA/OVA-PBS mice was deprived from mast cell progenitors. Hence, the number of bone marrow cells isolated from this group was much lower than the number of cells isolated from the other groups. Additionally, in repeated experiments, the very limited number of bone marrow cells which could be isolated from OVA/OVA-PBS animals did not raise any mast cells in culture. This finding might be caused by the inflammatory status of these animals since it has been described that during chronic airway inflammation in mice, mast cell progenitor cells as well as other myeloid progenitor cells from the bone marrow travel to the inflamed airways to develop into mast cells and other inflammatory cells resulting in low number of bone marrow cells [35, 36].

Long-term treatment of OVA/OVA mice treated with *B. breve* or *L. rhamnosus* lead to a significant inhibition of IgE-mediated degranulation in mast cells cultured *in vitro* from bone marrow from these animals. Interestingly, BMMC from *B. breve*-treated mice showed almost completely suppression of IgE-mediated degranulation as compared to BMMC cultured from bone marrow of OVA/Sal-PBS animals. The greatly reduced responsiveness of the cultured BMMC could not be explained by reduced maturation. Although *L. rhamnosus* treatment decreased the relative number of c-Kit-positive BMMC to some extent and also it tended to decrease the relative number of FcεRI-positive BMMC in OVA/OVA mice as compared to the OVA/Sal-PBS group, an even greater decrease was found in BMMC from the budesonide-treated group, while these BMMC are not compromised in their response to IgE-receptor cross-linking.

A previous study with a different strain of *L. rhamnosus* bacteria demonstrated inhibition of IgE-mediated mast cell degranulation in rats after oral treatment with *L. rhamnosus* [26]. Another study demonstrated inhibitory effects of *Lactobacillus* bacteria on IgE-mediated mast cell degranulation in a murine model for acute allergic inflammation [37]. However, the mechanism

by which these potentially beneficial bacteria reduced mast cell degranulation still needs further investigation. Since ingested *B. breve* and *L. rhamnosus* bacteria are expected to colonize the intestine, the place at which the interaction between the bacteria and mast cells has taken place still needs to be identified. Interestingly, the inhibitory effect of *B. breve* on mast cell degranulation could be mediated by a direct interaction between mast cells and bacteria. This is supported by the finding that *in vitro* co-culture of *B. breve* with BMMC obtained from bone marrow cells of "healthy" controls (OVA/Sal-PBS) or OVA/OVA-budesonide mice reduced antigen-specific response of BMMC and decreased degranulation by almost 50% compared to the control BMMC in these groups. However, the antigen-specific response of BMMC obtained from bone marrow cells of OVA/OVA-*B. breve* or OVA/OVA-*L. rhamnosus* mice was not attenuated by *in vitro* co-culture with *B. breve*. Co-culture with *B. breve* did not induce cell death.

The inhibitory effects by *B. breve* and *L. rhamnosus* on the mast cell progenitor cells in bone marrow are more likely to be caused by the induction of epigenetic changes, because during the differentiation of bone marrow into mast cells there is no contact with bacteria or bacterial components. This is supported by the observations that specific nutrients, such as probiotics, induce changes in gene expression during early development and that these changes were implicated in epigenetic changes in disease predisposition [38]. Additionally, a previous *in vitro* study demonstrated epigenetic effects of a different strain of *B. breve* on the intestinal mucosal immune system by reducing histone acetylation and enhancing DNA methylation [39].

Here we demonstrate that long-term treatment of chronic asthmatic mice with *B. breve* or *L. rhamnosus* suppresses IgE-mediated BMMC degranulation *in vitro*. Additionally, the observed inhibitory effects of *B. breve* and *L. rhamnosus* on the mast cell progenitor cells in bone marrow are likely to be caused by the induction of epigenetic changes. These preliminary findings suggest that inhibition of IgE-mediated mast cell degranulation might be a component of the systemic immunomodulatory effects of *B. breve* and *L. rhamnosus* and this may contribute to the anti-allergic effects of these beneficial bacteria. However, future studies are necessary to investigate which factors or components of the bacteria could be responsible for this inhibitory effect.

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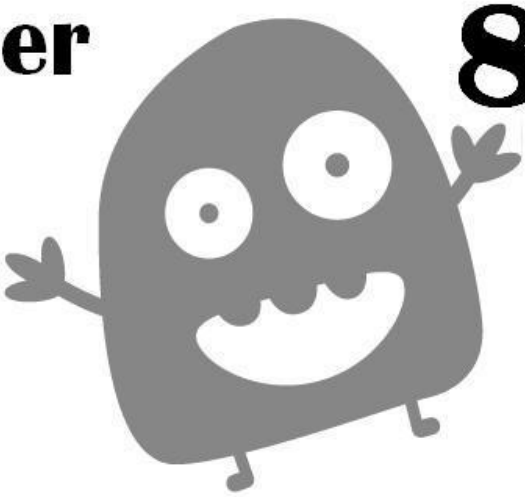
Chapter 8



General discussion

1. Inflammation and immunity in mild and severe allergic asthma

As described in **chapter 1**, asthma is a heterogeneous inflammatory disorder of the airways with different phenotypes varying in clinical, physiological and pathological features [1-3]. In **chapter 2** inflammation and immunity were investigated in experimental models for mild and severe asthma, respectively.

Higher total inflammatory cell number was observed in the bronchoalveolar lavage (BAL) fluid of allergen-sensitized and challenged mice in the severe model when compared to the mild model. Both models showed a major increase in eosinophil infiltration into the airways. No increase in the number of neutrophils was observed in the severe asthma model, possibly due to the fact that neutrophil accumulation is prominent in the BAL fluid of asthmatic subjects during acute severe asthma exacerbations [4]. Different mRNA expression profiles of T cell-related cytokines were observed in the mild and severe models for allergic asthma. A profound change, 13-fold, in *interleukin 10* (*Il10)* mRNA expression was observed in whole lung tissue of allergen-sensitized and challenged mice in the mild model. The expression of *Il4*, *Il5, Il6* and *Tnfα* were also increased in this model, but to a lesser extent. The increase in *Il10* expression might be necessary to limit the inflammation in the airways and to counter the effects of the other cytokines on disease progression as IL10 is a potent anti-inflammatory and can inhibit the synthesis of several inflammatory cytokines (including asthma-associated cytokines such as tumor necrosis factor-α (TNFα) and IL5) and gene presentation [5-8]. In the severe model, increased expression of *Il6*, *Il5*, and *Il10* was observed in allergen-sensitized and challenged mice. Yet, *interferon γ* (*Ifnγ*) showed the largest change in expression suggesting a shift of the immune balance from Th2 towards Th1 response. In severe asthmatics it was demonstrated that *in vitro* synthesis of IFNγ increases with asthma severity. However, it is also reported that IFNγ decreases asthma by decreasing the number of Th2 cells [6,9]. IL5 and IL6 were reported to enhance asthma by increasing the number of eosinophils and inflammation, respectively, and increased levels of IL5 and IL6 are found in the sputum of asthmatic subjects [6].

In mild and severe asthma different T cell-specific transcription factor mRNA expression profiles were observed in whole lung tissue of allergen-sensitized and challenged mice. In the mild model enhanced mRNA expression of the Treg-specific transcription factor *forkhead box P3* (*Foxp3*) was the most prominent. In the severe model, *Foxp3* and *Tbet,* Th1-specific

transcription factor, showed the highest expression. Interestingly, allergen sensitization and challenge resulted in a strong T reg response, and the expression of *Foxp3* was correlated with *Il10* expression in both models. Animal studies have shown that *Il10* produced by pulmonary dendritic cells (DCs) from mice exposed to ovalbumin induces the development of Treg cells through an effect on Foxp3 resulting in regulation/suppression of both Th2 and Th1 cells [7, 10]. In the mild model, *Foxp3* showed the largest change in expression when compared to the severe model. *Tbet*, on the other hand, showed the largest increase in mRNA expression in the severe model. Comparable expression levels of *Gata3,* the Th2-specific transcription factor, were found in allergen-sensitized and challenged mice in both models. *Gata3* is reported to inhibit both the *in vivo* and *in vitro Foxp3* gene induction by directly binding to the *Foxp3* promoter in mice [11]. Speculating on our findings in the mild model, an opposite action of *Foxp3* might also be possible, and a counter-regulatory mechanism of Treg/Th1 to suppress theTh2 immune response may occur. Our results, however, might also be explained by measurement of mRNA expression in mouse whole lung tissue instead of in peripheral blood mononuclear cells (PBMCs) as performed in asthmatics and/or measurement of mRNA expression instead of protein expression. The finding in the severe model might be explained by the fact that severe asthma frequently includes a Th1 component as well as a Th2 immune response [1]. In the mild model, *Tbet* mRNA expression was strongly correlated with the expression of innate cytokines (*Il2, Il6 and Tnfα*) and Th2 cytokines (*Il4, Il5 and Il13*), previously reported to enhance asthma in human, but not with Th1 cytokine (*Ifnγ*). This might be caused by the Treg response observed in this model. In the severe model, *Rorγt,* Th17-specific transcription factor, mRNA expression was significantly correlated with *Il6*. The findings that *Il2, Il4, Il5, Il13, Tnfα* mRNA expression was increased in mild asthma and *Il6* in both models and that no correlations were found between *Gata3* and Th2 cytokines mRNA expression might suggest a counter regulatory mechanism of Treg/Th1 to suppress the Th2 immune response as described previously.

To elaborate on the differential regulation of immunity in mild and severe asthma, mRNA expression profiles of different toll-like receptors (TLR) and nod-like receptors (NLR) were investigated. Interestingly, different TLR and NLR mRNA expression profiles were observed in mild and severe asthma. Low *Tlr2* expression was observed in whole lung tissue of ovalbumin-sensitized and/or challenged mice in the mild model. This is in line with human studies which have linked decreased *TLR2* mRNA expression to increased asthma susceptibility [12-14]. In the

severe model, increased expression of *Tlr2* in allergen-challenged only mice was observed. Additionally, *Tlr1* expression was significantly increased in allergen-sensitized and challenged mice in this model. Murine models of allergic asthma have shown that *Tlr2* heterodimers have the potency to both inhibit and promote the development of allergic immune responses [15].
Decreased *Tlr3* expression was observed in whole lung tissue of allergen-sensitized and challenged mice in both asthma models. To date, no direct associations between *Tlr3* expression and function and asthma have been reported yet. However, *Tlr3* was up-regulated in the spleen of rats in an antigen induced pulmonary inflammation model [16]. Due to differences in *Tlr3* expression measurement between the latter study and our study no hard conclusion can be made about the role of this receptor in mild and severe asthma. Additionally, further research is needed to investigate the implication of the decrease in *Tlr3* mRNA expression observed on the inflammatory status of the disease. The expression levels of *Tlr4, Tlr5* and *Tlr6* remained unchanged in both asthma models. Possibly, a plasmacytoid DCs response to allergen challenge increased *Tlr7* expression in the severe model [17]. The expression levels of *Tlr8, Tlr9* and *Tlr11, Tlr12, Tlr13* and *Nod2* remained unchanged in both asthma models. Decreased *Nod1* expression was observed in allergen-sensitized and challenged mice in the severe model. Despite the positive association between single nucleotide polymorphisms (SNPs) in the *Nod1* gene and susceptibility towards asthma, the exact mechanism on cellular or molecular level of these in relation to asthma pathology is still unknown [18-20]. The correlation of *Tlr1*, *Tlr3*, *Tlr6*, *Tlr9*, *Tlr11*, *Tlr13*, *Nod1*, and *Nod2 mRNA* expression with the total inflammatory cell number in the BAL fluid observed in the severe model might be caused by an increase in the number of inflammatory cells which express these receptors. As this does not apply to all TLRs, these receptors could also contribute to the increased sensitivity to inflammation/exacerbation since a small trigger can lead to an inflammatory cascade. The exact link between the found correlations and asthma pathogenesis remains to be elucidated.

2. Beneficial bacterial strains and inflammation in chronic allergic asthma

As reviewed in **chapter 1** beneficial bacteria as potential modulators of the intestinal microbiota and mucosal immune responses, have gained a lot of attention recently. In **chapter 3** the therapeutic effects of long-term treatment with two different beneficial bacterial strains

(*Bifidobacterium breve (B. breve)* and *Lactobacillus rhamnosus(L. rhamnosus)*) on inflammatory response in the lung was explored in a murine model for chronic allergic asthma. In this study a glucocorticoid, budesonide, was used as a reference treatment. It was demonstrated, for the first time in a chronic asthma model, that treatment with *B. breve* and *L. rhamnosus* was as effective as budesonide treatment in suppressing increased ovalbumin-induced neutrophil and eosinophil infiltration into the airways observed in the allergic group. These findings are in accordance with previous animal and human studies in which different strains of *Bifidobacteria* and *Lactobacilli*, and budesonide have been individually investigated in acute allergic asthma models [21-27]. Interestingly, the mRNA expression of TLRs and NLRs in mouse whole lung tissue was differentially regulated by the different treatments. Remarkably, *B. breve* treatment skewed the immune response toward Th1 by significantly increasing *Tlr9* expression, as TLR9 was shown to modulate the immune response in allergic asthma by skewing the increased Th2/Th1 balance towards Th1 [13, 28, 29]. Additionally, *L. rhamnosus* and budesonide treatments increased *Tlr9* expression, but not significantly. Allergen-sensitized and challenged, PBS-treated mice showed decreased *Tlr3* and *Nod1* expression. Whether this decreased expression of *Tlr3* and *Nod1* is pro-inflammatory or anti-inflammatory remains to be investigated. However, the increase in *Tlr3* expression by *L. rhamnosus* and budesonide treatments and the increase of *Nod1* expression by budesonide treatment suggest that the lowered *Tlr3* and *Nod1* measured in the asthmatic mice is pro-inflammatory in nature. Moreover, *L. rhamnosus* also decreased the expression of *Tlr4* observed in allergic mice. Whether this decrease in *Tlr4* expression caused by *L. rhamnosus* treatment is beneficial for the disease status of the animals need to be investigated. Hence, reduced TLR4 activation in asthmatic subjects was suggested to contribute to asthma by reducing the release of Th1 and anti-inflammatory cytokines [30]. In accordance with the Th2-biased response found in chronic asthma [21] decreased *Tbet* and *Foxp3* mRNA expression and high *Gata3*/*Tbet* ratio represented Th2 dominance in the chronic allergic mice. Importantly, *B. breve* treatment did not affect T-cell transcription factor mRNA expression, but significantly increased *Foxp3*/*Rorγt* and *Foxp3*/*Gata3* ratios and significantly decreased *Gata3*/*Tbet* ratio and thereby shifted the immune balance towards Th1 and Treg. A different strain of *Bifidobacterium*, *B. animalis*, showed similar effect on immune balance skewing in a preventative, acute mouse model for respiratory allergy [31]. Consistent with results from previous studies in asthmatic subjects [32], budesonide treatment encouraged regulatory responses by significantly increasing

the expression of *Foxp3*. Yet budesonide did not influence the *Gata3/Tbet, Foxp3/Rorγt, Foxp3/Gata3* and *Foxp3/Tbet* ratios. *L. rhamnosus* treatment showed only a moderate effect by significantly decreasing the *Gata3/Tbet* ratio, but did not affect *Tbet*, *Gata3*, *Foxp3* or *Rorγt* mRNA expression. Interestingly, all treatments increased the function of Treg cells in the blood of treated mice by significantly increasing the number of Foxp3-expressing Tregs. Hence, increased expression of Foxp3 in Treg cells was reported to be directly associated with increased function of Treg cells [33, 34].

In **chapter 3** it is also shown that the mRNA expression of T cell-specific cytokines is differentially regulated by the different treatments. Due to the chronic inflammatory status of control allergic mice and consistent with data from asthmatic subjects [6], the mRNA expression of the pro-inflammatory cytokines *Il1β* and *Il6* was significantly increased and the expression of *Il13* and *Il17* tended to be increased. *B. breve* treatment induced a regulatory response by significantly increasing the expression of the anti-inflammatory cytokine *Il10* the expression of which was tightly correlated with *Foxp3* expression. This regulatory response was supported by the tight correlation of *Tbet expression* with *Il12* and *Ifnγ* expression. Hence, IL12 is involved in the differentiation of Th1 cells and these cells suppress Th2 cells through the release of IFNγ [6]. *B. breve* treatment also increased the expression of the Th2-associated cytokine *Il4;* yet, the mRNA expression of *Gata3* was not decreased which might suggest that the increase in *Il4* expression is not due to increased number of Th2 cells. Budesonide treatment exhibited its anti-inflammatory effect [35] by significantly decreasing the expression of the pro-inflammatory cytokine *Tnfα*. *L. rhamnosus* treatment tended to reduce the pro-inflammatory response observed in the chronic allergic mice, by significantly decreasing the expression of *Il6*. Although the treatment did not raise *Il10* expression, the expression of *Foxp3* was tightly correlated with *Il10* expression.

3. Beneficial bacterial strains and airway hyper-responsiveness and remodeling in chronic allergic asthma

In addition to the anti-inflammatory effects of *B. breve* and *L. rhamnosus* described in **chapter 3**, the effects of long-term treatment of mice with these two beneficial bacterial strains on allergic asthma symptoms were further explored in **chapter 5**. In line with airway responses to methacholine, airway inflammation and remodeling found in chronic asthma [33, 36-38], chronic

allergic mice showed increased airway hyper-responsiveness, represented by increased lung resistance, increased total inflammatory cell number in the BAL fluid and increased airway inflammation score, infiltration of macrophage, eosinophil and neutrophil into the airways. Additionally, the number of goblet cells, collagenous connective tissue fibers, airway smooth muscle cells and proliferating cells was also increased in lung section of chronic allergic mice. The increased lung resistance in allergen-sensitized and challenged mice was suppressed by *L. rhamnosus* and budesonide treatment, but not by *B. breve*. Nonetheless, all treatments were effective in suppressing inflammatory cellular infiltration into the airways and particularly eosinophils and neutrophils in the BAL fluid. This is in accordance with findings from other studies in which different strains of *Bifidobacteria* and *Lactobacilli,* and budesonide have been individually investigated in acute allergic asthma models [22-26]. Additionally, all treatments reduced the airway inflammation score and decreased the number of collagenous connective tissue fibers and proliferating cells in treated mice. Budesonide treatment also reduced the number of goblet cells in the lung. As the effects of the different treatments on airway remodeling were only moderate, further investigation and quantitative histological and immunohistochemical analyses of the lung tissue sections are needed to examine the possible effects on other features of airway remodeling. Interestingly, increased mucosal mast cell activation, assessed by increased protein expression of mMCP-1, observed in serum of the chronic asthmatic mice was significantly suppressed by all treatments. This inhibitory effect of *B. breve* and *L. rhamnosus* might be galectin-9-mediated as described previously for *B. breve* [39]. Budesonide might suppress mast cell degranulation by upregulating the transcription and expression of negative regulators of these cells as described before [40].

Due to the inflammatory status of the animals, increased levels of IL2, IL4, IL6, IL17a, TNFα and IFNγ proteins were observed in anti-CD3-stimulated TLN cells from chronic asthmatic mice. Increased levels of IFNγ are found in individuals with severe asthma and acute exacerbation [6, 41]. In addition to the anti-inflammatory effects of *B. breve*, *L. rhamnosus* and budesonide treatments described so far in **chapter 3 and chapter 5**, decreased levels of IL2, IL4, IL6, IL17a and TNFα were found in allergen-sensitized and challenged mice, treated with *B. breve*, *L. rhamnosus* or budesonide. Interestingly, *L. rhamnosus* and budesonide treatments also reduced the levels of IFNγ, restoring it to normal levels, in treated allergic mice.

4. Mast cells in allergic asthma

In **chapter 6,** the role of mast cells in allergic asthma was reviewed. Mast cells are key players in various inflammatory disorders including allergic asthma. Cross-linking of the high affinity receptors for immunoglobulin E (IgE), FcεRI, on the surface of mast cells is necessary for the development of allergic reactions. This cross-linking results in degranulation of mast cells and the release of pro-inflammatory, vasoactive and nociceptive mediators. During the immediate phase of the allergic reaction, mast cells are involved in vasodilatation, edema formation and/or bronchoconstriction. The late phase response, on the other hand, is caused by mast cell mediators-induced infiltration and activation of other inflammatory cells [42, 43]. Additionally, in the context of asthma, the release of mast cell mediators can also induce smooth muscle contraction and mucus secretion, two key features of asthma [44]. Hence, mast cells degranulation and activation is detected in asthmatic lungs and various mast cell mediators are found in the BAL fluid of asthma patients [45]. Besides allergen-dependent activation, mast cells can also be activated by other triggers such as activation of neuropeptide receptors, c-Kit, TLRs, complement receptors or direct interaction with G-proteins [46-50]. Mast cells express various TLRs and the ligand for TLR4 was suggested to synergize with the cross-linking of allergen-IgE-FcεRI to enhance allergic inflammation in asthma [51, 52]. Besides their well-known harmful effects in allergic reactions, it is becoming more and more established that mast cells can also carry out a number of beneficial functions by acting as suppressors of allergic inflammation and/or the development of immunological tolerance [53, 54]. Mast cells suppress allergic inflammation by suppressing cytokine production by T cells and monocytes, and enhancing the ability of DCs to reduce T cell proliferation and cytokine production [55].

5. Beneficial bacterial strains and mast cell degranulation in chronic allergic asthma

Preliminary data described in **chapter 7**, demonstrated further evidence for the anti-allergic effects of long-term treatment of mice with *B. breve* and *L. rhamnosus* in chronic allergic asthma. It was shown that oral treatment of mice with *B. breve* and *L. rhamnosus,* previously demonstrated to inhibit mucosal mast cell degranulation by reducing mMCP-1 levels in serum of mice *in vivo* (**chapters 4 and 5**), leads to reduced responsiveness of bone marrow-derived mast

cells (BMMC) to antigen-IgE-mediated degranulation. An interesting finding was that the bone marrow of control chronic allergic mice was deprived from mast cell progenitors, as the number of bone marrow cells isolated from this group was very low and no BMMC could be obtained. This could be caused by the inflammatory status of these animals since it has been described that during chronic airway inflammation in mice, mast cell progenitor cells as well as other myeloid progenitor cells from the bone marrow travel to the inflamed airways to develop into mast cells and other inflammatory cells resulting in low number of bone marrow cells [56, 57]. Oral treatment of mice with *B. breve* and *L. rhamnosus* prevented the depletion of bone marrow cells during the allergic inflammation elicited in the airways of chronic allergic mice. These bacterial strains were equally active as treatment with the corticosteroid budesonide.

Long-term treatment of chronic asthmatic mice with *B. breve* and *L. rhamnosus* resulted in a significant inhibition of antigen-IgE-mediated degranulation in mast cells cultured *in vitro* from bone marrow from these animals. Interestingly, BMMC from *B. breve*-treated mice showed almost completely suppression of IgE-mediated degranulation as compared to BMMC cultured from bone marrow of "healthy" controls. This greatly reduced responsiveness of the cultured BMMC could not be explained by reduced maturation. *L. rhamnosus* treatment decreased the relative number of c-Kit-positive BMMC to some extent and it tended to decrease the relative number of FcεRI-positive BMMC in chronic allergic mice as compared to "healthy" controls. Yet, an even greater decrease was found in BMMC from the budesonide-treated group, while these BMMC are not compromised in their response to IgE-receptor cross-linking. The inhibitory effect of *L. rhamnosus* on degranulation is consistent with findings from a previous study with a different strain of *L. rhamnosus* bacteria which demonstrated inhibition of IgE-mediated mast cell degranulation in rats after oral treatment with *L. rhamnosus* [58]. Additionally, *Lactobacillus* bacteria were demonstrated to inhibit IgE-mediated mast cell degranulation in a murine model for acute allergic inflammation [59]. However, the mechanism by which these potentially beneficial bacteria reduced mast cell degranulation still needs further investigation. An interesting finding was that *in vitro* co-culture of *B. breve* with BMMC obtained from bone marrow cells of "healthy" controls or budesonide-treated chronic allergic mice reduced antigen-specific response of BMMC and decreased degranulation by almost 50% compared to the control BMMC in these groups. Yet, the antigen-specific response of BMMC obtained from bone marrow cells of *B. breve*- or *L. rhamnosus*-treated chronic allergic mice was

not attenuated by *in vitro* co-culture with *B. breve*. The inhibitory effects of *B. breve* and *L. rhamnosus* on mast cell degranulation are more likely to be caused by the induction of epigenetic changes, because during the differentiation of bone marrow into mast cells there is no contact with bacteria or bacterial components. A previous *in vitro* study demonstrated epigenetic effects of a different strain of *B. breve* on the intestinal mucosal immune system by reducing histone acetylation and enhancing DNA methylation [60]. It remains to be established how these bacteria influence degranulation, by inducing changes in mast cell progenitor phenotypes or by interfering with gene expression or signaling pathways in these cells. In addition, measurement of cytokines and arachidonic acid metabolites in BMMC-derived supernatant harvested after 24 hrs of co-culture with *B. breve* can provide further information about the possible effect of *B. breve* on the late phase reaction.

6. *Bifidobacterium breve* combined with non-digestible oligosaccharides in chronic allergic asthma

The effects of long-term treatment with a mixture of *Bifidobacterium breve* with short-chain(sc) fructo-oligosaccharides (FOS) and long-chain (lc) FOS and pectin-derived acidic-oligosaccharides (AOS), *Bb/scFOS/lcFOS/AOS*, on airway inflammation and remodeling in a murine model for chronic allergic asthma were described in **chapter 4**. We demonstrated for the first time that this specific mixture of *B. breve* and non-digestible oligosaccharides is effective at reducing chronic allergic inflammation and airway remodeling. Consistent with the airway inflammation and remodeling found in other murine models for chronic asthma [36], increased total inflammatory cell number and number of eosinophils and neutrophils was observed in the BAL fluid of the asthmatic mice in chronic allergic mice. Additionally, increased number of inflammatory cells and goblet cells, collagenous connective tissue fibers, airway smooth muscle cells and proliferating cells, key features of airway remodeling, were observed in lung sections of chronic allergic mice. Interestingly, *Bb/scFOS/lcFOS/AOS* treatment reduced the increased total number of inflammatory cells in the BAL fluid and reduced eosinphils infiltration into the airways of allergic mice. These findings are in line with the effects of *B. breve* on airway inflammation described in **chapter 3**. The effect of *Bb/scFOS/lcFOS/AOS* treatment on airway remodeling was moderate, as the treatment decreased the number of collagenous connective

tissue fibers and proliferating cells in lung sections of treated allergic mice, but did not attenuate other features of airway remodeling. However, other inhibitory effects of the treatment on airway remodeling features might be present but were minor to be detected with semi-quantitative histological and immunhistochemical analyses of the lung tissue sections.

The mRNA expression of *Tlr3*, *Tlr9* and *Nod1* was significantly decreased in the lungs of chronic allergic mice. *Bb/scFOS/lcFOS/AOS* treatment normalized the expression levels of *Tlr3* and *Nod1*. Based on the limited knowledge about the role of these two receptors in asthma pathogenesis and the findings described in **chapter 3**, this lowered expression in *Tlr3* and *Nod1* is possibly pro-inflammatory in nature. Interestingly, *Bb/scFOS/lcFOS/AOS* treatment skewed the immune response from Th2 towards Th1 by significantly increasing the *Tlr9* expression. Hence TLR9 was reported to modulate immunity by skewing the Th2-biased immune response towards Th1 cell [13, 28, 29]. Due to the chronic inflammatory status of the animals and in accordance with the Th2 responses described in previous studies of chronic asthma [36], chronic allergic mice showed Th2 dominance represented by high *Gata3/Tbet and* low *Foxp3/Gata3* ratios and decreased mRNA expression of *Tbet*. A shift of the immune balance towards Treg was induced by *Bb/scFOS/lcFOS/AOS* treatment with a significant increase in *Foxp3/Rorγt* and *Foxp3/Gata3* ratios. These effects of the treatment in the lung were confirmed by the detection of high Foxp3-expressing Treg cells in the blood of treated allergic animals suggesting increased function of Treg cells as described previously [33, 34]. Consistent with observations in the sputum and BAL fluid of asthmatic subjects [6], increased mRNA expression of *Il1β*, *Il6* and *Il17* was observed in the asthmatic mice. Nonetheless, the expression of the Th2 cytokine *Il5* was significantly decreased and no increase in *Gata3* mRNA expression was observed in whole lung tissue of chronic allergic mice. This contrast between our results and findings in asthmatic individuals might be explained by measurement of mRNA expression in mouse whole lung tissue instead of in the sputum or mucosal bronchial biopsies of asthmatic subjects. *Bb/scFOS/lcFOS/AOS* treatment restored the levels of the pro-inflammatory cytokines *Il1β, Il6* and *Tnfα* to normal levels, but decreased the expression of *Il12* and induced a Treg response by significantly increasing the expression of *Il10* and *Il23*. The decrease in *Il12* might be caused by the increased expression of the anti-inflammatory cytokine *Il10,* as IL10 is known to inhibit *Il12* p40 transcription [61]. Importantly, the strong anti-inflammatory effect of the *Bb/scFOS/lcFOS/AOS* treatment was confirmed by significant decreases in the production of

IL2, IL6 and TNFα protein, restoring them to normal levels, by T cells in the TLNs of treated allergic mice after aspecific activation with an anti-CD3 antibody *in vitro*. The production of IFNγ protein by T cells in the TLNs was normalized as decreased level of this cytokine was found in treated allergic mice. Increased levels of IFNγ are found in individuals with severe asthma and acute exacerbation [6, 41]. *Bb*/*scFOS*/*lcFOS*/*AOS* treatment significantly decreased the protein levels of mouse mast cell protease 1 (mMCP-1) systemically, in serum, as well as in the BAL fluid of treated allergic mice indicating a strong inhibition of allergic mucosal mast cell degranulation. The mechanism by which the *B. breve* and *L.rhamnosus* treatments inhibited mucosal mast cell degranulation needs further investigation. Yet, it might be galectin-9-mediated as recently it was shown that a combination of *Bifidobacterium breve* M-16V with non-digestible oligosaccharides (*Bb*-NDO) suppresses allergic symptoms, including mast cell degranulation, in mice and humans via the induction of galectin-9 [39]. In addition, *in vitro* studies using bone marrow-derived mast cells demonstrated that galectin-9 was responsible for the inhibition of antigen-IgE-induced mast cell degranulation induced by sera obtained from *Bb*-NDO-treated allergic mice [39]. Possibly, budesonide suppresses mast cell degranulation by up-regulating the transcription and expression of negative regulators of these cells, such as Src-like adaptor protein (SLAP), which in turn inhibit signaling in allergic-activated mast cells [40].

Concluding remarks

Immune and inflammatory responses are differentially regulated in mild and severe allergic asthma, as represented by the different mRNA expression profiles of TLRs, NLRs and T helper-specific cytokines and transcription factors. How these changes in expression of the different inflammatory markers determine asthma severity remains to be investigated. In the case of TLRs and NLRs it would be interesting to investigate the impact of the use of specific ligands on inflammation and disturbed immunity in the disease. These findings add to our understanding of the allergic characteristics of mild and severe allergic asthma which can contribute to the identification of phenotype-specific therapeutic targets.

Two different beneficial bacterial strains, *Bifidobacterium breve* M-16V and *Lactobacillus rhamnosus* NutRes1, demonstrated anti-allergic and anti-inflammatory properties by modulating

various parameters in a murine model for chronic allergic asthma. Long-term treatment with *B. breve* or *L. rhamnosus* reduced pulmonary airway inflammation and modulated the mRNA expression of different TLRs, NLRs, T helper-specific cytokines and transcription factors. However, among the two strains, *B. breve* was the most potent inducer of regulatory T cell responses, shown by increasing *Il10* and *Foxp3* transcription in lung tissue and augmenting the mean fluorescence intensity of Foxp3 in blood CD4+ T cells. Additionally, the anti-inflammatory properties of *B. Breve* were comparable with these of the reference treatment budesonide. Considering airway hyper-responsiveness, *L. rhamnosus* was as effective as budesonide in reducing the enhanced lung resistance in allergic mice. Yet, both strains were as effective as budesonide in inhibiting mast cell degranulation, reducing T cell activation and suppressing airway remodeling. An interesting finding was that long-term treatment of mice with *B. breve* and *L. rhamnosus* lead to reduced responsiveness of BMMC to IgE-mediated degranulation *in vitro*. Additionally, *in vitro* co-culture of *B. breve* with BMMC obtained from bone marrow cells of "healthy" controls or budesonide-treated, but not *B. breve*- or *L. rhamnosus*-treated, chronic allergic mice reduced antigen-specific response of BMMC and decreased degranulation by almost 50% compared to the control BMMC in these groups. The observed inhibitory effects of *B. breve* and *L. rhamnosus* on the mast cell progenitor cells in bone marrow are likely to be caused by the induction of epigenetic changes. Further studies are needed to explore the mechanism by which these bacteria influence mast cells degranulation. It is also worth measuring galectin-9 in BMMC-derived supernatant. Collectively, our findings show that *B. breve* and *L. rhamnosus* are effective in suppressing asthma by modulating inflammation and immunity and therefore may be beneficial in the management of chronic inflammation in allergic asthma.

When combined with a specific mixture of non-digestible oligosaccharides, *B. breve* showed strong anti-inflammatory and anti-allergic properties. Long-term therapeutic treatment of allergic mice with *Bb/scFOS/lcFOS/AOS* reduced 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. *Bb/scFOS/lcFOS/AOS* 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. Anti-inflammatory and anti-allergic properties were observed as well with *B. breve* alone. However, the setup of these experiments does not allow a scientifically relevant comparison of efficacy between these two treatments, as different forms of *B. breve* bacteria, glycerol stock vs. lyophilized powder, were used. Additionally, *B. breve* bacteria, for the different treatments, were grown in different media and harvested at different growth phases and these factors are known to influence the immunomodulatory effects of the bacteria [62, 63].

Altogether, the findings described in this thesis demonstrate various anti-inflammatory and anti-allergic properties of *B. breve* alone or in combination with a specific mixture of non-digestible oligosaccharides, and *L. rhamnosus*. These results highlight the therapeutic potential of these beneficial bacterial strains for chronic allergic asthma management. An interesting finding was that orally-administered beneficial bacteria modulated the immune responses at site of inflammation, in the lungs, as well as systemic. Lactic acid bacteria were suggested to induce Treg cells in the gut-associated lymphoid tissue (GALT) that can spread to the airways upon immune challenge and inflammation. Immune challenge in the airways seems to promote the trafficking of Treg cells, activated in the intestine, to the respiratory mucosa where they induce protective and anti-inflammatory responses as described previously in [64]. Further studies are needed to explore the mechanism by which the trafficking of cells is induced and regulated. Additionally, the effects of these beneficial bacterial strains on the gut mucosa need to be investigated.

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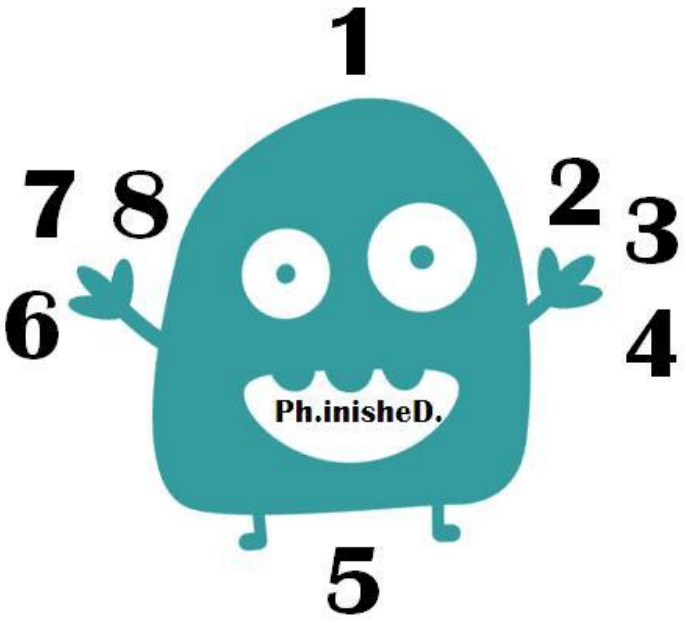
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Appendix

Nederlandse samenvatting

Dankwoord

Curriculum Vitae

List of Publications

Nederlandse samenvatting

Allergisch astma is een chronische aandoening van de luchtwegen. Aanvallen van kortademigheid, beklemming op de borst, piepende ademhaling en/of hoesten zijn kenmerkende symptomen van astma. Deze klachten ontstaan na blootstelling aan specifieke prikkels (= allergenen), zoals huisstofmijt, pollen en huisdieren. Bij astma is het afweersysteem ontregeld waarbij vele receptoren, zoals de toll-like receptoren (TLRs) en nod-like receptoren (NLRs), ontstekingscellen (waaronder eosinofiele granulocyten, mestcellen, macrofagen, T- en B-lymphocyten) en hun mediatoren (cytokinen en chemokinen) een rol spelen. De interacties tussen alle receptoren en cellen is zeer ingewikkeld en zal niet in detail in deze samenvatting worden besproken (zie hoofdstuk 1).

De aanhoudende chronische ontsteking in the luchtwegen draagt bovendien potentieel bij aan het ontstaan van structurele veranderingen in de wand van de luchtwegen wat remodeling (= vervorming) wordt genoemd. De prevalentie van allergische aandoeningen, zoals astma, nam sinds het eind van de vorige eeuw wereldwijd sterk toe. Ondanks de effectiviteit van de huidige behandeling van astma, β_2-agonisten al dan niet in combinatie met corticosteroïden, is deze therapie bij een hoog percentage astmapatiënten niet afdoende. Er is een grote behoefte aan nieuwe therapieën.

Inzicht in de verstoorde immuunrespons en de verschillende markers voor ontsteking, die een belangrijke rol spelen in astma, zijn essentieel voor de ontwikkeling van effectieve, veilige en potentiële behandelingsstrategieën voor deze ziekte. In eerdere studies is aangetoond dat veranderingen in de samenstelling van de micro-organismen in de darm (microbiota) de kans op het verkrijgen van astma verhogen. Gezondheidsbevorderende bacteriën, ook wel probiotica genoemd, en niet-verteerbare oligosacchariden zijn voedingssuplementen die immuun-modulerende eigenschappen hebben.

Het specifieke doel van het onderzoek in dit proefschrift was het verkrijgen van meer inzicht in de verstoorde immuunrespons, die chronisch allergisch astma kan veroorzaken, en de modulatie van deze respons en astmasymptomen door gebruik te maken van gezondheidsbevorderende bacteriën alleen of in combinatie met specifieke niet-verteerbare oligosacchariden. Hiertoe zijn zowel *in vitro* (in de reageerbuis) als *in vivo* (in muizen) studies uitgevoerd. In eerdere studies werden alleen korte termijn preventieve effecten van deze voedingscomponenten in allergisch

astma onderzocht. Daarom is er in dit proefschrift ook aandacht besteed aan de lange termijn therapeutische effecten van gezondheidsbevorderende bacteriën alleen of in combinatie met specifieke niet-verteerbare oligosacchariden in chronisch allergisch astma.

In **hoofdstuk 2** is de regulatie van ontsteking en de verstoorde afweer in muismodellen voor milde en ernstige vormen van allergisch astma onderzocht. Om dit te onderzoeken werden verschillende kenmerken van ontsteking in allergische muizen gemeten. Dit werd gedaan door het meten van de infiltratie van ontstekingscellen in de longen en de expressie van verschillende eiwitten (TLRs, NLRs, T-cel specifieke transcriptiefactoren en cytokinen) op mRNA-niveau in longweefsel van "astmatische" muizen. Hieruit is gebleken dat in milde en ernstige muismodellen van allergisch astma verschillende ontstekingsreacties een rol spelen.

Het effect van de gezondheidsbevorderende bacteriën *Bifidobacterium breve* en *Lactobacillus rhamnosus* op de ontstekingsreactie en de verstoorde immuunrespons in chronisch allergisch astma in muizen werd onderzocht in **hoofdstukken 3** en **5**. Budesonide, een corticosteroïde, werd in dit onderzoek gebruikt als een referentiebehandeling. Ons onderzoek toont aan dat het behandelen van chronisch allergisch "astmatische" muizen met *Lactobacillus rhamnosus* bacteriën de verhoogde longweerstand, een kenmerk voor astma, verlaagt in dezelfde mate als budesonide. Daarnaast is ook aangetoond dat in dit onderzoek gebruikte gezondheidsbevorderende bacteriën en corticosteroïde vergelijkbare effecten hebben op het remmen van de mestceldegranulatie (= vrijkomen van ontstekingsmediatoren), T cel activering en remodeling van de luchtwegen. De mRNA expressie van TLRs, NLRs en T cel specifieke cytokinen en transcriptiefactoren in longweefsel werd verschillend beïnvloed door de verschillende behandelingen. Een belangrijk bevinding was dat *Bifidobacterium breve* een sterk ontstekingsremmend effect heeft door de inductie van zogenaamde regulatoire T cel (Treg) reactie in de luchtwegen, maar ook in de periferie. Treg cellen spelen een essentiële rol in de onderdrukking van andere typen afweercellen, namelijk T helper cel type 1, 2 en 17. T helper 2 cellen zijn mogelijk verantwoordelijk voor het in stand houden van de allergische ontsteking in de luchtwegen bij astma. Deze bevindingen geven aan dat deze gezondheidsbevorderende bacteriën, in het bijzonder *Bifidobacterium breve*, mogelijk gebruikt kunnen worden voor het behandelen van chronisch allergisch astma.

In **hoofdstuk 4** is een onderzoek gedaan naar de therapeutische effecten van het concept *Bifidobacterium breve* (Bb) in combinatie met een specifiek mengsel van niet-verteerbare oligosacchariden (Bb/scFOS/lcFOS/AOS), op chronische ontsteking en remodeling van de luchtwegen in een muismodel voor allergisch astma. Het mengsel van niet-verteerbare oligosacchariden bestond uit korte keten en lange keten fructo-oligosacchariden (FOS) en van pectine afgeleide zure oligosacchariden (pAOS). Het is aangetoond dat naast het bevorderen van de groei van gezondheidsbevorderende bacteriën in de darm, deze niet-verteerbare oligosacchariden ook een direct immuun-modulerend effect hebben in de darmen. Behandelen van chronisch allergisch "astmatische" muizen, na sensibilisatie, met Bb/scFOS/lcFOS/AOS remde de luchtwegontsteking, verlaagde de remodeling van de luchtwegen, de activering van T cel en mestcel degranulatie, en beïnvloedde de mRNA expressie van TLRs, NLRs, en T cel specifieke cytokinen en transcriptiefactoren in longweefsel. Daarnaast werd ook een verhooogde Treg reactie gemeten. Dit geeft aan dat dit specifiek mengsel van *Bifidobacterium breve* en niet-verteerbare oligosacchariden immuunmodulerende eigenschappen heeft en mogelijk gebruikt kan worden voor het behandelen van chronische ontsteking in allergisch astma.

Een samenvatting van de huidige kennis van de rol van de mestcel in allergisch astma wordt nader belicht in **hoofdstuk 6**. Naast hun welbekende rol bij het ontstaan van allergische ontstekingsreacties, lijken mestcellen deze reacties ook te kunnen remmen.

In **hoofdstuk 7** is in een pilot-studie gedaan naar de effecten van *Bifidobacterium breve* en *Lactobacillus rhamnosus* op de rekrutering en functie van beenmergcellen in een muismodel voor chronisch allergisch astma. In het beenmerg zitten multipotente stamcellen van waaruit verschillende typen bloedcellen worden gemaakt. De corticosteroïde, budesonide, werd in dit onderzoek gebruikt als een referentiebehandeling. *In vivo* behandeling van "astmatische" muizen met gezondheidsbevorderende bacteriën, *Bifidobacterium breve* of *Lactobacillus rhamnosus*, resulteerde in een verlaagde antigeen specifieke degranulatie van beenmerg afgeleide mestcellen (BMMC) *in vitro*. *In vitro* co-cultuur van BMMC met *Bifidobacterium breve* verminderde de degranulatie van BMMC gekweekt uit het beenmerg van gezonde muizen of "astmatische" muizen behandeld met budesonide. Ons onderzoek toont aan dat *Bifidobacterium breve* en *Lactobacillus rhamnosus* bacteriën de rekrutering van beenmergcellen

en daarbij het tot stand komen van een ontstekingreactie voorkomen. Daarnaast is ook gevonden dat orale (via de mond) toediening van *Bifidobacterium breve* en *Lactobacillus rhamnosus* lange termijn veranderingen in de voorlopercellen van mestcellen in het beenmerg induceert. Een interessante bevinding was dat in muizen die met deze bacteriën werden behandeld, de allergische reactie sterk geremd werd. De onderliggende mechanismen waarmee deze effecten tot stand komen zijn onbekend. Het is mogelijk dat deze remmende werking van *Bifidobacterium breve* en *Lactobacillus rhamnosus* tot stand komt door de inductie van epigenetische veranderingen (veranderingen in genfunctie) in de voorlopercellen van mestcellen.

De onderzoeksresultaten beschreven in dit proefschrift laten zien dat in milde en ernstige muismodellen van allergisch astma verschillende kenmerken van ontsteking betrokken zijn. In dit proefschrift werden de therapeutische effecten van twee gezondheidsbevorderende bacteriële stammen, *Bifidobacterium breve* en Lactobacillus rhamnosus, voor het eerst in een muismodel voor chronisch allergisch astma onderzocht. Onze bevindingen laten zien dat deze bacteriën sterke allergie- en ontstekingsremmende eigenschappen hebben. Daarnaast is ook aangetoond dat *Bifidobacterium breve* in combinatie met een specifiek mengsel van niet verteerbare-oligosacchariden een remmend werking heeft op de allergische reactie in een muismodel voor chronisch astma. Dit benadrukt de therapeutische potentie van deze gezondheidbevorderende bacteriën als een behandelingsstrategie voor chronisch allergisch astma.

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Curriculum Vitae

Seil Sagar was born on the 15th of August, 1982, in Baghdad, Iraq. She grew up in Baghdad where she also attended primary school. In November 1997 Seil moved with her family to the Netherlands. She lived in Arnhem where she attended the International secondary school to learn the Dutch language (Mozaëk College). Seil attended high school for two years in Arnhem (VWO, Lorentz Lyceum) and then moved to Nijmegen from which she graduated in 2003. In the same year, she started her study Molecular Life Sciences at the Radboud University in Nijmegen. Seil finished her Bachelor in 2006 and her Master in 2008. Two days after graduation she started her PhD research project concerning the effect of beneficial bacteria and non-digestible oligosaccharides as a treatment for chronic allergic asthma in murine models in the research group of Prof. Gert Folkerts at the division of Pharmacology, Department of Pharmaceutical Sciences, Utrecht University; and Prof. Johan Garssen at the Department of Immunology, Danone Research, Centre for Specialised Nutrition in Wageningen. Seil performed her PhD research project within the framework of the Dutch Top Institute Pharma Project D1-101 'Exploitation of toll-like receptors in drug discovery'. Seil will continue her scientific career as a postdoc researcher in the group of Prof. Peter Deen at the Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Nijmegen, to study the role of the succinate receptor in chronic kidney disease.

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