Digesting the role of specific pre- and synbiotics in the prevention of house dust mite asthma

Thinking out of the lung

Kim Verheijden

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Thinking out of the lung

De preventieve effecten van specifieke pre- en synbiotica in huisstofmijt allergische astma

(met een samenvatting in het Nederlands)

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Everything happens for a reason

CONTENTS

CHAPTER ONE

General introduction

According to the World Health Organization (WHO), 235 million people suffer from asthma worldwide [1]. Asthma is a chronic pulmonary non-communicable disease that leads to inflammation in the airways, mucus hypersecretion and airway hyperresponsiveness (AHR) [2]. The prevalence of asthma is still rising, in high- as well as low-income countries. Among children, asthma is the most common non-communicable disease [1]. The latest definition of asthma from GINA (Global INitiative for Asthma) states that: "Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that can vary over time and in intensity, together with variable expiratory airflow limitation." Asthma can be classified as mild, moderate or severe [3]. The disease can be triggered by environmental stimuli (e.g. house dust mite (HDM)), airborne pollutants (e.g. tobacco smoke) and drugs (e.g. aspirin) [4]. Asthma can be classified as allergic asthma or non-allergic asthma. In allergic asthma antigen-specific immunoglobulin E (IgE)-antibodies are present in serum. Allergic asthma is a T helper 2 (Th2) mediated disease [2]. Non-allergic asthmatic patients have no elevated IgE and non-allergic asthma often develops later in life and is more common in women [5]. In non-allergic asthma, alveolar macrophage activation is more important compared to allergic asthma [6]. Asthma is a complex pulmonary disease that involves innate as well as adaptive immune responses, which lead to the production of various inflammatory mediators, such as cytokines and chemokines, and an influx of inflammatory cells in the lungs. Not only airborne environmental factors and drugs can influence the development of asthma, also dietary changes such as, a lower fiber and higher fat intake characteristic for nowadays Western diets, may have an effect [7,8]. It has been suggested that the Western diet influences the bacterial composition of the intestine resulting in a reduced diversity of bacterial species and as consequence, a dysbiosis of the microbiome [9,10]. To study the effects of dietary supplementation on the outcome of asthmatic disease, we investigated the effect of diets supplemented with non-digestible oligosaccharides (fermentable fibers) either or not combined with beneficial bacteria on the features of HDM-induced allergic asthma in a murine model. In this general introduction chapter the pathophysiology of asthma, current and future treatments, and new therapeutic and preventive dietary approaches (pre-, pro- and synbiotics) for allergic asthma will be discussed. Moreover, an outline of the thesis is presented.

Pathology of allergic asthma

The main cells that form a barrier for allergens are airway epithelial cells [11]. Allergens can directly trigger or stimulate airway epithelial cells, which leads to the release of different cytokines (Interleukin (IL)-33, IL-25, Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF) and Thymic Stromal Lymphopoietin (TSLP)) and chemokines (Chemokine (C-C motif) Ligand (CCL)5 and CCL20) [12]. These chemokines will attract immature dendritic cells (DC, antigen-presenting cells) to the lung, which will be activated by the above mentioned cytokines [13]. In response to these cytokines, also type 2 innate lymphoid cells (ILC2) will undergo proliferation and produce IL-4, -5 and -13 [14]. ILC2, also termed natural helper cells, are recently described innate immune cells that contribute to the initiation of allergic sensitization via release of these Th2 polarizing cytokines [15]. After processing of the antigen, the activated DC are capable of presenting antigenic peptides to naïve T cells and prime naïve T cells to differentiate into Th2 cells in the mediastinal lymph nodes. In response to CCL17 and CCL22, antigen-specific Th2 cells will migrate into the lung and after antigen exposure also produce IL-4, -5 and -13 [13]. IL-4 and IL-13 lead to isotype switching and development of antigen-specific B cells, which differentiate into IgE-producing plasma cells. IgE binds to the mast cells, and upon antigen binding, the mast cells are activated and will degranulate. Mediators such as prostaglandins and histamine as well as pre-stored and *de novo* produced cytokines (tumor necrosis factor-alpha (TNFα), IL-4, -5, -9 and -13) will be released leading to mucus hyper-secretion, infiltration of inflammatory cells, such as eosinophils, and bronchoconstriction [16]. Not only IL-4 is capable of activating mast cells, but also binding of IL-33 on mast cells can modulate maturation, degranulation and cytokine production [17]. IL-5 is important for eosinophil growth, maturation, activation, migration to tissue sites and survival, whereas IL-13 leads to goblet cell hyperplasia, increased mucus production and bronchial hyperreactivity [18-20]. IL-6 can be produced by different innate immune cells (e.g. DC, mast cells, neutrophils, macrophages) or secreted by non-leukocytes (e.g. endothelial and epithelial cells). IL-6 is known to be increased in pulmonary diseases, but its role remains unclear [21] (**Fig. 1**).

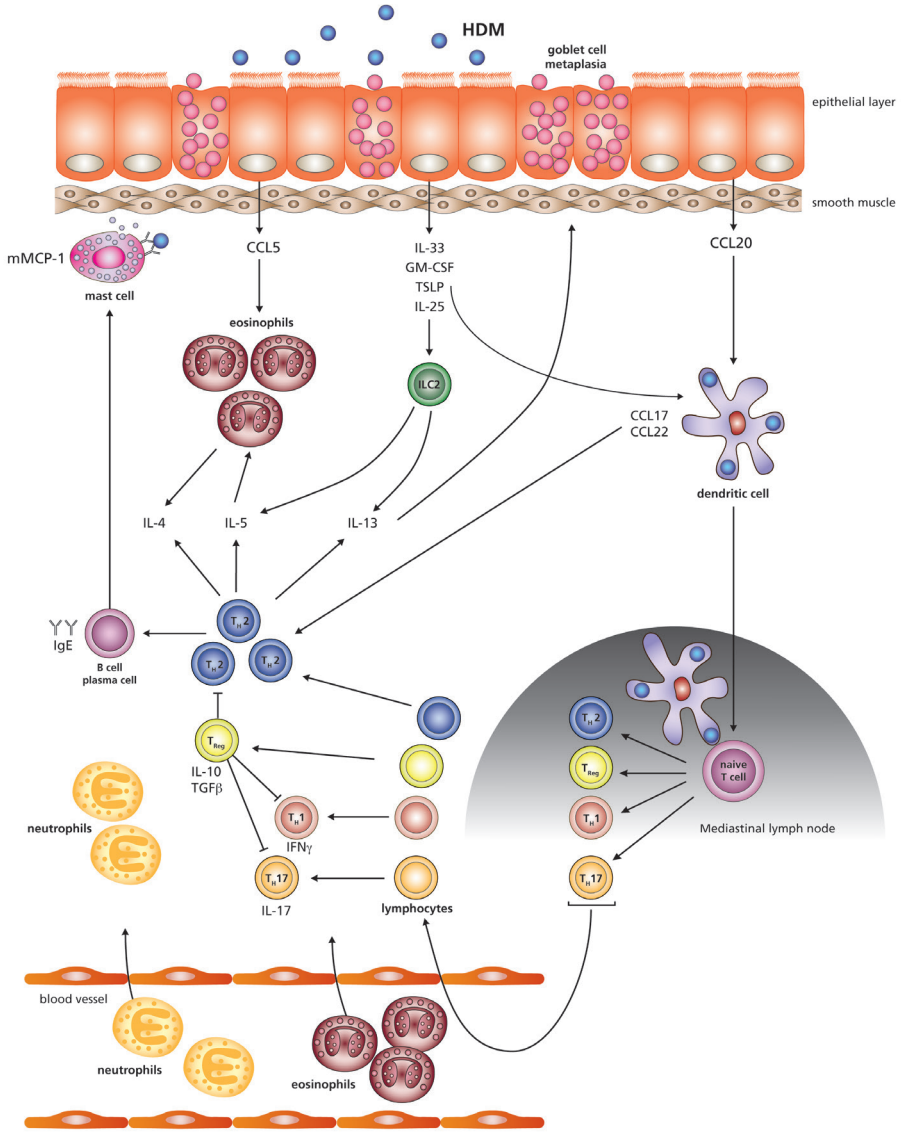

Fig. 1 Pathology of allergic asthma.

IL-33, GM-CSF, TSLP, IL-25, CCL5 and CCL20 are known to be secreted by the airway epithelium after the initial exposure to HDM. These cytokines and chemokines are able to activate DC and ILC2. CCL17 and CCL22 are secreted by activated DC, which can differentiate naïve T cells into Th2 cells in the lymph nodes and are chemo-attractants for Th2 cells. Th2 cells as well as ILC2 and mast cells are able to produce IL-5 and -13, which will attract eosinophils into the lung. IL-13 and -4 induce the differentiation of antigen-specific B cells into IgE-producing plasma cells. IgE binds to the mast cells leading to activation and degranulation of these cells resulting in release of mediators like histamine and mMCP-1. IL-13 also leads to goblet cell hyperplasia, increased mucus production and bronchial hyperreactivity.

Current treatments of asthma

Currently, the mostly used treatments for asthma are inhaled corticosteroids (e.g. budesonide) with or without long-acting β-agonist (e.g. salmeterol) which improve asthma symptoms and the frequency of exacerbations [22]. However, corticosteroids have considerable side effects, such as weight gain, reduced growth in children, muscle weakness and osteoporosis in elderly [23]. Biologic agents aiming to modulate cell signaling and immunological responses in asthma are important targets for improved treatment. The first biological drug approved for treatment of severe asthma was Omalizumab, a monoclonal antibody, which blocks the binding of IgE on the FcεRI of mast cells by binding to free IgE. This results in a decrease in free IgE serum levels, decreased degranulation of mast cells and suppression of new IgE production. As a consequence, allergic bronchial inflammation and exacerbations will be reduced. However, only patients with moderate to severe asthma are treated with this drug due to high costs [11,24,25]. Several monoclonal antibodies that target IL-5 are being studied in clinical trials. Mepolizumab was able to decrease eosinophil numbers in sputum and blood of allergic asthmatic patients, but had no effect on airway function [26,27], whereas Reslizumab showed no effect on exacerbations or asthma symptoms compared to the placebo group, but improved airway function [28-30]. The soluble recombinant IL-4R was able to reduce airway inflammation, although asthma symptoms and respiratory function were not improved [31,32]. A monoclonal antibody against IL-13, Lebrikizumab, improved airway function in patients with asthma [33]. Other drugs targeting different cytokines, like IL-33, IL-25, TSLP or GM-CSF, are still under development [11,34]. Although the above mentioned treatments with biologicals are effective in asthma, not all symptoms are decreased. Therefore, more research is needed to improve the therapeutic efficacy of asthma medication.

Microbiota

The 'hygiene hypothesis', which states that a lack of early microbial stimulation results in aberrant immune responses to innocuous antigens later in life, was the first interpretation for the increased development of allergies and asthma [35]. Recently, a new hypothesis was formed, the 'microbiota hypothesis', which means that changes in the gastrointestinal microbiota composition due to dietary changes and/or antibiotics use, may contribute to defects in mucosal immunologic tolerance [36]. Different animal studies indicate that the composition of the gut microbiota can affect systemic immune functions [37-40]. The intestinal microbiota is different in asthmatic children compared to healthy children. Asthmatic children have a low prevalence of *Bifidobacterium* species and a high prevalence of certain species of *Clostridium difficile* [41,42]. Bifidobacteria are known to have a beneficial effect on the immune homeostasis, whereas some species of *Clostridium difficile* are able to cause infections [43]. Development of allergic eczema in children is associated with a reduced gut microbial diversity in infancy [44]. Disruption of the gut microbiota in infancy and childhood may influence the development of allergic

diseases in later life [45]. There are also reports stating that in cesarean-delivered infants, the risk of developing asthma and atopy is higher due to changes in the microbiota caused by a reduced exposure to vaginal microbiota of the mother [46]. Optimizing the gut microbiome by increasing the amount of probiotic bacteria (e.g. Bifidobacteria) may reduce the susceptibility to the development of asthma [47]. Not only beneficial bacteria, but also non-digestible oligosaccharides (NDO) with or without beneficial bacteria might be able to adapt the microbiota in a positive manner. The NDO and/or beneficial bacteria can act as modulators of the intestinal microbiota and systemic immune responses and therefore have an effect on the development of asthma [48-50].

Prebiotics, non-digestible oligosaccharides

Human milk oligosaccharides (HMOS) are important constituents of breast milk and approximately 130 different oligosaccharides have been identified [51]. These human milk derived NDO have several functions, amongst them the support of development of the infants microflora. Specific NDO derived from vegetable sources or manufactured from dairy sources may be capable of mimicking some of the aspects of human milk NDO since they support selective growth of beneficial bacteria such as Bifidobacteria and Lactobacilli. These NDO are often referred to as prebiotics. Prebiotics are defined as "selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host well-being and health" [52]. Galacto-oligosaccharides (GOS), long-chain fructo oligosaccharides (lcFOS) and short-chain fructo oligosaccharides (scFOS) are examples of prebiotics. GOS can be synthesized from two sources, namely soy beans and lactose. GOS is produced via enzymatic conversion of lactose using β-galactosidase, resulting in a chain of galactoses coupled by β-glycosidic bonds with a degree of polymerization (DP) of 2-8, connected to a glucose molecule [53]. Inulin is processed from chicory roots and contains a chain of fructoses connected by β-glycosidic bonds with a DP from 2 to 60. scFOS is partially hydrolyzed inulin with a DP of 2 to 8. LcFOS is produced by physical removal of short-chain fructans from chicory inulin, which leads to a mixture of fructans with an average DP of 22 [54,55].

Prebiotics are non-digestible and selectively fermentable oligosaccharides. From infant studies it is known that GOS/lcFOS supplemented in formula milk survives passage of the gastrointestinal tract, since these prebiotics are still detectable in the feces. In addition, GOS/lcFOS is selectively fermented by Bifidobacteria and Lactobacilli. Indeed, it was shown that the intestinal microbiota of infants fed formula with added GOS/lcFOS had similar amounts of beneficial *Lactobacillus* and *Bifidobacterium* species compared to breastfed infants [56-58]. In infants at high risk for developing allergic disease and fed GOS/lcFOS, the cumulative incidence of atopic dermatitis was reduced by 50% compared to the placebo group [59]. This study continued and after 5 years these prebiotics still had a beneficial effect on the development of atopic dermatitis and allergic rhinoconjuctivitis [60,61]. Also, a combination of GOS/lcFOS/pectin-derived acidic oligosaccharide (pAOS)

prevented the development of atopic dermatitis in infants in a general population [62]. Not much is known about the effect of prebiotics on asthma [63]. However, in a murine OVA-induced asthma model, GOS/lcFOS/pAOS decreased airway inflammation and airway hyperresponsiveness (AHR) in asthmatic mice [64].

Probiotics, beneficial bacteria

Probiotics are defined as 'live microorganisms that, when administered in adequate amounts, confer a benefit on the host' [65]. Most frequently, *Lactobacillus* and *Bifidobacterium* species are used as probiotics as they are naturally part of the gut microbiota [66]. Different animal studies indicate a positive effect of probiotics on allergic airway disease in a preventive setting. *L. rhamnosus* and *L. reuteri* reduce AHR, airway inflammation, IgE levels in serum and IL-4, -5 and -13 levels in both serum and bronchoalveolar lavage fluid (BALF), while increasing $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cells in the spleen [67-70]. However, not all probiotics are effective. In a comparative study using 6 probiotic strains Hougee *et al.* showed that *B. breve* M-16V was most effective in suppressing OVA-induced asthma in mice. Hence, *B. breve* reduced AHR, the number of eosinophils and IL-4, -5 and IL-10 in BALF [71]. Probiotic milk (containing Bifidobacteria and Lactobacilli strains) consumption by Norwegian pregnant women slightly reduced the incidence of rhinoconjunctivitis and eczema in the offspring, but had no effect on the development of asthma, at 3 years of age [72]. Also no long-term effect (above 5 years) of *L. paracasei* or *L. reuteri* was observed on allergic rhinitis, asthma, eczema, food allergy, airway inflammation or IgE sensitization in children [73,74]. Although the results in animal models are promising, the effect of probiotics used in human studies so far have not revealed any preventive effect on asthma development.

Synbiotics, combination of pre- and probiotics

The combination of pre- and probiotics is called synbiotics, aiming to induce additive beneficial effects in the host [75]. The most used synbiotic, is a combination of GOS/lcFOS/*B. breve*. In murine food allergy models, it is shown that this synbiotic can increase serum galectin-9 levels, which regulates immune responses and tolerance and suppresses mast cell degranulation by IgE neutralization [76]. These synbiotics inhibit clinical relevant parameters like allergic skin responses and anaphylactic symptom scores in mice [77]. scFOS/lcFOS/pAOS/*B. breve* was able to decrease airway inflammation, T cell cytokine production and mast cell degranulation in an OVA-induced murine asthma model [78]. In human studies FOS in combination with *L. salivarius* was superior to FOS alone in treating moderate to severe childhood atopic dermatitis, however, this trial only had a duration of 10 weeks [79]. GOS/lcFOS/*B. breve* prevented asthma like symptoms in infants with atopic dermatitis, however, no effect was observed on atopic disease markers [80,81]. When *B. breve* M-16V and GOS/lcFOS were used in patients suffering from HDM-allergy and -asthma, Th2 cytokines (IL-4, -5 and -13) produced upon *ex vivo* allergen stimulation of peripheral blood mononuclear cells or as measured in serum, were reduced and the

peak expiratory flow was increased. Although in this study no effect on bronchial allergic inflammation in the BALF was demonstrated [82]. Still more clinical research needs to be done to investigate the beneficial effect of synbiotics on allergy and asthma.

Murine models of allergic asthma

To study the pathogenesis of asthma and/or new therapies, mouse models are commonly used. As mice do not develop asthma spontaneously, an allergen must be used to induce sensitization and asthmatic-like reactions. Although there is no model that mimics the entire asthma human phenotype [83,84]. Ovalbumin (OVA) derived from chicken egg is the most frequently used model to induce allergic asthma in mice, which leads to asthmatic responses and airway inflammation [85,86]. However, a disadvantage of this model is the use of an adjuvant (typically aluminum hydroxide) and the fact that humans are not sensitized to allergens via injections but mostly via inhalation (e.g. HDM, pollen, cockroach) [87]. Moreover, ovalbumin is not a physiological asthma trigger in humans. A model that is more related to the human situation is the HDM-induced allergic approach, since this is a natural allergen for 85% of the asthmatic patients [88,89]. The HDM-allergen is applied intra-tracheally (i.t.) or intranasally (i.n.) without an adjuvant, which mimics the human situation more realistically and induces AHR and airway inflammation [90,91]. The most widely used mouse strain in research of allergic inflammation is the BALB/c, as these mice are capable of developing a Th2 immunological response [92,93]. The use of other strains like C57BL/6 mice is increasing, as more research is done in transgenic and knock-out mice [94]. However, different outcomes of asthma characteristics can vary extensively between mouse strains and sexes [95-97].

Aim and outline of this thesis

Worldwide the prevalence of asthma is rising. Although there are some treatments which can improve asthmatic symptoms, the disease is still poorly controlled in a high percentage of asthmatics. Therefore, new strategies are needed to treat and/or prevent asthmatic disorders. As animal and human studies indicate that changes in the intestinal microbiota can influence the development of asthma, a balanced gut microbiota may help to protect against the development of allergic diseases. This thesis aims to investigate the mechanisms of immune responses in allergic asthma and the modulating effects of non-digestible oligosaccharides with or without beneficial bacterial strains on asthmatic symptoms in animal models.

Chapter One provides a general introduction of asthma, the pathology of asthma, current and future treatments and new therapeutic and preventive dietary approaches (pre-, pro- and synbiotics) for allergic asthma, including the outline of this thesis.

Chapter Two discusses the differences between two types of OVA-induced allergic airway inflammation: mild and severe. Also a direct comparison was investigated between non-invasive and non-ventilated unrestrained whole body plethysmography (Penh) and the invasive ventilated lung resistance (R_L) method. As R_L gave the most consistent results in both inflammation models this method was used to assess airway responsiveness in all the subsequent experiments.

Since HDM-induced asthma models are more related to the human situation than OVA-induced allergic asthma in mice, in **Chapter Three** this model was used to investigate the preventive effect of different oligosaccharides. In this chapter it is shown that different oligosaccharides act via different mechanisms to be effective on modulating the development of asthma in mice.

Not only different oligosaccharides are effective in reducing asthmatic features, also synbiotics can have beneficial effects on allergy symptoms induced by HDM in mice, which is described in **Chapter Four**. Here, a combination of scFOS/lcFOS with *B. breve* reduced the number of eosinophils and lymphocytes in BALF of HDM-allergic mice as well as IL-4 and IL-5 concentrations. However, the combination of GOS/lcFOS with *B. breve* was less potent in this model.

From **Chapter Three** it was concluded that GOS alone was capable of suppressing allergic symptoms in mice. Therefore in **Chapter Five**, the preventive effect of dietary intervention with GOS was compared to an intra-airway therapeutic treatment with budesonide, a glucocorticosteroid. Dietary intervention with GOS reduced airway eosinophil numbers and Th2 cytokine and chemokine concentrations in the lung as effective as budesonide.

Since GOS and budesonide showed similar effects on HDM-induced allergic asthma, a combination of these two interventions was used to test whether there is a more pronounced effect on allergic asthma. Indeed, in **Chapter Six** it is shown that after dietary intervention with GOS and treatment with budesonide, the eosinophilic inflammation in the lungs of HDM-allergic mice was largely abolished. Also, this combination was most effective in suppressing HDM-induced increases in different cytokines and chemokines in lung homogenates as well as the frequency of Th2 cells.

In **Chapter Five** and **Six** a relatively high dose of budesonide was used, therefore, in **Chapter Seven** different doses of budesonide were used with or without dietary intervention with GOS to determine if a more pronounced effect of the combination also at lower doses of budesonide is still present. The combination did suppress most of the allergic features without any harmful side effects, however, no additional beneficial effects were observed.

As shown in **Chapter Five**, GOS reduced IL-33 concentrations in the lung. Since IL-33 is important for the mucosal barrier and crucial for Th2 mediated host defense, the effect of GOS on IL-33 expression was investigated in an intestinal barrier dysfunction- and asthma-model in **Chapter Eight**. In this chapter, it is shown that dietary intervention with GOS mitigated IL-33 at the mucosal surfaces both in a murine model for intestinal barrier dysfunction as well as a HDM-induced asthma model, indicating that GOS may target allergies and inflammation associated with increased IL-33 expression.

Treg cells may play a role in down-regulating the development of asthma and allergic diseases. In **Chapter Nine** Treg cells were depleted by means of anti-CD25 treatment to examine the role of these cells in the protective effect of GOS on HDM-allergic asthma in mice. Dietary intervention with GOS was able to decrease the percentage of eosinophils and IL-33 and CCL5 concentrations in the lung of HDM-allergic mice. However, anti-CD25 treatment abrogated this protective effect of GOS. This indicates that GOS exerts its protective effect by improving Treg function.

Finally the results of all studies are summarized and discussed and future research directions are described in **Chapter Ten**.

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CHAPTER TWO

Measurement of airway function using invasive and non-invasive methods in mild and severe models for allergic airway inflammation in mice

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Abstract

In this study a direct comparison was made between non-invasive and non-ventilated unrestrained whole body plethysmography (Penh) (conscious animals) and the invasive ventilated lung resistance (R_L) method (anaesthetized animals) in both mild and severe allergic airway inflammation models. Mild inflammation was induced by intraperitoneal sensitization and aerosols of ovalbumin. Severe inflammation was induced by intraperitoneal sensitization using trinitrophenyl-ovalbumin, followed by intranasal challenges with IgE-allergen complexes. A significant increase in airway responsiveness to methacholine was observed in the mild inflammation group when R_L was measured. Significant changes in both R_L and Penh were observed in the severe inflammation groups. There was a significant increase in the number of inflammatory cells in the bronchoalveolar lavage fluid (BALF) in both the mild and severe inflammation animals. The enforced ventilation of the animals during the R_L measurement further increased the number of cells in the BALF. IL-2 and RANTES levels in the BALF were higher in the severe inflammation groups compared to the mild inflammation groups. Penh gave only reliable measurements during severe airway inflammation. Measuring R_L gave consistent results in both mild and severe allergic airway inflammation models, however, ventilation induced an additional cell influx into the airways.

Introduction

Asthma is characterized by airway hyperresponsiveness and inflammation. Airway inflammation is initiated and propagated by multiple inflammatory mediators such as lipid mediators, cytokines and chemokines [1,2]. To investigate airway function in preclinical models, both non-invasive and invasive analysis methods have been used. Unrestrained whole body plethysmography (Penh), a non-invasive method for measurement of airway responsiveness, has been used frequently but its validity is under debate. For this method the airway function is measured with enhanced pause (Penh), an empirical and dimensionless parameter [3]. Increased bronchoconstriction is considered to be paralleled with an increase in Penh. However, various experimental conditions resulting in a change of breathing pattern can also affect Penh [4]. Penh is viewed as a better indicator for control of breathing (as seen in respiratory patterns) rather than an indicator for mechanical lung function [2,5-7]. Respiratory patterns can be influenced by stress [8], heating or humidification of the chamber, affecting the measured signal [7,9]. Moreover, the outcome of unrestrained Penh may be dependent on the mouse strain used [7]. Furthermore, unrestrained Penh measures changes in the upper airway parts (nose) as well as in the lower airways - of particular importance in rodents as these animals are nose-breathers [10]. There are also practical advantages of Penh measurements because the animals do not need to be anaesthetized and do not need surgery for ventilation hence, the method is simpler than others and less time-consuming [5,6,11-13]. Also, the animal can be used for repeated measurements in time [6,11,13-15]. Invasive lung resistance measurement (R_L) is another method for measuring airway function. Although R_L measurements are considered to more accurately represent lung function, the method also has its limitations. First, the animals have to be anaesthetized which might change physiological parameters such as body temperature. A cannula also has to be placed into the trachea which could cause local mechanical stress. Moreover, the animals are artificially ventilated with a fixed volume which artificially influences the pattern of breathing and might have an effect on the homeostasis of the airways. The technique is also time-consuming [16] and terminal for the animals after measurement. There is, however, no stress for the animal during the experiment [10,12], and exposing the lower airways to allergens or agonists is more accurate via a cannula in the trachea. When the trachea is orally intubated, instead of with an incision, repetitive measurements can be conducted in spontaneously breathing mice [17-19]. The most important advantage of the resistance method is that it is a sensitive and specific measurement to analyze pulmonary mechanics [12,16]. Due to the continuous discussion on the measurement of lung function in rodents, a comparative study between the non-invasive measurement airway function (Penh) and the invasive measurement airway function (Resistance (R_L)) was conducted in mild and severe allergic airway inflammation models, which mimics some features of allergic asthma in humans.

Materials and methods

Mice

Male BALB/c mice (Charles River, The Netherlands), 6-8 weeks old (20 -25 g), were used in all experiments. Mice were maintained under standard laboratory conditions. Food and water were provided *ad libitum*. All animal experiments were conducted in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the University Utrecht.

Sensitization and airway challenge

Mild airway inflammation model

On days 0 and 7 mice were sensitized with ovalbumin (OVA; chicken egg albumin, grade V, Sigma, USA) or treated with saline. Active sensitization was conducted by two intraperitoneal injections of 0.1 mL alum-precipitated antigen, comprising 10 µg OVA absorbed into 2.25 mg alum (AlumImject; Pierce, USA). On days 35, 38 and 41 mice were exposed either to an OVA (1% ovalbumin in pyrogen-free saline, OVA group) or control solution (saline, SAL group) aerosol challenge for 30 min.. The aerosol was conducted in a plexiglass exposure chamber (5 L) coupled to a Pari LC Star nebulizer (PARI Respiratory Equipment, USA; particle size 2.5–3.1 µm) driven by compressed air at a flow rate of 6 L/min. [20] **(Fig. 1A)**. An overview of the groups included in this study is given in **Table 1**.

Table 1 Experimental groups included in the mild airway inflammation model.

Sensitization	Challenge	Group abbreviation
SAL	SAL	SAL-SAL
SAL	OVA	SAL-OVA
OVA	SAL	OVA-SAL
OVA	OVA	OVA-OVA

Severe airway inflammation model

On days 0 and 7 mice were sensitized with trinitrophenyl (TNP) conjugated-OVA or saline. Sensitization was conducted by two intraperitoneal injections of 0.1 mL alum-precipitated antigen, comprising 10 µg TNP-OVA absorbed into 2.25 mg alum (AlumImject) or saline. On days 14 - 20, mice were challenged daily by intranasal administration of a TNP-ovalbumin/IgE immune complex (2µg TNP-OVA plus 20µg DNP-specific IgE (clone H1 26.82)) or saline, as described by Pasquier [21,22] **(Fig. 1B)**. An overview of the groups included in this study is given in **Table 2.**

Table 2 Experimental groups included in the severe airway inflammation model.

Sensitization	Challenge	Group abbreviation
SAL	SAL	SAL---SAL
SAL	TNP-OVA-IgE	SAL---TNP-OVA-IgE
TNP-OVA	SAL	TNP-OVA---TNP-OVA-IgE
TNP-OVA	TNP-OVA-IgE	TNP-OVA---TNP-OVA-IgE

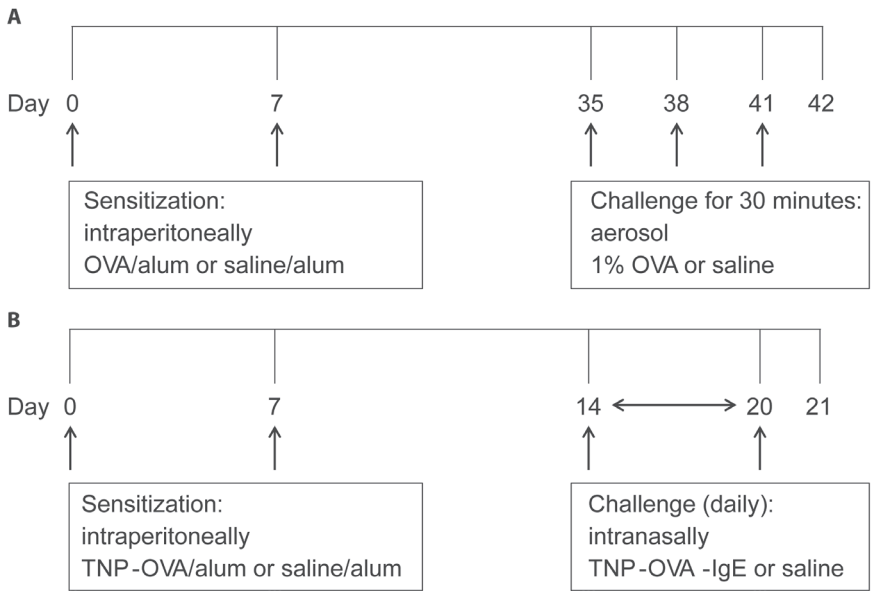

Fig. 1 Experimental scheme of the mild airway inflammation model (A) and severe airway inflammation model (B).

Measurement of airway responsiveness *in vivo*

Non-invasive measurement airway function (Penh)

Airway responsiveness was measured 24h after the last aerosol exposure by recording respiratory pressure curves using barometric unrestrained whole-body plethysmography (Buxco; EMKA Technologies, France) in response to inhaled methacholine (acetyl-β-methyl-choline chloride, Sigma, The Netherlands) in conscious unrestrained mice. Airway responsiveness was expressed as enhanced pause (Penh) as described in detail previously [23]. Briefly, mice were placed in a whole-body chamber and basal readings were obtained and averaged for a 3 min. period. Subsequently, increasing doses of methacholine (0 – 50 mg/mL), were aerosolized for 3 min., and readings were taken and averaged for 3 min. after each nebulization [24].

Invasive measurement airway function (Resistance (R_L))

The mice were intraperitoneally anaesthetized with KM-mix (containing Ketamine (Vetoquinol S.A., France; 125 mg/kg) and Medetomidine (Pfizer, The Netherlands; 0.4 mg/kg)). The animals were ventilated (O_2:air (1:2)) at a frequency of 150 beats/min. (TV = 0.3 mL). An anaesthesia-induced fall in body temperature was avoided by placing the animals in a heated box in which the body temperature was kept at 37°C. The mice were prepared for the measurement of lung parameters (pulmonary resistance (R_L)). Pressure was determined as follows: a small catheter was placed in the trachea of the mouse. This catheter was connected to a pressure transducer fixed on the box (EMKA Technologies, France) and transpulmonary pressure was determined by measuring pressure differences in the cannula in the trachea. Airflow and tidal volume were determined using a flow transducer fixed to the body box that measured flow differences inside the box. Increasing doses of methacholine (0 -50 mg/mL, 10% puff for 10 sec.) were administered by aerosol generated in a nebulizer (EMKA Technologies, France) connected in between the animal in the body box and the ventilator (EMKA Technologies, France). After the first dose of methacholine, pulmonary resistance (R_L) was measured for 3 min. and this procedure was repeated for all doses. R_L was yielded by dividing transpulmonary pressure by airflow at isovolume points. Data are presented as average R_L in cm H_2O/(mL/sec.) [25].

Bronchoalveolar lavage (BALF)

Mice were killed by an intraperitoneal overdose of pentobarbital (Nembutal™, Ceva Santé Animale, The Netherlands) after the airway responsiveness measurement. The trachea was trimmed free of connective tissue and a small incision was made for insertion of a cannula into the trachea. Lungs were lavaged with 1 mL of pyrogen-free saline (0.9% NaCl, 37°C) supplemented with protease inhibitor cocktail tablet. The supernatant of the first mL was used for cytokine and chemokine measurement. Afterwards the lungs were lavaged 3 times with 1 mL saline solution (0.9% NaCl, 37°C). The bronchoalveolar fluid (BALF) cells were centrifuged (400 g, 5 min.) and pellets of the 4 lavages were pooled, resuspended and total numbers of BALF cells were counted by use of a Bürker-Türk chamber. For differential BALF cell counts cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Switzerland). After coding, all cytospin preparations were evaluated by one observer using oil immersion microscopy. Cells were differentiated into macrophages, lymphocytes, neutrophils and eosinophils by standard morphology. At least 200 cells per cytospin preparation were counted and the absolute number of each cell type was calculated [26].

Measurement of cytokines and chemokines

A standard mouse cytokine 21-plex assay (GM-CSF, IFNγ, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-9, IP-10, KC, MCP-1, MIG, MIP-1α, MIP-2, RANTES and TNFα, Luminex; Biosource, Invitrogen, The Netherlands) was used to determine cytokine

and chemokine concentrations in the BALF (n = 4-5) according to the manufacturer's instructions [27]. The concentrations of these cytokines and chemokines were expressed as pg/mL.

Lung histology

After lung lavage, lungs were fixated with 10% formalin infusion through the cannula at a constant pressure of 25 cm H_2O. After at least 24h of fixation lungs were embedded in paraffin. After embedding, 5µm sections were cut and stained with hematoxylin/eosin (H&E) according to standard methods. Photomicrographs were taken with an Olympus BX50 microscope equipped with a Leica DFC 320 digital Camera [28,29].

Statistical analysis

Results are presented as the mean ± standard error of mean (SEM). Data were statistically analyzed using a One-Way ANOVA followed by a Bonferroni post hoc analysis. P values < 0.05 were considered to be significant. Statistical analyses were conducted using GraphPad Prism software (version 5.0).

Results

Airway function

Airway responsiveness was measured in conscious unrestrained mice (Penh) or in anaesthetized ventilated animals (R_L) exposed to inhaled methacholine 24h after the last OVA or saline challenge.

Measurement of airway function in the mild airway inflammation model

In unrestrained mice, basal airway resistance was significantly increased in the OVA-OVA group compared to the SAL-SAL group **(Fig. 2)**. Moreover, an increase in Penh was observed after administrating a low dose of methacholine to the OVA-OVA group compared to the SAL-SAL group. However, this effect was not observed using higher doses of methacholine. The Penh dose-dependently increased in the SAL-SAL group in response to methacholine inhalation to a maximum of 5.47 ± 1.63. The Penh in the OVA-OVA group was increased by 56% to a maximum of 8.54 ± 1.84, but this increase was not significant compared to the SAL-SAL group **(Fig. 2)**. In ventilated mice, basal airway resistance was not significantly different between the experimental groups **(Fig. 3)**. Methacholine slightly increased the R_L in the SAL-SAL group, while the R_L was significantly increased by 71% in the OVA-OVA group after methacholine (12.5-50 mg/mL) inhalation **(Fig. 3)**.

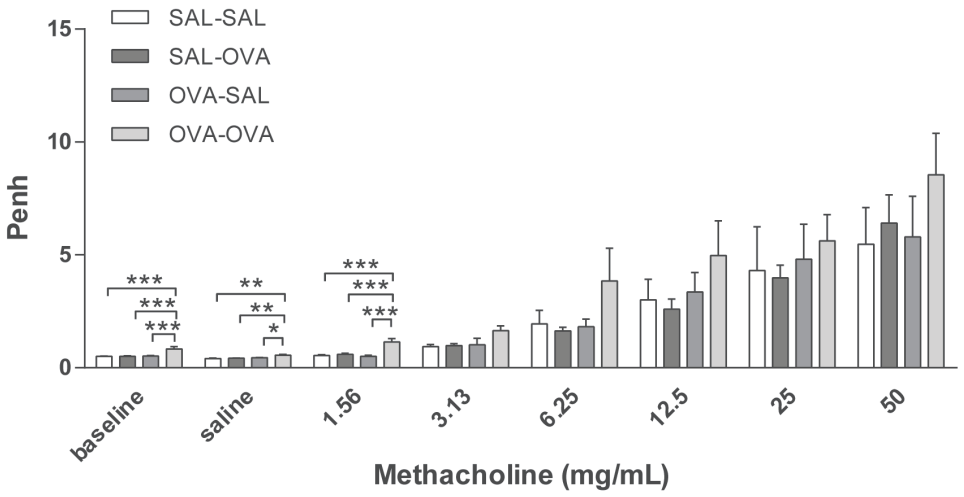

Fig. 2 Airway responsiveness (Penh) to methacholine in the mild airway inflammation model.
Airway responsiveness was measured in mice sensitized with saline or ovalbumin and challenged by aerosol with saline or ovalbumin. Values are expressed as mean ± SEM * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; using a One-Way ANOVA followed by a Bonferroni post hoc analysis, n = 5-9 mice/group.

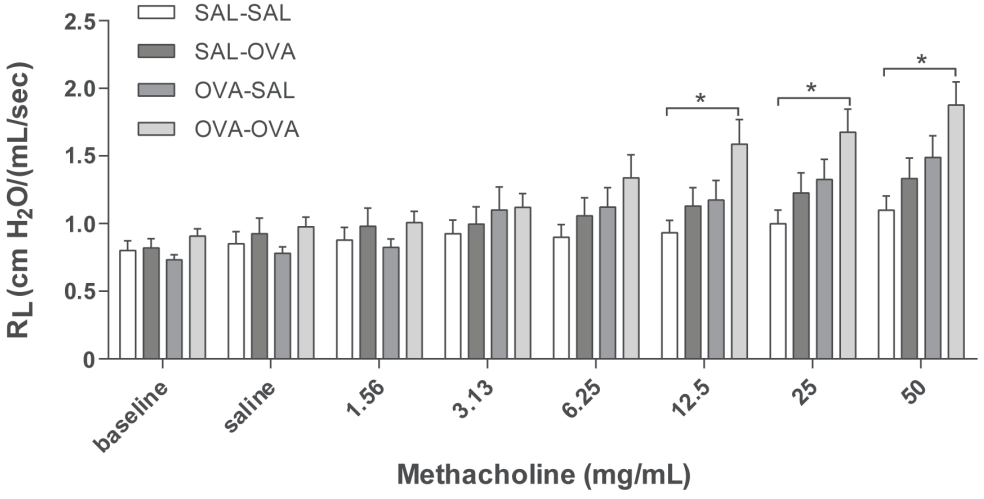

Fig. 3 Resistance measurement (R_L) to methacholine in the mild airway inflammation model.
Resistance was measured in ventilated mice sensitized with saline or ovalbumin and challenged by aerosol with saline or ovalbumin. Values are expressed as mean ± SEM * $P < 0.05$; using a One-Way ANOVA followed by a Bonferroni post hoc analysis, n = 7-9 mice/group.

Measurement of airway function in the severe airway inflammation model

Basal airway resistance was significantly increased in the TNP-OVA---TNP-OVA-IgE group compared to the SAL---SAL group. The Penh increased dose-dependently in the SAL---SAL group after methacholine inhalation **(Fig. 4)**. The Penh in the TNP-OVA---TNP-OVA-IgE group was significantly increased compared to the SAL---SAL group to a maximum of 12.33 ± 1.99 **(Fig. 4)**. The increase in Penh was 1.5 times higher compared to the sensitized and challenged animals with mild airway inflammation **(Fig. 2)**. Although the basal R_L did not differ in the OVA---OVA group of the mild model **(Fig. 3)**, basal R_L tended to increase in the severe model **(Fig. 5)**, reaching the level of significance after saline exposure. As in the mild model, methacholine slightly increased the R_L in the SAL---SAL group of the severe model. The R_L was significantly increased in the TNP-OVA---TNP-OVA-IgE group after the methacholine inhalation to a maximum of 1.76 ± 0.18 cm H_2O/(mL/sec.). The maximal increase in R_L to methacholine in the severe model was a level similar to the OVA-OVA group in the mild airway inflammation model, but the sensitivity was higher as evidenced by significant changes at lower doses of methacholine (**Fig. 3** & **Fig. 5**).

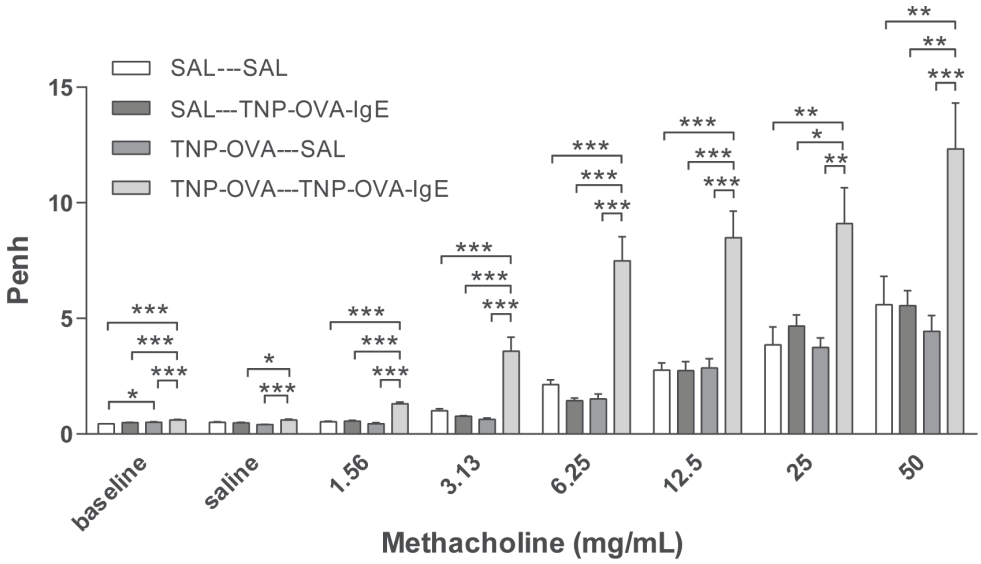

Fig. 4 Airway responsiveness (Penh) to methacholine in the severe airway inflammation model.

Airway responsiveness was measured in mice sensitized with saline or TNP-OVA and challenged intranasally with saline or TNP-OVA-IgE. Values are expressed as mean ± SEM * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; using a One-Way ANOVA followed by a Bonferroni post hoc analysis, n = 7-9 mice/group.

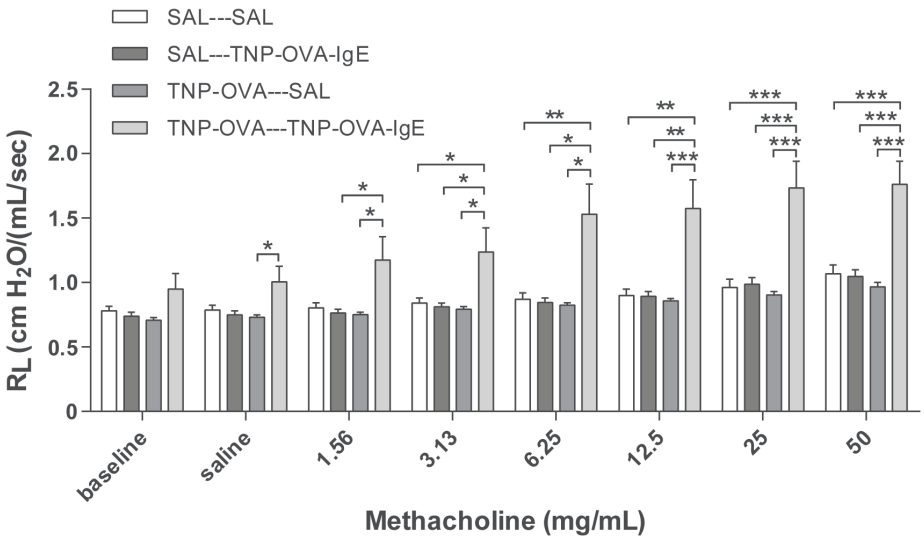

Fig. 5 Resistance measurement (R_L) to methacholine in the severe airway inflammation.

Resistance was measured in ventilated mice sensitized with saline or TNP-OVA and challenged intranasally with saline or TNP-OVA-IgE. Values are expressed as mean ± SEM * P< 0.05, ** P < 0.01, *** P < 0.001; using a One-Way ANOVA followed by a Bonferroni post hoc analysis, n = 9 mice/group.

Airway inflammation in the mild model

The influx of inflammatory cells (total and differentiated cell numbers) in the lungs was measured after lung function measurement. Total BALF cell numbers were four times higher in the OVA-OVA group compared to the SAL-SAL group after the Penh measurement (**Table 3**). This increase in total cells was mainly due to an increase in the number of eosinophils. Moreover, the number of lymphocytes in the OVA-OVA group was significantly increased. The total number of inflammatory cells after R_L measurement was nine times higher in the OVA-OVA group compared to the SAL-SAL group. Lymphocytes, neutrophils and eosinophils were all significantly increased in the OVA-OVA group. There were no differences between the SAL-SAL groups after measuring Penh or R_L. In contrast, after R_L measurement, the total number of BALF cells in the OVA-OVA group was three times higher compared to the OVA-OVA group after Penh measurement. This increase was mainly due to a significant increase in the number of eosinophils and to a lesser extent to an increase of lymphocytes and neutrophils (**Table 3**).

Table 3 Total and differential cell counts in bronchoalveolar lavage fluid in the mild airway inflammation model.

Bronchoalveolar cells (*10^4/mL) (mean±SEM)

	Groups	Total	Macrophages	Lymphocytes	Neutrophils	Eosinophils
Penh	SAL-SAL	23.9 ± 1.0	23.6 ± 1.0	0.1 ± 0.1	0.05 ± 0.03	0
	SAL-OVA	25.2 ± 2.4	25.0 ± 2.4	0.02 ± 0.01	0.2 ± 0.1	0
	OVA-SAL	26.5 ± 3.5	26.3 ± 3.5	0.08 ± 0.04	0.1 ± 0.1	0
	OVA-OVA	95.2 ± 30.6	35.4 ± 5.4	4.9 ± 2.0	9.1 ± 5.2	45.7 ± 20.1 *
R_L	SAL-SAL	31.9 ± 2.6	31.5 ± 2.5	0.07 ± 0.04	0.4 ± 0.1	0
	SAL-OVA	33.8 ± 3.8	33.5 ± 3.8	0.06 ± 0.04	0.3 ± 0.1	0
	OVA-SAL	31.9 ± 3.3	30.2 ± 2.9	0.2 ± 0.06	1.6 ± 0.28	0.4 ± 0.2
	OVA-OVA	291.8 ± 47.1 *** ###	50.1 ± 6.7	22.6 ± 4.7 *** ###	20.1 ± 2.9 *** #	199.0 ± 37.5 *** ###

Values are expressed as mean ± SEM * $P < 0.05$, *** $P < 0.001$; significantly different from the saline-saline group; # $P < 0.05$, ### $P < 0.001$ significant different from the OVA-OVA Penh group, using a One-Way ANOVA followed by a Bonferroni post hoc analysis.

Airway inflammation in the severe model

The total number of BALF cells after the Penh measurement in the SAL---SAL group of the severe model did not differ from the number of cells after the R_L measurement (**Table 4**) and showed a slight increase compared to Penh and R_L measurement in the mild model (**Table 3**). Daily intranasal administration of TNP-OVA-IgE from day 14 - 20 of the SAL group significantly increased the number of BALF cells whereas intranasal saline administration had no effect. This increase was mainly due to an increase in macrophages and neutrophils and not caused by changes in the number of eosinophils (**Table 4**). The total number of BALF cells was increased in the TNP-OVA---TNP-OVA-IgE group compared to the SAL---SAL group after the Penh measurement (**Table 4**). The total number of BALF cells was five times higher than in the mild OVA-OVA group (**Table 3**). This increase was mainly due to an increase in macrophages and eosinophils. The total number of BALF cells consisted for nearly 65% of eosinophils. Again the number of inflammatory cells was the highest in the severe group after ventilation of the animals (**Table 4**).

Cytokine measurements

Inflammatory cytokines were measured in the BALF of the experimental groups. There was no relation between the cytokine profile or amount of cytokine protein and ventilation versus no ventilation. Therefore, in contrast to the number of inflammatory cells, the ventilation procedure did not influence the type and amount of cytokines produced. However, the levels of IL-2 and RANTES were increased in BALF in the severe

airway inflammation model compared to the mild model (**Fig. 6A** & **6B**, respectively). In both models, GM-CSF, IFNγ, IL-12p70, IL-13, IL-17 and MIP-2 were below detection limit. All other cytokines and chemokines are depicted in **Table 5** and **6**.

Table 4 Total and differential cell counts in bronchoalveolar lavage fluid in the severe airway inflammation model.

		Bronchoalveolar cells (*10^4/mL) (mean±SEM)				
	Groups	**Total**	**Macrophages**	**Lymphocytes**	**Neutrophils**	**Eosinophils**
Penh	SAL---SAL	43.1 ± 8.1	42.9 ± 8.1	0.03 ± 0.03	0	0
	SAL---TNP-OVA-IgE	156.8 ± 23.3 ***	125.1 ± 16.8 ***	1.89 ± 0.8	29.8 ± 8.4 **	0
	TNP-OVA ---SAL	42.7 ± 4.4	42.1 ± 4.4	0.27 ± 0.2	0.25 ± 0.1	0
	TNP-OVA ---TNP-OVA-IgE	552.8 ± 24.7 ***	185.7 ± 9.7 ***	9.63 ± 2.7 ***	2.66 ± 1.6	354.8 ± 26.2 ***
R_L	SAL---SAL	43.1 ± 6.3	42.8 ±6.3	0.09 ± 0.1	0.23 ± 0.1	0
	SAL---TNP-OVA-IgE	209.8 ± 31.6 **	148.75 ± 21.3 **	4.6 ± 1.8	56.5 ± 14.4 ***	0
	TNP-OVA ---SAL	42.2 ± 5.6	41.8 ± 5.6	0.2 ± 0.1	0.1 ±0.1	0
	TNP-OVA ---TNP-OVA-IgE	667.5 ± 56.9 ***	211.9 ± 37.5 ***	26.5 ± 6.2 *** ###	9.2 ± 5.5	419.9 ± 35.0 *** #

Values are expressed as mean ± SEM ** $P < 0.01$, *** $P < 0.001$; significantly different from the saline---saline group; # $P < 0.05$, ### $P < 0.001$ significant different from the TNP-OVA---TNP-OVA-IgE Penh group, using a One-way ANOVA followed by a Bonferroni post hoc analysis.

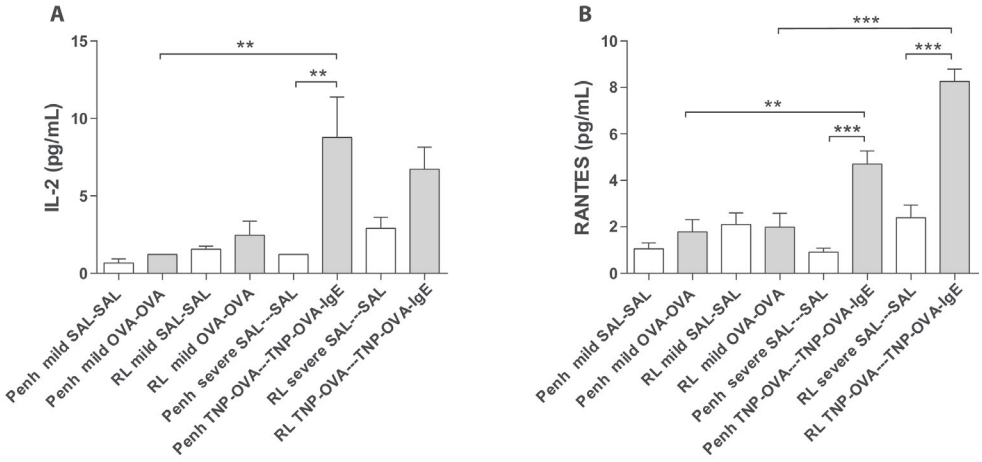

Fig. 6 Cytokine measurement in BALF.

Cytokines were measured in mice sensitized with saline or ovalbumin and challenged by aerosol with saline or ovalbumin (mild) and mice sensitized with saline or TNP-OVA and challenged intranasally with saline or TNP-OVA-IgE (severe) after Penh measurement or R_L measurement. **(A)** IL-2 concentration in pg/mL, **(B)** RANTES concentration in pg/mL. Values are expressed as mean ± SEM ** $P < 0.01$, *** $P < 0.001$ using a One-Way ANOVA followed by a Bonferroni test, n = 4-5 mice/group.

Table 5 Chemokine and cytokine concentration in bronchoalveolar lavage fluid in the mild airway inflammation model.

	Penh			
pg/mL	**SAL-SAL**	**SAL-OVA**	**OVA-SAL**	**OVA-OVA**
IL-10	n.d.	n.d.	17.8 ± 10.6	n.d.
IL-12p40	n.d.	n.d.	n.d.	n.d.
IL-1b	n.d.	n.d.	n.d.	n.d.
IL-2	0.67 ± 0.26	1.21 ± 0.08	n.d.	n.d.
IL-4	n.d.	n.d.	n.d.	130.30 ± 75.96
IL-5	n.d.	n.d.	n.d.	133.50 ± 53.23
IL-6	6.13 ± 4.91	n.d.	n.d.	3.90 ± 2.68
IL-9	49.04 ± 32.71	94.25 ± 61.92	n.d.	n.d.
IP-10	n.d.	n.d.	n.d.	5.92 ± 0.05
KC	37.92 ± 9.03	22.16 ± 4.85	29.06 ± 7.80	143.90 ± 40.57
MCP-1	n.d.	n.d.	n.d.	n.d.
MIG	n.d.	5.35 ± 1.71	n.d.	3.80 ± 1.59
MIP-1α	n.d.	n.d.	n.d.	83.37 ± 52.98
MIP-2	n.d.	n.d.	n.d.	n.d.
RANTES	1.06 ± 0.25	1.38 ± 0.39	1.03 ± 0.23	1.78 ± 0.52
TNFα	1.57 ± 0.33	2.06 ± 0.42	1.98 ± 0.03	1.65 ± 0.19

	R_L			
pg/mL	**SAL-SAL**	**SAL-OVA**	**OVA-SAL**	**OVA-OVA**
IL-10	n.d.	13.13 ± 6.53	n.d.	n.d.
IL-12p40	n.d.	n.d.	n.d.	n.d.
IL-1b	n.d.	n.d.	n.d.	n.d.
IL-2	1.55 ± 0.20	1.45 ± 0.28	0.91 ± 0.27	2.46 ± 0.91
IL-4	n.d.	n.d.	7.06±3.62	48.98 ± 12.80
IL-5	n.d.	n.d.	35.85 ± 21.21	107.60 ± 71.93
IL-6	n.d.	n.d.	n.d.	n.d.
IL-9	n.d.	n.d.	41.45 ± 22.24	n.d.
IP-10	n.d.	n.d.	n.d.	74.58 ± 16.05

KC	35.43 ± 3.81	61.52 ± 9.12	74.17 ± 13.99	267.9 ±92.75
MCP-1	n.d.	n.d.	n.d.	35.51 ± 16.98
MIG	n.d.	5.40 ± 1.80	2.02 ± 0.61	118.90 ± 41.71
MIP-1α	n.d.	n.d.	n.d.	132.80 ± 3.26
MIP-2	n.d.	n.d.	n.d.	n.d.
RANTES	2.11 ± 0.50	2.18 ± 0.22	1.67 ± 0.34	1.99 ± 0.60
TNFα	1.72 ± 0.23	2.21 ± 0.25	1.69 ± 0.10	1.66 ± 0.29

n.d. not detectable

Table 6 Chemokine and cytokine concentration in bronchoalveolar lavage fluid in the severe airway inflammation model.

	Penh			
pg/mL	**SAL---SAL**	**SAL--- TNP-OVA-IgE**	**TNP-OVA---SAL**	**TNP-OVA--- TNP-OVA-IgE**
IL-10	n.d.	n.d.	n.d.	n.d.
IL-12p40	n.d.	n.d.	n.d.	n.d.
IL-1b	n.d.	n.d.	n.d.	n.d.
IL-2	n.d.	n.d.	n.d.	7.26 ± 2.52
IL-4	n.d.	n.d.	n.d.	5.51 ± 1.86
IL-5	n.d.	n.d.	n.d.	28.67 ± 14.7
IL-6	n.d.	n.d.	n.d.	n.d.
IL-9	n.d.	n.d.	n.d.	n.d.
IP-10	n.d.	n.d.	n.d.	42.00 ± 6.08
KC	85.40 ± 22.11	28.24 ± 6.70	39.90 ± 2.66	169.30 ± 58.66
MCP-1	n.d.	n.d.	n.d.	n.d.
MIG	n.d.	n.d.	n.d.	30.40 ± 4.71
MIP-1α	n.d.	n.d.	n.d.	79.70 ± 38.74
RANTES	0.91 ± 0.17	1.68 ± 0.35	0.91 ± 0.21	4.70 ± 0.57
TNFα	2.23 ± 0.18	2.41 ± 0.56	2.12 ± 0.01	1.62 ± 0.16

pg/mL	R_L			
	SAL---SAL	**SAL--- TNP-OVA-IgE**	**TNP-OVA---SAL**	**TNP-OVA--- TNP-OVA-IgE**
IL-10	7.83 ± 3.06	n.d.	16.23 ± 9.86	6.12 ± 4.90
IL-12p40	4.93 ± 2.19	n.d.	n.d.	33.87 ± 10.30
IL-1b	5.98 ± 2.94	n.d.	n.d.	9.56 ± 5.65
IL-2	2.91 ± 0.71	1.28 ± 0.35	2.52 ± 0.94	6.72 ± 1.42
IL-4	n.d.	n.d.	n.d.	5.14 ± 2.06
IL-5	n.d.	n.d.	n.d.	41.69 ± 26.33
IL-6	n.d.	n.d.	n.d.	n.d.
IL-9	n.d.	n.d.	n.d.	n.d.
IP-10	n.d.	101.40 ± 25.48	n.d.	11.6 ± 5.61
KC	33.1 ± 8.04	38.36 ± 7.59	14.55 ± 1.19	92.89 ± 29.16
MCP-1	n.d.	n.d.	n.d.	28.29 ± 18.00
MIG	5.48 ± 1.82	289.20 ± 84.17	1.59 ± 0.26	11.43 ± 6.10
MIP-1α	27.62 ± 17.25	n.d.	n.d.	n.d.
RANTES	2.40 ± 0.54	0.99 ± 0.36	2.73 ± 0.55	8.26 ± 0.52
TNFα	2.40 ± 0.34	1.49 ± 0.23	1.74 ± 0.26	2.10 ± 0.71

n.d not detectable

Discussion

In this study, a comparison was made between the body plethysmography (Penh) and resistance (R_L) measurements, to analyse murine airway function in two models for allergic airway inflammation. Although often referred to as a model for asthma, these animal models do not reflect all pathophysiological mechanisms in asthma patients [30]. Therefore, the models used in this manuscript are referred to as models of allergic airway inflammation. We demonstrated that the Penh measures are not as pronounced in the mild model as compared to R_L measures. However, an additional increase in inflammatory cells was found during the R_L measurement, which was most likely due to enforced pulmonary ventilation. In the severe inflammation model the increase in Penh was more pronounced compared to the R_L. Using the bodyplethysmographic analysis of Penh, the total number of BALF cells in the OVA-OVA group increased three times compared to the SAL-SAL group in the mild inflammation model. The increase was mainly due to an increase in eosinophils. Eosinophilic inflammation is a characteristic of allergic asthma [1]. In severe

asthma, the numbers of eosinophils, macrophages, lymphocytes and neutrophils are higher compared to mild asthma [1]. In agreement, in the severe inflammation model, the total number of BALF cells after TNP-OVA sensitization and TNP-OVA-IgE challenge was more than five times higher compared to the OVA-OVA group in the mild inflammation model. The number of eosinophils in the severe airway inflammation model was more than seven times higher than in the mild inflammation model, as demonstrated earlier by Zuberi *et al.* and Sagar *et al*. [22,31]. After ventilation of the animals and R_L measurements, the total number of BALF cells was higher compared to Penh, both in the mild (OVA-OVA group) and severe inflammation model (TNP-OVA---TNP-OVA-IgE group). In the severe airway inflammation model this increase was not as high as in the mild inflammation model. Potentially due to the number of BALF cells already at a maximum level caused by the process: ventilator-induced lung injury (VILI). Cannizarro *et al.* [32] and Zhang *et al.* [33] demonstrated that mechanical ventilation increases the total number of BALF cells in BALB/c mice. In the mild inflammation model, only a slight but not significant increase in the Penh in the OVA-OVA group was found. Resistance of the upper airways may influence the outcome of changes in the lower airways [10], explaining this anomaly. In contrast, a pronounced airway hyperresponsiveness was observed in the severe inflammation model. This indicates that Penh measurements might only be useful to investigate airway function under conditions of severe inflammation. When the airway responsiveness was measured using the R_L method, a significant increase in both the mild and severe allergic airway inflammation model was recorded. Strikingly, ventilation causes a significant increase in the number of BALF cells in the mild model, but not in the severe model. The number of inflammatory cells does not correlated with the airway hyperresponsiveness. From studies it is known that airway hyperresponsiveness to bronchoconstrictor agents does not correlate with inflammation, but indirect stimuli, like hypertonic saline does [30]. Histology of the lungs showed that more cells are present in mice with mild allergic airway inflammation after the R_L measurement as compared to these after Penh measurement (**Fig. 7**). The increase in the number of BALF cells in the ventilated animals of the mild inflammation model could not be explained by changes in chemokines or cytokines, but significantly higher levels of IL-2 and RANTES were observed in the severe model compared to the mild model. IL-2 can be produced by epithelial cells and eosinophils [34] and increased levels are found in the BALF of patients with symptomatic asthma [34,35]. These experiments are in line with our observation that more eosinophils were present in the severe model. The eosinophilia might be further promoted by RANTES. RANTES is a CC chemokine involved in the chemoattraction of T lymphocytes, monocytes and eosinophils [34,36]. Increased levels of RANTES are present in the BALF obtained from asthmatic patients [34,36,37] and blocking antibodies against RANTES are able to inhibit airway inflammation in a murine model of allergic airway disease [36]. In conclusion, in models with mild inflammation, body plethysmography for the determination of the airway hyperresponsiveness may not be as reliable as measurements of resistance which provided a more accurate analysis compared with previous studies. In severe models with

more pronounced airway inflammation, both body plethysmography and measurement of R_L may be used to analyze airway function. Along with the invasive procedure, a disadvantage of the R_L method could be the ventilation-induced increase in BALF cell numbers under mild inflammatory conditions.

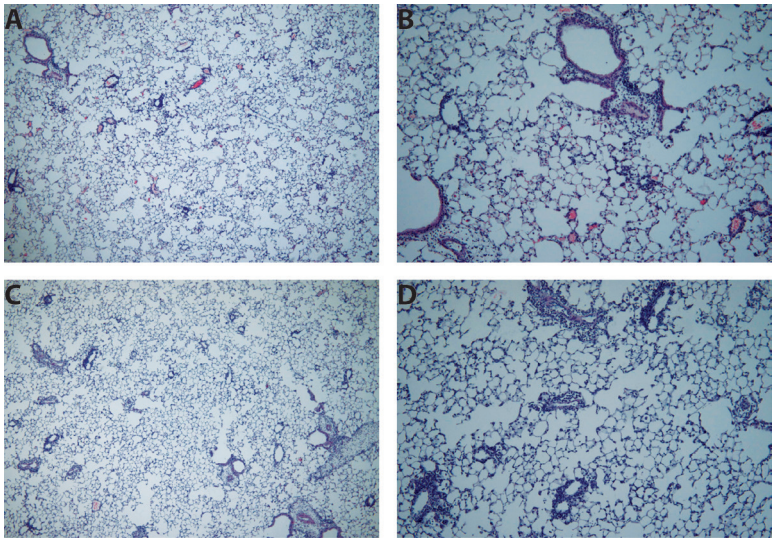

Fig. 7 Lung histology in the mild airway inflammation model.
Lungs were stained with H&E for histomorphometric analysis of the inflammation in mice sensitized with ovalbumin and challenged by aerosol with ovalbumin (mild) after Penh **(A, B)** or resistance measurement **(C, D)**. Magnification 40x **(A, C)** or 100x **(B, D)**.

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CHAPTER THREE

The effects of galacto-oligosaccharides and different oligosaccharides mixtures on house dust mite induced asthma in mice: a preliminary proof of concept study

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Abstract

Background: There is an increased interest in dietary oligosaccharides to prevent or reduce allergic disease symptoms. House dust mites (HDM) can induce allergic asthma, which is associated with airway hyperresponsiveness (AHR) and airway eosinophilia.
Aim: To study the effects of different dietary oligosaccharides on HDM-induced allergic asthma in mice.
Methods: On day 0, BALB/c mice were intranasally (i.n.) sensitized with 1 µg HDM and challenged i.n. on days 7 to 11 with PBS or 10 µg HDM while being fed a diet containing 1% w/w mixtures of galacto-oligosaccharides (GOS) and long-chain fructo-oligosaccharides (FOS) (GF) (ratio 9:1) or short-chain fructo-oligosaccharides and long-chain fructo-oligosaccharides (FF) (1:1) from day -14 to 14. Other groups were fed 1% w/w of GOS alone or in combination with sialyllactose (GS) (1:1). On day 14, AHR to metacholine, inflammatory cell influx in bronchoalveolar lavage fluid (BALF) and chemokine/cytokine concentrations in lung homogenates were analyzed.
Results: HDM-sensitization and -challenge resulted in AHR and a significant increase in BALF leukocyte numbers. The AHR was significantly decreased by dietary intervention with GOS, and FF showed a similar trend, although not significant. GF and GS had no effect on AHR. In contrast to GS, all other oligosaccharide diets significantly reduced the number of eosinophils in the BALF. GOS reduced the concentrations of CCL5 and IL-13, while FF reduced CCL5 and GF tended to reduce IL-6 in lung homogenate supernatants. In general, IL-6 concentrations were positively correlated with the number of leukocytes in the BALF of HDM-allergic mice and IL-13 concentrations were positively correlated with the number of lymphocytes and the AHR.
Conclusion: In this model GOS, GF and FF were able to decrease eosinophilic inflammation, whereas GOS also inhibited AHR and IL-13 concentrations in lung homogenates. Different types of dietary oligosaccharides fed during the sensitization and challenge phase seem to have different effects on several features of HDM-allergic asthma.

Introduction

During the past years, there is an increased interest in dietary oligosaccharides to prevent or reduce allergic disease symptoms. Several studies indicate that the gut microbiome can influence the immune function in the gut, but also systemically and even in the lungs. Several data demonstrate that the development of allergies and asthma is associated with changes in the gut microbiome [1-3]. Non-digestible oligosaccharides (NDO) are selectively fermentable components. The prebiotic capacities contribute to selectively support the growth and/or activity of lactobacilli and bifidobacteria [4-6]. Examples of NDO are galacto-oligosaccharides (GOS; degree of polymerization (DP) ranging from 2 to 8) and long-chain (lc) fructo-oligosaccharides (lcFOS), which are neutral oligosaccharides that are used in medical nutrition and e.g. infant formula, mostly in a 9:1 ratio. In addition to lcFOS (DP ≥23), also short-chain (sc) FOS (DP ranging from 2 to 8) are interesting NDO. scFOS has a similar size distribution as GOS, but it origins from a vegetable source (inulin) instead of a dairy source used to produce GOS (cow's milk-derived lactose). It has been shown that these neutral NDO might have some similarities in terms of functions as compared to specific neutral oligosaccharides present in human milk, although they are far from identical [7]. Human milk oligosaccharides may protect against the development of allergies [7,8]. As human milk also contains acidic oligosaccharides besides the neutral NDO, an acidic oligosaccharide, sialyllactose was developed [9].

It is know from animal studies that NDO or mixtures of NDO with beneficial bacteria (synbiotics) can reduce allergic symptoms in asthma and food allergy [10-12]. However, not all NDO have the same effectiveness in different animal models for allergy. We previously showed that GOS was able to reduce allergic symptoms in a murine house dust mite (HDM)-induced asthma model [13], whereas Kerperien *et al.* did not find an effect of GOS alone in a cow's milk allergy mouse model, but only if GOS was combined with lcFOS [14]. Also in clinical studies, positive effects of NDO or synbiotics have been observed in patients suffering from atopic dermatitis, rhinoconjunctivitis and allergic asthma [15,16]. The effect of sialylated oligosaccharides in human studies on respiratory infections and inflammatory diseases is still limited [9]. Airway hyperresponsiveness (AHR) and airway inflammation involving mainly eosinophils, are important features of asthma [17]. As HDM is a major inducer of allergic asthma in humans [18], in this study, the effects of galacto-oligosaccharides alone (GOS) or in combination with sialyllactose (GS), and mixtures of galacto- and fructo-oligosaccharides (GF, FF) on AHR, pulmonary inflammation and lung chemokine and cytokine concentrations were analyzed and compared in a murine model for HDM-induced asthma. The results of the effects of GOS have already been published previously [13].

Materials and methods

Mice

All animal experiments were conducted in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Utrecht University (DEC 2013. II.01.003). Six- to eight- week old male BALB/c mice (Charles River, The Netherlands) were housed under bio-contained sterile conditions using HEPA® filtered isocages® (Tecniplast, Italy). Food and water were provided *ad libitum*.

HDM-induced murine asthma model

On day 0 mice were intranasally (i.n.) sensitized with 1 µg HDM (Greer Laboratories, USA) and challenged i.n. with PBS (control, HDM-PBS) or 10 µg HDM (HDM-HDM) on days 7 to 11. Mice were fed a control diet based on the synthetic AIN-93G composition, a diet containing 1% w/w galacto-oligosaccharides (GOS; Vivinal® GOS syrup with approximately 59% galacto-oligosaccharides, 21% lactose, 19% glucose, and 1% galactose on dry matter (dry matter of 75%; DP 2 - 8); FrieslandCampina Domo, The Netherlands), a diet containing a 1% w/w 9:1 mixture of galacto-oligosaccharides and long-chain fructo-oligosaccharides (Orafti ® HP with approximately ~100% inulin, DP ≥23; Beneo, Belgium) (GF), a diet containing 1% w/w 1:1 mixture of short-chain fructo-oligosaccharides (Orafti ® P95, with approximately 95% oligofructose content, DP 2-8; Beneo, Belgium) and long-chain fructo-oligosaccharides (FF) or a diet containing 0,5% w/w galacto-oligosaccharides (Vivinal® GOS syrup) in combination with 0,5% sialyllactose (sialyllactose powder consisting of approximately 61% sialyllactose, 22% other carbohydrates, 8% protein, 4% minerals and 4% moisture; FrieslandCampina Domo, the Netherlands) (GS). Carbohydrates in Vivinal® GOS were isocalorically compensated in the control diet by means of exchange against cellulose (for GOS), lactose (for lactose), and dextrose (for glucose). Sialyllactose was exchanged against cellulose as well. In case of the fructo-oligosaccharides, carbohydrates were isocalorically compensated in the control diet by means of exchange against cellulose (for FOS). Diets were given two weeks prior to sensitization and provided ad libitum during the entire experimental period [13] (**Fig. 1**). Since this was a preliminary proof of concept study, not all the appropriate control groups were included. Lung function was measured on day 14, using invasive measurement of dynamic resistance (EMKA Technologies, France) in response to increasing doses of methacholine (acetyl-β-methyl-choline chloride, Sigma-Aldrich, The Netherlands). Data are presented as average lung resistance (R_L) in cm H_2O/(mL/sec.) [19].

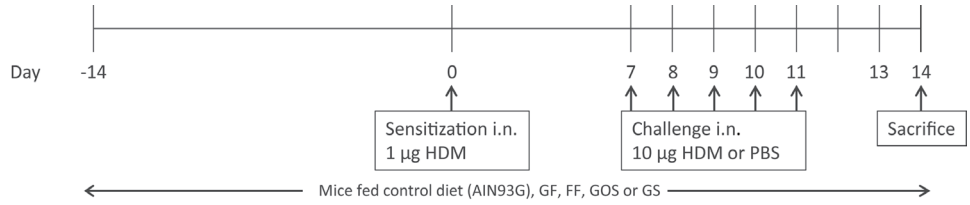

Fig. 1 Protocol HDM-induced asthma model.

On day 0, BALB/c mice were sensitized intranasally (i.n.) with house dust mite (HDM) and challenged i.n. from day 7 to 11 with HDM or PBS. Mice were fed the control diet (AIN93G, contr), a diet containing 1% w/w GOS, a diet containing a 1% w/w 9:1 mixture of galacto-oligosaccharides and long-chain fructo-oligosaccharides (GF), a diet containing 1% w/w 1:1 mixture of short-chain fructo-oligosaccharides and long-chain fructo-oligosaccharides (FF) or a diet containing 1% w/w 1:1 galacto-oligosaccharides and sialyllactose (GS). Dietary interventions started two weeks prior to sensitization and continued during the entire experiment. Mice were sacrificed on day 14.

Bronchoalveolar lavage

After sacrificing the mice, lungs were lavaged with pyrogen-free saline (0.9% NaCl, 37°C). The bronchoalveolar lavage fluid (BALF) cells were centrifuged (400 g, 5 min.), total numbers of BALF cells were counted using a Bürker-Türk chamber (magnification 100x). Cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Düdingen, Switzerland) for the differentiation of BALF cell counts. The number of lymphocytes and eosinophils were scored with light microscopy [20].

Preparation of lung homogenates

After homogenization in 1% Triton X100 (Sigma-Aldrich)/PBS containing protease inhibitor (Complete Mini, Roche Diagnostics, Germany) lung homogenates were centrifuged (14,000 rpm, 10 min.). The supernatants were stored at -20°C until further use. Protein concentrations were measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific, USA). Lung homogenates were diluted to a final concentration of 1 mg protein/mL [21,22].

Cytokine and chemokine measurement in lung homogenates

A standard Th1/Th2/Th17 assay (IL-2, -4, -6, -10, TNFα, IFNγ; BD Biosciences, The Netherlands) was used to determine cytokine concentrations in lung homogenates according to the manufacturer's instructions. Only the IL-6 concentration was assessed since all other cytokines concentrations were below the detection limit. CCL5, CCL17 and CCL22 were measured with a DuoSet ELISA (R&D Systems, USA), IL-13 with a Ready-SET-Go!® ELISA (eBioscience, USA). The concentrations of these cytokines and chemokines were expressed as pg/mg protein in lungs [13].

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Data were statistically analyzed using a One-Way ANOVA followed by a Bonferroni's multiple comparisons test. $P < 0.05$ were considered significant. Statistical analyses were conducted using GraphPad Prism software (version 6.04). When data was not normally distributed statistics were analyzed after square root transformation (BALF cells).

Results

Dietary intervention with GOS reduces airway hyperresponsiveness in HDM-allergic mice and FF shows a similar trend

Lung resistance was measured, in order to investigate the lung function in HDM-allergic mice, . On baseline level there were no differences between the experimental groups (**Fig. 2**). Airway resistance was dose-dependently increased after methacholine aerosol. In HDM-HDM mice fed the control diet an increase was observed in the lung resistance compared to the HDM-PBS control group. Dietary intervention with GOS decreased lung resistance, FF showed the same tendency whereas the GF and GS diet did not affect HDM-induced AHR in this murine model (**Fig. 2**).

HDM-induced airway inflammation is decreased by dietary intervention with GOS, GF or FF

BALF was examined to study the airway inflammation in HDM-allergic mice after dietary intervention with the different (mixtures of) oligosaccharides. The total number of inflammatory cells was significantly increased in HDM-HDM mice fed the control diet compared to HDM-PBS mice (**Fig. 3A**), which was mainly due to a significant increase in the number of eosinophils and lymphocytes (**Fig. 3B-C**). Dietary intervention with GOS tended to decrease the total number of BALF cells ($P=0.07$) caused by a significant decrease in the number of eosinophils (**Fig. 3A-B**). GF or FF significantly reduced the number of eosinophils, whereas GS did not suppress the number of eosinophils (**Fig. 3B**). Lymphocyte counts showed a similar pattern (**Fig. 3C**).

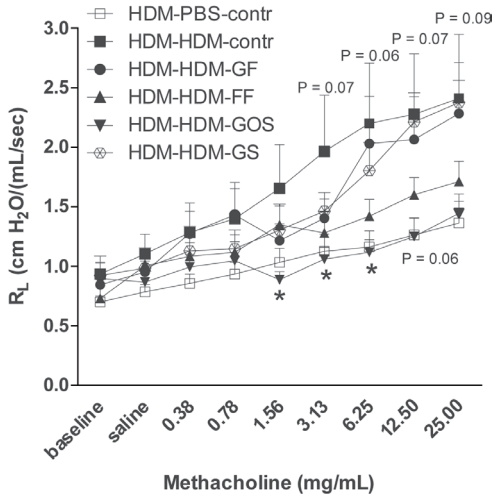

Fig.2 HDM-induced lung resistance decreased after dietary intervention with GOS, FF showed same tendency.

Airway resistance (R_L) was measured in response to increasing doses of methacholine on day 14. HDM-PBS: HDM-sensitized and PBS-challenged mice, HDM-HDM: HDM-sensitized and -challenged mice. Contr: control diet; GOS: 1% GOS diet; GF: galacto-oligosaccharide and long-chain fructo-oligosaccharide diet; FF: short-chain fructo-oligosaccharide and long-chain fructo-oligosaccharide diet; GS: 0.5% galacto-oligosaccharides and 0.5% sialyllactose diet. Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$ compared to the HDM-HDM-contr, n = 7-9 mice/group.

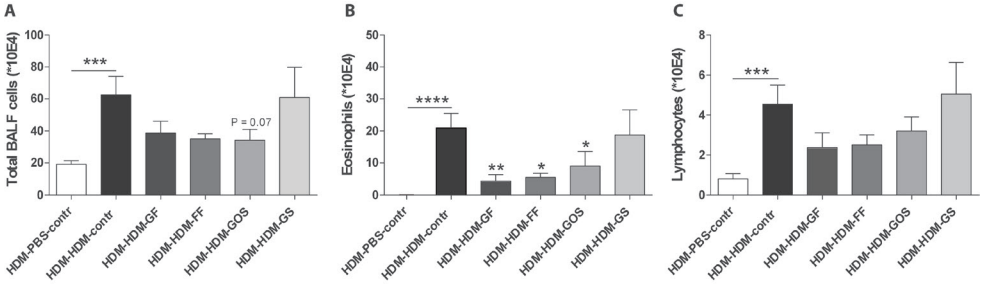

Fig. 3 Dietary intervention with GF and FF tends to reduce HDM-induced eosinophilic airway inflammation.

Inflammatory cell differentiation in the BALF of HDM-allergic mice. HDM-PBS: HDM-sensitized and PBS-challenged mice, HDM-HDM: HDM-sensitized and -challenged mice. Contr: control diet; GOS: 1% GOS diet; GF: galacto-oligosaccharide and long-chain fructo-oligosaccharide diet; FF: short-chain fructo-oligosaccharide and long-chain fructo-oligosaccharide diet; GS: 0.5% galacto-oligosaccharide and 0,5% sialyllactose diet. Total BALF cells (A), absolute number of eosinophils (B) and lymphocytes (C). Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to the HDM-HDM-contr, n = 7-9 mice/group.

Effects of different dietary NDO on Th2 type cytokines and chemokines in lungs of HDM-allergic mice

Although CCL5 concentrations in the supernatants of lung homogenates of HDM-HDM mice fed the control diet were not significantly increased compared to the HDM-PBS mice, dietary intervention with GOS and FF reduced the CCL5 concentrations (**Fig. 4A**). A significant increase was observed in CCL17, CCL22, IL-6 and IL-13 concentrations in lung homogenates of HDM-HDM mice fed the control diet compared to HDM-PBS mice (**Fig. 4B-E**). CCL17 and CCL22 concentrations were not significantly affected after dietary intervention with GOS, GF or FF (**Fig. 4B-C**). IL-6 concentrations tended to decrease after dietary intervention with GF (P = 0.10; **Fig. 4D**) and IL-6 concentrations in supernatants of lung homogenates were positively correlated with the total number of BALF cells (**Fig. 4E**). In addition, IL-13 concentrations were significantly decreased after GOS intervention whereas GF and FF did not affect the HDM-induced increase in IL-13 (**Fig. 4F**). IL-13 was positively correlated with the number of lymphocytes in the BALF (**Fig, 4G**) and lung resistance measurement, respectively (**Fig. 4H**). Since GS did not show any effect on AHR and BALF numbers, chemokines and cytokines were not measured in lung homogenates.

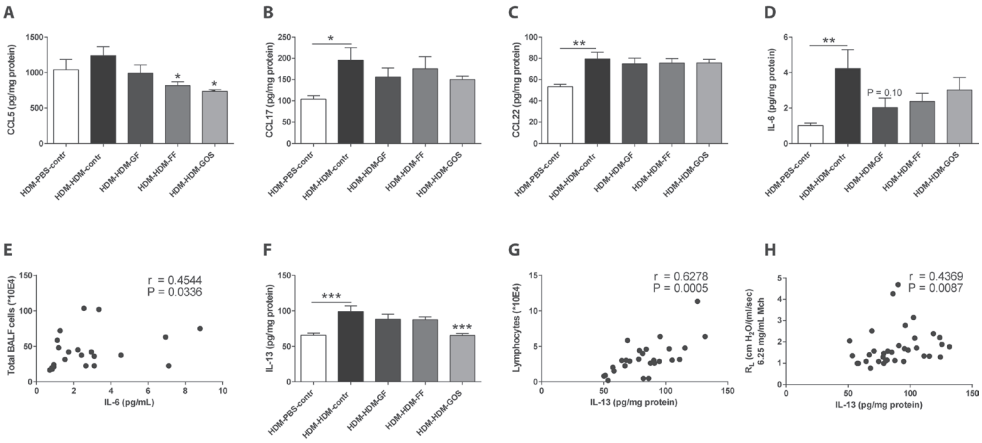

Fig. 4 Effects of different oligosaccharides on chemokines and cytokines in lungs of HDM-allergic mice.

CCL5 (**A**), CCL17 (**B**), CCL22 (**C**), IL-6 (**D**) and IL-13 (**F**) concentrations were measured in supernatant of lung homogenates. Correlation of IL-6 in lung homogenates and the total number of BALF cells (**E**), IL-13 and the number of lymphocytes (**G**) and IL-13 and lung resistance measurement (H). HDM-PBS: HDM-sensitized and PBS-challenged mice, HDM-HDM: HDM-sensitized and -challenged mice. Contr: control diet; GOS: 1% GOS diet; GF: galacto-oligosaccharide and long-chain fructo-oligosaccharide diet; FF: short-chain fructo-oligosaccharide and long-chain fructo-oligosaccharide diet. Results are shown as mean ± SEM. Statistical significance of differences by One-Way ANOVA and post hoc Bonferroni's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to HDM-HDM-contr, n = 6 mice/group. Correlation was analyzed using the Spearman correlation test.

Discussion

In this preliminary study, dietary intervention with galacto-oligosaccharides or different mixtures of NDO were used to compare their effects on AHR, pulmonary inflammation in the BALF and Th2 cell-associated cytokines and chemokines concentrations in the lungs of HDM-induced allergic mice. As human asthma is associated with the common allergen house dust mite, (HDM) in the current study an animal model for HDM-induced asthma was used [23,24]. Upon methacholine exposure, airway responsiveness was increased in HDM-HDM mice fed the control diet compared to HDM-PBS control mice. Moreover, in HDM-HDM allergic mice total inflammatory BALF cell numbers were significantly increased compared to HDM-PBS control mice represented by an enhanced number of eosinophils and lymphocytes. Dietary intervention with GOS significantly decreased AHR development in HDM-allergic mice and FF showed a similar tendency, although not significant. The total inflammatory cell number tended to decrease after dietary intervention with GOS ($P = 0.07$). Although GF did not affect AHR, like FF and GOS, it significantly reduced the eosinophil influx, whereas GS did neither affect AHR nor airway inflammation. In Chapter Four of this thesis, effects of combinations of GF or FF with *Bifidobacterium breve* (*BB*) are shown. When *BB* was combined with GF, the positive effect on total BALF cells and especially on eosinophils and lymphocytes, was abolished. Combining FF with *BB* was very effective in reducing allergic airway inflammation and appeared to further enhance the positive effect of FF [25]. Not much research has been conducted on the effects of sialyllactose. In an influenza infection murine model, intranasal administrations of 3'-sialyllactose worsened the influenza outcomes leading to an increased lung inflammation response, whereas sialic acid reduced disease symptoms [26,27]. However, this administration is different from the current application via the diet. The chemical three dimensional structures and compositions of GOS and GS could be responsible for the different effects induced by these components. Since 1% GOS is composed of a galactose chain connected to a glucose and GS is composed of 0.5% GOS and 0.5% sialyllactose composed of 61% sialyllactose and 22% other carbohydrates which may not have added to the protective mechanism of GOS. The remaining 0.5% GOS might be too low to generate its effect on the inflammatory response in the HDM-induced allergic model.

CCL5 is produced by epithelial cells after allergen stimulation with HDM and capable of attracting immune cells, such as dendritic cells (DC), and it plays a pivotal role in eosinophil recruitment to the lungs [28,29]. From clinical studies it is known that CCL5 is present in the airways and BALF of asthmatic patients [30]. Indeed, CCL5 concentrations were induced in HDM-HDM mice fed the control diet, whereas dietary intervention with GOS or FF, did decline CCL5 concentrations significantly. As CCL5 induces eosinophil recruitment to the lungs, the reduced number of eosinophils in mice fed FF or GOS may be a consequence of the decrease in CCL5 concentrations in the lung homogenates.

However, this was not observed in mice fed the GF diet which also showed a significantly lowered BALF eosinophil number without any decrease in CCL5 concentrations. This may indicate that GF acts via a different mechanism than FF or GOS alone in dampening eosinophil influx.

It is difficult to comprehend the difference between GOS and GF. One can only speculate why the addition of 10% lcFOS changes the beneficial effects of GOS. GOS and GF may influence the microbiota in a different manner, resulting in higher concentrations of short chain fatty acids (SCFA), which can suppress airway inflammation and have anti-inflammatory properties [31]. In HDM-mice fed a high-fiber diet, SCFA were increased and the number of eosinophils and Th2 related cytokines were dampened [32]. More research needs to be done to find the exact mechanism of the mode of action of the different oligosaccharides and their mixtures.

CCL17 and CCL22 can be secreted by DC after allergic sensitization and are increased in sputum and BALF of asthmatic patients [33-35]. The use of specific antibodies against CCL17 in a murine asthma model ameliorated airway eosinophilia and AHR induction was prevented after neutralization of CCL22 [36]. Furthermore, an increase in CCL17 and CCL22 was observed in the HDM-HDM mice fed the control diet compared to HDM-PBS mice, whereas none of the dietary interventions significantly affected CCL17 and CCL22 concentrations in the lungs of HDM-allergic mice.

IL-6 is a cytokine that can be produced by different cell types, like T cells and macrophages and is produced in high amounts by epithelial cells in asthmatic patients [37]. Although the exact role of IL-6 in the asthma pathology is not fully understood, in this current study a significant increase in IL-6 concentration in supernatants of lung homogenates of HDM-HDM mice was observed. Dietary intervention with GF tended to inhibit the development of the HDM-induced IL-6 production. Moreover, IL-6 was positively correlated with the total number of BALF cells.

Another important cytokine, is the Th2 related cytokine IL-13, which can activate macrophages and contributes to the development of AHR [38,39]. In IL-13-deficient mice and mice treated with anti-IL-13, AHR and mucus production were decreased [40,41]. Also human asthmatics have increased levels of IL-13 in sputum and BALF compared to healthy controls [42,43]. In this study, dietary intervention with GOS decreased IL-13 concentrations in the lung. From murine asthma models it is known that IL-13 plays a central role in inducing AHR after HDM-sensitization and -challenge [44]. As IL-13 concentrations are decreased after dietary intervention with GOS, this might be linked to the inhibition of the development of AHR in HDM-allergic mice. Indeed, a positive correlation between IL-13 and AHR in the HDM-allergic groups has been demonstrated in the current study.

In conclusion, dietary intervention with GOS alone was most effective in reducing HDM-induced AHR and pulmonary IL-13 concentrations in mice, these effects were lost when 10% of GOS was replaced by long-chain FOS (GF) or when 50% of GOS was replaced by sialyllactose (GS). However, like GOS, GF as well as FF reduced airway eosinophilia, and GOS as well as these mixtures of oligosaccharides show different effects on different parameters. This may indicate that each oligosaccharide (mixture) acts via a different mode of action or that the different oligosaccharides may influence each other. Hence, interventions with GOS alone and possibly certain mixtures of oligosaccharides may have potential clinical applications for the prevention or reduction of symptoms in asthmatic disease. Hence, more research on the optimal dose of these oligosaccharides is needed to elucidate the beneficial effects in HDM-allergic asthma.

Acknowledgments

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CHAPTER FOUR

The development of allergic inflammation in a murine house dust mite asthma model is suppressed by synbiotic mixtures of non-digestible oligosaccharides and *Bifidobacterium breve* M-16V

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Abstract

Purpose: The incidence and severity of allergic asthma is rising and novel strategies to prevent or treat this disease are needed. This study investigated the effects of different mixtures of non-digestible oligosaccharides (NDO) combined with *Bifidobacterium breve* M-16V (*BB*) on the development of allergic airway inflammation in an animal model for house dust mite (HDM)-induced allergic asthma.

Methods: BALB/c mice were sensitized intranasally (i.n.) with HDM and subsequently challenged (i.n.) with PBS or HDM while being fed diets containing different oligosaccharide mixtures in combination with *BB* or an isocaloric identical control diet. Bronchoalveolar lavage fluid (BALF) inflammatory cell influx, chemokine and cytokine concentrations in lung homogenates and supernatants of *ex vivo* HDM-restimulated lung cells were analyzed.

Results: The HDM-induced influx of eosinophils and lymphocytes was reduced by the diet containing the short-chain and long-chain fructo-oligosaccharides and *BB* (FF*BB*). In addition to the HDM-induced cell influx, concentrations of IL-33, CCL17, CCL22, IL-6, IL-13 and IL-5 were increased in supernatants of lung homogenates or BALF and IL-4, IFNγ and IL-10 were increased in restimulated lung cell suspensions of HDM-allergic mice. The diet containing FF*BB* reduced IL-6, IFNγ, IL-4 and IL-10 concentrations, whereas the combination of galacto-oligosaccharides and long-chain fructo-oligosaccharides with *BB* (GF*BB*) was less potent in this model.

Conclusion: These findings show that synbiotic dietary supplementation can affect respiratory allergic inflammation induced by HDM. The combination of FF*BB* was most effective in the prevention of HDM-induced airway inflammation in mice.

Introduction

Allergic asthma is a chronic disease that affects around 235 million people worldwide. Asthma is not only a health problem for developed countries but also for developing countries and the prevalence is still increasing [1]. The disease is characterized by impaired lung function as well as airway inflammation containing high numbers of eosinophils [2]. House dust mite (HDM) is one of the well-known allergens that can trigger allergic diseases such as asthma [3]. The inflammatory response, mainly the recruitment of eosinophils to the airway tissue and the production of cytokines and chemokines, is initiated by HDM-allergen specific Th2 cells [4]. During sensitization, allergens can directly trigger or stimulate airway epithelial cells, which subsequently release different cytokines (e.g. IL-33) and chemokines (e.g. CCL20) [5]. In response to IL-33, group 2 innate lymphoid cells (ILC2) will proliferate and produce IL-4, -5 and -13 [6]. In addition, IL-33 also induces the maturation of dendritic cells (DC) having Th2 inducing polarizing capacities [7]. These specific DC release Th2 inducing chemokines such as CCL17 and CCL22. The activated DC take up the HDM allergen and induce the development of naïve T cells into antigen-specific Th2 cells in the local lymph nodes. Subsequently, these Th2 cells migrate back to the lung mucosal tissue. After encountering DC presenting the antigen in the airways, these effector Th2 cells will release IL-4, IL-5 and IL-13 [5], which can trigger allergic symptoms and eosinophilic inflammation [8]. Novel preventive and/or therapeutic approaches are needed to prevent and/or treat asthmatic disorders, while current treatment is still not sufficient and has considerable side-effects. Results from animal and human studies suggest that changes in the intestinal microbiota can contribute to the development of asthma. Indeed different animal studies have shown that the gut microbiota affects systemic immune function [9-12]. Since the composition of the microbiota is important for a balanced immune response, adapting the microbiota using oligosaccharides either or not combined with beneficial bacteria may help to protect against the development of allergic disease. For example, treatment with *B. breve* M-16V (*BB*) was found to suppress airway inflammation and treatment with *L. rhamnosus* also reduced lung resistance in a murine ovalbumin-induced asthma model [13]. In addition, to the direct use of probiotics, also specific non-digestible oligosaccharides such as galacto-oligosaccharides (GOS) and short-chain and long-chain fructo-oligosaccharides (scFOS and lcFOS, respectively) can be administered to support growth and/or activity of bifidobacteria and lactobacilli [9, 14, 15]. In previous studies, we have shown that GOS alone are capable of suppressing HDM-induced airway hyperresponsiveness (AHR), airway eosinophilia and Th2 related cytokine and chemokine concentrations in the lung [16]. Furthermore, the combination of GF*BB* or FF*BB* with acidic oligosaccharides reduced allergic responses in food allergic mice and suppressed airway inflammation in a mouse model for ovalbumin-induced asthma, respectively [17, 18]. In mice affected with food allergy GF*BB* was more effective than GF or *BB* alone [17]. Peak expiratory flow was increased and the production of systemic Th2 cytokines (IL-4, -5 and -13) was reduced when GF*BB* was used in patients suffering

from HDM-induced allergy and asthma. However, in this study no effect on bronchial allergic inflammation was demonstrated [19]. GOS are prepared from lactose derived from cow's milk. FOS, obtained from chicory root, provide a vegetable source of prebiotic oligosaccharides and may be used as an alternative for GOS in the synbiotic mixture. The aim of this study was to investigate the effect of two different synbiotic mixtures, GF*BB* and FF*BB*, on pulmonary inflammation, cytokine and chemokine concentrations in lungs and BALF of HDM-induced asthmatic mice.

Materials and methods

Mice

Six- to eight-week-old, specific pathogen-free, male BALB/c mice (Charles River, The Netherlands) were housed under bio-contained sterile conditions using HEPA® filtered isocages® (Tecniplast, Italy). Food and water were provided *ad libitum*. All animal experiments were conducted in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Utrecht University (DEC 2013.II.01.003).

Murine HDM-induced asthma model

While under isoflurane anaesthesia, BALB/c mice were intranasally (i.n.) sensitized with 1 µg HDM/40 µL PBS (Greer Laboratories, USA) on day 0 and challenged once a day for 5 consecutive days (day 7 to 11) with PBS (Phosphate Buffered Saline, control, HDM-PBS) or 10 µg HDM/40 µL PBS (HDM-HDM) [20]. Mice were *ad libitum* fed the control diet (Research Diet Services, The Netherlands) (AIN93G, contr), a diet containing a 1% w/w 9:1 mixture of galacto-oligosaccharides (Vivinal® GOS syrup with approximately 59% galacto-oligosaccharides, 21% lactose, 19% glucose, and 1% galactose on dry matter (dry matter of 75%); FrieslandCampina Domo, The Netherlands) and long-chain fructo-oligosaccharides (lcFOS, Orafti ® HP with approximately ~100% inulin, DP ≥23; Beneo, Belgium) (GF) or a 1% w/w 1:1 mixture of short-chain fructo-oligosaccharides (Orafti ® P95, with approximately 95% oligofructose content, DP 2-8; Beneo, Belgium) and long-chain fructo-oligosaccharides (FF). Carbohydrates in Vivinal® GOS were compensated isocalorically in the control diet by means of exchange against cellulose (for GOS), lactose (for lactose), and dextrose (for glucose). In case of the fructo-oligosaccharides, carbohydrates were compensated isocalorically in the control diet by means of exchange against cellulose (for FOS). Both GF and FF diets contained 2x10E9 colony forming units/g (2% w/w) *Bifidobacterium breve* M-16V (*BB*) (Morinaga milk Industry, Japan). The GF*BB* and FF*BB* diets were given two weeks prior to sensitization and were continuously provided throughout the entire experimental period. Mice were sacrificed on day 14 (**Fig. 1A**).

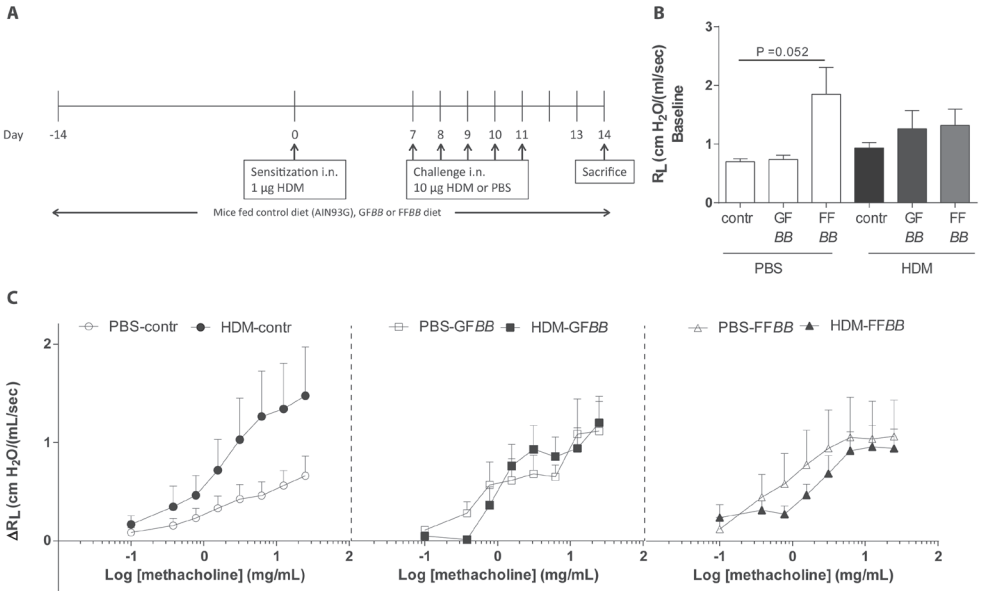

Fig. 1 Treatment protocol of the allergic house dust mite asthma model and lung resistance measurement. Male BALB/c mice were sensitized intranasally (i.n.) with house dust mite (HDM) on day 0 and were challenged i.n. for 5 consecutive days with HDM or PBS. Mice were fed the control diet (AIN93G, contr), a diet containing a 1% w/w 9:1 mixture of galacto-oligosaccharide and long-chain fructo-oligosaccharide (GF) or 1% w/w 1:1 mixture of short-chain fructo-oligosaccharide and long-chain fructo-oligosaccharide (FF) both in combination with 2x10E9 colony forming units/g *Bifidobacterium breve* M-16V (*BB*) (2% w/w). The GF*BB* and FF*BB* interventions started two weeks prior to sensitization and continued during the entire experiment. All mice were sacrificed on day 14 (**A**). Basal airway resistance (R_L) (**B**) and ΔR_L after baseline correction in response to increasing doses of methacholine was measured on day 14 (**C**). PBS: HDM- sensitized and PBS-challenged mice (white bars), HDM: HDM-sensitized and -challenged mice (grey bars). Contr: control diet, GF*BB*: mixture of galacto-oligosaccharide and long-chain fructo-oligosaccharide with *Bifidobacterium breve* M-16V diet, FF*BB*: mixture of short-chain fructo-oligosaccharide and long-chain fructo-oligosaccharide with *Bifidobacterium breve* M-16V diet.

Airway responsiveness measurement

Mice were anaesthetized with a mixture of Ketamine (Vetoquinol S.A., France; 125 mg/kg) and Medetomidine (Pfizer, The Netherlands; 0.4 mg/kg), intraperitoneally (i.p.). EMKA invasive measurement of dynamic resistance (EMKA Technologies, France) in response to increasing doses of methacholine (acetyl-β-methyl-choline chloride, Sigma-Aldrich, The Netherlands) (0-25 mg/mL, 10% puff for 10 sec.) was used to determine the lung function on day 14 [13]. Basal airway resistance values of each individual mouse as measured prior to metacholine exposure were deducted from the resistance as measured upon metacholine exposure (ΔR) (**Fig 1B-C**).

Bronchoalveolar lavage

Mice were sacrificed on day 14 using an i.p. overdose of pentobarbital (600 mg/kg, Nembutal™, Ceva Santé Animale, The Netherlands). Lungs were lavaged with 1 mL of pyrogen-free saline (0.9% NaCl, 37°C) supplemented with protease inhibitor cocktail tablet (Complete Mini, Roche Diagnostics, Germany). The supernatant of the first mL was used for cytokine and chemokine analyses, followed by three lavages with 1 mL saline solution (0.9% NaCl, 37°C). The BALF cells were centrifuged (400 g, 5 min.) and pellets of the four lavages were pooled; total numbers of BALF cells were counted using a Bürker-Türk chamber (magnification 100x). Cytospin were stained with Diff-Quick (Merz & Dade A.G., Switzerland) for differential BALF cell counts. Numbers of macrophages, lymphocytes, neutrophils and eosinophils were scored using light microscopy [21].

Preparation of lung homogenates

Lung samples were homogenized in 1% Triton X-100 (Sigma-Aldrich)/PBS containing protease inhibitor (Complete Mini) using a Precellys 24 tissue homogenizer (Bertin Technologies, France). Homogenates were centrifuged at 14,000 rpm for 5 min. and supernatants were collected. The protein concentration of each sample was assayed using the Pierce BCA protein assay kit standardized to BSA according to the manufacturer's protocol (Thermo Fisher Scientific, USA). The homogenates were diluted to a final concentration of 1 mg protein/mL [22, 23].

***Ex vivo* lung restimulation with house dust mite**

Lung cell suspensions were prepared by cutting the lung into small pieces and by adding digestion buffer, containing DNase I and Collagenase A (Roche Diagnostics, Germany), for 30 min. The digestion was stopped using fetal calf serum (FCS, Hyclone Laboratories, USA). The lung pieces were transferred towards a 70 µm nylon cell strainer (BD Biosciences, The Netherlands) and rinsed with 10 mL RPMI. Cells were washed and resuspended in RPMI-1640 culture medium (Lonza, USA) supplemented with 10% heat-inactivated FCS and 0.1% penicillin-streptomycin solution (Sigma-Aldrich). Total number of cells was calculated using a Beckman Z1 coulter ® Particle Counter (Beckman, USA). Lung cells (4 X 10^5 cells/well) were cultured in a Greiner bio-one CellSTAR 96-well U-bottom plate (Greiner Bio-One B.V., The Netherlands) in medium with or without 50 µg/mL HDM. The supernatant was harvested after 4 days of culture at 37°C in 5% CO_2 and stored at -20° [13].

Measurement of cytokines

A standard Th1/Th2/Th17 assay (BD Biosciences, The Netherlands) was used to determine cytokine concentrations in lung homogenates and supernatants of lung restimulation according to the manufacturer's instructions. IL-33, CCL17, CCL20 and CCL22 were measured with a DuoSet ELISA (R&D Systems), IL-13 and IL-5 with a Ready-SET-Go!® ELISA (eBioscience, USA). The concentrations of these cytokines were expressed as pg/mg protein in lung homogenates and pg/mL in BALF and restimulation supernatants.

Statistical analysis

Results are presented as mean ± standard error of mean (SEM). Data were statistically analyzed by One-Way ANOVA and post hoc Bonferroni's multiple comparisons test. $P < 0.05$ were considered significant. Statistical analyses were conducted using GraphPad Prism software (version 6.04).

Results

Dietary intervention with FF*BB* reduces pulmonary eosinophilic inflammation in HDM-allergic mice

Airway hyperresponsiveness (AHR) to metacholine aerosols was measured, at baseline no differences were observed between the PBS- and HDM-challenged mice fed the different diets (**Fig. 1B**). In the PBS-challenged mice fed the FF*BB* diet the baseline airway resistance tended to increase compared to PBS mice fed the control diet (**Fig. 1B**), however metacholine exposure did not significantly enhance hyperreactivity in these mice as compared to control diet fed mice (**Fig. 1C**). HDM-challenged mice showed a higher AHR response upon metacholine exposure than PBS-challenged mice but this did not reach significance (**Fig. 1C**). HDM-challenge in GF*BB* or FF*BB* fed mice did not increase the AHR response compared to PBS-challenged mice fed the similar diet (**Fig. 1C**). Furthermore the AHR of HDM-challenged mice fed GF*BB* or FF*BB* remained below the AHR of HDM-challenged mice fed the control diet albeit this did not reach significance (**Fig. 1C**).

BALF was examined to study the inflammatory cell influx into the airways of HDM-allergic mice upon dietary intervention with control diet or the synbiotic diets (**Fig. 2A**). The total number of inflammatory cells in the BALF of HDM-HDM mice fed the control diet, was significantly increased when compared to HDM-PBS mice (**Fig. 2A**). The HDM-challenge induced an increase in the number of eosinophils and lymphocytes. The same tendency was shown for macrophages (**Fig. 2B-D**) compared to the PBS-challenged group. Dietary intervention with FF*BB* reduced the total number of inflammatory cells in HDM-allergic mice compared to the HDM-HDM group fed the control diet (**Fig. 2A**), this reduction was mainly due to a decrease in the number of eosinophils and lymphocytes (**Fig. 2B-C**). Dietary intervention with GF*BB* did not suppress the total number of BALF cells (**Fig. 2A**) or the differentiated cell numbers (**Fig. 2B-D**). In this group the number of neutrophils was significantly increased compared to the HDM-PBS control group fed the GF*BB* diet (**Fig. 2E**).

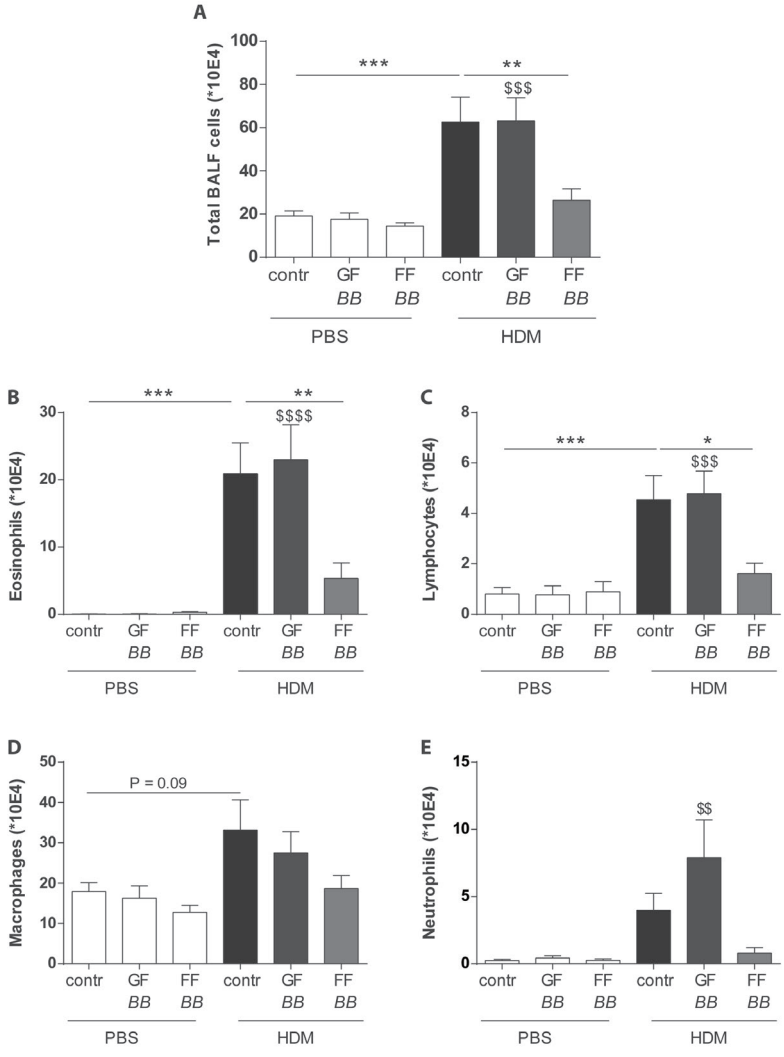

Fig. 2 Dietary intervention with FF*BB* reduced pulmonary eosinophilic inflammation in the lungs of HDM-allergic mice.

Differentiation of inflammatory cell infiltration in the BALF of HDM-allergic mice. PBS: HDM- sensitized and PBS- challenged mice (white bars), HDM: HDM-sensitized and -challenged mice (grey bars). Contr: control diet, GF*BB*: mixture of galacto-oligosaccharide and long-chain fructo-oligosaccharide with *Bifidobacterium breve* M-16V diet, FF*BB*: mixture of short-chain fructo-oligosaccharide and long-chain fructo-oligosaccharide with *Bifidobacterium breve* M-16V diet. Total BALF cells (**A**), absolute number of eosinophils (**B**), lymphocytes (**C**), macrophages (**D**) and neutrophils (**E**). Results are shown as mean ± SEM. Statistical significance of differences by One-Way ANOVA and post hoc Bonferroni's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to HDM-HDM, $^{\$\$}$ $P < 0.01$, $^{\$\$\$}$ $P < 0.001$, $^{\$\$\$\$}$ $P < 0.0001$ compared to HDM-PBS group of corresponding diet, n = 6-9 mice/group.

Effect of synbiotics on inflammatory and Th2 type cytokines and chemokines in lungs of HDM-allergic mice

IL-33, CCL20, CCL17 and CCL22 were significantly increased in supernatants of lung homogenates of HDM-HDM mice fed the control diet compared to the HDM-PBS control group. Dietary interventions with the different synbiotics did not affect these cytokines and chemokines (**Fig. 3A-D**). IL-6 and IL-13 concentrations in the supernatant of the lung homogenates and IL-5 in BALF of HDM-HDM mice fed the control diet showed a significant increase compared to the HDM-PBS control group (**Fig. 4A, C, D**). Dietary intervention with FF*BB* significantly decreased the HDM-allergy-induced increase in IL-6 (**Fig. 4A**). IL-6 concentrations in lung tissue correlated positively with the total number of BALF cells (**Fig. 4B**, r = 0.5011, P = 0.0288). Dietary interventions with the different synbiotics did not affect this HDM-induced increase in pulmonary IL-13 (**Fig. 4C**), but tended to decrease the concentrations of IL-5 in the BALF (**Fig. 4D**). IL-5 concentrations in the BALF were positively correlated with the number of lymphocytes (**Fig. 4E**, r = 0.8257, P = 0.0003) and the number of eosinophils in the BALF, respectively (**Fig. 4F**, r = 0.7292, P = 0.0028).

Synbiotic diets suppress T cell activity in HDM-allergic mice

To investigate the effects of different synbiotics on allergen-specific cytokine secretion of lung tissue, cell suspensions were *ex vivo* restimulated with HDM. The concentrations of IL-10, IFNγ and IL-4 were significantly increased upon HDM-stimulation of lung cells of the HDM-HDM mice fed the control diet, whereas HDM-PBS mice showed no increase compared to medium-stimulation (**Fig. 5A-C**). Dietary intervention with GF*BB* and FF*BB* significantly decreased the concentrations of IL-10, while FF*BB* also decreased IL-4 and IFNγ (**Fig. 5A-C**). Although upon HDM-stimulation IL-17A concentrations were increased in the lung cells of HDM-HDM mice fed the control diet, both dietary interventions showed no effect (data not shown).

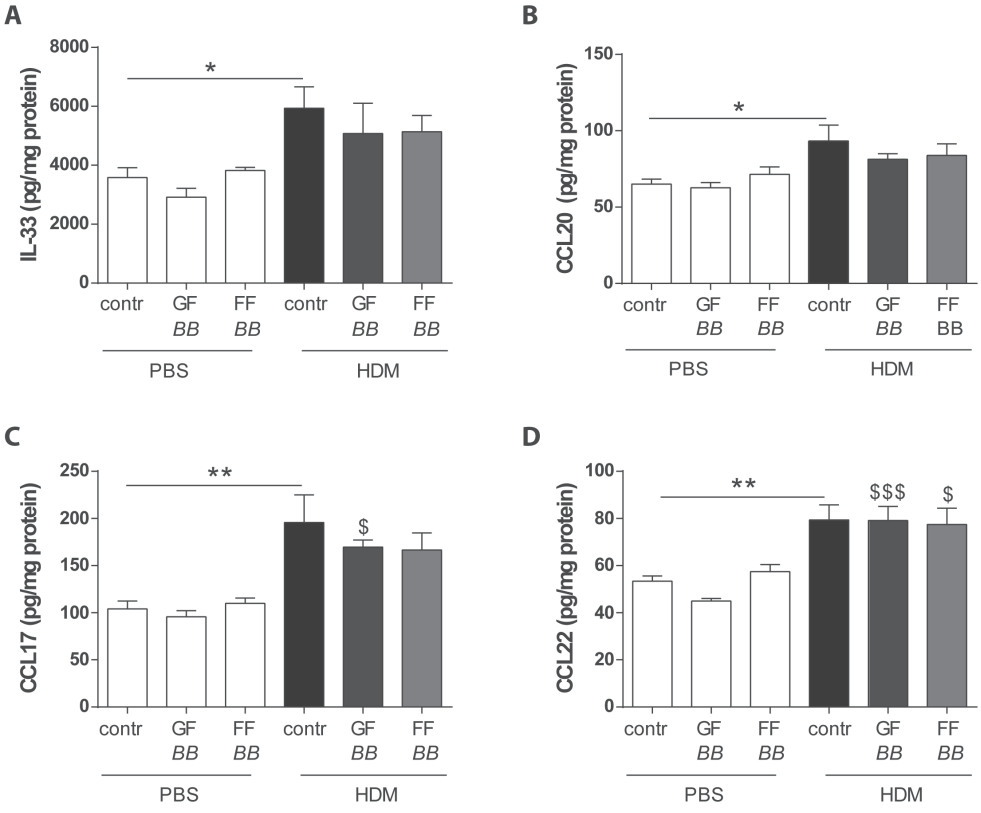

Fig. 3 IL-33, CCL20, CCL17 and CCL22 in lungs of HDM-allergic mice.

IL-33 (**A**), CCL20 (**B**), CCL17 (**C**) and CCL22 (**D**) concentrations were measured in supernatant of lung homogenates. PBS: HDM-sensitized and PBS-challenged mice (white bars), HDM: HDM-sensitized and -challenged mice (grey bars). Contr: control diet, GF*BB*: mixture of galacto-oligosaccharide and long-chain fructo-oligosaccharide with *Bifidobacterium breve* M-16V diet, FF*BB*: mixture of short-chain fructo-oligosaccharide and long-chain fructo-oligosaccharide with *Bifidobacterium breve* M-16V diet. Results are shown as mean ± SEM. Statistical significance of differences by One-Way ANOVA and post hoc Bonferroni's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$ compared to HDM-HDM, $^{\$}$ $P < 0.05$, $^{\$\$\$}$ $P < 0.001$ compared to corresponding PBS group of diet, n = 5 mice/group.

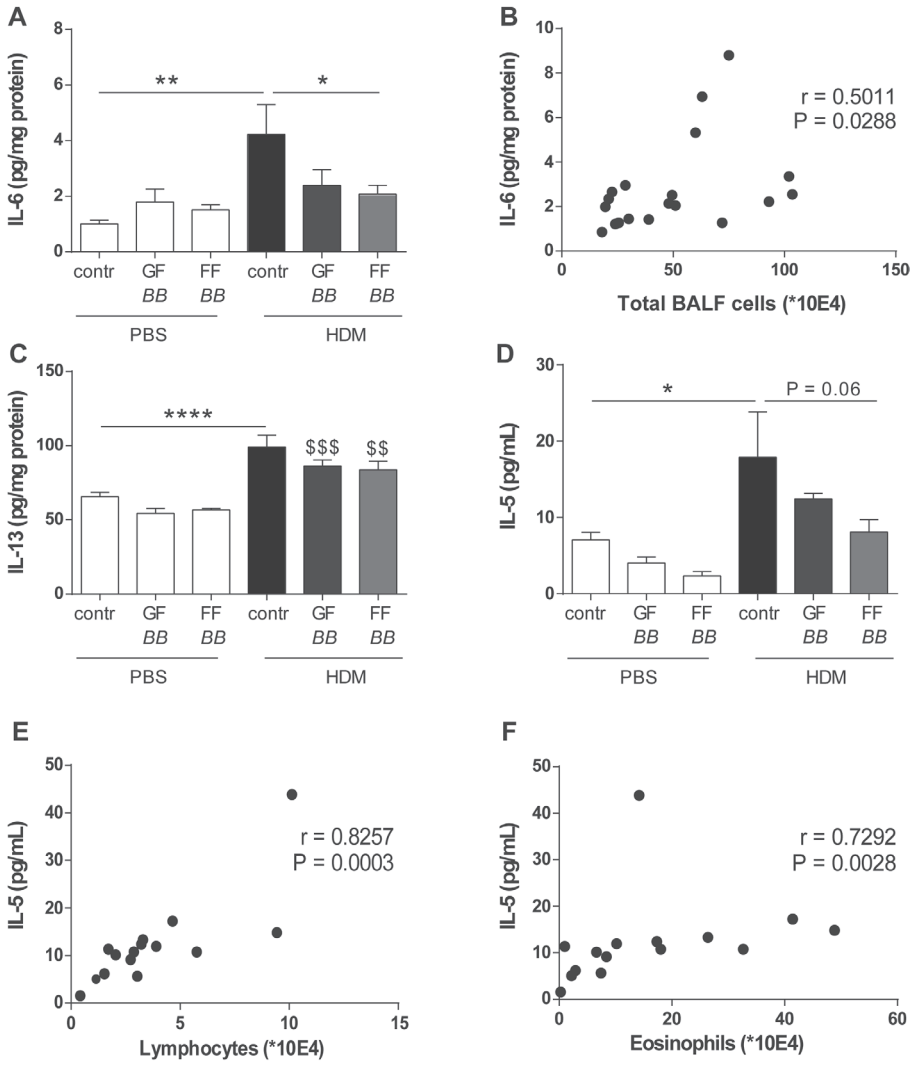

Fig. 4 IL-6, IL-13 and IL-5 in lungs of HDM-allergic mice.

IL-6 (**A**), IL-13 (**C**) concentrations were measured in supernatant of lung homogenates. IL-5 (**D**) was measured in the BALF. Correlation of IL-6 in lung homogenates and the total number of BALF cells (**B**), IL-5 in the BALF and the number of lymphocytes (**E**) and IL-5 in the BALF and the number of eosinophils (**F**). PBS: HDM-sensitized and PBS-challenged mice (white bars), HDM: HDM-sensitized and -challenged mice (grey bars). Contr: control diet, GF*BB*: mixture of galacto-oligosaccharide and long-chain fructo-oligosaccharide with *Bifidobacterium breve* M-16V diet, FF*BB*: mixture of short-chain fructo-oligosaccharide and long-chain fructo-oligosaccharide with *Bifidobacterium breve* M-16V diet. Results are shown as mean ± SEM. Statistical significance of differences by One-Way ANOVA and post hoc Bonferroni's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$ compared to HDM-HDM, $^{\$\$}$ $P<0.01$, $^{\$\$\$}$ $P < 0.001$ compared to corresponding PBS group of diet, n = 5-9 mice/group. Correlation was analyzed using the Spearman correlation test.

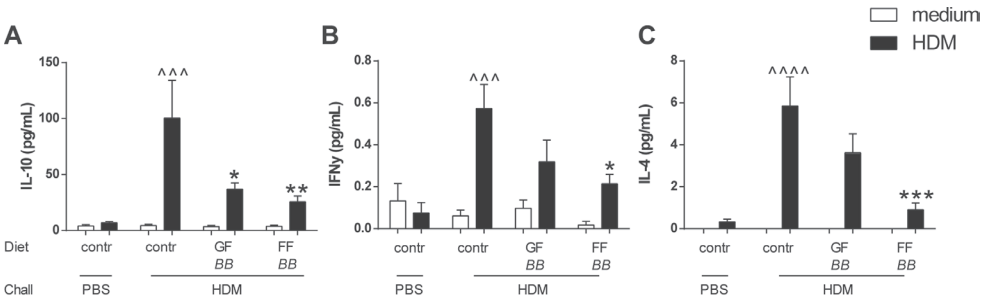

Fig. 5 Synbiotic diets suppress antigen-specific T cell activation in HDM-allergic mice.

Lung cell suspensions were *ex vivo* restimulated with medium (white bars) or HDM (grey bars) for 4 days (37°C, 5% CO_2). IL-10 (**A**), IFNγ (**B**) and IL-4 (**C**) concentrations were measured in the supernatants. PBS: HDM- sensitized and PBS-challenged mice, HDM: HDM-sensitized and -challenged mice. Contr: control diet, GF*BB*: mixture of galacto-oligosaccharide and long-chain fructo-oligosaccharide with *Bifidobacterium breve* M-16V diet, FF*BB*: mixture of short-chain fructo-oligosaccharide and long-chain fructo-oligosaccharide with *Bifidobacterium breve* M-16V diet. Results are shown as mean ± SEM. Statistical significance of differences by One-Way ANOVA and post hoc Bonferroni's multiple comparisons test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared to HDM-HDM, ^^^ $P < 0.001$, ^^^^$P<0.0001$ compared to HDM-PBS, n = 6-7 mice/group.

Discussion

This is the first study in which anti-inflammatory effects of different oligosaccharide mixtures (GF or FF) in combination with *Bifidobacterium breve* M-16V (*BB*) (synbiotics) were investigated on the development of HDM-induced allergic inflammation in a murine model appropriate to mimic human allergic asthma. murine models for HDM-allergic asthma are commonly used to mimic the human features of asthma, since HDM is one of the most common allergens associated with human allergic asthma [24, 25]. In this study, the effects of the synbiotic diets on AHR remain unclear, whereas strong protective effects on airway inflammation were observed in HDM-allergic mice. Total inflammatory BALF cell numbers were significantly increased in HDM-HDM allergic mice when compared to HDM-PBS control mice. In contrast to GF*BB*, dietary intervention with FF*BB* significantly decreased the total inflammatory cell number. This decrease was mainly due to reduction in the number of eosinophils and lymphocytes compared to HDM-HDM mice fed the control diet. Although FF*BB* was not able to reduce epithelial and DC related cytokines (IL-33) and chemokines (CCL20, CCL17 and CCL22) known to contribute to allergic sensitization, FF*BB* decreased the pro-inflammatory cytokine IL-6. IL-6 was found to correlate with the number of total inflammatory cells in the BALF. Furthermore, FF*BB* reduced Th2 related cytokines (IL-4 and IL-5) and the Th1 related cytokine IFNγ concentrations in lung homogenates or in supernatants of *ex vivo* restimulated lung cell suspensions. GF*BB* was less effective, but showed a similar pattern with regard to these parameters, and both FF*BB* and the GF*BB* diet reduced IL-10 concentrations after *ex vivo* restimulation of lung cells with HDM.

The data show that in particular the FF*BB* diet was capable of suppressing airway inflammation, whereas it did not suppress Th2 driving mediators such as IL-33, CCL17 and CCL22. Epithelial cells are known to secrete IL-33 upon HDM-stimulation, which activates DC [26] and is a chemoattractant for Th2 cells [27, 28]. IL-33 has also been shown to be increased in bronchial biopsies of asthmatic patients compared to non-asthmatic patients [29]. In the present study, a significant increase in IL-33 concentrations was observed in lung homogenates obtained from HDM-allergic mice, no evidence was found that the dietary interventions modulated this increase.

Another relevant chemokine that is produced by the epithelium is CCL20 which is responsible for the attraction of immature DC to the lung [26, 30]. From human studies, it is known that CCL20 levels are increased in asthmatic patients compared to healthy controls, which is even more pronounced after allergen challenge [31]. In the current study, a significant increase in CCL20 in lung homogenates of the HDM-HDM mice compared to the HDM-PBS mice fed the control diet was found which remained unaffected by the synbiotic diets.

In patients suffering from atopic asthma it was shown that after a challenge with HDM the concentrations of CCL17 were also increased in the BALF [32]. CCR4 and its ligand CCL17 are up-regulated in the airways of asthmatic patients after challenge and contribute to the Th2 cell recruitment in asthma [32, 33]. However, CCR4 is not only a ligand for CCL17, it also binds CCL22 with an even higher affinity [34]. CCR4 is expressed by regulatory T cells, mast cells and Th2 cells and is known to play a pivotal role in allergic diseases. In bronchial biopsies of asthmatic patients most of the T lymphocytes were CCR4 positive [35]. In the current study, a significant increase in CCL17 and CCL22 was found in lung tissue of the HDM-HDM mice compared to the HDM-PBS mice fed the control diet. However, the synbiotic diets did not affect these pulmonary concentrations of CCL17 and CCL22. Overall, these results indicate that although the FF*BB* diet effectively reduced lung eosinophilia and lymphocyte influx, the diet did not suppress the release of Th2 polarizing mediators by epithelial cells and/or DC.

IL-6 is not only produced by inflammatory cells like DC and macrophages, but also by lung epithelial cells after stimulation with an allergen. The murine model for HDM-induced allergic asthma showed elevated pulmonary IL-6 concentrations which is in line with human studies that showed elevated IL-6 levels in BALF, sputum and serum from asthmatic patients [36, 37]. Dietary intervention with FF*BB* significantly decreased the concentration of IL-6 in lung homogenates, which correlated with the observed decrease in the influx of inflammatory cells. Hence, the decrease in IL-6 by the FF*BB* diet may relate to the protective effect of this diet on lung inflammatory responses as seen in this animal model.

Besides the pro-inflammatory IL-6, IL-13 and IL-5 were also investigated. These allergy-driven cytokines are secreted by antigen-specific Th2 cells and mast cells, as well as by ILC2. IL-13 is also capable of triggering eosinophils and macrophages [38] and IL-5 is a Th2 cytokine which is essential for the maturation and activation of eosinophils [39]. The concentration of IL-13 in lung tissue and IL-5 in the BALF were significantly increased in HDM-allergic mice compared to control mice. This effect corresponds to human studies which showed that IL-13 levels were increased in sputum of asthmatic patients [38, 40, 41]. Furthermore, IL-5 mRNA levels are increased in bronchial biopsies of asthmatic patients compared to healthy controls [42]. Since IL-5 is essential for eosinophil maturation and eosinophils are important in asthma, animal and human studies showed that eosinophilia in blood and BALF can be reduced with monoclonal antibodies against IL-5 [39, 43]. Although FF*BB* did not reduce the concentration of IL-13 in lung homogenates, it showed a strong tendency to decrease the concentration of IL-5. Moreover, IL-5 in BALF was positively correlated with eosinophil and lymphocyte numbers. The reduction of inflammatory cell influx by FF*BB* may be a consequence of mechanisms involving the suppression of challenge-induced IL-6 and IL-5 secretion by lung inflammatory cells.

The FF*BB* diet suppressed lymphocyte influx and indeed *ex vivo* antigen-specific restimulation of lung cell suspensions showed reduced Th1 (IFNγ) and Th2 (IL-4) type cytokine release by lung cells of HDM-challenged mice fed the FF*BB* diet. IFNγ is a Th1 cytokine which is found to be increased in BALF and peripheral blood of asthmatic patients. In mouse models, IFNγ is increased at mRNA levels in the airway epithelium [44]. IL-4 is one of the key players in the development of allergic asthma. A high IL-4 milieu is necessary for the differentiation of Th2 cells that in turn can also produce IL-4 upon antigen binding [26]. In patients with asthma increased IL-4 protein levels were found in BALF and serum [45]. When patients with mild asthma were nebulized with IL-4, an elevation of eosinophil number in the sputum was observed. In addition, it was shown that after nebulizing anti-IL-4 antibodies, eosinophilic inflammation was reduced in mice [46]. Since both allergen-induced IFNγ and IL-4 were decreased after restimulation *ex vivo* with HDM in the FF*BB* group, these results support the hypothesis that the influx of inflammatory cells into the lung tissue upon the previous *in vivo* allergen challenge was reduced by the diet.

The FF*BB* diet may be capable of reducing the allergen-specific effector response, which can include regulatory cytokines like IL-10. Upon *ex vivo* HDM-stimulation, IL-10 was also decreased in FF*BB* fed mice. Besides FF*BB* also GF*BB* reduced IL-10, whereas IFNγ and IL-4 were not significantly lowered by GF*BB*. This may imply that GF*BB* acts via a different mechanism of action in particular targeting HDM-induced increase in IL-10 or that FF*BB* is more effective in dampening the inflammatory cascade.

Hence, the current study shows a protective effect of dietary FF*BB* in HDM-induced airway inflammation in mice when provided before and during allergic sensitization and challenge. FF*BB* did not affect IL-33 and CCL20 induction which are known to initiate the development and activation of CCL17 and CCL22 producing Th2 polarizing DC contributing to allergic sensitization. However, FF*BB* may have reduced the influx of eosinophils and allergen specific T cells by suppressing local secretion of IL-6 and IL-5 by inflammatory cells via currently unknown mechanisms (**Fig. 6**).

Despite the strong effect of the FF*BB* diet on airway inflammation, it remains to be assessed whether FF*BB* may improve the clinical outcome of asthma since the effects of FF*BB* on the AHR remained unclear in the current study. Future studies are needed to further optimize the effectivity of FF*BB* in prevention and/or treatment of HDM-induced asthma. This may include an earlier introduction of the diet prior to sensitization and dose optimization. Furthermore, combining FF with other bifidobacteria or lactobacillus strains may be considered. Indeed, in other mice studies positive effects of *Lactobacillus rhamnosus* have been observed on AHR and lung inflammation [13, 47]. A combination of FF with this or other bacteria might have beneficial effects on allergic asthmatic features. The mode of action of FF*BB* in suppressing lymphocyte and eosinophil influx hereby dampening the allergic asthma pathway is currently unclear. We hypothesize that compared to GF*BB*, FF*BB* changes the microbiota in a different manner and via these changes differentially affects the mucosal and systemic immune response. One of the ways by which this may occur is via altering the levels or patterns of short-chain fatty acids (SCFA) produced upon bacterial fermentation of the oligosaccharides in the intestine. These SCFA become available in the blood stream and have anti-inflammatory properties by acting via the GPR41 (FFA2) and GPR43 (FFA3) receptors and are known to suppress airway inflammation [48]. Indeed, SCFA have been shown to dampen asthmatic responses in HDM-allergic mice fed a high-fiber diet by lowering the number of eosinophils and lymphocytes and the concentration of Th2 related cytokines [49]. However, whether this also underlies the mechanism of FF*BB* needs to be further elucidated. The promising results of our study suggest the potential clinical application of this intervention and in future studies the use of FF*BB* as an adjunct therapy to budesonide treatment, a corticosteroid used to treat asthma in humans, may be considered as well.

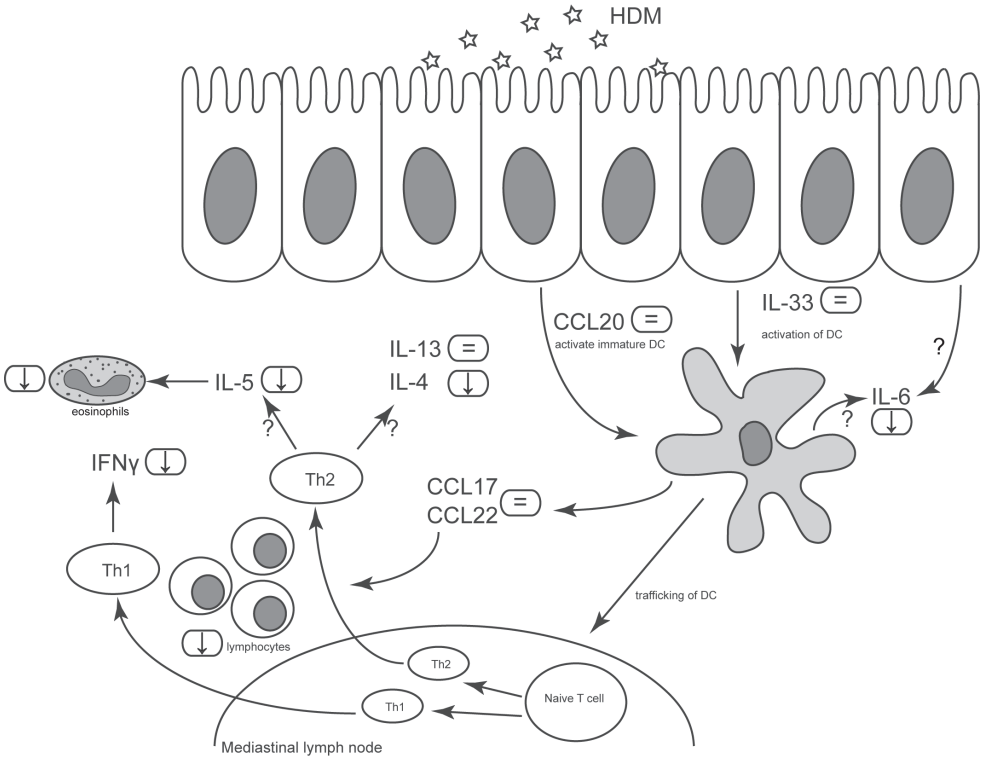

Fig. 6 Overview of the effects of the synbiotic diet FF*BB*.

After the initial exposure to HDM, CCL20 is secreted by the airway epithelium, which will activate immature dendritic cells (DC). IL-33, also secreted by the epithelium, activates the DC. Both CCL20 and IL-33 concentrations in lung homogenate supernatants were not affected by the FF*BB* diet. Activated DC secrete CCL17 and CCL22, which are chemoattractants for Th2 cells, and will traffic to the mediastinal lymph nodes to differentiate naïve T cells into Th2 cells. The FF*BB* diet did not affect CCL17 and CCL22 release by these DC. In contrast, IL-6, which can be secreted by lung epithelial cells or inflammatory cells such as DC, was suppressed which correlated with the reduced inflammatory cell influx. Mainly lymphocyte and eosinophil influx was lowered by the FF*BB* diet. Amongst other cells, Th2 cells secrete IL-13, IL-4 and IL-5 and Th1 cells secrete IFNγ. Although IL-13 remained high, the FF*BB* diet reduced IL-4 and IFNγ. Furthermore, FF*BB* tended to suppress IL-5 concentrations which correlated with the reduced number of lymphocytes and eosinophils upon dietary intervention.

In conclusion, dietary intervention with a synbiotic supplementation can suppress pulmonary inflammation in HDM-asthmatic mice, although the FF*BB* showed a more beneficial effect on the management of HDM-induced allergic asthma compared to GF*BB*. The mode of action of FF*BB* in suppressing airway eosinophilia and lymphocyte numbers hereby dampening the allergic asthma pathway needs to be further elucidated, but the promising results of our study suggest the potential clinical application of this intervention.

Acknowledgments

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CHAPTER FIVE

Dietary galacto-oligosaccharides prevent airway eosinophilia and hyperresponsiveness in a murine house dust mite-induced asthma model

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Abstract

Background: Allergic asthma is strongly associated with the exposure to house dust mite (HDM) and is characterized by eosinophilic pulmonary inflammation and airway hyperresponsiveness (AHR). Recently, there is an increased interest in using dietary oligosaccharides, also known as prebiotics, as a novel strategy to prevent the development of, or reduce, symptoms of allergy.
Aim: We investigated the preventive capacity of dietary galacto-oligosaccharides (GOS) compared to an intra-airway therapeutic treatment with budesonide on the development of HDM-induced allergic asthma in mice.
Methods: BALB/c mice were intranasally sensitized with 1 µg HDM on day 0 followed by daily intranasal challenge with PBS or 10 µg HDM on days 7 to 11. Two weeks prior to the first sensitization and throughout the experiment mice were fed a control diet or a diet containing 1% GOS. Reference mice were oropharyngeally instilled with budesonide (500 µg/kg) on days 7, 9, 11, and 13, while being fed the control diet. On day 14, AHR was measured by nebulizing increasing doses of methacholine into the airways. At the end of the experiment, bronchoalveolar lavage fluid (BALF) and lungs were collected.
Results: Sensitization and challenge with HDM resulted in AHR. In contrast to budesonide, dietary intervention with 1% GOS prevented the development of AHR. HDM-sensitization and -challenge resulted in a significant increase in BALF leukocytes numbers, which was suppressed by budesonide treatment and dietary intervention with 1% GOS. Moreover, HDM-sensitization and -challenge resulted in significantly enhanced concentrations of IL-6, CCL17, IL-33, CCL5 and IL-13 in lung tissue. Both dietary intervention with 1% GOS or budesonide treatment significantly decreased the HDM-induced increased concentrations of CCL5 and IL-13 in lung tissue, while budesonide also reduced the HDM-enhanced concentrations of IL-6 and CCL17 in lung tissue.
Conclusion: Not only did dietary intervention with 1% GOS during sensitization and challenge prevent the induction of airway eosinophilia and Th2 related cytokines and chemokines in the lung equally effective as budesonide treatment, it also prevented AHR development in HDM-allergic mice. GOS might be useful for the prevention and/or treatment of symptoms in asthmatic disease.

Introduction

Asthma is a complex disease from which the exact underlying immunological processes are still not fully understood [1]. According to the World Health Organization, 235 million people suffer from asthma worldwide and it is a common disease among children [2]. In asthma, the Th2 immune response leads to eosinophilic inflammation in the airways, mucus hypersecretion and airway hyperresponsiveness (AHR) [3]. During sensitization, an antigen activates the airway epithelial cells that in turn, via the release of several chemokines (e.g. CCL5) and cytokines (e.g. IL-33), results in the activation of innate immune cells such as dendritic cells (DC). Innate immune cells release Th2 polarizing cytokines, such as CCL17 and CCL22. {{158 Hammad,H. 2008}}In particular, high concentrations of IL-33 are expressed in epithelial cells of asthmatic patients [4,5]. Activated DC take up the allergenic protein and present their peptides to naïve T cells in draining lymph nodes. The naïve T cells develop into antigen-specific Th2 cells [6]. Upon subsequent antigen challenges, antigen-specific Th2 cells are activated to release IL-4, IL-5 and IL-13, resulting in the development of allergen-specific IgE producing plasma cells and generation and infiltration of eosinophils [7-11]. Allergen-specific IgE binds to mast cells in the airways and a second exposure to antigen results in the degranulation of mast cells. The influx of inflammatory cells (eosinophils, Th2 cells and mast cells) and production of mediators (TNFα, IL-4, IL-5, IL-6, IL-13, IL-33) ultimately leads to acute bronchoconstriction, increased mucus production and AHR [11-13]. Currently, the treatment of asthma focuses on symptom relief only, using long-acting beta-agonists with or without glucocorticosteroids, which is considered highly effective and safe. However, in many patients, the disease remains poorly controlled [14]. Long-term treatment with glucocorticosteroids can also have considerable side effects, such as weight gain, muscle weakness, reduced growth in children and osteoporosis in elderly [15]. In severe asthma patients long-term treatment can even induce glucocorticosteroid-resistance [16]. Therefore, novel preventive and/or therapeutic approaches are needed. Recent experiments have demonstrated a substantial influence of the gut microbiota on immune function beyond the gut. Development of asthma and allergies might even be due to the changes in gut microbiota [17-19]. Galacto-oligosaccharides (GOS) are non-digestible carbohydrates with prebiotic capacity, meaning that they selectively support growth and/or activity of bifidobacteria and lactobacilli. These bacteria are associated with a positive health benefit [17,20]. In addition, *in vivo*, *in vitro*, as well as clinical research has shown benefits of GOS on the digestive and immune health [21-23]. Various animal studies have shown a preventive effect of non-digestible oligosaccharides on allergic diseases. In food allergic mice, a combination of GOS/long-chain fructo-oligosaccharides (lcFOS) with *Bifidobacterium breve* M-16V was able to reduce allergic responses [24]. Van de Pol *et al.* used the same combination in patients with asthma and showed an increased peak expiratory flow, but no effect was seen on bronchial inflammation [25]. Vos *et al.* used a combination of GOS, lcFOS, and pectin-derived acidic oligosaccharides (AOS) in

an ovalbumine-induced asthma mouse model and showed a significant suppression of the airway inflammation and AHR [26]. In a murine OVA-induced chronic asthma model, Sagar *et al.* showed a decrease in pulmonary inflammation and airway remodeling after long-term treatment with scFOS/lcFOS/AOS in combination with *Bifidobacterium breve* [27]. Also treatment with *Bifidobacterium breve* alone was as effective as budesonide in reducing airway remodeling, but not in reducing lung resistance [28]. The development of allergic asthma is strongly associated with the exposure to house dust mite (HDM) [29]. For this reason, this study uses a HDM-induced allergic asthma model to study the preventive effect of dietary GOS on the AHR, pulmonary inflammation and lung cytokine concentrations in comparison with the therapeutic treatment budesonide.

Materials and methods

Mice

Male BALB/c mice (Charles River, The Netherlands), 6- to 8-week old (20-25 g), were used in all experiments. Mice were housed under bio-contained sterile conditions using HEPA® filtered isocages® (Tecniplast, Italy). Food and water were provided *ad libitum*. All animal experiments were conducted in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Utrecht University (DEC 2013.II.01.003).

HDM murine asthma model

While under isoflurane anaesthesia, BALB/c mice were intranasally (i.n.) sensitized with 1 µg HDM/40 µL PBS (Greer Laboratories, USA) on day 0 and challenged daily on days 7 to 11 with PBS (control, HDM-PBS) or 10 µg HDM/40 µL PBS (HDM-HDM) [30]. From day -14 to 14, mice were fed a control diet (AIN93G, contr) or the same diet containing 1% w/w GOS (Vivinal® GOS syrup with approximately 59% galacto-oligosaccharides, 21% lactose, 19% glucose, and 1% galactose on dry matter (dry matter of 75%); FrieslandCampina Domo, The Netherlands). Carbohydrates in Vivinal® GOS were compensated isocalorically in the control diet by means of cellulose (for GOS), lactose (for lactose), and dextrose (for glucose). A separate control and HDM-allergic group were treated with budesonide as a reference treatment while being fed the control diet. After induction of a light isoflurane anaesthesia, mice were instilled oropharyngeally, with budesonide (500 µg/kg, Sigma-Aldrich, The Netherlands) on days 7, 9 and 11, 6 h prior to the daily challenge and on day 13, 24 h prior to the assessment of airway responsiveness to methacholine (**Fig. 1**) [28,31]

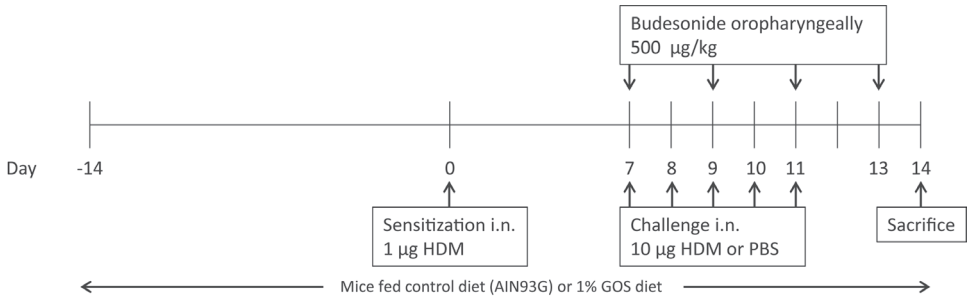

Fig. 1 Time schedule of the house dust mite allergic asthma model.

Male BALB/c mice were sensitized intranasally (i.n.) with house dust mite (HDM) on day 0 and were challenged on days 7 to 11 i.n. with HDM or PBS. Mice were fed control diet (AIN93G, contr) or 1% w/w GOS from day -14 to 14. In a separate set of animals, control mice and HDM-allergic mice were oropharyngeally instilled with budesonide on days 7, 9, 11 and 13. All mice were sacrificed on day 14.

Airway responsiveness measurement

Mice were intraperitoneally (i.p.) anaesthetized with a mix containing Ketamine (Vetoquinol S.A., France; 125 mg/kg) and Medetomidine (Pfizer, The Netherlands; 0.4 mg/kg). Lung function was assessed using EMKA invasive measurement of dynamic resistance (EMKA Technologies, France) in response to increasing doses of methacholine (acetyl-β-methyl-choline chloride, Sigma-Aldrich, The Netherlands) (0-25 mg/mL, 10% puff for 10 sec.). Data are presented as average lung resistance (R_L) in cm H_2O/(mL/sec.) [28].

Bronchoalveolar lavage

Mice were killed with an i.p. overdose of pentobarbital (600 mg/kg, Nembutal™, Ceva Santé Animale, The Netherlands) after the airway responsiveness measurement. A small incision was made in the trachea to insert a cannula. Lungs were lavaged with 1 mL of pyrogen-free saline (0.9% NaCl, 37ºC) supplemented with protease inhibitor cocktail tablet (Complete Mini, Roche Diagnostics, Germany). The supernatant of the first mL was used for cytokine and chemokine measurement. Afterwards, the lungs were lavaged 3 times with 1 mL saline solution (0.9% NaCl, 37ºC). The bronchoalveolar lavage fluid (BALF) cells were centrifuged (400 g, 5 min.) and pellets of the 4 lavages were pooled, resuspended and total numbers of BALF cells were counted using a Bürker-Türk chamber (magnification 100x). For differential BALF cell counts, cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Switzerland). Numbers of macrophages, lymphocytes, neutrophils and eosinophils were scored with light microscopy [32].

Preparation of lung homogenates

In brief, lung samples were homogenized in 1% Triton X100 (Sigma-Aldrich)/PBS containing protease inhibitor (Complete Mini) using a Precellys 24 tissue homogenizer (Bertin Technologies, France) 3 times for 10 sec. at 6,000 rpm with a minimum of 5 min. cooling period on ice in between. Homogenates were centrifuged at 14,000 rpm for 5 min., supernatants collected and stored at -20^{o}C until further use. The protein concentration of each sample was assayed using the Pierce BCA protein assay kit standardized to BSA according to the manufacturer's protocol (Thermo Fisher Scientific, USA). The homogenates were diluted to a final concentration of 1 mg protein/mL [33,34].

Measurement of cytokines

A standard Th1/Th2/Th17 assay (IL-2, -4, -6, -10, TNFα, IFNγ; BD Biosciences, The Netherlands) was used to determine cytokine concentrations in lung homogenates according to the manufacturer's instructions. Only the IL-6 concentration was assessed since all other cytokines concentrations were below the detection limit. IL-33, CCL5 and CCL17 were measured with a DuoSet ELISA (R&D Systems, USA), IL-13 and IL-5 with a Ready-SET-Go!® ELISA (eBioscience, USA). The concentrations of these cytokines were expressed as pg/mg protein in lungs and pg/mL in BALF.

Statistical analysis

Results are presented as mean ± standard error of mean (SEM). Data were statistically analyzed using a One-Way ANOVA followed by a Bonferroni's multiple comparisons test. $P < 0.05$ were considered significant. Statistical analyses were conducted using GraphPad Prism software (version 6.04).

Results

Airway hyperresponsiveness abrogates upon dietary intervention with 1% GOS in HDM-allergic mice

Airway hyperresponsiveness (AHR) as a measure of lung function upon HDM-exposure was determined in mice that were fed the control diet or the 1% GOS diet or those treated with budesonide. The basal airway responsiveness (0.70 ± 0.05 cm H_2O/(mL/sec.) in HDM-PBS-control group did not differ between the experimental groups. Moreover, an aerosol with saline (as a control for methacholine) did not change basal lung resistance (R_L 0.79 ± 0.06 H_2O/(mL/sec.) in the HDM-PBS control group. HDM-HDM mice fed the control diet showed a significant increase in airway hyperresponsiveness (6.25-25 mg/mL of methacholine) compared to the HDM-PBS control group. Dietary intervention with 1% GOS resulted in a significant inhibition of AHR, reducing it back to control level, in HDM-allergic mice. Treatment with budesonide did not significantly affect AHR in HDM-HDM mice (**Fig. 2**).

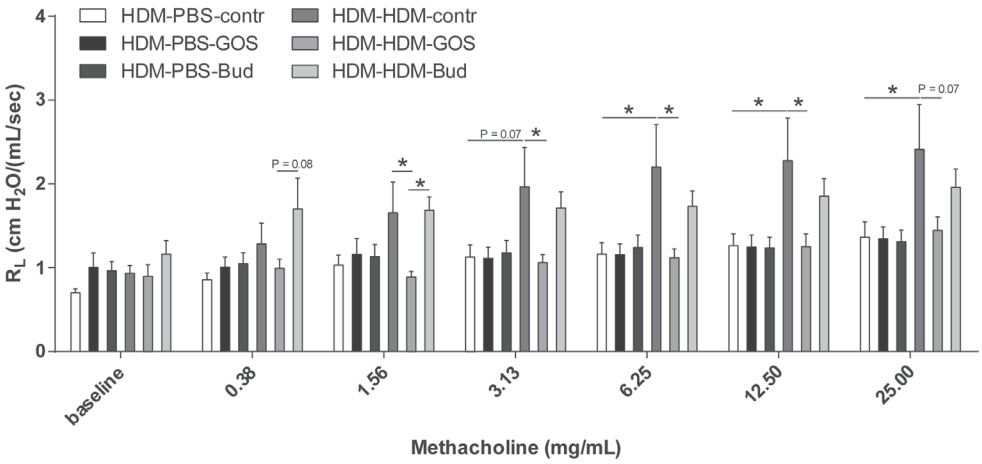

Fig 2. Airway hyperresponsiveness was abrogated upon dietary intervention with 1% GOS in HDM-allergic mice.

Airway resistance (R_L) in response to increasing doses of methacholine on day 14. HDM-PBS: HDM-sensitized and PBS-challenged mice, HDM-HDM: HDM-sensitized and -challenged mice. Contr: control diet; GOS: 1% GOS diet; Bud: budesonide treatment. Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, n = 7-8 mice/group.

Dietary intervention with 1% GOS reduces pulmonary eosinophilic inflammation in the lungs of HDM-allergic mice

To investigate the inflammatory cell influx into the airways of HDM-allergic mice upon dietary intervention with 1% GOS or intra-airway treatment with budesonide, BALF was examined (**Fig. 3A**). The total number of inflammatory cells in the BALF of HDM-HDM mice fed the control diet was significantly increased (**Fig. 3A**), which was mainly due to an increase in the number of eosinophils and macrophages, and there was also a significant increase in the number of lymphocytes and neutrophils (**Fig. 3B-E**) compared to the HDM-PBS control group. Dietary intervention with 1% GOS reduced the total number of BALF cells and number of eosinophils (> 57%, P = 0.057) in HDM-allergic mice (**Fig. 3B**). Treatment with budesonide significantly reduced the total number of inflammatory cells in HDM-allergic mice compared to the HDM-HDM group fed the control diet (**Fig. 3A**), which was due to a significant decrease in eosinophil and lymphocyte numbers (**Fig. 3B, 3E**).

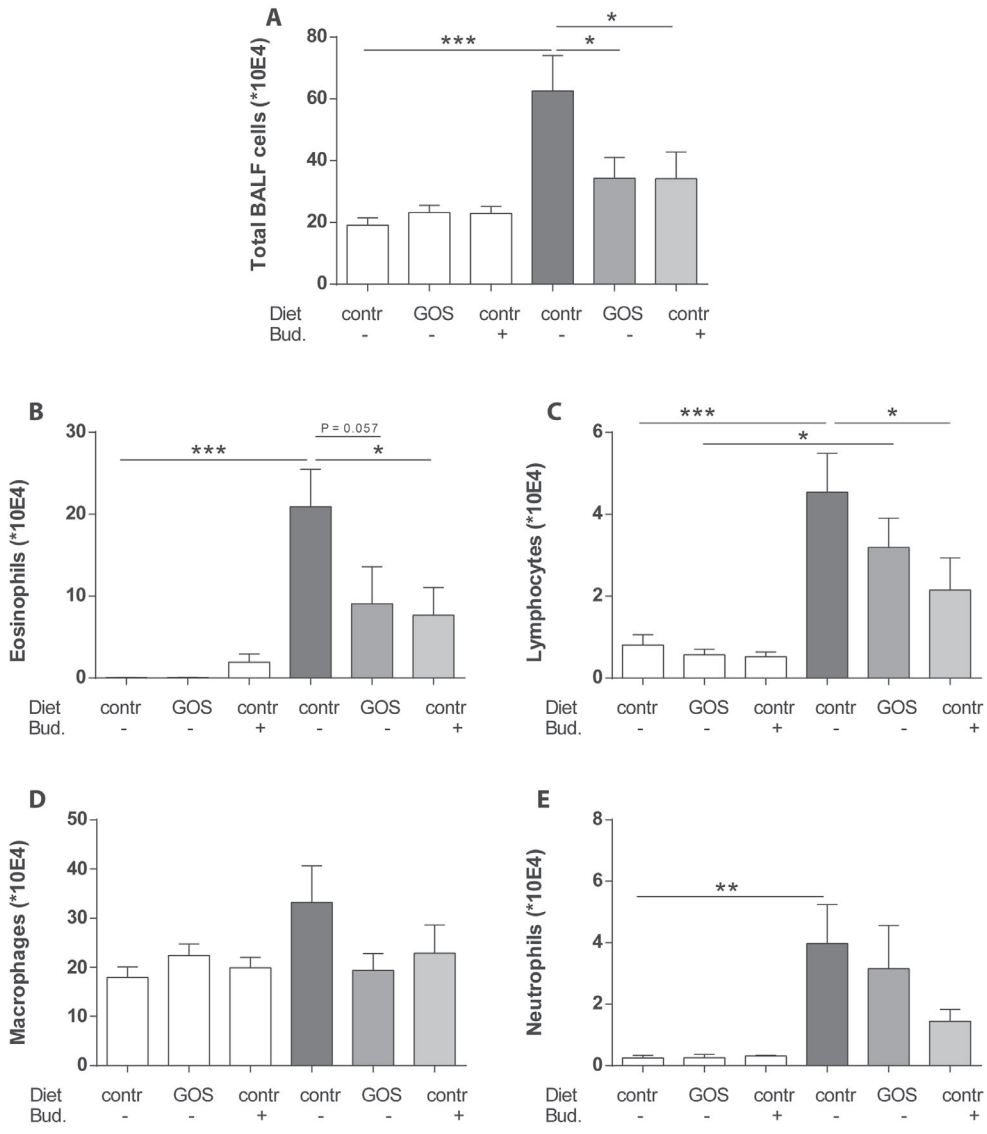

Fig. 3 Dietary intervention with 1% GOS reduced pulmonary eosinophilic inflammation in the lungs of HDM-allergic mice.

Infiltration of inflammatory cells in the BALF of house dust mite allergic mice. HDM-PBS: HDM- sensitized and PBS-challenged mice (white bars), HDM-HDM: HDM-sensitized and -challenged mice (grey bar). Contr: control diet, GOS: 1% GOS diet, Bud: budesonide treatment. Total BALF cells (**A**), absolute number of eosinophils (**B**), lymphocytes (**C**), macrophages (**D**) and neutrophils (**E**). Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $n = 7$-9 mice/group.

The effect of 1% GOS on enhanced concentrations of IL-6, CCL17, IL-33, CCL5 and IL-13 in lungs of HDM-allergic mice

In order to determine the effect of 1% GOS on pulmonary IL-6 concentrations, lungs were homogenized and IL-6 concentration was measured in the supernatant. The lung tissue concentration of IL-6 was significantly increased in HDM-HDM mice fed the control diet compared to the HDM-PBS control group. After dietary intervention with 1% GOS, IL-6 concentrations were not significantly changed in the lung tissue of HDM-allergic mice compared to controls. Treatment with budesonide of HDM-HDM mice significantly decreased the IL-6 concentration when compared with non-treated HDM-allergic mice (**Fig. 4A**). In addition, CCL17 pulmonary concentrations were significantly increased in HDM-HDM mice fed the control diet compared to HDM-PBS control groups. Treatment with budesonide significantly decreased the CCL17 concentration when compared with non-treated HDM-allergic mice, 1% GOS showed a reduction of > 20% (**Fig. 4B**). Moreover, the IL-33 concentration was significantly increased in supernatants of HDM-HDM mice compared to the HDM-PBS control group. In the 1% GOS treated HDM-allergic mice, IL-33 was not changed while it was still enhanced in the budesonide-treated HDM-allergic group (**Fig. 4C**). After dietary intervention with 1% GOS, CCL5 concentrations were significantly decreased in HDM-allergic mice when compared to the non-treated groups, respectively. Treatment with budesonide also significantly decreased the CCL5 concentration in the supernatant of lung tissue obtained from HDM-allergic and HDM-PBS control mice. However, budesonide treatment already had an effect on CCL5 concentrations in HDM-PBS mice (**Fig. 4D**). The concentration of the Th2 cytokine IL-13 in the lungs was significantly increased in HDM-HDM mice fed a control diet compared to the HDM-PBS control group. Both 1% GOS and budesonide significantly decreased the HDM-allergy-induced increase in IL-13 (**Fig. 4E**). Moreover, IL-13 concentrations in lung homogenates of HDM-HDM mice positively correlated with the number of lymphocytes (**Fig. 4F**).

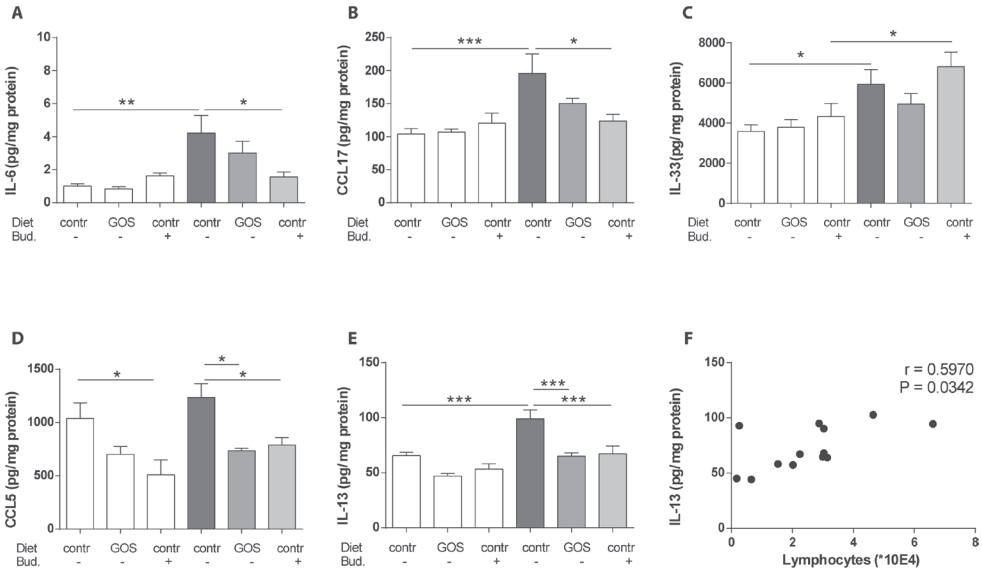

Fig. 4 IL-6, CCL17, IL-33, CCL5 and IL-13 concentrations in lungs of HDM-allergic mice.

IL-6 (**A**), CCL17 (**B**), IL-33 (**C**), CCL5 (**D**), and IL-13 (**E**) concentrations were measured in supernatant of lung homogenates. Correlation of IL-13 and the number of lymphocytes (**F**). HDM-PBS: HDM-sensitized and PBS- challenged mice (white bars), HDM-HDM: HDM-sensitized and -challenged mice (grey bar). Contr: control diet, GOS: 1% GOS diet, Bud: budesonide treatment. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n = 6-8 mice/group. Correlation was analyzed using the Spearman correlation test.

Discussion

The aim of this study was to investigate the preventive effect of 1% dietary GOS on lung function and pulmonary inflammation in a murine model of HDM-induced allergic asthma. As is usually done for potentially new preventive and/or therapeutic agents, we compared the effectiveness with a golden standard reference treatment, the corticosteroid budesonide. To understand the underlying pathophysiology of the disease, animal models for allergic asthma are used. Here, a murine model for HDM-allergic asthma was used that mimics human features of asthmatic disease such as HDM-induced AHR, airway inflammation and pulmonary cytokine release [11,35,36]. Airway responsiveness upon methacholine exposure in HDM-allergic mice was significantly increased when compared to HDM-PBS control mice. Moreover, total inflammatory cell numbers were significantly increased in the BALF of HDM-allergic mice when compared to control mice. Budesonide treatment showed no significant decrease on the development of AHR whereas dietary

intervention with 1% GOS prevented AHR development in HDM-allergic mice. However, both interventions suppressed airway inflammation in HDM-allergic mice. Both 1% GOS and budesonide were effective in the suppression of airway inflammation and decreased the number of eosinophils and macrophages. Furthermore, 1% GOS prevented the increase of CCL17 and IL-33 and significantly decreased CCL5 and IL-13 concentrations in the lungs. To our knowledge, this is the first study to demonstrate that dietary intervention with 1% GOS during sensitization and challenge is as effective as treatment with budesonide on allergic HDM-asthma symptoms in a murine model. IL-6 concentrations have been reported to be increased in serum, BALF and sputum of asthmatic patients [12,37]. This increase was also observed in the murine model of HDM-induced allergic asthma. Budesonide, but not 1% GOS, normalized HDM-allergy-induced increase of IL-6 concentrations to the control level. This effect of budesonide is in agreement with clinical studies which demonstrate that glucocorticosteroids decrease IL-6 concentrations in BALF of asthmatic patients in association with decreased activation and recruitment of inflammatory cells in asthma [38]. In our study, budesonide decreased the number of inflammatory cells as well. Besides being released by macrophages and T cells, IL-6 is highly expressed by epithelial cells obtained from allergic asthma patients [39]. However, the exact role of IL-6 in asthma pathology is not fully understood and has to be further elucidated. In patients with asthma, CCL17 has been found in increased concentrations in serum and BALF [40]. Previously, an important role for CCR4 and its ligand CCL17 in Th2 cell recruitment has been demonstrated in asthma [41]. Budesonide significantly decreased the HDM-allergy-induced concentrations of CCL17, whereas 1% GOS showed a reduction of > 20%. The latter is in agreement with studies from Leung *et al.*, who found higher concentrations of CCL17 in the serum of non-steroid-treated asthmatic children compared with steroid-treated asthmatic children [42]. IL-33 is known to contribute to AHR since animal studies demonstrated that intranasally administered IL-33 results in an AHR-association with eosinophilia, goblet cell hyperplasia, and accumulation of IL-4, -5 and -13 in the lungs [43,44]. IL-33, produced by epithelial cells after allergen stimulation, is a chemoattractant for Th2 cells and can activate mast cells to release CXCL8, IL-5, -6, and -13 [45]. Indeed, IL-33 may be involved in human asthmatic disease, since it is increased in BALF of moderate asthma patients as compared to mild asthma patients or controls [46]. Typically, the concentration of IL-33 in the lung tissue obtained in our study was significantly enhanced in HDM-allergic and budesonide-treated mice compared to the control mice. In these budesonide-treated mice, the AHR was still evident. Indeed, Deckers *et al.* also demonstrated that budesonide had no effect on IL-33 concentrations in asthmatic patients [47]. However, in HDM-allergic mice fed 1% GOS, the HDM-induced increase in IL-33 in lung tissue was prevented. Since IL-33 is one of the factors contributing to AHR [43,44], this could relate to the 1% GOS-induced abrogation of the AHR response of these mice. CCL5 is produced at high concentrations within the airway epithelium of human asthmatics and in turn will target eosinophils to the airways [48]. In this study, both dietary 1% GOS as well as budesonide treatment showed a significant decrease in

CCL5 concentrations in lung tissue of HDM-allergic mice. Dampening of pulmonary CCL5 concentrations could be the mechanism by which GOS and budesonide treatment induces an abrogation of eosinophil infiltration in the lungs of HDM-allergic mice. In humans, the release of the Th2 cytokine IL-5 leads to activation of the eosinophil/basophil lineage. Increased eosinophil cell influx and AHR are strongly associated in asthmatic patients and can be provoked after inhalation of IL-5. However, there are many studies in humans demonstrating that treatment with anti-IL-5- specific antibodies reduced the number of eosinophils in sputum and blood of mild and severe asthmatic patients, although the AHR was not affected [49]. Thus, inflammatory pathways underlying IL-5 alone are not sufficient for the development of AHR in allergic asthma. In our study, IL-5 tended to be increased in the BALF of HDM-allergic mice whereas this did not occur after dietary intervention with 1% GOS or treatment with budesonide (**Supp. data Fig. 1**). Indeed, IL-5 in BALF was positively correlated with eosinophil numbers (**Supp. data Fig. 1**). The concentration of another Th2 cytokine, IL-13, in lung tissue was significantly increased in HDM-allergic mice compared to the control mice. These data are in agreement with previous studies that show a central role for IL-13 in generating the murine allergic AHR following sensitization and challenge of HDM [50]. Many of the processes involved in allergic asthma can be directed to IL-13. Besides being secreted by Th2 cells, IL-13 is also secreted by mast cells and innate lymphoid cells. In the current study, IL-13 concentrations in lung tissue were positively correlated with BALF lymphocyte numbers, suggesting this subset to be an important source of IL-13. IL-13 also triggers macrophage and eosinophil activation which, in turn, can contribute to AHR [51]. Furthermore, IL-13 is increased in BALF and bronchial biopsy specimens of asthmatic patients and known to be inhibited by glucocorticoids [51,52]. Both dietary 1% GOS as well as budesonide treatment showed a significant decrease in IL-13 concentrations in lung homogenates of HDM-allergic mice, hereby dampening a major contributor to asthmatic inflammation. In previous studies, *Bifidobacterium breve* or *Lactobacillus rhamnosus*, either or not combined with specific oligosaccharides, suppressed airway inflammation in a murine model for OVA-induced chronic asthma [27,28]. Our studies show similar effects with GOS alone in an acute model for HDM-induced asthma. As shown in earlier studies with dietary oligosaccharides, it is known that they have a positive effect on the composition of microbiota [21-23]. A potential mechanism of GOS could be that by changing the microbiota, immunomodulation via intestinal epithelial signaling occurs leading to systemic effects resulting in a decreased HDM-immune response, as has been suggested by several studies [17,20]. In conclusion, in our study budesonide suppressed inflammatory cell numbers and cytokine concentrations of IL-6, CCL17, CCL5 and IL-13 in HDM-allergic mice. However, budesonide did not modulate the HDM-allergy induced AHR and increased the pulmonary tissue concentrations of IL-33. Interestingly, dietary intervention with 1% GOS prevented the development of AHR and suppressed airway eosinophilia in HDM-allergic mice. Moreover, 1% GOS prevented the increase in IL-33 and abrogated the HDM-induced CCL17, CCL5 and IL-13 release in the lungs of HDM-allergic mice. Dietary

intervention with 1% GOS may be beneficial in the prevention of HDM-induced allergic asthma, and may offer a potential novel strategy with less side effects than current therapeutic treatments. However, more research is needed to demonstrate this beneficial effect. In addition, the mechanism of the immune modulating functions of 1% GOS needs to be further elucidated, as well as the most effective dose.

Acknowledgments

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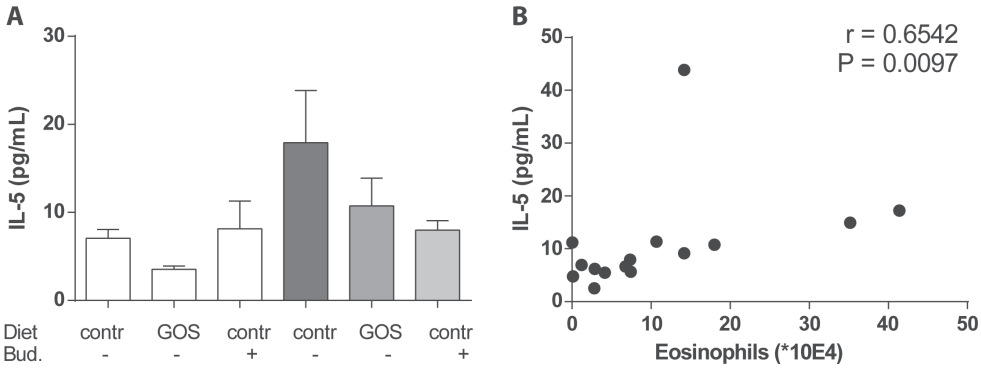
Supplementary data

Measurement of IL-5 in BALF

IL-5 concentrations were measured with a Ready-SET-Go!® ELISA (eBioscience, San Diego, CA, USA). The concentration of this cytokine was expressed as pg/mL.



Supplementary Fig. 1 IL-5 concentrations in the BALF of HDM-allergic mice.

IL-5 concentration (**A**) was measured in the BALF. Correlation of IL-5 and the number of eosinophils (**B**). HDM-PBS: HDM-sensitized and PBS-challenged mice (white bars), HDM-HDM: HDM-sensitized and -challenged mice (grey bar). Contr: control diet, GOS: 1% GOS diet, Bud: budesonide treatment. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. Correlation was analyzed using the Spearman correlation test.

CHAPTER SIX

Dietary galacto-oligosaccharides improve the effectiveness of budesonide treatment on airway inflammation in house dust mite-induced asthma in mice

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Abstract

Background: Dietary non-digestible galacto-oligosaccharides (GOS) suppress murine house dust mite (HDM)-induced asthma.
Objective: To study combined dietary GOS and budesonide treatment on HDM-induced asthma in mice.
Methods: BALB/c mice were sensitized and challenged with HDM while fed a control or 1 - 2.5% w/w GOS diet, either or not oropharyngeally instilled with budesonide. Airway resistance (AHR) was determined and airway inflammation was studied in bronchoalveolar lavage fluid (BALF) and lung.
Results: HDM-allergy increased AHR and BALF eosinophils and lymphocytes, budesonide treatment suppressed this in control or GOS fed mice. The GOS diet also reduced airway eosinophilia and combined with budesonide treatment this eosinophilic inflammation was largely abolished. This was associated with effective suppression of HDM-induced increase in IL-33, CCL17, CCL22 and IL-13 in lung homogenates and reduced lung Th2 cell frequency.
Conclusion: Dietary intervention using GOS may be a novel way to further improve the efficacy of anti-inflammatory drug therapy in asthma.

Introduction

Asthma is a chronic disease affecting 300 million people worldwide. The disease is characterized by airway hyperresponsiveness, airway narrowing and airway inflammation containing high numbers of eosinophils [1]. House dust mites (HDM) are common allergens that can induce allergic diseases like asthma and allergic rhinitis [2]. Different cytokines (e.g. IL-33 and GM-CSF) and chemokines (e.g. CCL20) can be released by airway epithelial cells after stimulation with HDM contributing to allergic sensitization [3]. IL-33 and GM-CSF are capable of activating dendritic cells (DC) and group 2 innate lymphoid cells (ILC2), which produce IL-5 and IL-13 [4]. Furthermore, immature DC are attracted to the lung by CCL20, and in turn are activated by Th2 driving mediators [5]. After activation, DC release chemokines such as CCL17 and CCL22. These activated DC prime naïve T cells in the mediastinal lymph nodes to develop into Th2 cells. In response, Th2 cells will migrate to the lung and like ILC2, produce IL-5 and IL-13, which can trigger eosinophilic inflammation and airway hyperresponsiveness (AHR) [3,5,6]. Long-acting beta-agonists with or without glucocorticosteroids are the most commonly used drugs for asthma. Consistent use can improve asthma symptoms (e.g. hyperresponsiveness and severity of exacerbations) [7,8]. Nevertheless, when the drug is discontinued the effects of inhaled corticosteroids rapidly disappear. Also, long-term treatment with glucocorticosteroids can have considerable side effects, such as weight gain, reduced growth in children and muscle weakness [7,8]. Current treatment is still not sufficient, therefore, novel preventive and/or therapeutic approaches are needed for asthmatic disorders. The gut microbiota has a substantial influence on systemic immune function. Different animal and human studies showed that the development of asthma can be caused by changes in the intestinal microbiota [9-12]. Specific non-digestible oligosaccharides such as galacto-oligosaccharides (GOS) are selectively fermented in the intestine, resulting in support of growth and/or activity of bifidobacteria and lactobacilli [9,13,14]. Non-digestible oligosaccharides can have a preventive effect on allergic diseases. In different murine ovalbumin (OVA) asthma models, a combination of GOS/long-chain fructo-oligosaccharides (lcFOS)/pectin-derived acidic oligosaccharides (AOS) suppressed airway inflammation and hyperreactivity in asthmatic mice [15,16]. Also, from rat studies it is known that GOS suppressed allergic airway eosinophilia in ovalbumin-sensitized rats [17]. Clinical studies showed that a mixture of 9:1 GOS/lcFOS still has a protective effect against allergic manifestations until 5 years of age for allergic rhinoconjunctivits and atopic dermatitis [18]. This mixture of GOS/lcFOS in combination with *Bifidobacterium breve* M-16V also reduced Th2 cytokine production and increased peak expiratory flow in patients suffering from asthma [19]. GOS and budesonide have shown similar effects [20]. In the present study the effectiveness of combined dietary intervention with GOS and treatment with budesonide on AHR and pulmonary inflammation was investigated in a murine HDM-induced allergic asthma model.

Methods

Mice

Male BALB/c mice (Charles River, The Netherlands), 6 to 8-week old were housed under bio-contained sterile conditions using HEPA ® filtered isocages ® (Tecniplast, Italy). Food and water were provided *ad libitum*. All animal experiments were conducted in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Utrecht University (DEC 2013.II.08.090).

HDM murine asthma model

While under isoflurane anesthesia, BALB/c mice were intranasally (i.n.) sensitized with PBS in presence or absence of 1 µg HDM (Greer Laboratories, USA) and challenged i.n. with PBS or 10 µg HDM on days 7 to 11. Animals were fed a diet (AIN-93G, control diet) containing 0, 1 or 2.5% w/w GOS (Vivinal® GOS syrup with approximately 59% galacto-oligosaccharides, 21% lactose, 19% glucose, and 1% galactose on dry matter (dry matter of 75%); FrieslandCampina Domo, The Netherlands). Carbohydrates in Vivinal® GOS were compensated isocalorically in the control diet by means of exchange against cellulose (for GOS), lactose (for lactose), and dextrose (for glucose), from day -14 to day 14 [21]. On day 7, 9 and 11 budesonide (500 µg/kg, Sigma-Aldrich, The Netherlands) was either or not instilled oropharyngeally, after induction of a light isoflurane anesthesia, 6 h prior to the daily challenge andon day 13, 24 h prior to sacrifice on day 14 [20,22] (**Fig. 1**).

Airway responsiveness measurement

EMKA invasive measurement of dynamic resistance (EMKA Technologies, France) in response to increasing doses of methacholine (acetyl-β-methyl-choline chloride, Sigma-Aldrich) (0 - 25 mg/mL, 10% puff for 10 sec.) was used to measure lung function in anaesthetized mice. Data are presented as average lung resistance (R_L) in cm H_2O/(mL/sec.) [23].

Bronchoalveolar lavage

Lungs were lavaged with 1 mL of pyrogen-free saline (0.9% NaCl, 37°C) supplemented with protease inhibitor cocktail tablet (Complete Mini, Roche Diagnostics, Germany). This was followed by 3 lavages with 1 mL saline solution (0.9% NaCl, 37°C). The BALF cells were centrifuged (400 g, 5 min.) and pellets of the lavages were pooled and total numbers of BALF cells were counted using a Bürker-Türk chamber (magnification 100x). For differential BAL cell counts, cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Switzerland). Numbers of lymphocytes and eosinophils were scored with light microscopy [24].

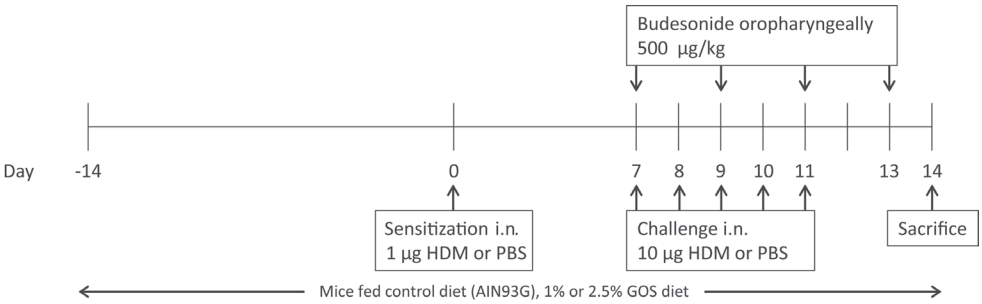

Fig. 1 Induction of house dust mite allergy in mice.

BALB/c mice were intranasally (i.n.) sensitized with PBS or house dust mite (HDM) on day 0 and challenged on days 7 to 11 intranasally with PBS or HDM. Mice were fed control diet (AIN93G, contr) or 1% or 2.5% w/w GOS from day -14 to 14 and either or not oropharyngeally instilled with budesonide on days 7, 9, 11 and 13. All mice were sacrificed on day 14.

Serum preparation

After sacrifice, blood samples were collected by cardiac puncture. The blood was coagulated 30 min. at room temperature and centrifuged at 14,000 rpm for 10 min.. Serum samples were stored at -20°C until further use.

Preparation of lung homogenates

Lung samples were homogenized in 1% Triton X100 (Sigma-Aldrich)/PBS containing protease inhibitor (Complete Mini, Roche Diagnostics) using a Precellys 24 tissue homogenizer (Bertin Technologies, France). Homogenates were centrifuged at 14,000 rpm for 10 min, supernatants were collected and stored at -20°C until further use. The protein concentration was measured using the Pierce BCA protein assay kit standardized to BSA according to the manufacturer's protocol (Thermo Fisher Scientific, USA). The homogenates were diluted to a final concentration of 1 mg protein/mL prior to cytokine or chemokine measurements [25,26]

Lung restimulation with house dust mite *ex vivo*

Lung cell suspensions were prepared after enzymatic digestion of the lungs using digestion buffer, containing DNase I and Collagenase A (Roche Diagnostics), for 30 min. The digestion was stopped by adding fetal calf serum (FCS, Hyclone Laboratories, USA). The lung pieces were passed through a 70 µm filter and rinsed with 10 mL RPMI. Cells were washed and resuspended in RPMI 1640 culture medium (Lonza, USA) supplemented with 10% heat-inactivated FCS and 0.1% penicillin-streptomycin solution (Sigma-Aldrich). Lung cells (4 x 10^5 cells/well) were cultured in medium with or without 50 µg/mL HDM. The supernatant was harvested after 4 days of culture at 37°C in 5% CO_2 and stored at -20°C until further analysis [27].

Lung T cell subsets assessed by flow cytometry

Aspecific background was blocked using PBS blocking buffer containing 1% BSA and 5% FCS for 30 min. 5 x 10^5 cells were plated per well and incubated at 4ºC for 30 min. with different antibodies against CD4-PerCP Cy5, CD69-FITC, GATA3-PE, Tbet-eFLUOR660, RORγt-PE (eBioscience, USA), CD8a-APC Cy7 (BD, The Netherlands) and matching isotype controls were used. Cells were permeabilized for intracellular staining using fixation/ permeabilization buffer set, according to manufacturer's protocol (eBioscience). Flow cytometry was conducted using FACS Canto II (BD) and analyzed using Flowlogic Software (Inivai Technologies, Australia) [28].

Measurement of cytokines and chemokines

IL-33, GM-CSF, CCL17, CCL20 and CCL22 were measured with a DuoSet ELISA (R&D Systems, Abingdon, United Kingdom), IL-13, IL-5 and mMCP-1 with a Ready-SET-Go!® ELISA (eBioscience) all according to manufacturer's protocol. Cytokine concentrations in supernatants of lung cell restimulation were determined by a standard IL-13 flex set (BD Biosciences). The concentrations of these mediators were expressed as pg/mg protein in supernatants of lung homogenates and pg/mL in restimulation supernatants and serum.

Histology

Lungs were fixed with 10% formalin via a cannula inserted in the trachea at a constant pressure of 25 cm H_2O. After 24 h of fixation the lungs were embedded in paraffin and 5 µm sections were cut. Sections were stained with hematoxylin & eosin, 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 scored as follow: score 0 no inflammation, score 1 mild inflammation, score 2 moderate inflammation, score 3 severe inflammation [29].

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Data were statistically analyzed using a one-way ANOVA followed by a Bonferroni's multiple comparisons test. $P < 0.05$ were considered significant. Statistical analyses were conducted using GraphPad Prism software (version 6.04).

Results

Dietary intervention with GOS in combination with budesonide reduces lung resistance in HDM-allergic mice

Lung resistance was measured to investigate the lung function in HDM-asthmatic mice. There were no differences between the experimental groups at basal level. Methacholine dose-dependently increased lung resistance. A significant increase in lung resistance

(6.25 – 12.5 mg/mL of methacholine) was observed in HDM-mice fed the control diet compared to the PBS control group. The GOS diet did not affect this, whereas budesonide alone tended to prevent the increase in airway resistance. However, only in budesonide treated HDM-allergic mice fed the 1% or 2.5% GOS diet the lung resistance was significantly reduced compared to HDM-allergic mice fed the control diet (**Fig. 2**)

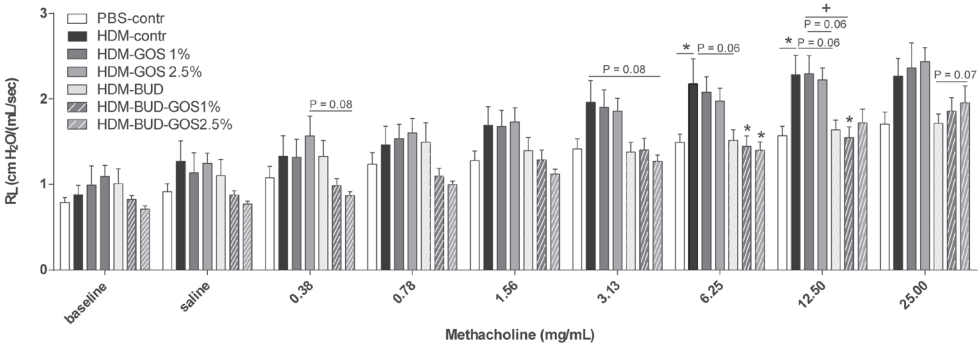

Fig. 2 Dietary intervention with GOS in combination with budesonide decreased lung resistance in HDM-allergic mice.

Airway resistance (R_L) in response to increasing doses of methacholine on day 14. PBS: PBS-sensitized and -challenged mice, HDM: HDM-sensitized and -challenged mice. Contr: control diet; GOS 1%: 1% GOS diet; GOS 2.5%: 2.5% GOS diet; Bud: budesonide treatment. Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$ compared to the HDM-contr group, $^{+}$ $P < 0.05$, n = 7-8 mice/group.

Dietary intervention with GOS in combination with budesonide is most effective in reducing eosinophil numbers in BALF

To study the extent of airway inflammation in HDM-allergic mice upon dietary intervention with 1% or 2.5% GOS or intra-airway treatment with budesonide or a combination, the BALF was examined. The total number of inflammatory cells was significantly increased in the HDM-mice compared to the PBS group both fed the control diet (**Fig. 3A**), comprising a significant increase in the number of lymphocytes and eosinophils (**Fig. 3B-C**). Dietary intervention with 1% or 2.5% GOS significantly reduced the number of total BALF cells and eosinophils, whereas treatment with budesonide reduced total BALF cells, eosinophils and lymphocyte numbers. When GOS and budesonide were combined, the amount of eosinophils almost returned to baseline levels (**Fig. 3C**). An increase of inflammatory cells was also observed in lung histology sections of the HDM-mice compared to the PBS-mice fed the control diet (**Fig. 3D-E**). Dietary intervention with 1% or 2.5% GOS, treatment with budesonide or the combination of both showed a decrease in inflammation score (**Fig. 3D-I**).

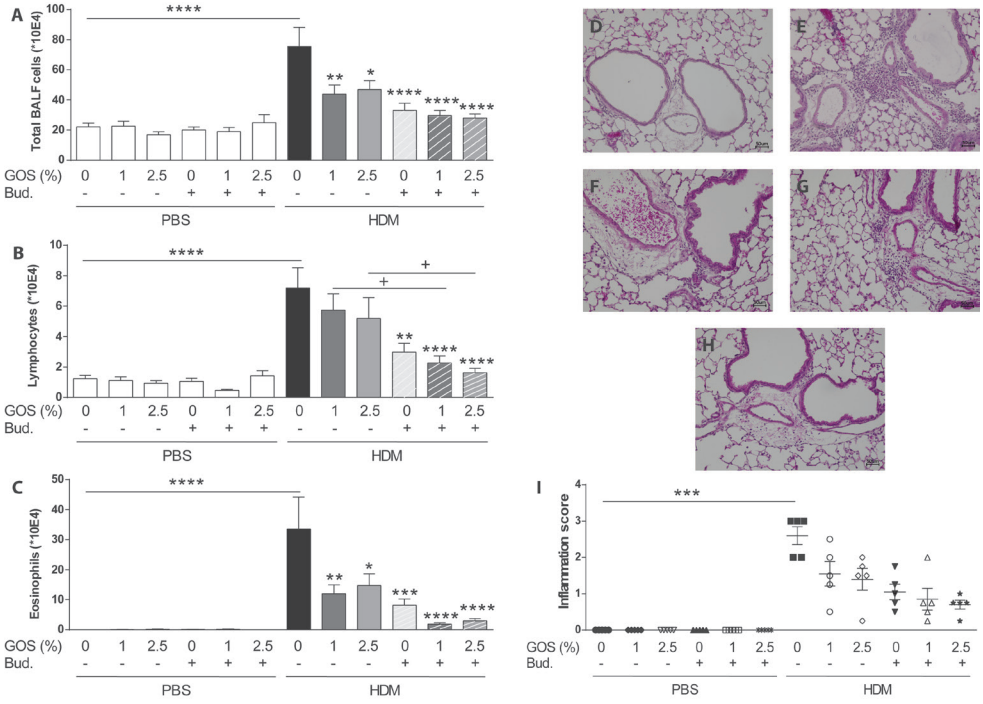

Fig. 3 Combination of dietary GOS and budesonide was most effective in reducing eosinophilic inflammation in the lungs of HDM-allergic mice.

Inflammatory cell influx in the BALF of HDM-allergic mice. PBS: PBS- sensitized and PBS- challenged mice (white bars), HDM: HDM -sensitized and -challenged mice (grey bars). Contr: control diet, GOS: 1% GOS or 2.5% GOS diet, Bud: budesonide. Total BALF cells (**A**), absolute number of lymphocytes (**B**), eosinophils (**C**). Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to the HDM-contr group, $^{+}$ $P < 0.05$ n = 8-9 mice/group. Representative photomicrographs of the lungs stained with H&E. PBS- control diet (**D**), HDM-control diet (**E**), HDM- control diet and budesonide treatment (**F**), HDM-1% GOS diet (**G**), HDM-1% GOS diet and budesonide treatment (**H**), inflammation score of histological photomicrographs; score 0 no inflammation, score 1 mild inflammation, score 2 moderate inflammation, score 3 severe inflammation (I). Results are shown as mean ± SEM. Statistical significance of differences was tested using Kruskall Wallis test. Magnification 200x, BALF n = 8-9 mice per group, histology n = 5 mice/group.

Dietary intervention with GOS in combination with budesonide decreases serum mMCP-1 and chemokine and cytokine concentrations in lung homogenates

mMCP-1 concentrations were measured in serum to examine the effect on mucosal mast cell degranulation. The expression of mMCP-1 in the HDM control group tended to increase compared to the PBS control group. Dietary intervention with 1% GOS or 2.5% GOS, as well as budesonide treatment or the combination of both, significantly decreased the levels of mMCP-1 (**Fig. 4A**). IL-33 was significantly increased compared to

PBS control mice in the supernatants of lung homogenates of HDM-mice fed the control diet. Dietary GOS or budesonide treatment did not affect this but the combination of both significantly decreased IL-33 concentrations in HDM-allergic mice (**Fig. 4B**). GM-CSF and CCL20 did not increase in HDM-allergic mice compared to the PBS control mice fed the control diet (**Fig. 4C-D**). However, in budesonide treated mice fed the GOS diet the concentrations GM-CSF and CCL20 were reduced in both the control as well as the HDM-allergic mice (**Fig. 4C-D**). CCL17 and CCL22 were significantly increased in HDM-allergic mice compared to the PBS control group (**Fig. 4E-F**). Dietary intervention with 1% GOS tended to decrease CCL17 and CCL22 concentrations, whereas budesonide treatment reduced CCL22 (**Fig. 4E-F**). However, when dietary GOS and budesonide treatment were combined, a significant decrease in CCL17 and CCL22 concentrations was observed both in the HDM-allergic mice as well as the PBS control mice (**Fig. 4E-F**).

Dietary intervention with GOS in combination with budesonide decreases pulmonary IL-13 and IL-5 concentrations

Dietary intervention with GOS in combination with budesonide treatment suppressed Th2 driving chemokines CCL17 and CCL22, therefore Th2 type cytokines in lung homogenate supernatants were measured. IL-13 concentrations were significantly increased in the HDM group compared to the PBS control group fed the control diet (**Fig 5A**), this was also observed after *ex vivo* restimulation of lung cell suspensions with HDM (**Fig. 5B**). Only in budesonide treated mice fed the GOS diet IL-13 concentrations were significantly reduced in supernatants of lung homogenates and the same tendency was shown upon HDM restimulation of lung cells (**Fig. 5A-B**). IL-13 concentrations in lung homogenates of HDM-mice correlated positively with the number of lymphocytes (**Fig. 5C**). Although IL-5 concentrations were not significantly increased in HDM-mice, in mice fed the 1% or 2.5% GOS diet while treated with budesonide, IL-5 concentrations were significantly decreased (**Fig. 5D**). A positive correlation between IL-5 concentration and lymphocytes (**Fig. 5E**) as well as eosinophils (**Fig. 5F**) was observed in the lungs of HDM-mice.

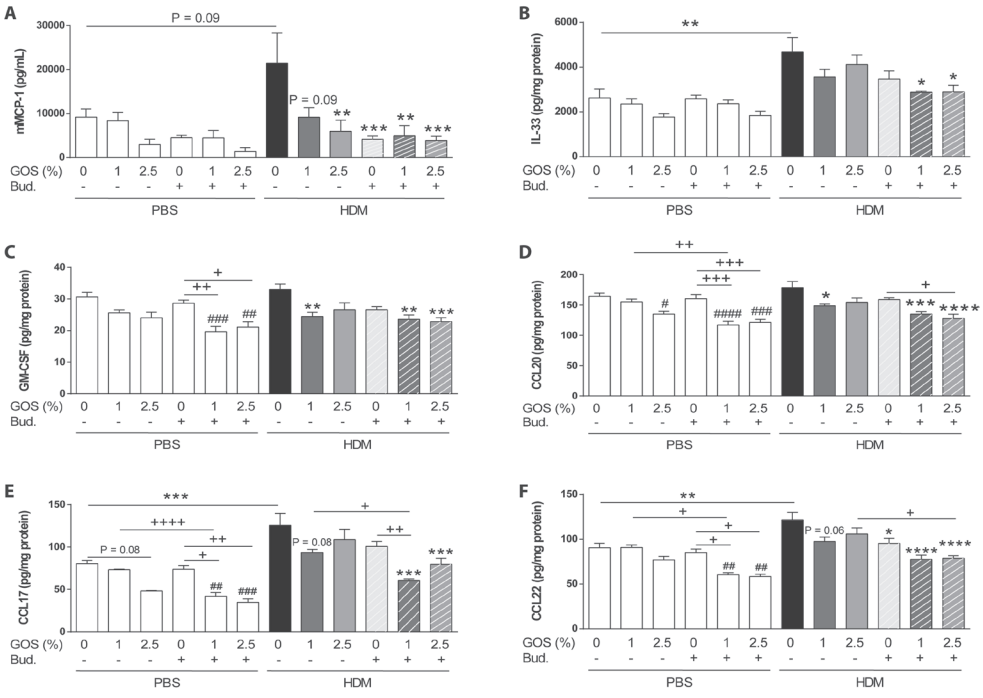

Fig. 4 Combination of dietary GOS and budesonide suppresses mMCP-1 serum concentration and Th2 driving mediators in lung homogenates of HDM-allergic mice.

mMCP-1 (**A**) (pg/mL in serum) and IL-33 (**B**), GM-CSF (**C**), CCL20 (**D**), CCL17 (**E**), CCL22 (**F**) (pg/mg protein in supernatant of lung homogenates) were measured. PBS: PBS- sensitized and -challenged mice (white bars), HDM: HDM-sensitized and -challenged mice (grey bars). Contr: control diet, GOS: 1% GOS or 2.5% GOS diet, Bud: budesonide. Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to the HDM-contr group, $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$, $^{++++}P < 0.0001$ and $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ compared to the PBS-contr group, n = 6 mice/group.

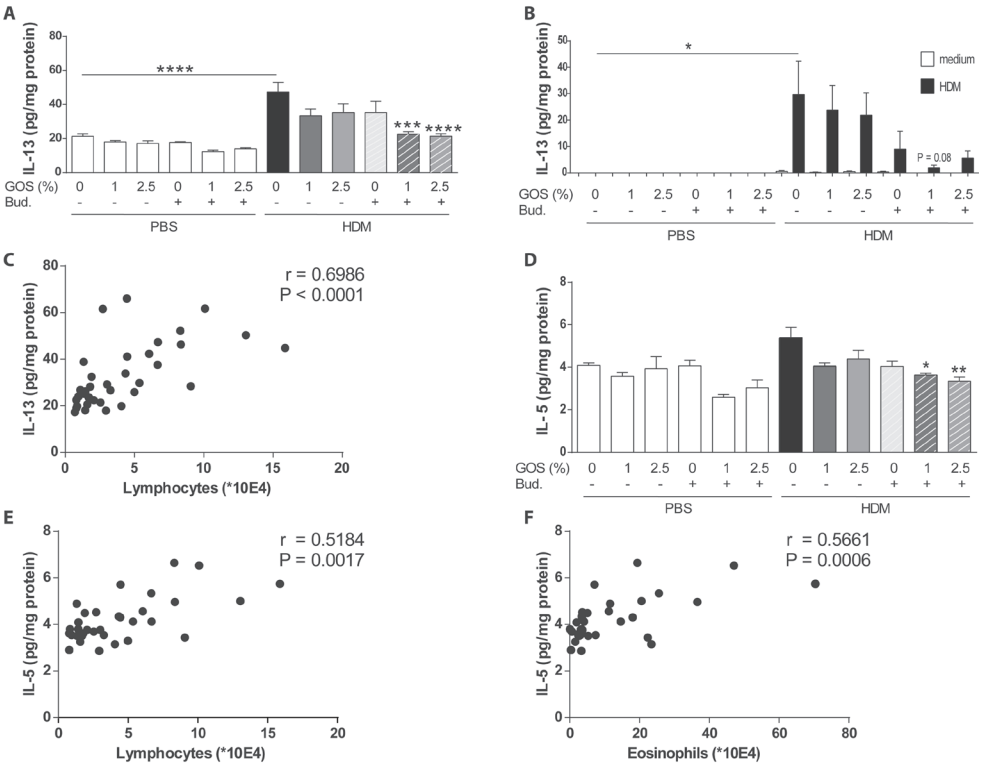

Fig. 5 Combination of dietary GOS and budesonide reduced IL-13 and IL-5 concentrations in the lungs of HDM-allergic mice.

IL-13 concentrations were measured in supernatants of lung homogenates (pg/mg protein) (**A**) and in supernatants of lung cell suspensions upon ex vivo HDM restimulation (pg/mL) (**B**), IL-5 (pg/mg protein) in supernatants of lung homogenates (**D**). Correlation of IL-13 concentration in lung homogenates and the number of lymphocytes between the HDM-groups (**C**), IL-5 concentration in lung homogenates and the number of lymphocytes (**E**) and the number of eosinophils between the HDM-groups (**F**). PBS: PBS- sensitized and - challenged mice (white bars), HDM: HDM-sensitized and -challenged mice (grey bars). Contr: control diet, GOS: 1% GOS or 2.5% GOS diet, Bud: budesonide. Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.001$, **** $P < 0.0001$ compared to the HDM-contr group $n = 6$ mice/group. Correlation was assessed using the Spearman correlation test.

Decrease in frequency of activated T helper cells and Th2 and Th17 cells after dietary intervention with 2.5% GOS combined with budesonide

The expression of the early activation marker CD69 was increased in $CD4^+$ T helper (Th) cells in lungs of HDM-mice compared to the PBS control mice. After treatment with budesonide in the mice fed the control or GOS diet a decreased frequency of activated Th cells was observed (**Fig. 6A**). The frequency of $GATA3^+CD4^+$ Th2 cells was significantly increased in HDM-allergic mice fed the control diet compared to the PBS control group (**Fig. 6B**). Where GOS and budesonide alone did not affect this, there was a strong tendency toward decrease after dietary intervention with 2.5% GOS combined with budesonide (**Fig. 6B**). Although the frequency of $CD4^+ROR\gamma t^+$ Th17 cells or $CD4^+Tbet^+$ Th1 cells was not significantly increased in HDM-allergic mice compared to PBS control mice, a tendency toward a decrease in Th17 cell frequency was observed when mice were treated with 2.5% GOS combined with budesonide (**Fig. 6C-D**).

Discussion

This study was performed to examine the effects of a dietary intervention with 1% or 2.5% GOS provided before and during HDM-sensitization and -challenge on the anti-inflammatory actions of the glucocorticosteroid budesonide. Dietary intervention with GOS improved the effectiveness of budesonide therapy in most features of the HDM-asthma model. Murine HDM-allergic asthma models are commonly used to mimic some of the human features of asthma as they show AHR, airway inflammation and pulmonary cytokine release [30,31]. As we have shown in a previous study, GOS showed some similarities to budesonide in suppression of allergic features in a murine model for HDM-induced allergic asthma [20]. However, while in the previous study 1% GOS decreased AHR, no effect was observed in this study, whereas it did reduce airway eosinophilia, inflammation in the lungs, mast cell degranulation and suppress allergy related chemokine concentrations in lung tissue of HDM-allergic mice (for an overview see **Fig. 7**). By contrast, budesonide showed a strong tendency to reduce AHR which is in agreement with effects observed in OVA-induced chronic asthma models in which budesonide also decreased AHR [32,33]. However, considering the multiple factors capable of provoking AHR, combining dietary intervention using GOS with budesonide treatment may be capable of enhancing the effectiveness of budesonide in suppressing AHR. Indeed, in the current study this combination significantly reduced AHR in HDM-allergic mice and almost completely abolished eosinophil numbers, lymphocyte numbers and inflammation as observed using lung histology. This indicates that dietary intervention with GOS amplifies the effect of treatment with budesonide on AHR and inflammation.

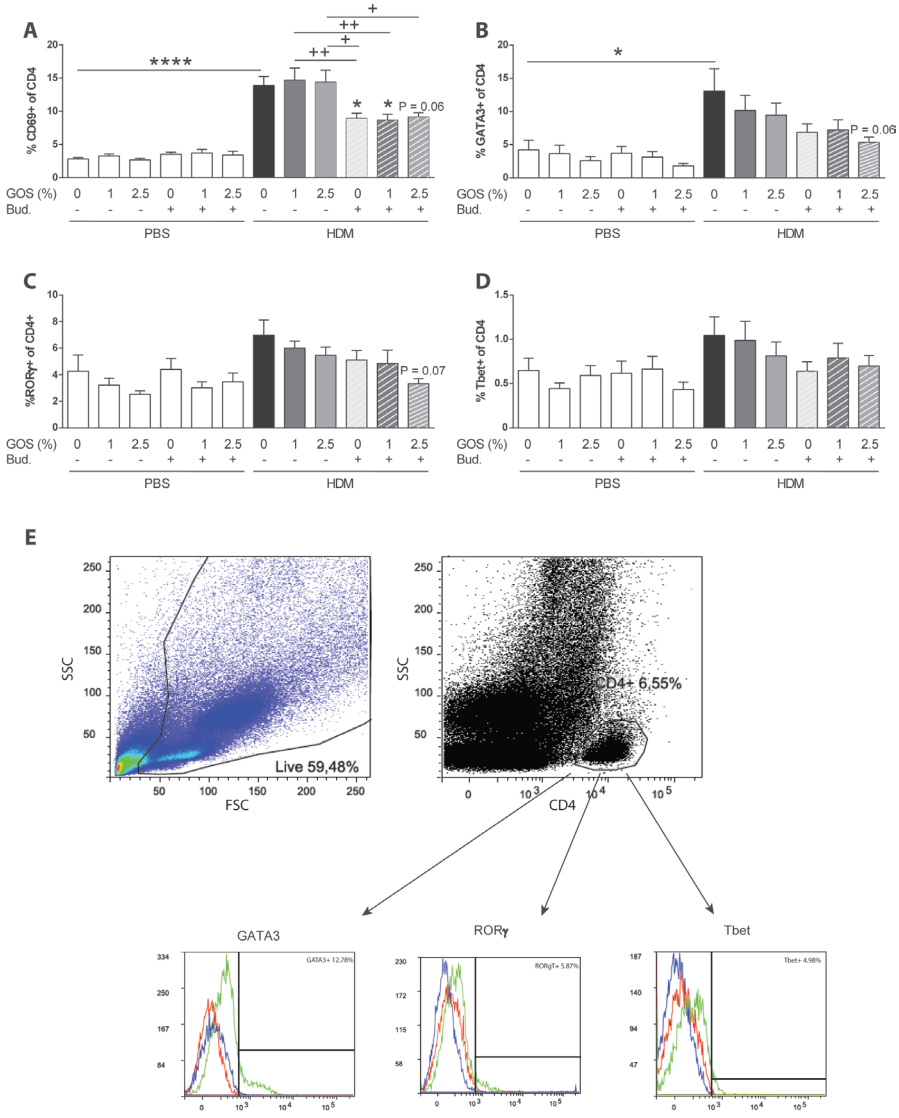

Fig. 6 The frequency of activated Th cells and Th2 and Th17 cells decreases after dietary intervention with 2.5% GOS combined with budesonide.

Representative dot plots and histograms of gating strategy of lung T helper cell subsets (**E**). Lymphocytes were gated based on FSC-SSC pattern, and T helper cells were gated based on expression of CD4. Within the $CD4^+$ population the frequency of GATA3 (Th2 cells), RORγt (Th17 cells) and Tbet (Th1 cells) was analyzed. In the histogram the blue line represents FMO control, red line isotype control and green line MFI of the specific antibody. Percentage of activated $CD4^+$ cells (**A**), $GATA3^+$ of $CD4^+$ cells (**B**), $ROR\gamma t^+$ of $CD4^+$ cells (**C**), and $Tbet^+$ of $CD4^+$ cells (**D**) was calculated. Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, **** $P < 0.0001$ compared to the HDM-contr group, $^+$ $P < 0.05$, $^{++}$ $P < 0.01$, n = 6 mice/group.

After allergen stimulation, IL-33 and GM-CSF are produced by bronchial epithelial cells and they are capable of activating mast cells, dendritic cells and ILC2 and are a chemo-attractant for Th2 cells [34-37]. In the airway tissue of asthmatics, mast cell numbers are increased and also an increase in activation and recruitment of Th2 lymphocytes and mast cell proteases is observed [38]. Indeed, the mucosal mast cell derived mMCP-1 concentration was significantly increased in HDM-allergic mice, whereas both dietary interventions with GOS and/or treatment with budesonide decreased this. Hence, both the diet as well as budesonide were capable of suppressing mast cell degranulation. In the airways of asthmatic patients, the level of IL-33, mainly expressed by bronchial epithelial cells, is increased [39,40]. Dietary intervention with GOS or treatment with budesonide slightly decreased IL-33 concentrations, which is in line with our previous observations [20], while in asthmatic patients budesonide had no effect on IL-33 levels [41]. However, when mice were fed the GOS diet and treated with budesonide, a significant decrease of IL-33 concentrations was observed in lung homogenate supernatants of HDM-allergic mice. As both mediators are important for activating DC and ILC2, reducing these cytokines may result in decreased HDM-sensitization and/or allergic symptoms. Bronchial epithelial cells are also capable of producing CCL20 and GM-CSF, which can attract immature DC to the lung [5,42]. CCL20 levels in these cells are upregulated after exposure to inflammatory stimuli [43], also in asthmatic patients CCL20 levels were increased [44]. GM-CSF is induced upon exposure of the bronchial epithelium to HDM and upstream of the allergic sensitization cascade [4,45]. In the current study, both GM-CSF and CCL20 were not enhanced in the HDM-allergic mice. However, typically in the GOS fed and budesonide treated mice these factors were reduced not only in the allergic but also in the control mice. Beyond CCL20 and GM-CSF, also increased levels of CCL17 were found in BALF, plasma/serum and sputum of patients with asthma [46-48]. DC that drive allergic sensitization are an important source of CCL17 as well as CCL22. These chemokines are both CCR4 ligands [49], and involved in allergic responses as CCR4 is expressed by allergen induced Th2 lymphocytes, which are attracted to the airways in humans [50-53]. In a murine asthma model, CCL22 neutralization with a specific antibody prevented the induction of AHR, whereas specific antibodies against CCL17 decreased airway eosinophilia and ameliorated AHR [54]. Both chemokines play an important role in the pathophysiology of asthma. Dietary intervention with 1% GOS tended to reduce both CCL17 and CCL22, while budesonide reduced CCL22. However the combination of the GOS diet and budesonide treatment was most effective in inhibiting both chemokines in HDM-allergic mice as well as in control mice (**Fig. 7**). This indicates that dietary GOS facilitates budesonide treatment in its capacity to further decrease allergy driving chemokines IL-33, GM-CSF, CCL20, CCL17 and CCL22, derived from both airway epithelial cells and DC.

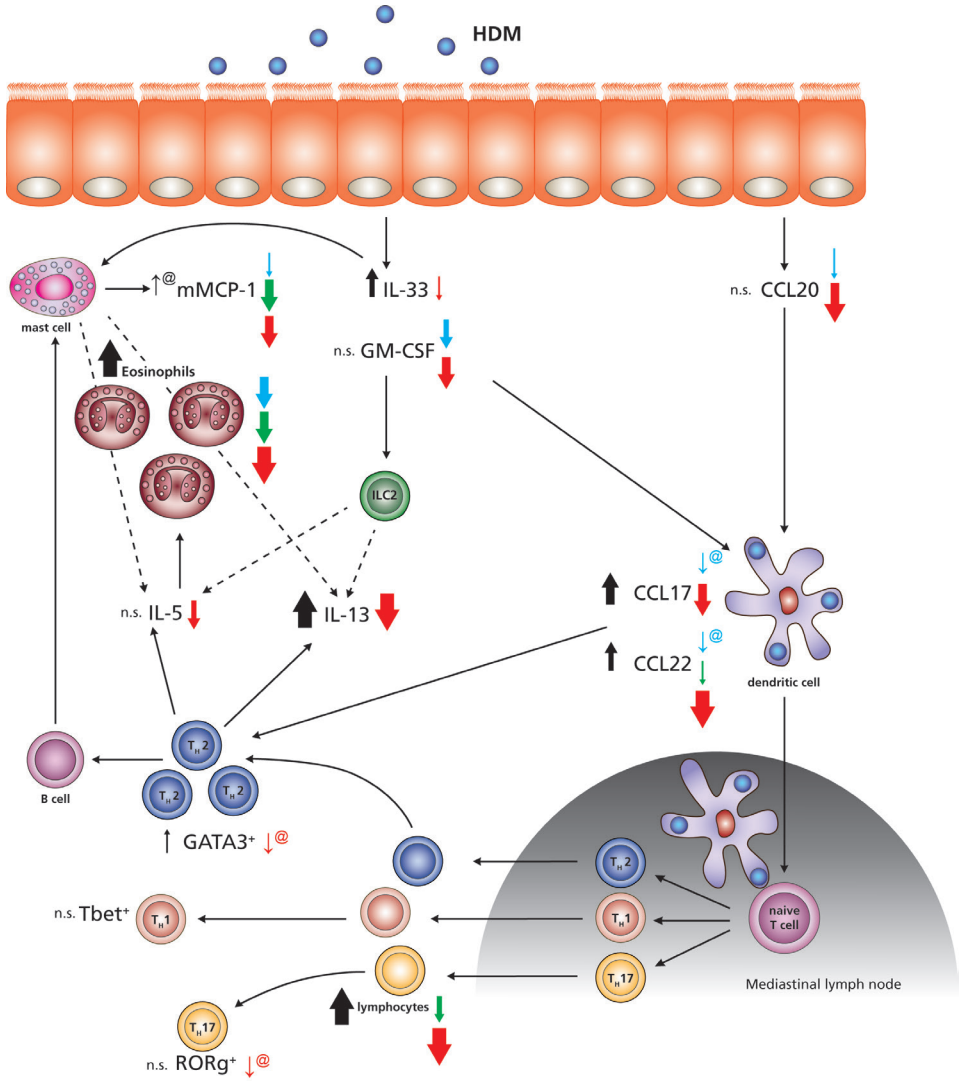

Fig. 7 Overview of the effects of the dietary intervention with GOS with or without glucocorticosteroid budesonide treatment.

After the initial exposure to HDM, IL-33, GM-CSF and CCL20 are known to be secreted by the airway epithelium, which will activate DC and ILC2. Both GM-CSF and CCL20 concentrations in lung homogenate supernatants were decreased after dietary intervention with GOS. Combination of the GOS diet with budesonide treatment enhanced this effect and also reduced IL-33 concentrations. CCL17 and CCL22 are secreted by activated DC, which can differentiate naïve T cells into Th2 cells and are chemo-attractants for Th2 cells. The GOS diet tended to decrease CCL17 and CCL22 concentrations, budesonide reduced CCL22 concentrations, whereas the combination showed a more pronounced suppression of both chemokines. Th2 cells as well as ILC2 and mast cells are able to produce IL-5 and IL-13. Concentrations of both cytokines were reduced after the combination >>

>> of GOS with budesonide. The number of lymphocytes and eosinophils was decreased by treatment with budesonide, whereas GOS alone suppressed the number of eosinophils. The combination of dietary GOS with budesonide treatment effectively suppressed the number of both leukocyte subtypes. mMCP-1 concentrations were decreased using dietary intervention with GOS or budesonide treatment as well as the combination of both. Only dietary intervention with GOS in combination with budesonide tended to suppress the Th2 and Th17 frequency in lung cell suspensions. Black: HDM-HDM effect, blue: effect of dietary intervention with GOS, green: effect of treatment with budesonide, red: effect of dietary intervention with GOS in combination with budesonide. $\downarrow^{@}\uparrow^{@}$ tendency to decrease or increase, $\downarrow\uparrow$ $P<0.05$, $\downarrow\uparrow$ $P<0.01$, $\downarrow\uparrow$ $P<0.001$, $\downarrow\uparrow$ $P<0.0001$ compared to HDM-HDM-mice fed the control diet.

As far as we know, such a mechanism has not been described before and may be part of the mechanism by which dietary GOS is capable of enhancing the effectiveness of budesonide treatment. Future studies may investigate the possibility of lowering the effective dose of glucocorticosteroids when provided together with a GOS diet. Anti-inflammatory therapies still have considerable side-effects, and lowering the dose of glucocorticosteroids may have beneficial effects for asthmatic patients. T lymphocytes are important in the development of asthma. Activated T helper cells were increased in OVA-sensitized and -challenged mice compared to control mice, and a positive correlation between AHR and CD69+ Th cells was shown [55]. In the current study budesonide treatment alone or in combination with a GOS diet showed a decrease in both AHR and activated T cell frequency. The reduction in allergy related mediators by budesonide treatment in GOS fed HDM-mice may have resulted in a reduced Th2 cell influx. Indeed Th2 type cytokine concentrations of IL-13 and IL-5 were lower in GOS fed budesonide treated HDM-allergic mice.

Antigen-specific Th2 cells, ILC2s and mast cells secrete IL-13 and IL-5 [34,56,57]. IL-13 can activate macrophages and contribute to AHR, whereas IL-5 is important for activation and survival of eosinophils [57,58]. In mild asthmatics, an increase in IL-13 mRNA levels was found in the BALF upon allergen challenge [59] and in bronchial biopsies of asthmatic patients, IL-5 mRNA levels were increased compared to healthy controls [60]. The decreased AHR in mice fed GOS and treated with budesonide may relate to the reduction in IL-13 levels in lung tissue. GATA3 is necessary for differentiation of naïve T cells into Th2 cells but also for the development and function of ILC2 [56,61]. In asthmatic patients the GATA3 expression in T cells was five times higher compared to healthy controls, whereas also bronchial epithelial cells express high levels of GATA3 [62]. However dietary intervention with GOS in combination with budesonide not only abrogated IL-13 concentrations in the lung homogenates, but also after *ex vivo* antigen specific restimulation of lung cell suspensions. This reduction of HDM-induced IL-13 release indicates that the generation and/or attraction of HDM-specific Th2 was reduced. Indeed in this study dietary intervention with 2.5% GOS combined with budesonide treatment showed a strong tendency toward decrease in frequency of $GATA3^{+}CD4^{+}$ Th2

cells. As GATA3 is known to play a crucial role in the production of IL-5 and IL-13 by Th2 cells [56], the reduced IL-13 may be explained, at least in part, by the reduction in Th2 cells present in the lung tissue. Besides its tendency in reducing the Th2 cell frequency, budesonide treatment in mice fed 2.5% GOS also tended to decrease the percentage of RORγt$^+$ CD4$^+$ Th17 cells [63], known to be increased in asthmatic patients [64].

In conclusion, budesonide treatment was most effective in reducing HDM-induced release of Th2 type chemokines and cytokines in mice fed a GOS diet in association with effective suppression of inflammatory cell influx, mast cell degranulation and AHR in these mice. Hence, dietary intervention using GOS may be a novel way to further improve the effectiveness of glucocorticosteroid drug therapy in asthma.

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CHAPTER SEVEN

Dietary galacto-oligosaccharides suppress airway eosinophilia and Th2 type inflammation either or not in combination with budesonide treatment in house dust mite-allergic mice

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Abstract

Background: House dust mite (HDM)-induced allergic asthma is characterized by airway hyperresponsiveness (AHR) and eosinophilic inflammation. Budesonide is frequently used as treatment in a clinical setting. However, dietary galacto-oligosaccharides (GOS) may suppress allergic inflammation as well, based on preclinical studies.
Aim: To study the effect of preventive dietary intervention with GOS on the therapeutic efficacy of different doses budesonide in a murine HDM-induced allergic asthma model.
Methods: BALB/c mice were intranasally (i.n.) HDM- or PBS-sensitized and -challenged while fed a GOS (1% w/w) or control diet. Budesonide (500, 100 or 20 µg/kg) was either or not oropharyngeally instilled during the challenge phase. AHR was measured, leukocyte subtypes were determined in the bronchoalveolar lavage fluid (BALF) and cytokines and chemokines were measured in lung cell supernatants. Lung inflammatory dendritic cells (DC) and T helper cell subtypes were phenotyped by means of flow cytometry.
Results: HDM-increased AHR and BALF eosinophil numbers. Budesonide treatment at all dosages tested, either or not combined with dietary GOS, suppressed these asthma features, whereas GOS alone reduced eosinophil numbers. The highest dose of budesonide reduced inflammatory DC, CCL17, CCL22 and CCL20. GOS or lower doses of budesonide either or not combined with GOS did not significantly suppress these features, although they did reduce CCL20 concentrations. Furthermore, GOS or budesonide as well as the combination of both reduced the Th2 cell frequency. GOS or the highest dose of budesonide reduced IL-4 secretion of lung cells upon *ex vivo* restimulation. IL-13 and IL-10 followed the same pattern.
Conclusion: The therapeutic treatment with budesonide suppressed AHR, pulmonary eosinophilia and Th2 type inflammation in a murine model for HDM-allergic asthma. Preventive dietary intervention with GOS had similar effects on the pulmonary inflammation. In this study, GOS did not significantly promote the therapeutic efficacy of budesonide.

Introduction

House dust mite (HDM) is an allergen that can trigger allergic diseases such as allergic rhinitis and asthma [1]. Around 300 million people worldwide suffer from asthma and the prevalence is still increasing. Asthma is characterized by airway inflammation containing high numbers of eosinophils and airway hyperresponsiveness (AHR) [2]. After stimulation of the epithelial cells with HDM, different cytokines and chemokines (e.g. CCL20) can be released [3]. CCL20 will attract immature dendritic cells (DC) to the lung and the DC will accordingly be activated by different cytokines [4]. These cytokines will induce the proliferation of innate lymphoid type 2 cells (ILC2), which in turn will produce cytokines like IL-4 and -13 [5]. Activated DC prime naïve T cells to differentiate into Th2 cells and regulatory T cells (Treg) in the local lymph nodes. The Th2 cells migrate from the lymph node to the lung in response to CCL17 and CCL22 and will produce IL-4 and -13, which contribute to AHR and eosinophilic inflammation [3,4,6]. Treg cells are able to inhibit Th2 cell responses in asthmatic patients [7]. The most common used treatment for asthma are inhaled glucocorticosteroids (e.g. budesonide) with or without long-acting β-agonists. These drugs are able to reduce asthma symptoms and the frequency of asthma exacerbations [8]. A disadvantage of using corticosteroids is the considerable induction of side effects, such as reduced growth in children, weight gain and osteoporosis in elderly [9,10]. Since current treatment is still not sufficient, optimal and novel approaches are needed to prevent and/or treat asthma. The development of asthma can be associated by changes in the intestinal microbiome, as has been demonstrated in different animal and human studies [11-14]. Galacto-oligosaccharides (GOS), which are non-digestible oligosaccharides, are selectively fermentable and have prebiotic capacities that support the activity and/or growth of bifidobacteria and lactobacilli in the intestinal tract [11,15,16]. Clinical evidence showed that GOS support digestive and immune function [17]. Non-digestible oligosaccharides either alone or combined with beneficial bacteria (e.g. *Bifidobacterium breve* M-16V) are capable of reducing allergic symptoms in cow's milk allergic mice and ovalbumin-induced asthmatic mice and rats [18-21]. Van de Pol *et al.* observed a reduced systemic Th2 cytokine production and an increase in peak expiratory flow in patients suffering from HDM-allergy and asthma after the intake of GOS in combination with long-chain fructo-oligosaccharides and *Bifidobacterium breve* M-16V, but no effect was found on bronchial allergic inflammation although proinflammatory cytokines such as IL-5 were affected [22]. In previous studies, it was demonstrated that GOS are capable of reducing Th2 related cytokine concentrations and HDM-induced airway eosinophilia in the lungs of mice [23]. In this study, it was investigated whether preventive dietary intervention with GOS can promote the therapeutic efficacy of different doses of budesonide (20, 100 and 500 µg/kg) in a murine HDM-induced allergic asthma model.

Materials and methods

Mice

Six-week old male BALB/c mice (Charles River, The Netherlands) were housed under bio-contained sterile conditions using HEPA ® filtered isocages ® (Tecniplast, Italy). Food and water were provided *ad libitum*. All animal experiments were conducted in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Utrecht University (DEC 2014.II.04.026).

Murine HDM-induced asthma model

Anaesthetized mice were intranasally (i.n.) sensitized with PBS or 1 µg HDM (Greer Laboratories, USA) on day 0 and challenged i.n. with PBS (control, PBS) or 10 µg HDM on days 7 to 11 while being fed a diet (AIN-93G) containing 0 or 1% w/w GOS (Vivinal® GOS syrup with approximately 59% galacto-oligosaccharides, 21% lactose, 19% glucose, and 1% galactose on dry matter (dry matter of 75%); FrieslandCampina Domo, The Netherlands), from day -14 till day 14. Carbohydrates in Vivinal® GOS were compensated isocalorically in the control diet by means of exchange against cellulose (for GOS), lactose (for lactose), and dextrose (for glucose) [23,24]. On day 7, 9 and 11 budesonide (500, 100, 20 µg/kg, Sigma-Aldrich, The Netherlands) was either or not instilled oropharyngeally, after isoflurane anesthesia, 6 h prior to the daily challenge and on day 13, 24 h prior to sacrifice on day 14 [25] (**Fig. 1**). In general, in different murine models for allergic asthma dosages of 100 - 600 µg/kg budesonide are frequently used [26-28]. Thus, doses budesonide used in our experiments can be regarded as low to high. On day 14, lung function was measured using invasive measurement of dynamic resistance (EMKA Technologies, France) in response to increasing doses of methacholine (acetyl-β-methyl-choline chloride, Sigma-Aldrich, The Netherlands). Data are presented as average lung resistance (R_L) in cm H_2O/(mL/sec.) [29].

Bronchoalveolar lavage

Lungs were lavaged 4 times with 1 mL of pyrogen-free saline (0.9% NaCl, 37ºC). The BALF cells were centrifuged (400 g, 5 min.) and pellets were pooled, total numbers of BALF cells were counted using a Bürker-Türk chamber (magnification 100x). Cytospin preparations were made and stained with Diff-Quick (Merz & Dade A.G., Switzerland) for the differentiation of BALF cell counts. The number of eosinophils was scored using light microscopy [30].

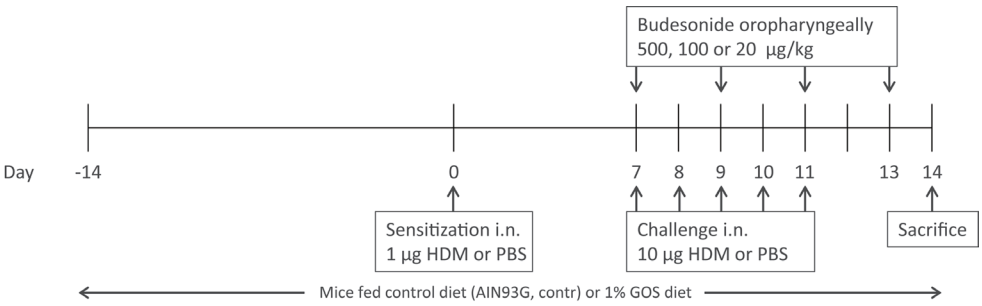

Fig. 1 Murine HDM-induced asthma model.

Mice were sensitized intranasally (i.n.) with PBS or HDM on day 0 and challenged i.n. with PBS (PBS) or HDM (HDM) on days 7 - 11 while being fed a diet (AIN-93G, contr.) containing 0 or 1% w/w GOS from day -14 till day 14. On day 7, 9, 11 and 13 budesonide (500, 100 or 20 µg/kg) was either or not instilled oropharyngeally. Mice were sacrificed on day 14.

Preparation of lung homogenates

Lung samples were homogenized in 1% Triton X100 (Sigma-Aldrich)/PBS containing protease inhibitor (Complete Mini, Roche Diagnostics, Germany). After centrifugation (14,000 rpm, 10 min.), supernatants were collected and stored at -20°C until further use. The Pierce BCA protein assay kit (Thermo Fisher Scientific, USA) was used to measure protein concentrations. Lung homogenates were diluted to a final concentration of 1 mg protein/mL [31,32].

Ex vivo lung restimulation with house dust mite

Lung cell suspensions were prepared after enzymatic digestion. Cells were washed and resuspended in RPMI 1640 culture medium (Lonza, USA) supplemented with 10% heat-inactivated FCS and 0.1% penicillin-streptomycin solution (Sigma-Aldrich). Lung cells (4 x 10^5 cells/well) were restimulated with or without 50 µg/mL HDM. After 4 days, supernatants were harvest and stored at -20°C until further analysis [33].

Lung T cell subsets and inflammatory DC assessed by flow cytometry

Single lung cell suspensions of lung cells were obtained. Cells were blocked using blocking buffer containing 1% BSA and 5% FCS in PBS for 30 min. 5 x 10^5 cells were plated per well and incubated at 4°C for 30 min. with antibodies against CD4-PerCP CY5.5, GATA3-PE, Tbet-eFLUOR660, Foxp3-APC, CD11b-PE, CD11c-PerCP CY5.5, MHCII-APCeFLUOR780, Ly6C-PE CY7 (eBioscience, The Netherlands) or matching isotype controls. Cells were permeabilized for intracellular staining. Flow cytometry was conducted using FACS Canto II (BD) and analyzed using Flowlogic Software (Inivai Technologies, Australia) [34].

Measurement of chemokines and cytokines

CCL17, CCL20 and CCL22 were measured with a DuoSet ELISA (R&D Systems, USA) according to manufacturer's protocol. Cytokine concentrations in supernatants of lung restimulation were determined using a standard IL-13 flex set or a standard Th1/Th2/Th17 assay (BD Biosciences, The Netherlands). The concentrations of these cytokines were expressed as pg/mg protein in lungs and pg/mL in restimulation supernatants.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Data were statistically analyzed using a one-way ANOVA followed by a Bonferroni's multiple comparisons test. $P < 0.05$ were considered significant. Statistical analyses were conducted using GraphPad Prism software (version 6.04).

Results

Budesonide treatment either or not combined with dietary GOS abrogates AHR in HDM-allergic mice

To investigate lung function in HDM-asthmatic mice, lung resistance was measured after nebulizing increasing doses of methacholine. A significant increase in lung resistance was observed in HDM-control mice compared to PBS-control mice. Treatment with all doses budesonide either or not combined with dietary GOS showed a significant decrease in lung resistance compared to HDM-mice fed the control diet (**Fig. 2A**). Furthermore, a significant increase in eosinophil numbers was observed in HDM-control mice compared to PBS-control mice (**Fig. 2B**). Dietary GOS or budesonide (all doses) reduced the number of eosinophils compared to HDM-control mice. The other groups showed the same tendency (**Fig. 2B**).

Highest dose budesonide treatment reduces inflammatory DC and CCL17 and CCL22 concentrations in the lungs of HDM-allergic mice

The frequency of inflammatory DC tended to increase in HDM-allergic mice compared to PBS-control mice and was significantly decreased after treatment with the highest dose of budesonide used (**Fig. 3A**). GOS alone showed no effect on inflammatory DC and did not enhance effectiveness of any doses of budesonide. CCL20, CCL17 and CCL22 were significantly increased compared to the PBS-control group in lung homogenates supernatants of HDM-control mice, (**Fig. 3B-D**). CCL20 concentrations were decreased after all budesonide doses used for treatment either or not combined with dietary GOS (**Fig. 3B**). Only the highest dose of budesonide treatment reduced CCL17 and CCL22 concentrations, whereas GOS alone showed no effect and did not improve the effect of the lower doses of budesonide (**Fig. 3B-D**).

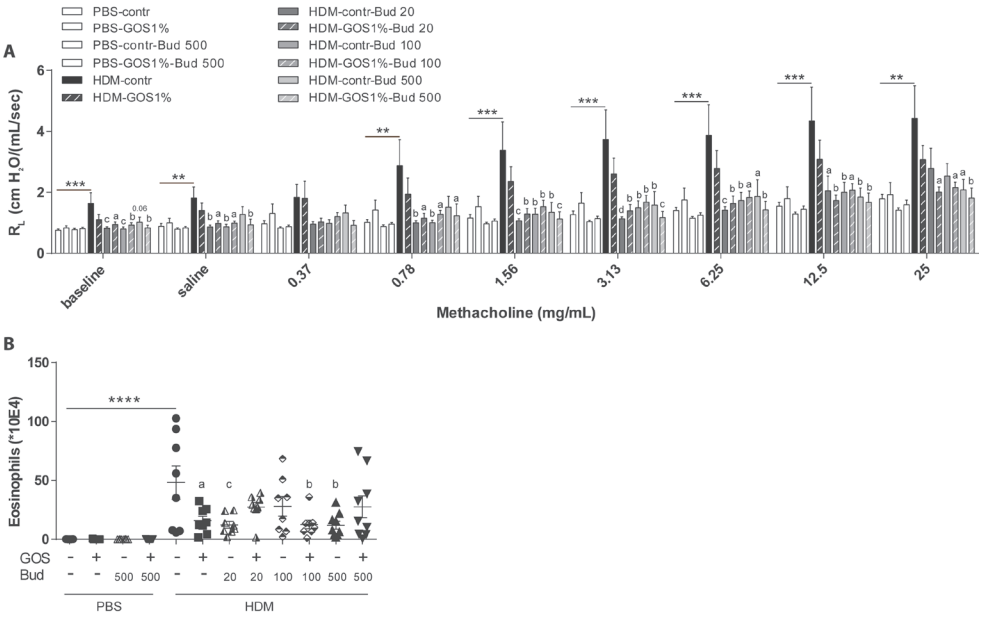

Fig. 2 Lung resistance in HDM-control mice was normalized after budesonide treatment either or not combined with dietary GOS.

On day 14 airway resistance (R_L) was measured in response to increasing doses of methacholine (**A**) and eosinophilic inflammation was counted in the BALF (**B**). PBS: PBS-sensitized and -challenged mice, HDM: HDM-sensitized and -challenged mice. GOS: 1% GOS diet; Bud: budesonide treatment (500, 100 or 20 µg/kg). Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to the PBS control group, [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$, [d] $P < 0.0001$ compared to the HDM-control group, n = 7-9 mice/group.

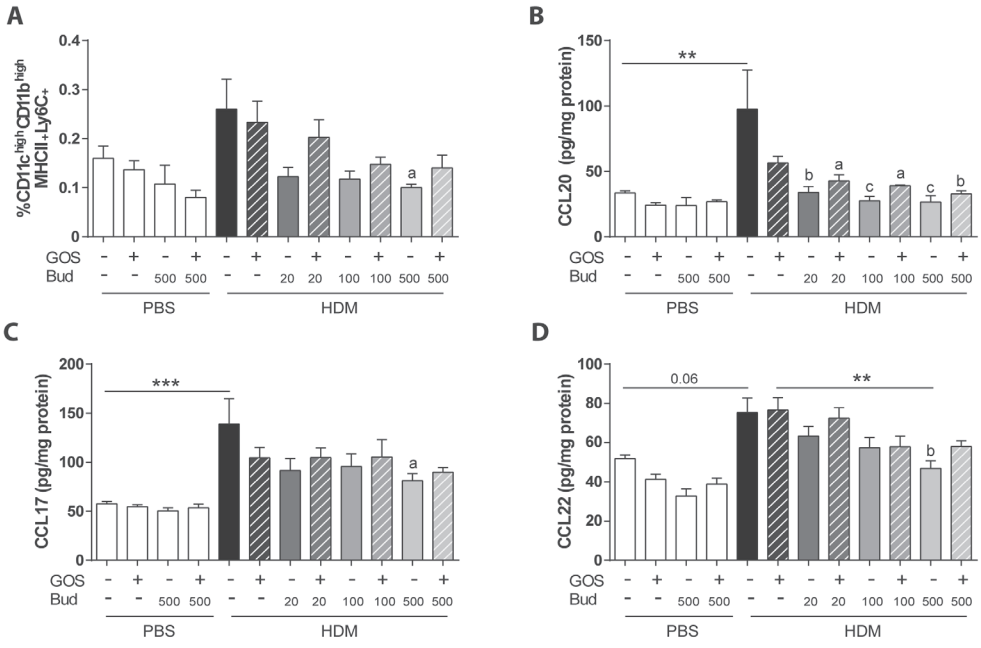

Fig. 3 Reduced frequency of inflammatory DC and related chemokines in the lungs after budesonide treatment.

Cells were gated on FSC-SSC pattern and expression of CD11b and CD11c. Co-expression of inflammatory DC was analyzed (**A**). Chemokines CCL20 (**B**), CCL17 (**C**) and CCL22 (**D**) (pg/mg protein) were measured in the supernatants of lung homogenates. PBS: PBS-sensitized and -challenged mice, HDM: HDM-sensitized and -challenged mice. GOS: 1% GOS diet; Bud: budesonide treatment (500, 100 or 20 µg/kg). Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. ** $P < 0.01$, *** $P < 0.001$ compared to the PBS control group, [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$ compared to the HDM-control group, n = 3-4 mice/group for DC subsets, n = 5-6 mice/group for chemokine concentrations.

Th2 cell frequency decreases after dietary intervention with GOS and/or budesonide treatment

In HDM-control mice the frequency of Th2 cells was significantly increased compared to the PBS-control mice (**Fig. 4A)**. The GOS diet and all doses of budesonide treatment either or not combined with dietary GOS effectively decreased the percentage of Th2 cells (**Fig. 4A**). The Th1 cell frequency was not increased in HDM-control mice and was also not significantly influenced by the interventions (**Fig. 4B**). In addition, the frequency of Treg cells was significantly increased in HDM-control mice compared to PBS-control mice. Treatment with budesonide decreased the frequency of Treg cells, but not in mice fed the GOS diet (**Fig. 4C**). IL-4 and -10 concentrations in lung homogenate supernatants were significantly increased in the HDM-control group compared to the PBS-control

group after *ex vivo* restimulation with HDM, whereas IL-13 tended to increase (**Fig. 4D-F**). The GOS diet or the highest dose of budesonide treatment decreased the concentration of IL-4 and a similar pattern was found for IL-13 and IL-10 (**Fig. 4D-F**).

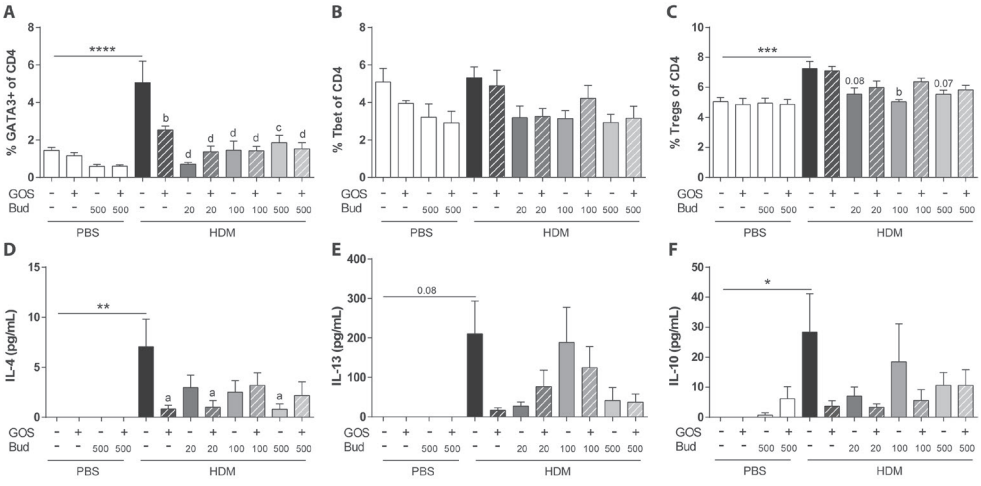

Fig. 4 Th2 cell frequency and Th2 related cytokines decrease after budesonide treatment and/or dietary intervention with GOS.

Lymphocytes were gated on FSC-SSC pattern, and expression of CD4. Co-expression of Th2 cells (**A**), Th1 cells (**B**) and Treg cells (**C**) were analyzed. IL-4 (**D**), IL-13 (**E**) and IL-10 (**F**) concentrations (pg/mL) were measured in supernatants of lung cells after *ex vivo* restimulation with HDM. PBS: PBS-sensitized and -challenged mice, HDM: HDM-sensitized and -challenged mice. GOS: 1% GOS diet; Bud: budesonide treatment (500, 100 or 20 µg/kg). Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared to the PBS control group, [a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.001$, [d] $P < 0.0001$ compared to the HDM-control group, n = 5-6 mice/group.

Discussion

The aim of this study was to investigate whether preventive dietary intervention with GOS can promote the therapeutic efficacy of different doses of budesonide (20, 100 and 500 µg/kg) in a murine HDM-induced allergic asthma model. Effects on AHR, pulmonary inflammation, chemokine and cytokine concentrations, inflammatory DC and different T cell subtypes in the lung have been analyzed. Human features of asthma, like AHR, pulmonary cytokine release and airway inflammation can be mimicked in murine models of HDM-allergic asthma [35,36]. In the current study, all doses of budesonide used either or not combined with dietary GOS reduced AHR significantly. From literature it

is known that budesonide treatment in chronic OVA-challenged mice decreased AHR as well [37,38]. Although GOS alone did not suppress AHR in this study, eosinophil numbers were decreased after dietary intervention with GOS. This was comparable to the treatment with the highest dose of budesonide as shown in a previous study [23]. GOS alone did not significantly suppress AHR in this study, but showed a similar declining pattern as in a previous study where a significant reduction of AHR was found upon dietary intervention with GOS [23]. Eosinophil numbers were decreased after dietary intervention with GOS like treatment with the highest dose of budesonide, which was similar to a previous study [23].

One of the first innate immune cells that will come in contact with HDM in the airways are DC which are very well known for their involvement in the pathophysiology of asthma [39]. Increased numbers of DC have been observed after allergen exposure in murine models of asthma [40,41]. Th2 effector cells and cytokine production were abolished when lung DC in CD11c-DTR transgenic mice were depleted [42]. After depletion of DC in OVA-sensitized mice, the AHR was abrogated and the asthma phenotype was lost as well [43]. It is also known that asthmatic patients have increased numbers of intraepithelial DC in the airways compared to healthy controls [44]. Furthermore, segmental challenge increases the pDC and mDC in BALF of allergic asthmatics as well [45]. In HDM-allergic mice the frequency of inflammatory DC ($CD11c^{high}CD11b^{high}MHCII^{+}Ly6C^{+}$) tended to be increased. Treatment with the highest dose of budesonide effectively abrogated this. The latter is in agreement with clinical studies in which corticosteroids were able to reduce DC numbers in asthmatic patients [44]. In contrast to the effect of budesonide, dietary intervention with GOS did not dampen the inflammatory DC influx. Furthermore, dietary GOS could not enhance the effectiveness of the lower doses of budesonide in reducing the percentage of inflammatory DC. Epithelial cell-derived CCL20 is a chemokine that attracts immature DC to the lung [4,46] and was found to be increased in asthmatic patients compared to healthy controls [47]. In the current study, CCL20 concentrations were significantly increased in HDM-control mice compared to PBS-control mice. All used doses of budesonide significantly reduced CCL20 either or not combined with GOS. Although lowering the doses of budesonide treatment was less effective in suppressing inflammatory DC it did suppress the chemokine involved in the attraction of DC. Also a significant increase in CCL17 and CCL22 was observed in lung tissue of HDM-control mice and the highest dose budesonide treatment used was able to reduce CCL17 and CCL22 concentrations. Indeed, CCL17 and CCL22 link to the pathology of HDM-asthma. After challenge with HDM, the concentration of CCL17 was increased in the BALF of patients suffering from atopic asthma. Mast cells, regulatory T cells and Th2 cells express CCR4, which is a receptor for CCL17 and CCL22 [48]. The majority of the T lymphocytes were CCR4 positive in bronchial biopsies of asthmatic patients [49]. Similar to our observations in the HDM-control mice, inhaled budesonide showed a reduced CCL17 expression in epithelium of bronchial biopsies from human asthmatic patients, while high doses of corticosteroids also reduced the CCL22 concentrations in biopsies of asthmatic children

[50,51]. Overall, these results indicate that the highest dose of budesonide used resulted in decreased CCL20 and inflammatory DC frequency, coinciding with reduced CCL17 and CCL22 production in the lungs. However, GOS or lowering the dose of budesonide treatment either or not combined with GOS were not capable of significantly reducing CCL17 and CCL22, although a similar reducing pattern compared to CCL20 was observed.

For the differentiation of $CD4^+$ T cells into Th2 cells, GATA3 is important [52,53]. GATA3 expression in T cells of asthmatic patients is higher compared to healthy controls [54]. Furthermore, Th2 cells contribute to the induction of asthmatic symptoms since they are able to produce Th2 cytokines and attract eosinophils [55]. In this study, dietary GOS, either dosages of budesonide or the combination of both, effectively suppressed the Th2 cell frequency in the HDM-control mice. From literature it is known that children with moderate to severe asthma have higher numbers of Tregs than patients with mild asthma [56], whereas in adults $CD4^+CD25^+$ Treg were higher in blood of mild asthmatics compared to severe asthmatics and healthy controls [57]. In this study, the frequency of Treg cells was significantly increased in the HDM-control group compared to the PBS-control group and is decreased after the treatment with budesonide. In contrast, dietary intervention with GOS suppressed the Th2 cell frequency in the lung of HDM-control mice whereas the percentage of Treg cells remained unaffected. As Treg are known to suppress allergic inflammation, the GOS diet may enhance the Treg over Th2 cell balance in association with its protective effect in allergic asthma. GOS, however, was not able to prevent the Treg decrease by budesonide in the co-treated groups. IL-4 is important for the differentiation of Th2 cells, which in turn can produce IL-4 upon antigen presentation by DC [4]. IL-4 deficient mice are protected from developing asthma by a defect in eosinophil recruitment and eosinophilic inflammation was reduced after nebulizing with anti-IL-4 antibodies [58,59]. Also in patients with asthma, IL-4 levels were increased in serum and BALF [60]. Budesonide treatment and dietary GOS were able to decrease IL-4 concentrations in lung cell suspensions after *ex vivo* antigen specific restimulation with HDM. Another Th2 cytokine, IL-13, contributes to AHR and activates macrophages [61,62]. After treatment with anti-IL-13 antibodies, mucus production and AHR were decreased in mice, this was also shown in IL-13 deficient mice after allergen challenge [63-65]. In asthmatic patients IL-13 levels were increased in the airways, BALF and sputum [66,67]. In this study, budesonide treatment or dietary GOS tended to decrease IL-13 concentrations after *ex vivo* restimulation with HDM. Furthermore, a similar pattern was observed for IL-10 which expression is also higher in asthmatic patients compared to healthy controls.

In conclusion, therapeutic treatment with budesonide suppressed AHR, eosinophilia and Th2 type inflammation. Preventive dietary intervention with GOS had similar effects on pulmonary inflammation since it reduced eosinophilic inflammation and the Th2 cell frequency in the lungs of HDM-allergic mice. In this study, GOS did not significantly promote the therapeutic efficacy of budesonide.

Acknowledgments

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CHAPTER EIGHT

Inflammation-induced expression of the alarmin IL-33 can be suppressed by galacto-oligosaccharides

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Abstract

Background: The alarmin interleukin-33 (IL-33) and its receptor ST2 play an important role at mucosal barrier tissues and seem to be crucial for Th2 cell mediated host defense. Galacto-oligosaccharides (GOS), used in infant formulas, exhibit gut and immune modulatory effects. To enhance our understanding of the immunomodulatory capacity of GOS, this study investigated the impact of dietary GOS intervention on IL-33 and ST2 expression related to intestinal barrier dysfunction or asthma.

Methods: B6C3F$_1$ and BALB/c mice were fed a control diet with or without 1% GOS. To simulate intestinal barrier dysfunction, B6C3F$_1$ mice received a gavage with the mycotoxin deoxynivalenol (DON). To mimic asthma-like inflammatory airway responses, BALB/c mice were sensitized on day 0 and challenged on days 7-11 with house dust mite (HDM). Intestines or lungs were collected for IL-33 and ST2 analysis by qRT-PCR, immunoblotting and immunohistochemistry.

Results: Dietary GOS counteracted the DON-induced IL-33 mRNA expression and changed the IL-33 distribution pattern in the mouse small intestine. The IL-33 mRNA expression was positively correlated to the intestinal permeability. A strong positive correlation was also observed between IL-33 mRNA expression in the lung and the number of BALF cells. Reduced IL-33 protein levels, altered IL-33 distribution, and decreased ST2 mRNA expression levels were observed in the lungs of HDM-allergic mice after GOS intervention.

Conclusions: Dietary GOS mitigated IL-33 at the mucosal surfaces in a murine model for intestinal barrier dysfunction and HDM-induced asthma. This promising effect of GOS may open new avenues to use GOS not only as a prebiotic in infant nutrition, but also as a functional ingredient that targets inflammatory processes and allergies associated with IL-33 expression.

Introduction

Non-digestible oligosaccharides, such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS), are currently added to infant milk formulas to achieve an intestinal microbiota composition more similar to that of breastfed infants [1]. Experimental evidence clearly indicates that infants given infant formula enriched with oligosaccharides, particularly GOS, showed a significant increase in Bifidobacteria and Lactobacilli in the microbiota, which resembles that of breastfed infants. From other studies we know that growth of pathogens is reduced [2]. Besides their effects on the intestinal flora, oligosaccharides can also modulate the activity of the immune system and regulate natural immune mechanisms [3]. Interestingly, a reduction in the incidence of allergic manifestations and infections was observed after nutritional application with prebiotic oligosaccharides (90% GOS/10% lcFOS) [4, 5]. Recent work from our group demonstrated that GOS also have microbiota-independent properties on intestinal epithelial cells [6, 7]. The homeostasis of the epithelial inflammatory response within the intestinal epithelium can be regulated by the axis of interleukin-33 (IL-33) and its receptor ST2, which belongs to the IL-1/TLRs receptor superfamily [8]. In recent years, scientific interest in IL-33 has grown, since this cytokine seems to be an indicator of Th2 mediated host defense and plays an important role in mucosal barrier tissues, such as the intestine and the airway surface, where it functions as an endogenous danger signal in response to tissue damage [9]. Moreover, increased expression of IL-33 and its receptor ST2 has been reported in asthma and ulcerative colitis patients related to pro-inflammatory effects [8, 10, 11].

Since there are indications that GOS can modulate barrier and immune functions and directly interact with epithelial cells, this study aimed to investigate whether the cytokine IL-33 and its receptor ST2 can be affected by the prebiotic GOS. An acute model for intestinal barrier dysfunction and a house dust mite (HDM)-induced allergic asthma model with an inflammation-induced expression of IL-33 were used to test our hypothesis. Dietary intervention with GOS mitigated the inflammation-induced expression of the alarmin IL-33 in these two different murine models, while a decreased ST2 mRNA expression was observed in lungs of HDM-allergic mice fed a GOS diet.

Materials and methods

Animal studies

All *in vivo* experiments were conducted in compliance with the guidelines of Ethical Committee on the use of Laboratory Animals of the Utrecht University (DEC 2012.III.02.012 and 2013.II.01.003). Male B6C3F$_1$ and male BALB/c mice (6-8 weeks old) obtained from Charles River (The Netherlands) were housed under controlled conditions in standard laboratory cages or under bio-contained sterile conditions using HEPA® filtered isocages® (Tecniplast, Italy), respectively. The present data were obtained from further analyses of samples from recently published studies [6, 12].

Animals were fed a control diet (AIN-93G) with or without 1% w/w GOS (Vivinal® GOS syrup with approximately 59% galacto-oligosaccharides, 21% lactose, 19% glucose, and 1% galactose on dry matter (dry matter of 75%); FrieslandCampina Domo, The Netherlands) from day -14 to 0 (DON gavage study) and from day -14 to 14 (asthma study). Carbohydrates in Vivinal® GOS were compensated isocalorically in the control diet by means of cellulose (for GOS), lactose (for lactose), and dextrose (for glucose). Food and water were provided *ad libitum*.

For the DON gavage study (**Supplementary Fig. 1**), DON (D0156; Sigma, USA) was administered at a dose of 25 mg/kg body weight (BW) by a single oral gavage to B6C3F$_1$ mice at day 0; control mice received sterile PBS. Six hours after the gavage, mice were sacrificed by cervical dislocation and the distal small intestine was collected for mRNA isolation and immunohistochemistry. For the asthma study (**Supplementary Fig. 1**), BALB/c mice were intranasally (i.n.) sensitized with 1 µg house dust mite (HDM)/40 µL PBS (Greer Laboratories, USA) under isoflurane anaesthesia on day 0 and i.n. challenged daily on days 7 to 11 with PBS (control) or 10 µg HDM/40 µL PBS. At day 14, mice were sacrificed by an intraperitoneal overdose of pentobarbital (600 mg/kg, Nembutal™, Ceva Santé Animale, The Netherlands) and the lungs were collected for mRNA isolation, western blot analysis and immunohistochemistry.

Fluorescein isothiocyanate (FITC)-dextran permeability assay

To assess intestinal permeability changes, the intestinal permeability to 4 kDa FITC-dextran (Sigma-Aldrich, USA) was measured as described previously [13]. Briefly, 2 h after DON administration, all mice received FITC-dextran (500 mg/kg BW) by an oral gavage. Four hours after the FITC-dextran gavage, blood was obtained by heart puncture directly after cervical dislocation, and the appearance of FITC-dextran in blood serum was measured with a spectrofluorometer (FLUOstar Optima; BMG Labtech, Germany).

Bronchoalveolar lavage

The trachea of the mice (asthma model) were cannulated and lungs were lavaged four times with 1 mL saline solution (0.9% NaCl, 37°C). The bronchoalveolar lavage fluid (BALF) cells were centrifuged (400 g, 5 min.) and total number of BALF cells were counted using a Bürker-Türk chamber. Differential cell counts were performed on cytospin preparations stained by DiffQuick (Dade, Switzerland).

qRT-PCR

Gene expression was determined by quantitative RT-PCR, as described previously [13]. In brief, distal small intestine and lung tissue samples were homogenized in RNA lysis buffer with β-mercaptoethanol and RNA was extracted using spin columns according to manufacturer's instructions (Promega, USA). cDNA was prepared from 1 µg RNA using the iScriptTM cDNA Synthesis kit (Bio-Rad, USA). qRT-PCR was performed using the MyIQ single-colour real-time PCR detection system (Bio-Rad, USA) with iQSYBR Green Supermix (Bio-Rad, USA) and IL-33 and ST2 primers were derived from the NCBI GenBank and manufactured commercially (Eurogentec, Belgium). IL-33: forward: 5'-TTATGAAGCTCCGCTCTGGC-3'; reverse: 5'-CCAAAGGCAAAGCACTCCAC-3' and ST2 forward: 5'-CAAGTAGGACCTGTGTGCCC-3'; reverse: 5'-CGTGTCCAACAATTGACCTG-3'. The relative amounts of gene expression were standardized and calculated by the expression of house-keeping control gene (ß-actin) as an internal standard, using the $2^{-\Delta\Delta Ct}$ method.

Immunoblotting

Total protein extracts were prepared as described previously [12]. Equal protein amounts were separated by SDS-PAGE, blotted onto PVDF membranes and analyzed with goat anti-mouse IL-33 (R&D systems, USA, mouse monoclonal, AF3626, 1:500), rabbit anti-ST2 (Abcam, England, rabbit polyclonal, ab25877, 1:1000) or rabbit anti-ß-actin (Cell Signaling, USA, rabbit monoclonal, #4970, 1:4000). Appropriate horseradish peroxidase-conjugated secondary antibodies (Dako, Denmark) were used for detection by enhanced chemiluminescence (Amersham Biosciences, The Netherlands). The band intensity was acquired by a GS710 calibrated imagine densitometer (Bio-Rad, USA).

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded distal small intestine (Swiss roll) and lung tissue using the IL-33 antibody (R&D systems, USA, mouse monoclonal, AF3626, 1:500) or ST2 antibody (Abcam, England, rabbit polyclonal, ab25877, 1:1200). For antigen retrieval, the slides were boiled in 10 mM citrate buffer (pH 6.0) for 10 min. in a microwave [14]. Digital images were acquired using an Olympus BX50 microscope (Olympus Europa GmbH, Germany) equipped with a Leica 320 digital camera (Leica Microsystems, Germany). No staining was detected in negative controls, in which the primary antibody was omitted (**Supplementary Fig. 2**).

ELISA

IL-33 and ST2 concentrations in BALF were measured by ELISA using the Mouse IL-33 ELISA set (R&D Systems, USA, DY3626) and Mouse ST2 ELISA set (R&D Systems, USA, DY1004) according to manufacturer's instructions.

Statistical analysis

Statistical analyses were performed by using GraphPad Prism 6.0 (Graphpad, USA). Differences between groups were statistically determined by using One-way ANOVA followed by a Bonferroni multiple comparison test. Spearman's rank tests were conducted for analyses of correlation. Results were considered to be statistically significant when $P < 0.05$.

Results

IL-33 mRNA expression is correlated to the intestinal permeability changes and to the number of BALF cells

In the murine model for intestinal barrier dysfunction, a strong positive correlation was observed between the IL-33 mRNA expression levels in the mouse distal small intestine and intestinal permeability to 4 kDa FITC-dextran ($r = 0.6634$, $P = 0.002$) (**Fig. 1A**). In the HDM-induced asthma model, IL-33 mRNA expression levels in the lung positively correlated with the number of total BALF cells ($r = 0.5424$, $P = 0.0008$) (**Fig. 1B**). Differential analysis of the BALF cells showed an increase in eosinophils, but also the number of lymphocytes and neutrophils was significantly elevated in HDM-allergic mice compared to control mice (**Supplementary Table 1**).

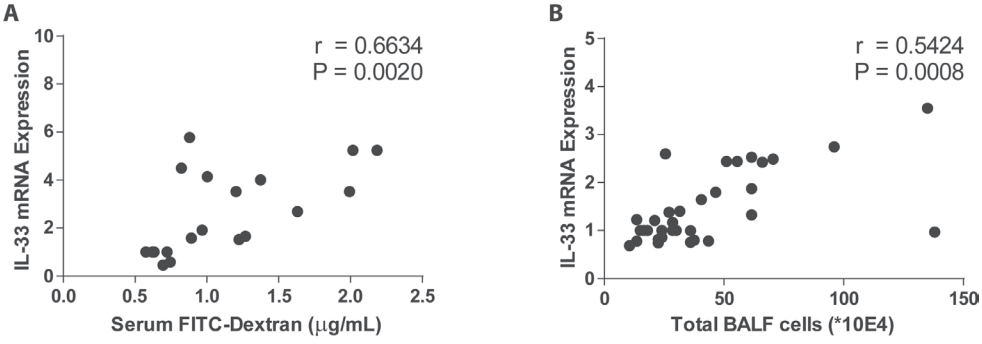

Fig. 1 IL-33 mRNA expression is correlated to the intestinal permeability and to the number of BALF cells. Correlation of IL-33 mRNA expression in the distal small intestine and the intestinal permeability to 4 kDa FITC-dextran in the murine model for DON-induced intestinal barrier dysfunction (**A**). Correlation of IL-33 mRNA expression in the lungs and the total amount of BALF cells in the HDM-induced asthma model (**B**). Correlation was analyzed using the Spearman correlation test.

Dietary intervention with GOS counteracts the DON-induced IL-33 mRNA expression and distribution pattern in distal part of the mouse small intestine

The prominent increase in IL-33 mRNA expression in the distal small intestine observed after DON gavage was prevented by GOS, since the IL-33 mRNA levels in DON-treated animals fed a GOS diet were significantly lower than in the DON-treated animals given a control diet (**Fig. 2A**).

The immunohistochemical staining confirmed that the IL-33 production was increased in the distal small intestine after DON gavage (**Fig. 2C**) compared to the control mice fed a control or GOS diet (**Fig. 2B** and **2D**). The most pronounced differences were observed in the epithelial layer around the villi. The GOS diet prevented this DON-induced IL-33 production in the distal small intestine and a lower amount of IL-33-expressing epithelial cells was observed (**Fig. 2E**). Related to the IL-33 mRNA expression, the increased ST2 mRNA expression in the distal small intestine of DON-treated animals was reduced in DON-treated animals fed a GOS diet, however this decrease was not significantly different (**Fig. 2F**). The ST2 immuno-histochemical staining depicted in **Fig. 2G-J** showed a strong expression pattern in the cytoplasm of the intestinal epithelial cells and ST2 was also detected in scattered lamina propria mononuclear cells. Similar patterns of ST2 expression for all experimental groups were observed (**Fig. 2G-J**).

Dietary intervention with GOS reduces IL-33 and ST2 mRNA expression and IL-33 protein levels in the lungs of HDM-allergic mice

The increase in IL-33 mRNA expression in the lungs of HDM-allergic mice was partly reduced by dietary intervention with GOS (**Fig. 3A**), however, this decrease was not statically significant. The same trend was observed for the IL-33 concentration in BALF (**Fig. 3B**). Moreover, the IL-33 protein levels in lung tissue homogenates of GOS-treated HDM-allergic mice were significantly decreased compared with non-treated HDM-allergic mice (**Fig. 3C**). Comparable with the western blot data, the immuno-histochemical staining indicated that in lungs of HDM-allergic mice more IL-33 expressing cells were present (**Fig. 3E**) compared to the control mice fed a control or GOS diet (**Fig. 3D-F**). Dietary intervention with GOS caused a decrease in IL-33 expression in the lungs of HDM-allergic mice compared to the non-treated HDM-allergic mice (**Fig. 3G**). Furthermore, a clear increase in ST2 mRNA expression was observed in the lungs of HDM-allergic mice, which was significantly reduced in HDM-allergic mice fed a GOS diet (**Fig. 4A**). The ST2 levels in BALF (**Fig. 4B**) and the ST2 protein levels in the lung (**Fig. 4C**) did not significantly differ between the experimental groups, which was confirmed by an immuno-histochemical staining showing the airway epithelium as primary source for ST2 (**Fig. 4D-G**).

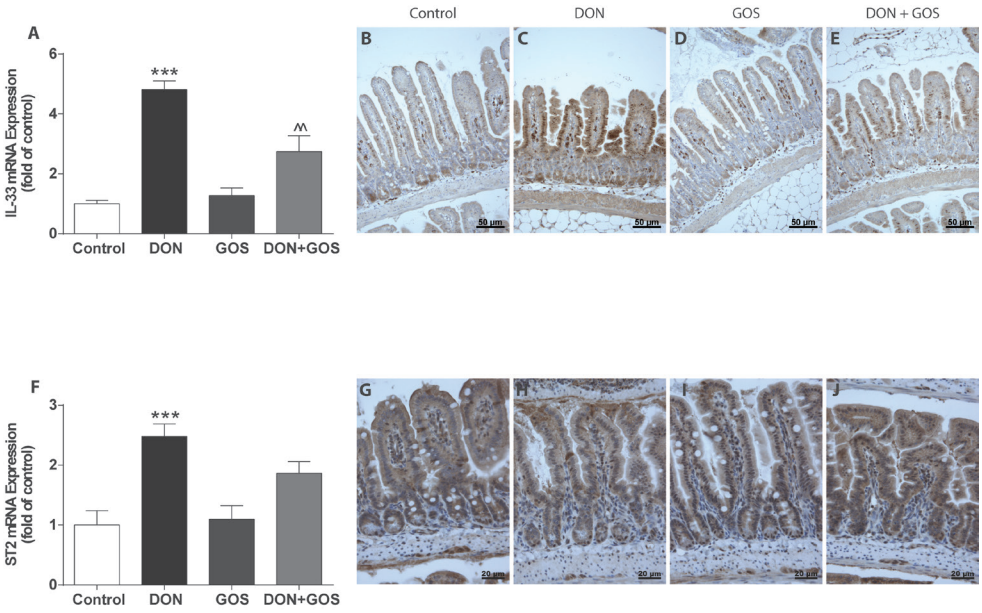

Fig. 2 Dietary intervention with GOS counteracts the DON-induced IL-33 mRNA expression and distribution pattern in distal part of the mouse small intestine.

Mice were fed a control diet or a diet supplemented with GOS for 2 weeks followed by an oral gavage with DON (25 mg/kg BW). 6 h after the DON challenge the mRNA levels of IL-33 and ST2 were measured by qRT-PCR (**A, F**). Results are expressed as IL-33 mRNA expression (fold of control)(qRT-PCR, normalized to β-actin) as mean ± SEM. (*** $P < 0.001$; significantly different from the control group ^^ $P < 0.01$; significantly different from the DON-treated animals). n = 5-6 animals/experimental group. For immunohistochemistry, Swiss-rolled paraffin sections obtained from distal small intestine were stained with anti-IL-33 (**B, C, D, E**) and anti-ST2 (**G, H, I, J**) antibodies as described in Materials and Methods. Magnification IL-33 staining: 200x and ST2 staining: 400x.

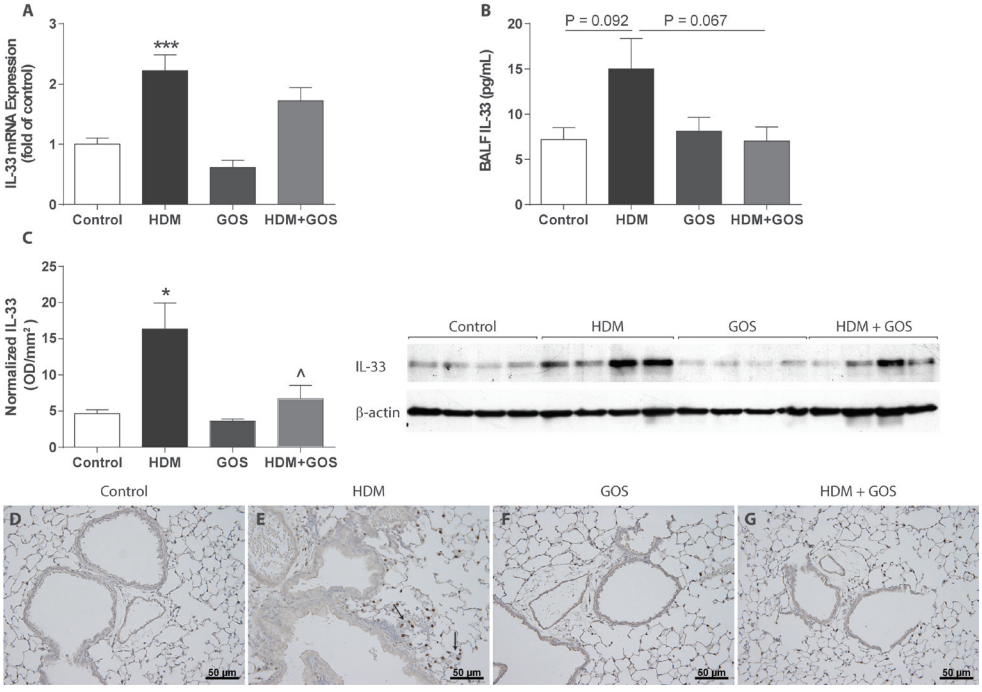

Fig. 3 Dietary intervention with GOS reduces IL-33 protein levels and expression in the lungs of HDM-allergic mice.

Mice fed a control diet or a diet supplement with GOS from day -14 to 14, were sensitized with HDM on day 0 and were challenged on days 7 to 11 with HDM or PBS (control). IL-33 mRNA expression in lungs (**A**), IL-33 concentration in BALF (**B**) and IL-33 protein levels in lungs (**C**) were measured and results are expressed as IL-33 mRNA expression (fold of control)(qRT-PCR, normalized to β-actin), pg/ml BALF (ELISA) or OD/mm^2 (Western blot, normalized to β-actin) as mean ± SEM. (* $P < 0.05$; *** $P < 0.001$; significantly different from the control group, ^ $P < 0.05$; significantly different from the HDM-allergic animals). n = 7-9 animals/experimental group (qRT-PCR and ELISA). For immunohistochemistry, lung sections were stained with anti-IL-33 antibody (**D, E, F, G**) as described in Materials and Methods (Magnification 200x).

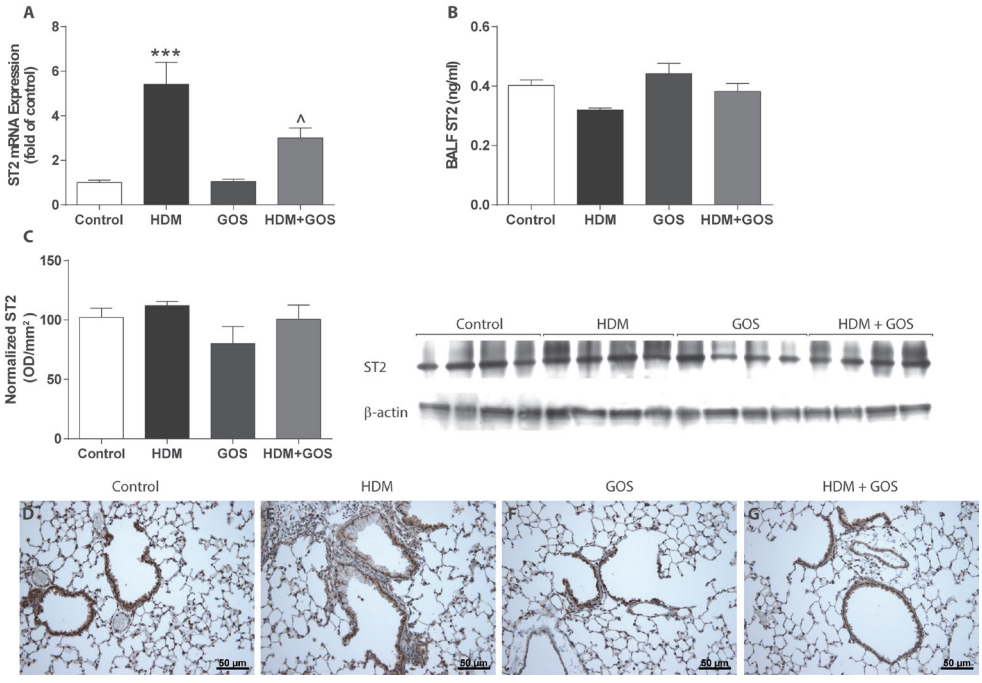

Fig. 4 Dietary intervention with GOS reduces ST2 mRNA expression in the lungs of HDM-allergic mice.

Mice fed a control diet or a diet supplement with GOS from day -14 to 14, were sensitized with HDM on day 0 and were challenged on days 7 to 11 with HDM or PBS (control). ST2 mRNA expression in lungs (**A**), ST2 concentration in BALF (**B**) and ST2 protein levels in lungs (**C**) were measured and results are expressed as ST2 mRNA expression (fold of control)(qRT-PCR, normalized to β-actin), pg/ml BALF (ELISA) or OD/mm^2 (Western blot, normalized to β-actin) as mean ± SEM. (*** $P < 0.001$; significantly different from the control group, ^ $P < 0.05$; significantly different from the HDM-allergic animals). n = 7-9 animals/experimental group (qRT-PCR and ELISA). For immunohistochemistry, lung sections were stained with anti-ST2 antibody (**D, E, F, G**) as described in Materials and Methods (Magnification 200x).

Discussion

IL-33 is a member of the IL-1 cytokine family with a dual function as it activates various immune cells through the IL-33 receptor ST2 and acts as an intracellular factor with transcriptional properties [15]. Subsequently, IL-33 functions as an important danger signal in the cellular response to tissue damage and epithelial cells at mucosal barrier sites constitutively express IL-33 [16]. Specific effects in the gut can be exerted by IL-33, since mice injected intraperitoneally (i.p.) with recombinant IL-33 demonstrated an increase in permeability of the mucosal barrier, intestinal inflammation and hypertrophy and hyperplasia of goblet cells [17, 18].

In our study, the mycotoxin DON serves as a reliable and reproducible model of intestinal barrier dysfunction [13, 19] and a strong positive correlation was observed between IL-33 mRNA expression in the mouse distal small intestine and the intestinal permeability induced by the mycotoxin DON. Besides IL-33, the tight and adherens junctions are critical for the maintenance of intestinal barrier integrity [20-22]. Furthermore, IL-33 impaired the epithelial barrier function as observed in a human colonic epithelial Caco-2 monolayer and in mice treated with exogenous IL-33, whereas IL-33 injected i.p. exacerbated sodium (DSS)-induced colitis in mice [23, 24]. Observations in IL-33$^{-/-}$ mice suggest that IL-33 deficiency leads to delayed local inflammation and tissue damage during experimental colitis [25]. On the other hand, it has been published recently that IL-33 promotes regulatory T-cell function in the intestine. IL-23, an important pro-inflammatory cytokine in the pathogenesis of inflammatory bowe disease (IBD), inhibits this regulatory T-cell responsiveness to IL-33 [26, 27]. Furthermore, IL-33 was prominently present in inflamed colon tissue, mainly localized to the surface epithelium and in crypt cells, in patients with Crohn's disease and ulcerative colitis [8, 28, 29]. In this study, a similar distribution pattern of IL-33 was observed in the mouse small intestine after disrupting the intestinal barrier with DON. Besides the increase in IL-33 expression observed by an immuno-histochemical staining, a 5-fold increase was observed in IL-33 mRNA levels in the mouse distal small intestine after a DON gavage, while other cytokines and chemokines, such as IFNγ, IL-1α, IL-1ß, IL-4, IL-6, and TNFα remained unchanged as described by Akbari et al. [6].

The IL-33/ST2 system plays apparently an important role in IBD [29-31] and Pastorelli et al. [28] described that a specific imbalance between IL-33 and ST2 may play a pathogenic role in ulcerative colitis (UC), since ST2 is decreased in UC colonic epithelium, while IL-33 is markedly increased in active UC. In the current study, no effect of the DON gavage on the ST2 distribution in the distal small intestine was observed, however, the ST2 mRNA expression was significantly increased in the distal small intestine of DON-treated animals.

The DON-induced increase in IL-33 was mitigated by dietary intervention with GOS as observed in IL-33 mRNA levels as well as by IL-33 distribution in the intestine, while GOS did not affect the DON-induced ST2 expression. Recently, we observed that GOS directly protects the intestinal barrier function by maintaining tight junctions (TJ) proteins and modulating CXCL8 responses in a human Caco-2 cell monolayer and in a mouse model for intestinal barrier dysfunction. However, the DON-induced hypermeability of the intestines for FITC-dextran (4 kDa) was not altered by GOS [6].

Since IL-33 is implicated in Th2 type responses required for the development of allergic inflammation, the effect of dietary GOS on IL-33 expression was also investigated in a murine HDM-induced asthma model. In this model, the IL-33 mRNA expression levels in the lung positively correlated with the number of total BALF cells. Previous studies suggested that IL-33 and ST2 are both associated with the development and maintenance of allergic asthma and are correlated with disease severity [32, 33]. The IL-33-induced production of pro-inflammatory cytokines has been suggested as a critical event that aggravates asthma [34] while treatments with anti-IL-33 monoclonal antibody have been reported to inhibit allergen-induced airway inflammation, Th2 cytokine production and mucus hypersecretion in mice [35]. Intranasally challenged IL-33-deficient mice show impaired IL-5 and IL-13 production from group 2 innate lymphoid cells (ILC2), lung inflammation and Th2 cell differentiation [36, 37]. Administration of blocking anti-ST2 antibodies or ST2-Ig fusion protein to allergic mice abrogated the Th2 mediated inflammatory response [38]. Contradictory results are described for T1/ST2-deficient mice, since Hoshino et al. [39] displayed normal Th2 responses in these animals, while Townsend et al. [40] showed reduced levels of IL-4 and IL-5.

In line with the data of the intestinal barrier dysfunction model, dietary intervention with GOS resulted in lower IL-33 and ST2 levels and an altered IL-33 distribution in the lungs of HDM-allergic mice. In addition, the increased concentration of the Th2 cytokine IL-13 in the lung of HDM-allergic mice was significantly decreased by dietary intervention with GOS and the same trend was observed for the IL-5 concentration in the BALF, however this was not significantly different (**Supplementary Table 2**) [12].

The mode of action of GOS is complex and still not entirely understood. The reduced IL-33 expression exerted by GOS might result from alterations in the composition of the microbiota, since initially, GOS was considered as a typical prebiotic supporting the growth of Lactobacillus and Bifidobacterium spp. in the large intestine [1, 41]. GOS not only stimulates these bacteria, but affects the whole intestinal flora by production of short-chain fatty acids, like butyrate and by decreasing the pH [1, 42]. It is known that butyrate exerts anti-inflammatory properties explaining the desirable effects of various oligosaccharides [43]. In turn, different immune-related, anti-allergic and anti-inflammatory effects were observed *in vivo* after GOS/lcFOS supplementation, suggesting

a positive effect on mucosal immunity via suppression of the Th2 type responses, a down-regulation of total immunoglobulin levels and an induction of Th1 and Treg cell polarization [4, 5, 44, 45].

Moreover, GOS seem to exert direct, microbiota-independent effects on the immune system by directly interacting with epithelial and immune cells as indicated by *in vitro* experiments [3, 6, 7, 46]. Although direct interaction with Toll-like receptor-4 (TLR4), has been hypothesized [46], the direct effect of GOS on TJ assembly in Caco-2 cells [6] indicates the involvement of other mechanisms as well, since Caco-2 do not express TLR4. Furthermore, different galectins have distinct binding specificities for binding oligosaccharides [47] and indeed previous investigations could show that dietary GOS enhanced the serum galectin-9 levels, which are involved the regulation of immune responses and tolerance induction, leading to a suppression of allergic symptoms in mice and humans [48].

These findings can be considered as a first indication of a systemic modulatory effect of GOS, and are now supported by our findings that GOS suppresses IL-33, an alarmin that is produced at different mucosal surfaces. The parallel response of intestinal repair mechanisms and anti-allergic properties of GOS, may hence be attributable to the systemic effects of signaling molecules like galectins and specific cytokines like IL-33. Further research is needed to investigate whether GOS directly interacts with the IL-33/ST2 system or whether it prevents intestinal barrier disruption and allergic asthma by altering the microbiota composition, which indirectly leads to a decreased IL-33 production.

In conclusion, dietary intervention with GOS mitigated the important immunomodulator IL-33 in mouse intestines observed in a model for intestinal barrier dysfunction and in murine lungs in a HDM asthma model, which is not necessarily associated with the ST2 expression. These preclinical experiments warrant studies on its clinical relevance and to unravel the mechanism behind this effect.

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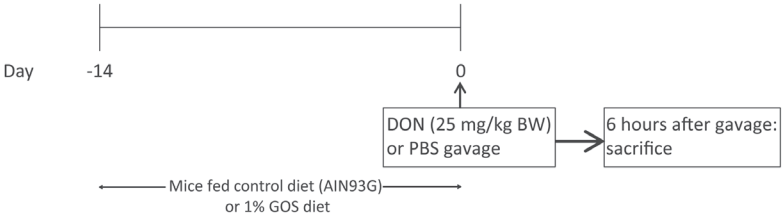
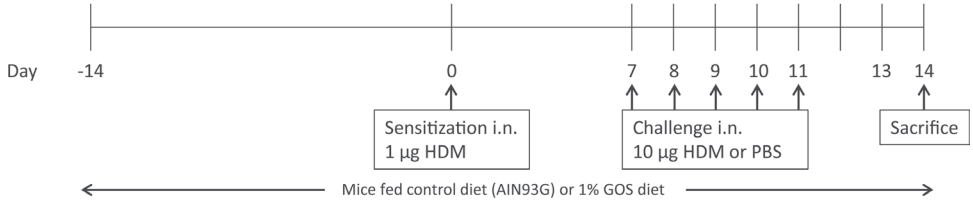
Supplementary data

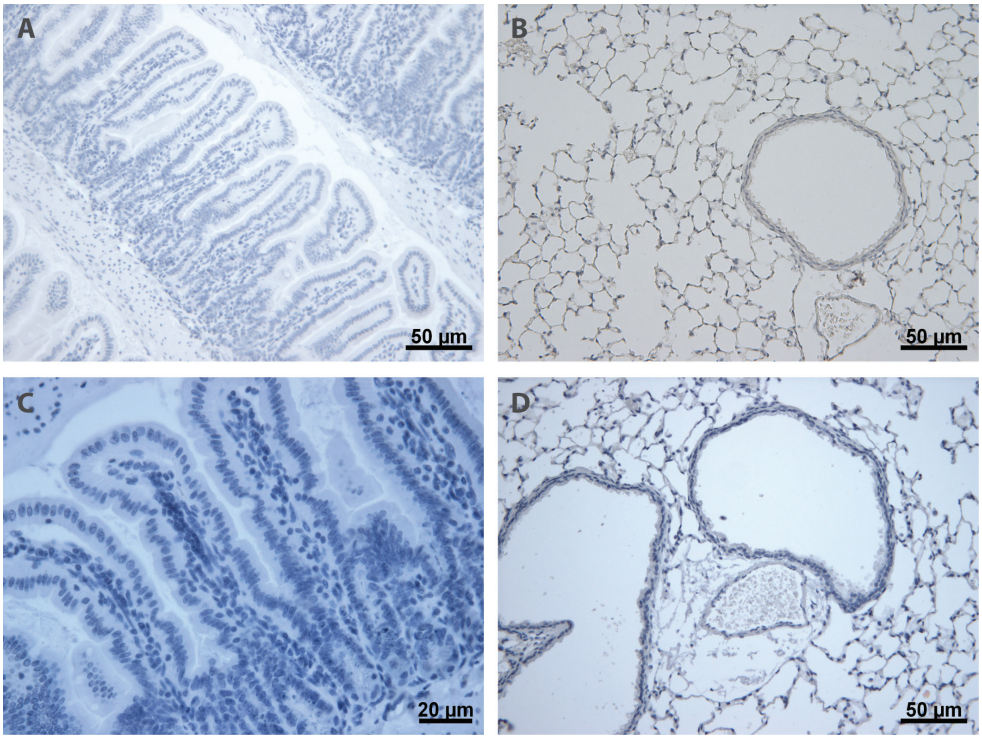
Mouse model for intestinal barrier dysfunction:



Mouse model for house dust mite-induced allergic asthma:



Supplementary Fig. 1 Mouse model for intestinal barrier dysfunction and mouse model for HDM-induced allergic asthma.



Supplementary Fig. 2 Negative controls immunohistochemistry.

No staining was detected in negative controls, in which the primary antibody was omitted. Negative control of IL-33 staining in distal small intestine (**A**), negative control of IL-33 staining in lungs (**B**), negative control of ST2 staining in distal small intestine (**C**), negative control of ST2 staining in lungs (**D**).

Supplementary Table 1 BALF cell count and differentiation in the HDM-allergic model.

	Mean (*10E4) ± SEM			
	Control	**HDM**	**GOS**	**HDM + GOS**
Macrophages	17.95 ± 2.15	33.19 ± 7.46	22.38 ± 2.35	19.36 ± 3.44
Lymphocytes	0.90 ± 0.25	4.54 ± 0.95***	0.57 ± 0.14	2.71 ± 0.60
Neutrophils	0.25 ± 0.08	3.97 ± 1.28*	0.26 ± 0.10	3.16 ± 1.41
Eosinophils	0.03 ± 0.03	20.92 ± 4.53***	0.05 ± 0.03	9.06 ± 4.53 (P = 0.09)
Total	**19.13 ± 2.37**	**62.63 ± 11.48*** **	**23.25 ± 2.32**	**34.29 ± 6.78#**

* P < 0.05; *** P < 0.001 significantly different from the control animals, # P < 0.05 significantly different from the HDM-allergic animals.

Supplementary Table 2 Cytokine concentrations in the HDM-allergic model.

	Mean ± SEM			
	Control	**HDM**	**GOS**	**HDM + GOS**
IL-13 (pg/mg protein)	65.57 ± 3.02	99.07 ± 8.01 ***	46.91 ± 2.41	65.25 ± 2.82 ##
IL-5 (pg/mL)	7.07 ± 0.99	17.92 ± 5.91	3.53 ± 0.39	10.75 ± 3.13

IL-13 (pg/mg protein) measured in lung homogenates and IL-5 (pg/mL) measured in BALF. *** P < 0.001 significantly different from the control animals, ## P < 0.05 significantly different from the HDM-allergic animals.

CHAPTER NINE

Regulatory T cell depletion abolishes the protective effect of dietary galacto-oligosaccharides on eosinophilic airway inflammation in house dust mite-induced asthma in mice

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This chapter is submitted for publication

Abstract

Background: In a murine model for house dust mite (HDM)-induced asthma, dietary non-digestible galacto-oligosaccharides (GOS) have been shown to suppress allergic symptoms. Previously, $CD25^+$ regulatory T-cells (Treg) were found to contribute to allergy protection induced by non-digestible oligosaccharides, but their function in allergic asthma is still not completely understood.

Aim: To examine the effect of anti-CD25 Treg depletion in a murine HDM-induced asthma model and to study the contribution of Treg in the protective effect of dietary intervention with GOS.

Methods: BALB/c mice were intranasally (i.n.) sensitized with PBS or 1 µg HDM and accordingly challenged with PBS or 10 µg HDM on days 7-11 while being fed a control or a 1% w/w GOS diet. Treg were depleted by two intraperitoneal injections with anti-mouse CD25 antibody (anti-CD25, PC61) on day -1 and day 6. On day 14, T helper (Th) cell subtypes in lung and spleen of control diet fed anti-CD25-treated mice were analyzed by flow cytometry and cytokines were measured in restimulated lung cell supernatants. In all mice, leukocyte subtypes were analyzed in the bronchoalveolar lavage fluid (BALF) and interleukin 33 (IL-33) and chemokines were measured in lung homogenate supernatants.

Results: Anti-CD25 treatment depleted $CD25^+Foxp3^+$ Treg in lung and spleen of control and HDM-allergic mice, while the frequency of activated T helper cells and Th2 cells increased. This phenomenon was associated with increased IL-10, IL-4 and IL-13 concentrations in supernatants of *ex vivo* restimulated lung cells. BALF leucocyte numbers and percentages of eosinophils and lymphocytes were increased in HDM-allergic mice but remained unaffected by the anti-CD25 treatment. The GOS diet decreased airway eosinophilia, this protective effect was lost in anti-CD25 treated mice. A similar pattern was observed for the percentage of lymphocytes and neutrophils. In lung homogenate supernatants of HDM-allergic mice, IL-33 and CCL5 concentrations were increased compared to controls, to a similar extent in HDM-allergic mice either or not treated with anti-CD25. Dietary intervention with GOS abrogated the increase in IL-33 which was abolished by the anti-CD25 treatment, CCL5 showed the same tendency.

Conclusion: Treg depletion enhances pulmonary Th2 cell frequency and cytokine release in HDM-induced asthma in mice but does not further increase pulmonary inflammation. Dietary intervention with GOS decreased airway eosinophilia and IL-33 concentrations in the lung, which was abrogated by Treg depletion. This indicates that dietary intervention with GOS has a beneficial effect on the prevention of HDM-induced allergic asthma by supporting pulmonary Treg function.

Introduction

Allergic asthma is a chronic inflammatory disease occurring as a result of Th2 immune responses. High pulmonary IL-4, -5 and -13 responses, airway hyperresponsiveness (AHR) and eosinophilic inflammation are major characteristics for allergic asthma [1]. Around 300 million people worldwide suffer from asthma and the incidence continues to rise in developed countries [2]. Th1 cells were first assumed to be able to down-regulate Th2 cells, however, research has shown that regulatory T cells (Treg) also contribute to down-regulate the development of allergic diseases and asthma [3]. Natural or inducible Treg express both Foxp3 and CD25 and represent up to 10% of $CD4^+$ T cells [4]. Previous animal studies have shown that $CD4^+CD25^+$ Treg can control allergic airway diseases [5-7]. Several studies have been conducted to investigate the role of Treg in asthmatic patients. However, results differ between adults and children and between airway tissue and blood [8]. Children with moderate-severe asthma showed more Treg than pediatric patients with mild asthma and both had significantly higher mRNA expression of Foxp3 than control subjects [9]. In adults $CD4^+CD25^+$ lymphocyte levels were higher in blood of mild asthmatics compared to severe asthmatics and healthy controls [10]. In contrast, Smyth et al. reported that airway Treg numbers increase with more severe disease in bronchoalveolar lavage fluid (BALF) of asthmatic patients [11]. This could counter balance the exacerbated inflammatory response but Treg function in asthma is still not fully understood.

In a whey- or casein-induced murine model for food allergy a diet containing non-digestible oligosaccharides (NDO) was able to reduce allergic symptoms. Treg depletion abrogated the protective effect of the NDO diet, indicating that NDO may act via the induction of functional Treg [12,13].

Therefore, an increased interest in using NDO to prevent the development or reduce symptoms in allergic diseases has increased. Galacto-oligosaccharides (GOS) are specific NDO, which are selectively fermentable in the intestine, with beneficial effects on the growth and/or the activity of Lactobacilli and Bifidobacteria [14-16]. NDO, either or not in combination with beneficial bacteria, reduce allergic symptoms not only in murine models of food allergy, but also in asthma [17-19]. In a HDM-induced allergic asthma model we found that GOS was capable of reducing allergic features of asthma [20]. Furthermore, clinical studies showed a protective effect of a mixture of oligosaccharides in the development of atopic dermatitis in young infants [21] and combined with beneficial bacteria the Th2 cytokine production was decreased upon treatment of adult asthmatic patients [22]. As GOS has a protective effect on allergic features in a HDM-induced asthma model, we examined the contribution of $CD25^+$ Treg in the preventive effect of dietary GOS on asthma-associated airway inflammation using *in vivo* anti-CD25 depletion in a murine model for HDM-allergic asthma.

Materials and methods

Mice

Six-to-eight week old male BALB/c mice (Charles River, The Netherlands) were housed under bio-contained sterile conditions using HEPA ® filtered isocages ® (Tecniplast, Italy). Food and water were provided *ad libitum*. All animal experiments were conducted in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Utrecht University (DEC 2014.II.04.027).

HDM murine asthma model and anti-CD25 depletion

Mice were fed a diet containing 0 (AIN-93G, control diet) or 1% w/w GOS (Vivinal® GOS syrup with approximately 59% galacto-oligosaccharides, 21% lactose, 19% glucose, and 1% galactose on dry matter (dry matter of 75%); FrieslandCampina Domo, The Netherlands). Carbohydrates in Vivinal® GOS were isocalorically compensated in the control diet by means of exchange against cellulose (for GOS), lactose (for lactose), and dextrose (for glucose). The diets were provided two weeks prior to sensitization and continued during the whole experiment, from day -14 to day 14. Mice were intranasally (i.n.) sensitized with PBS in presence or absence of 1 µg HDM (Greer Laboratories, USA) and challenged i.n. with PBS or 10 µg HDM on days 7 to 11, while under isoflurane anesthesia. One day before sensitization (day -1), control or 1% GOS fed mice were either or not intraperitoneal (i.p.) injected with 200 µL (1 mg/mL) rat anti-mouse CD25 (anti-CD25, PC61, monoclonal antibody, Bioceros, The Netherlands) for *in vivo* depletion of $CD25^+$ Treg. A second treatment was carried out 1 day prior to challenge (day 6) [13]. Mice were sacrificed on day 14 [20,23] (**Fig. 1**).

Bronchoalveolar lavage

Lungs were lavaged for 4 times with 1 mL of pyrogen-free saline (0.9% NaCl, 37°C). The BALF cells were centrifuged (400 g, 5 min.) and total numbers of BALF cells were counted using a Bürker-Türk chamber (magnification 100x). For differential BALF cell counts, cytospin preparations were stained with Diff-Quick (Merz & Dade A.G., Switzerland). Numbers of eosinophils, lymphocytes and neutrophils were scored with light microscopy [24].

Preparation of lung homogenates

A Precellys 24 tissue homogenizer (Bertin Technologies, France) was used to homogenize lung samples in 1% Triton X100 (Sigma-Aldrich, The Netherlands)/PBS containing protease inhibitor (Complete Mini, Roche Diagnostics, Germany). Homogenates were centrifuged at 14,000 rpm for 10 min., supernatants were collected and stored at -20°C until further use. Protein concentration was measured using the Pierce BCA protein assay kit standardized to BSA (Thermo Fisher Scientific, USA). Homogenates were diluted to a final concentration of 1 mg protein/mL prior to cytokine or chemokine measurements [25,26].

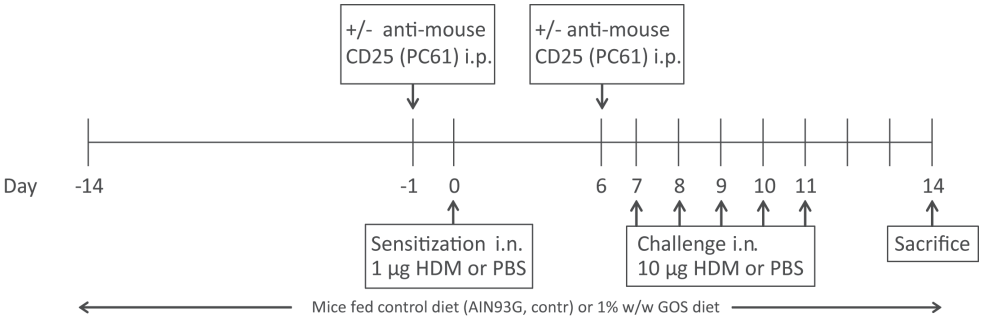

Fig. 1 Schematic overview of the experimental set-up.

BALB/c mice were intranasally (i.n.) sensitized with PBS or HDM on day 0 and i.n. challenged from day 7 to 11. On day -1 and day 6 mice were i.p. injected with 200 µL (1 mg/mL) rat anti-mouse CD25 (PC61) mAb. Mice were fed the control diet (AIN93G, contr) or a 1% w/w GOS diet two weeks prior to sensitization and continued during the entire experiment. Mice were sacrificed on day 14.

Ex vivo lung restimulation with house dust mite

After enzymatic digestion of the lungs using digestion buffer, containing DNase I and Collagenase A (Roche Diagnostics, Germany), for 30 min., lung cell suspensions were prepared. Digestion was stopped by adding fetal calf serum (FCS, Hyclone Laboratories, USA). Lung cells were washed and resuspended in RPMI-1640 culture medium (Lonza, USA) supplemented with 10% heat-inactivated FCS and 0.1% penicillin-streptomycin solution (Sigma-Aldrich, The Netherlands). Lung cells (4 x 10^5 cells/well) were cultured in medium with or without 50 µg/mL HDM and supernatant was harvested after 4 days of culture at 37°C in 5 % CO_2 and stored at -20°C until further analysis [27].

Lung T cell subsets and inflammatory dendritic cells assessed by flow cytometry

PBS-blocking buffer containing 1% BSA and 5% FCS for 30 min. was used to block aspecific background. Per well, 5 x 10^5 cells were plated and incubated at 4°C for 30 min. with different antibodies against CD25-AlexaF488, Foxp3-APC, CD4-PerCP Cy5, CD69-FITC, GATA3-PE, Tbet-eFLUOR660 (eBioscience, The Netherlands), or matching isotype controls. Cells were permeabilized for intracellular staining using the Foxp3 staining buffer set, according to manufacturer's protocol (eBioscience, The Netherlands). Flow cytometry was conducted using FACS Canto II (BD, The Netherlands) and analyzed using Flowlogic Software (Inivai Technologies, Australia) [28].

Measurement of cytokines and chemokines

CCL5, IL-33 and CCL20 were measured with a DuoSet ELISA (R&D Systems, USA), IL-13 with a Ready-SET-Go!® ELISA (eBioscience, The Netherlands) all according to manufacturer's protocol. Cytokine concentrations in supernatants of lung cell restimulation were determined by a standard IL-13 flex set and Th1/Th2/Th17 kit (BD Biosciences, The

Netherlands). The concentrations of these mediators were expressed as pg/mg protein in supernatants of lung homogenates and pg/mL in restimulation supernatants.

Statistical analysis

Data are presented as mean ± standard error of mean (SEM). Data were statistically analyzed using a One-way ANOVA or Two-way ANOVA followed by a Bonferroni's multiple comparisons test. $P < 0.05$ were considered significant. Statistical analyses were conducted using GraphPad Prism software (version 6.04). If required, data were transformed to normalize data distribution for analysis (BALF cells).

Results

Anti-CD25 antibody treatment diminishes the frequency of Treg and enhances activated T helper and Th2 cells

To confirm Treg depletion upon anti-CD25 treatment, lung cell suspensions and splenocytes from control diet fed mice were phenotyped. Anti-CD25 treatment depleted Treg in the spleen and lung of PBS control mice. The Treg depletion was associated with an increased frequency of activated T helper cells and Th2 cells in the spleen but not in the lung. In the spleen of HDM-allergic mice these findings were similar (**Fig. 2A-D**). In the lung, a significant increase in $CD25^+Foxp3^+CD4^+$Treg was observed in HDM-mice compared to PBS-mice, which was not observed in the spleen (**Fig. 2A & 2E**), anti-CD25 treatment declined the percentage Treg with more than 73% in lung cells of HDM-mice (**Fig. 2E**). Also the frequency of activated T helper cells was significantly increased in lungs of HDM-mice compared to PBS-mice and further enhanced after Treg depletion (**Fig. 2F**). Although the frequency of $GATA3^+CD4^+$ Th2 cells was not enhanced in the lungs of HDM-mice it significantly increased after Treg depletion (**Fig. 2G**). No differences were observed in the frequency of $Tbet^+CD4^+$ Th1 cells in both spleen and lung cells, with or without anti-CD25 treatment (**Fig. 2D** & **2H**).

Pulmonary IL-10, IL-4 and IL-13 increase after Treg depletion

Lung cell suspensions were *ex vivo* restimulated with medium or HDM to examine the effect of anti-CD25 treatment-induced Treg depletion on allergen-specific cytokine secretion. In control mice no cytokine release was observed, whereas the cells of HDM-mice showed a HDM-specific increase in IL-10, IL-4 and IL-13 release (**Fig. 2I-K**). Treg depletion, however, increased the IL-10, IL-4 and IL-13 concentrations of medium exposed cells to the same level as HDM-exposed cells, in PBS control as well as HDM-allergic mice (**Fig. 2I-K**). Typically, anti-CD25 treatment increased IFNγ concentrations only after HDM restimulation in both PBS control and HDM-allergic mice, but not after medium stimulation (**Fig. 2L**).

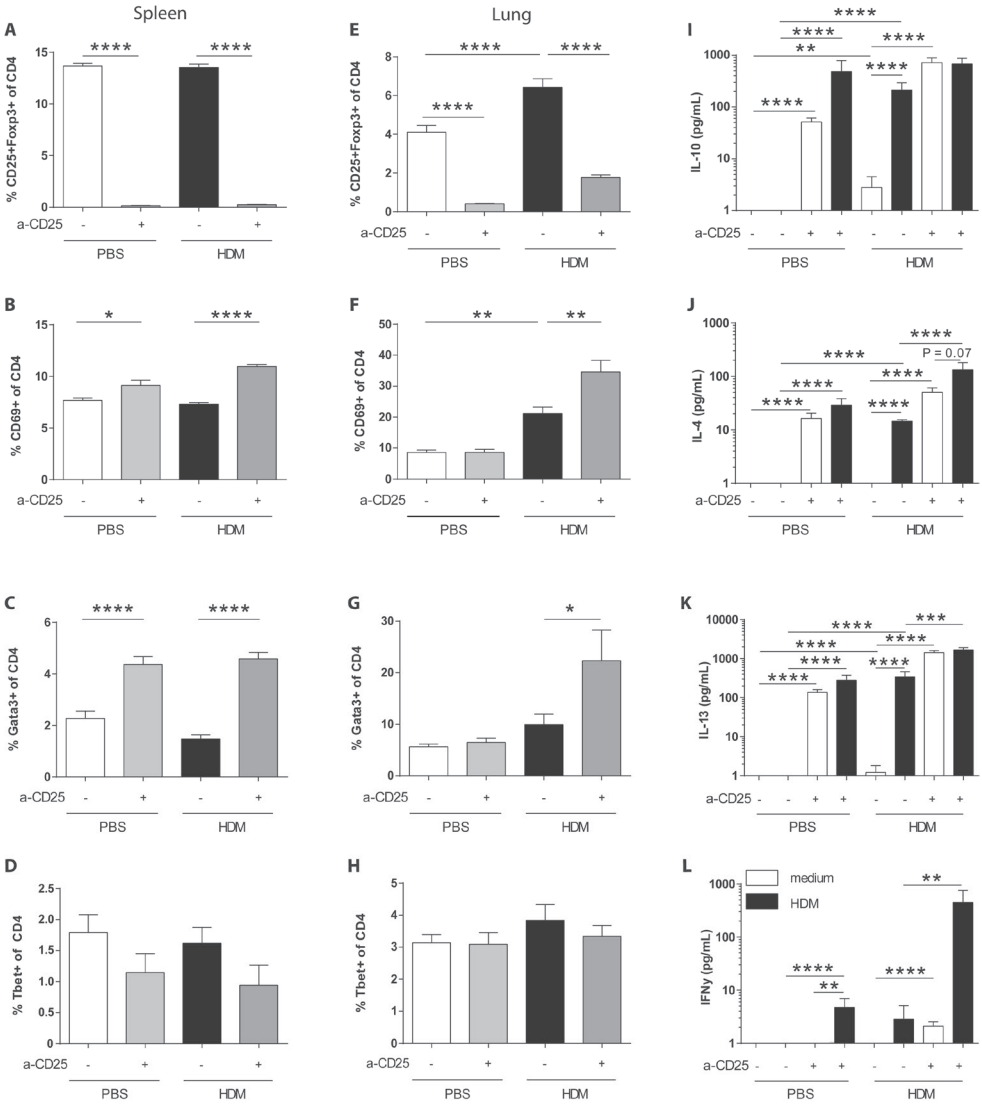

Fig. 2 Anti-CD25 treatment diminishes the frequency of Treg and enhances activated T helper and Th2 cells while increasing pulmonary IL-10, IL-4 and IL-13 concentrations.

Flow cytometric analysis of the percentage of $CD25^+Foxp3^+$ of $CD4^+$ cells in spleen (**A**) and lung (**E**), activated $CD4^+$ cells in spleen (**B**) and lung (**F**), $GATA3^+$ of $CD4^+$ cells in spleen (**C**) and lung (**G**), $Tbet^+$ of $CD4^+$ cells in spleen and (**D**) and lung (**H**). IL-10 (**I**), IL-4 (**J**), IL-13 (**K**) and IFNγ (**L**) were measured in supernatants of lung cell suspensions upon *ex vivo* HDM-restimulation (pg/mL). Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA (Fig. A-H) or Two-Way ANOVA (Fig. I-L). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, $n = 6$ mice/group.

Treg depletion abolishes the suppressive effect of dietary GOS on airway eosinophilia

BALF was examined to study the extent of airway inflammation in HDM-allergic mice upon dietary intervention with control diet or GOS diet and the effect of Treg depletion. The total number of BALF cells was significantly increased in HDM-mice compared to PBS-mice fed the control diet. Anti-CD25 treatment of HDM-allergic mice did not affect this and resulted in a similar increase in total BALF cell numbers compared to control mice treated with anti-CD25 (**Fig. 3A**). Dietary intervention with GOS did not show an effect on total BALF cells (**Fig. 3A**).

In HDM-allergic mice the percentage of eosinophils was significantly increased compared to the PBS control mice and remained unaffected high upon anti-CD25 treatment (**Fig. 3B**). Dietary intervention with GOS significantly reduced the percentage of eosinophils in HDM-allergic mice, and this effect was lost after treatment with anti-CD25 antibody. Anti-CD25 treatment in GOS fed HDM-mice resulted in a significant increase in percentage of eosinophils compared to their corresponding PBS group (**Fig. 3B**). The percentages of lymphocytes and neutrophils were also increased in HDM-allergic mice compared to PBS control mice and Treg depletion did not affect this (**Fig. 3C-D**). Dietary intervention with GOS did not significantly suppress the percentages of lymphocytes and neutrophils (**Fig. 3C-D**), however, anti-CD25 treatment significantly increased the percentage lymphocytes and neutrophils in mice fed the GOS diet (**Fig. 3C-D**). In the PBS control mice, the GOS diet reduced the percentage of lymphocytes which was abrogated by Treg depletion (**Fig 3C**), and Treg depletion also increased the percentage of neutrophils in this group (**Fig 3D**).

Treg depletion abrogates the suppressive effect of dietary GOS on IL-33 concentrations in lung homogenates

In lung homogenate supernatants of HDM-allergic mice fed the control diet, IL-33 and CCL5 concentrations were increased compared to PBS-mice fed the control diet, and anti-CD25 treatment did not affect this (**Fig. 4A-B**). Dietary intervention with GOS reduced the IL-33 concentration and the same tendency was observed for CCL5, these protective effects were lost upon Treg depletion (**Fig. 4A-B**). CCL20 concentrations were increased in HDM-allergic mice fed the control diet compared to PBS-mice and further increased upon anti-CD25 treatment (**Fig. 4C**). Dietary intervention with GOS did not show an effect on CCL20 concentrations in HDM-allergic mice (**Fig. 4C**).

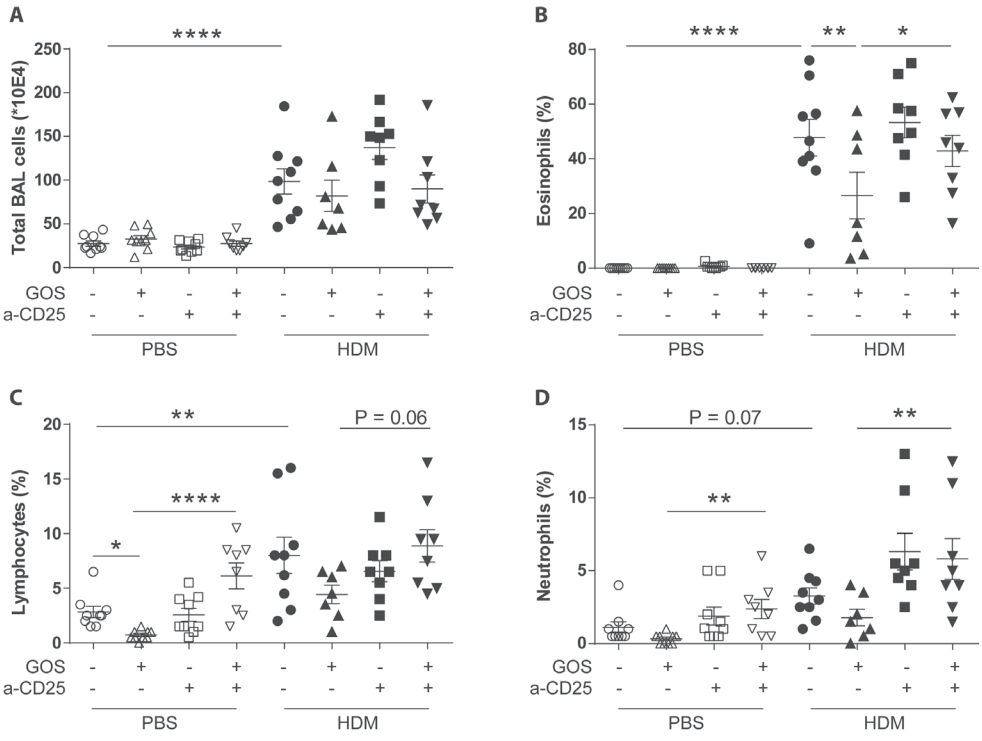

Fig. 3 Depletion of Treg abrogates the suppressive effect of GOS on eosinophilic inflammation in BALF.
Airway inflammatory cells in the BALF of HDM-allergic mice. Total number of BALF cells (**A**), percentage of eosinophils (**B**), lymphocytes (**C**) and neutrophils (**D**). Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, n = 7-9 mice/group.

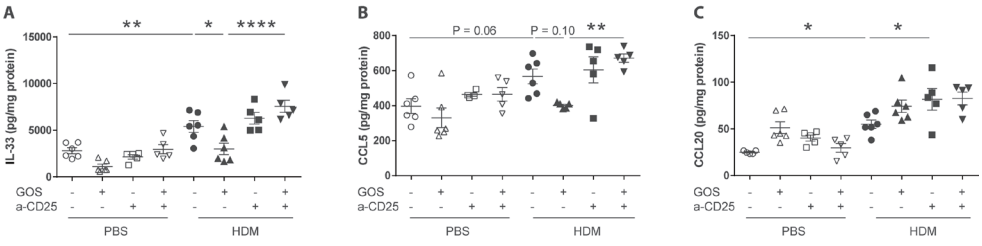

Fig. 4 GOS diet induced suppression of IL-33 and CCL5 in lung homogenates is lost by Treg depletion.
IL-33 (**A**), CCL5 (**B**) and CCL20 (**C**) (pg/mg protein) were measured in the supernatants of lung homogenates. Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$, n = 5-6 mice/group.

Discussion

This study was conducted to examine the effect of anti-CD25 Treg depletion on allergic responses after dietary intervention with galacto-oligosaccharide (GOS) in a murine model for house dust mite (HDM)-allergic asthma. Although CD25 is not a specific marker for Treg, anti-CD25 treatment is commonly used to deplete Treg *in vivo* [29]. To confirm whether the depletion of Treg occurred after anti-CD25 treatment, we analyzed lung and spleen cells and found a significant reduction in $CD25^{+}Foxp3^{+}$ $CD4^{+}$ Treg. The latter is in agreement with different animal studies in which anti-CD25 treatment was able to reduce the number of Treg [6,30]. From human studies, it is known that Treg can suppress Th2 responses [31,32]. Since Treg were almost completely abolished after anti-CD25 treatment, an increase in activated T helper cells and Th2 cell frequency was observed in both lung and spleen cells of HDM-allergic mice. Th2 cells are known to produce different cytokines like IL-4 and IL-13 [33,34]. Indeed, increased IL-4 and -13 concentrations were measured in restimulated lung cell supernatants after anti-CD25 treatment both in control as well as HDM-allergic mice. This indicates that Treg have a general suppressive role in Th2 type cytokine secretion not only in asthma but also constitutively in the lung. Besides the increase in Th2 cytokines, Treg depletion also resulted in an increase in IL-10 production. It is known, that $CD25^{+}$ $CD4^{+}$ Treg are not the only secretors of IL-10, but also many other cells (e.g. dendritic cells, lymphocytes and macrophages) are capable of secreting this cytokine [35,36]. These cellular sources other than Treg may explain the significant increase in IL-10 release by lung cells of anti-CD25 treated mice. By contrast to the increase in Th2 cell frequency, no differences were observed in Th1 frequency in anti-CD25 treated mice. Typically, the Th1 type IFNγ concentration in restimulated supernatants of lung cells was increased in a HDM-dependent manner. However this did not only occur in HDM-allergic mice, but also in control mice, which may reflect HDM memory cells that were already present in control mice due to environmental exposure in the animal facilities. Treg depletion may have resulted in loss of suppression rendering the Th1 cells more active. Taken these results together, we can conclude that anti-CD25 treatment prior to sensitization and challenge is capable of selective depletion of Treg in control and HDM-allergic mice showing a functional role of these cells in suppression of T-cell responses in the lung.

Next, we examined the effect of dietary intervention with GOS on pulmonary inflammation and studied the contribution of Treg in the protective effect of this diet. In previous studies, dietary GOS was shown to dampen airway eosinophilia, whereas the frequency of Treg in the lung was unaltered compared to HDM-allergic mice fed the control diet [20]. However, in murine models for food allergy it was shown that Treg were functionally involved in the protective effect of an oligosaccharide diet [12,13].

Total BALF, eosinophils and lymphocytes were increased in HDM-allergic mice compared to PBS control mice and neutrophils showed the same tendency. Although anti-CD25 treatment enhanced Th2 cell frequency and T-cell activation in the lungs of HDM-allergic mice fed the control diet, Treg depletion did not further increase pulmonary inflammation since the total BALF cell number, and percentage of eosinophils, lymphocytes and neutrophils were not increased compared to HDM-allergic mice not treated with anti-CD25. Also in the PBS control mice fed the control diet, Treg depletion did not result in airway inflammation. These data are supported by previous mice studies that showed no effect or a modest increase in pulmonary eosinophil numbers after Treg depletion [7,37]. Dietary intervention with GOS significantly decreased the percentage of eosinophils in HDM-allergic mice, which is in line with our previous study [20]. This protective effect was lost upon Treg depletion. This implies that the mechanism by which GOS is able to reduce airway eosinophilia in HDM-allergic mice is mediated via Treg. Although the GOS diet did not significantly decrease the percentage of lymphocytes and neutrophils in HDM-allergic mice, the percentages of these cells were increased upon anti-CD25 treatment similar to the eosinophils.

In HDM-allergic mice, GOS did not significantly suppress the percentage of lymphocytes in the BALF, although in the control mice the GOS diet did reduce this number and was again abrogated by Treg depletion. Hence, GOS may be capable of suppressing the basal percentage of lung lymphocytes acting via Treg. Currently, it remains to be elucidated how this is being established, but by enhancing Treg function, GOS may be capable of lowering basal chemokine release such as IL-33 and CCL5 by lung epithelial cells or innate immune cells resulting in reduced chemo-attraction of lymphocytes. Indeed, increased levels of IL-33 and CCL5 were observed in the airways of asthmatic patients [38-40]. Although concentrations of IL-33 and CCL5 followed the same pattern as the percentage of lymphocytes in the lung, the current study could not prove this hypothesis since GOS did not significantly reduce IL-33 and CCL5 in the PBS control mice. HDM is known to induce IL-33 and CCL5 release by airway epithelial cells [34]. Indeed, in HDM-allergic mice, IL-33 was enhanced compared to control mice and CCL5 showed the same tendency. In HDM-allergic mice, dietary intervention with GOS decreased IL-33 and tended to decrease CCL5. The latter is in agreement with previous studies, in which we showed a decrease in CCL5 and IL-33 concentrations in supernatants of lung homogenates of HDM-allergic mice after dietary intervention with GOS [20].

Furthermore, anti-CD25 treatment did not influence IL-33 and CCL5 concentrations of control or HDM-allergic mice but did abolish the suppressive effect of dietary intervention with GOS on IL-33 and CCL5. Hence, Treg facilitate the GOS-induced suppression of these epithelial cell mediators in HDM-allergic mice. CCL5 is produced by several cell types (e.g.

macrophages, mast cells, basophils), but is also known to be produced by/and attract eosinophils to the airways of asthmatic patients [40,41] and eosinophils are activated by IL-33 [42]. Therefore, the suppression of airway eosinophilia by dietary GOS may be caused by a Treg mediated dampening of CCL5 and IL-33 concentrations in the HDM-allergic mice (**Fig. 5**).

The suppression of CCL5 and IL-33 by GOS was rather selective since GOS did not reduce CCL20 concentrations in lung of HDM-allergic mice. The chemokine CCL20 is also produced by airway epithelial cells and increased in concentration in asthmatic patients [43-45]. CCL20 concentrations were significantly increased in HDM-allergic mice and even further increased after anti-CD25 treatment. Although IL-33 and CCL20 are both produced by airway epithelial cells, only CCL20 is affected by anti-CD25 treatment in HDM-allergic mice fed the control diet. Hence, the loss of Treg and/ or the increase in activated T cells due to the anti-CD25 treatment may have resulted in an increase in the production of CCL20 from the epithelial cells. However, dietary intervention with GOS did not affect CCL20 concentrations in HDM-allergic mice either or not treated with anti-CD25. Furthermore, HDM-induced CCL5 and IL-33 release may involve a different pathway than that for CCL20 and the latter being less sensitive for suppressive effects of GOS in HDM-allergic mice.

In conclusion, these results indicate that Treg are constitutively involved in the suppression of T cell activation in the lung and dampen T cell activation and Th2 influx in allergic asthma in mice. However, Treg as such are not capable of suppressing airway eosinophilia, and IL-33 and CCL5 release. By contrast, dietary intervention with GOS suppresses airway eosinophilia and IL-33 concentrations in supernatants of lung homogenates via a mechanism mediated by Treg. This indicates that dietary intervention with GOS has a beneficial effect on the prevention of HDM-induced allergic asthma by supporting pulmonary Treg function.

Acknowledgments

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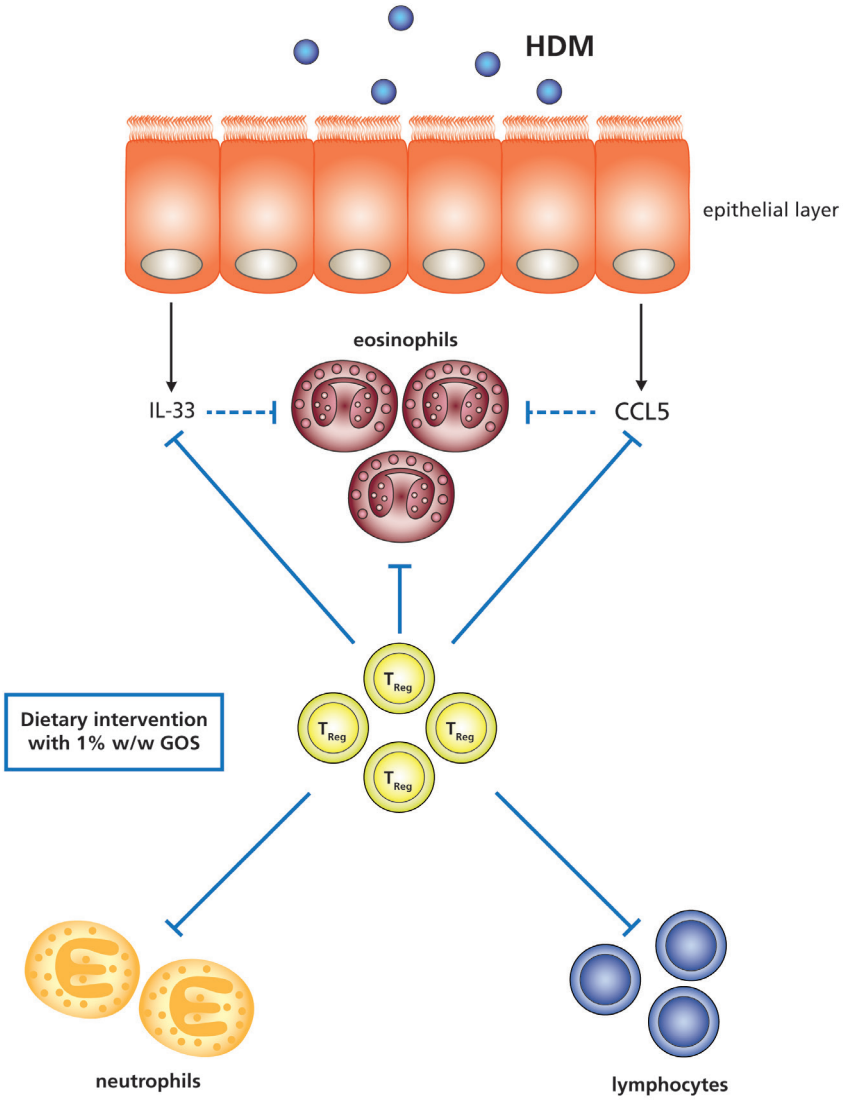

Fig. 5 The preventive effect of dietary intervention with GOS via a mechanism mediated by Treg.
IL-33 and CCL5 are secreted by the airway epithelium after HDM- exposure. After dietary intervention with GOS, airway eosinophilia, IL-33 and CCL5 concentrations are suppressed via a Treg mediated mechanism. A similar pattern was observed on neutrophil and lymphocyte numbers. Treg depletion abolishes this protective effect of GOS. CCL5 is produced by/ and attracts eosinophils to the airway, whereas IL-33 activates neutrophils [40-42]. Reducing CCL5 and IL-33 may lead to less attracted and/ or activated eosinophils.

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CHAPTER TEN

Summurazing discussion

Human milk non-digestible oligosaccharides (HMOS) are a major constituent of breast milk that can have beneficial effects on host well-being and health [1,2]. Dietary non-digestible oligosaccharides (NDO) such as galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) are known for their capacity to mimic some of the functional aspects of these HMOS. NDO are known as prebiotics since they are capable of selectively modulating the microbiota, which results in higher amounts of commensal *Lactobacillus* and *Bifidobacterium* species [3-5]. Patients suffering from allergic asthma have a different intestinal microbiota composition compared to healthy subjects [6-8]. Therefore, dietary intervention with NDO targeting the gut microbiome may be beneficial in the prevention and/or treatment of allergic asthma.

Different animal and human studies have demonstrated that NDO have beneficial effects on the immune system. Dietary intervention with GOS/long-chain (lc)FOS/pectin-derived acidic oligosaccharide (pAOS) or short-chain (sc)FOS/lcFOS/pAOS/*Bifidobacterium breve* has been shown to reduce airway inflammation and airway hyperresponsiveness (AHR) in a murine ovalbumin (OVA)-induced allergic asthma model [9,10]. In addition, when formula milk provided to infants at high risk for the development of allergy was supplemented with GOS/lcFOS, the incidence of infectious disease and atopic dermatitis was reduced [11]. This beneficial effect sustained even 5 years after the dietary intervention with these prebiotics, which took place directly after birth up until 6 month of age [12,13]. In addition, also in adult patients suffering from house dust mite (HDM) allergy and asthma, beneficial effects were found combining GOS/lcFOS with the probiotic strain *Bifidobacterium breve* (*B. breve*), as T helper 2 cell (Th2)-associated cytokines were decreased and lung function was improved (assessed by increased peak expiratory flow) [14].

In this thesis, results are presented on the possible beneficial effects of dietary interventions with several NDO either or not in combination with a beneficial bacterium (*B. breve*) on the development of airway allergy using a murine HDM-induced asthma model. The following dietary interventions were studied: a diet containing GOS alone, a combination of GOS with sialyllactose (GS), a combination of GOS/lcFOS (GF) or a combination of lcFOS and scFOS (FF) either or not combined with *B. breve* M-16V (GF*BB*, FF*BB*). In addition, in several studies we compared the effects of dietary intervention with a standard therapeutic glucocorticosteroid treatment on HDM-allergic asthma features in mice. Knowledge generated from these studies may provide new strategies to reduce allergic asthmatic symptoms in humans.

Lung function measurements

As described in **Chapter One**, asthma is a complex disease that involves innate as well as adaptive immune responses. Different animal models have been used to induce asthmatic-like reactions, although no model mimics the entire asthma phenotype found in humans [15,16]. In **Chapter Two**, two different types of OVA-induced allergic airway inflammation models are described: a mild and a severe inflammation model, respectively. Due to the continuous scientific discussion and debate on lung function measurements in mice, different lung function measurement protocols have been compared, non-invasive and non-ventilated unrestrained whole body plethysmography (Penh) and the invasive ventilated lung resistance (R_L) method. A more pronounced increase in total bronchoalveolar lavage fluid (BALF) cell numbers was observed in the OVA-sensitized and -challenged mice in the severe model compared to the mild model. Total BALF cells were higher in both the mild and severe model after R_L measurement compared to Penh. The results indicate that ventilation of mice during lung function measurements induces an inflammatory response in the lung. However, in the severe model this increase was not as pronounced as in the mild model, probably due to the fact that the number of BALF cells was already at the maximum level. From literature it is known that mechanical ventilation increases the total number of BALF cells in mice [17,18].
In both models the increase in BALF cells was mainly due to a significant increase in the number of eosinophils, which is characteristic for allergic asthma [19]. Severe allergic airway inflammation is known to have higher numbers of eosinophils compared to mild allergic airway inflammation [20,21]. Only severe pulmonary inflammation resulted in a pronounced AHR to metacholine assessed by Penh and R_L measurements. AHR in the mild airway inflammation model was only detectable using R_L measurements. These results indicate that Penh measurements might only be useful to study airway function under conditions of severe allergic inflammation in the lungs of mice. A disadvantage of the R_L measurement is the ventilation-induced increase in BALF cell numbers under mild inflammatory conditions. Despite this restriction, in our next experiments we have used the R_L measurement to assess airway reactivity since this measurement showed the best window of effect in both the mild and severe airway inflammation model.

As described in **Chapter One**, a major disadvantage of OVA-induced allergic asthma models in mice, is the use of adjuvants, commonly aluminum hydroxide, which may already promote the development of Th2 type responses by itself and may alter the mechanisms of allergen sensitization [22-24]. HDM is an important allergen in asthma and has been recognized to be involved in at least 85% of the asthmatic patients [25,26]. Therefore, we developed a murine model for HDM-induced allergic asthma based on the approach described by Kool *et al.* in which mice are sensitized for HDM upon intranasal exposure in absence of an adjuvant [27]. The HDM-induced murine model for allergic asthma is more relevant for the human situation. In this murine model we have investigated the

effects of dietary interventions with different oligosaccharides either or not combined with beneficial bacteria on the pulmonary inflammation as well on the airway reactivity. Importantly, in several studies, we have compared the effects of dietary intervention with a traditional therapeutic glucocorticosteroid treatment on HDM-allergic asthma features in mice. Finally, we examined the effect of glucocorticosteroid treatment in HDM-allergic mice either or not fed an oligosaccharide diet as well.

Dietary interventions to prevent or treat HDM-induced allergic asthma

Airway inflammation and airway hyperresponsiveness

Eosinophils were one of the first cell types described to be involved in allergic asthma [28] and about 50% of allergic asthmatic patients suffer from predominant eosinophilic inflammation [29]. In the murine model for HDM-induced allergic asthma, we observed an increase in the number of eosinophils in the BALF compared to PBS-sensitized and -challenged mice **(Chapter Three - Seven and Nine)**. Dietary interventions with GOS, GF, FF, or FF*BB* **(Chapter Three - Seven and Nine)** were able to inhibit the development of pulmonary eosinophilia in HDM-allergic mice, whereas GF*BB* or GS did not affect this in this model **(Chapter Four)**. Previously, GOS was used in a rat model for allergic asthma. Also in these OVA-sensitized and -challenged rats it was observed that GOS suppressed allergic airway eosinophilia [30]. Furthermore, in a murine OVA-induced asthma study, Sagar *et al.* used lcFOS/scFOS/pAOS in combination with *BB* and found reduced airway inflammation resulting in reduced airway remodeling [10]. Hence previous studies showed promising effects of dietary intervention with specific prebiotics or a synbiotic combination in OVA-induced animal models, and our studies expanded on these , but focused on HDM-induced asthma.

As a reference treatment, we compared the effects of dietary interventions with a standard therapeutic approach in allergic asthma: the glucocorticosteroid, budesonide. Previous studies in OVA-induced asthmatic mice showed that budesonide is able to decrease the number of eosinophils [31]. We are the first to describe the anti-inflammatory effect of budesonide in a murine model for HDM-induced allergic asthma. Budesonide specifically inhibited the HDM-induced eosinophilia and AHR **(Chapter Five)**, although the effect on AHR was not consistently found in all our studies (**Chapter Five - Seven**). When dietary intervention with GOS was combined with budesonide treatment the HDM-induced eosinophilia was almost completely abolished, suggesting that GOS might improve the effectiveness of budesonide **(Chapter Six)**. The dietary intervention with GOS did not further reduce eosinophil numbers in HDM-allergic mice treated with lower doses of budesonide **(Chapter Seven)**, however, in **Chapter Seven** also dietary intervention with GOS in combination with the high dose budesonide treatment did not improve the therapeutic effectiveness as was observed in **Chapter Six**. A possible explanation for this

discrepancy may be the more pronounced inflammatory response present in the HDM-allergic mice of which data are shown in **Chapter Seven** as compared to those studied in **Chapter Six**. Currently it is unknown what has caused the difference in magnitude of the inflammatory response in these mice, but environmental factors may have influenced and may have interfered with the capacity of GOS and/or budesonide to suppress airway inflammation.

Besides GOS also dietary interventions with GF, FF or FF*BB* were able to decrease the eosinophilic inflammation in HDM-allergic mice. However only the FF*BB* diet or treatment with budesonide was able to reduce the number of HDM-induced lymphocytes in the BALF **(Chapter Four - Six).** Hence in future studies it would be interesting to study whether the combined effect of dietary intervention with FF*BB* and budesonide treatment, alike combining a GOS diet with budesonide treatment will result in a more pronounced inhibitory effect of budesonide on the HDM-induced increase of lymphocytes **(Chapter Six)**. Budesonide is known to suppress Treg [32], which have a suppressive function that might be involved in limiting the pulmonary inflammation. Different from budesonide, GOS did not suppress the frequency of Treg when dampening the inflammatory response and may even support their function. The effects of GOS and budesonide on Treg cells will be discussed later on in this chapter.

Besides pulmonary inflammation, represented by high numbers of eosinophils and lymphocytes in the BALF, AHR is an important clinical feature of allergic asthma [19]. In HDM-allergic mice an increase in AHR upon metacholine exposure was observed compared to PBS mice **(Chapter Three - Seven and Nine)**. Dietary intervention with GOS was able to reduce the development of AHR **(Chapter Three and Five)**. However, this GOS effect was not observed in the experiments described in **Chapter Six, Seven** and **Nine**. This may be caused by the fact that multiple factors are capable of enhancing the susceptibility to provoke AHR, such as humidity and environmental stimuli [22]. Furthermore, like humans not all mice may act the same on drugs or dietary intervention with NDO. Also budesonide treatment alone decreased HDM-allergic AHR **(Chapter Six - Seven)**, which was not the case in **Chapter Five**. From literature it is known that budesonide treatment can decrease AHR in chronic OVA-allergic mice [33,34]. However, considering the multiple factors capable of provoking AHR, combining dietary intervention using GOS with budesonide treatment may be capable of enhancing the effectivity of budesonide in suppressing AHR. Indeed, we did observe a pronounced decrease in AHR after combining dietary GOS with budesonide treatment **(Chapter Six - Seven)**.

All other dietary NDO interventions were not capable of significantly decreasing AHR in HDM-allergic mice despite their suppressive effect on airway inflammation **(Chapter Three - Four)**. This indicates that the mechanistic background of the protective effect

of dietary intervention with GOS may differ from that of the other interventions. One of the hypothesis is that GOS modifies the intestinal microbiome in a different manner in comparison with the other used NDO, resulting in a more optimal composition to indirectly modulate airway function for example by the introduction of a different short-chain fatty acid (SCFA) fermentation profile, contributing to immune homeostasis.

Besides the preventive effect of GOS we investigated the treatment effects of GOS in the HDM-induced allergic model also (unpublished data, see **Fig. 1** of this Chapter). In this study mice were sensitized intranasally (i.n.) on day 0 and challenged i.n. on day 7 to 11 with PBS or HDM. Prior to challenge, mice were fed a control diet or a diet containing GOS from day 5 to day 14. However, in the treatment setting using the current experimental protocol, GOS was not able to affect the enhanced numbers of eosinophils and lymphocytes in the BALF of HDM-allergic mice whereas dietary supplementation with GOS prior to sensitization did reduce airway eosinophilia (**Fig. 1A-B**). However, the HDM-induced neutrophilia was significantly decreased in HDM-mice treated with the GOS diet in a preventive and therapeutic way (**Fig. 1C**). These results suggest that a therapeutic dietary intervention with GOS might be effective in patients suffering from neutrophilic asthma, in which neutrophils are important also during exacerbations [35]. The AHR measured in HDM-allergic mice treated with the GOS diet was not significantly decreased compared to HDM-mice fed the control diet, the AHR of the GOS fed HDM-allergic mice was not significantly different compared to PBS-sensitized and -challenged control mice either (**Fig. 1D**). Hence, this suggests some effectivity on the HDM-induced AHR of dietary intervention with GOS in a preventive and treatment setting as well. Future studies are warranted to further investigate therapeutic options with dietary GOS or other NDO either or not in combination with budesonide on AHR and airway eosinophilia in HDM-induced allergic asthma.

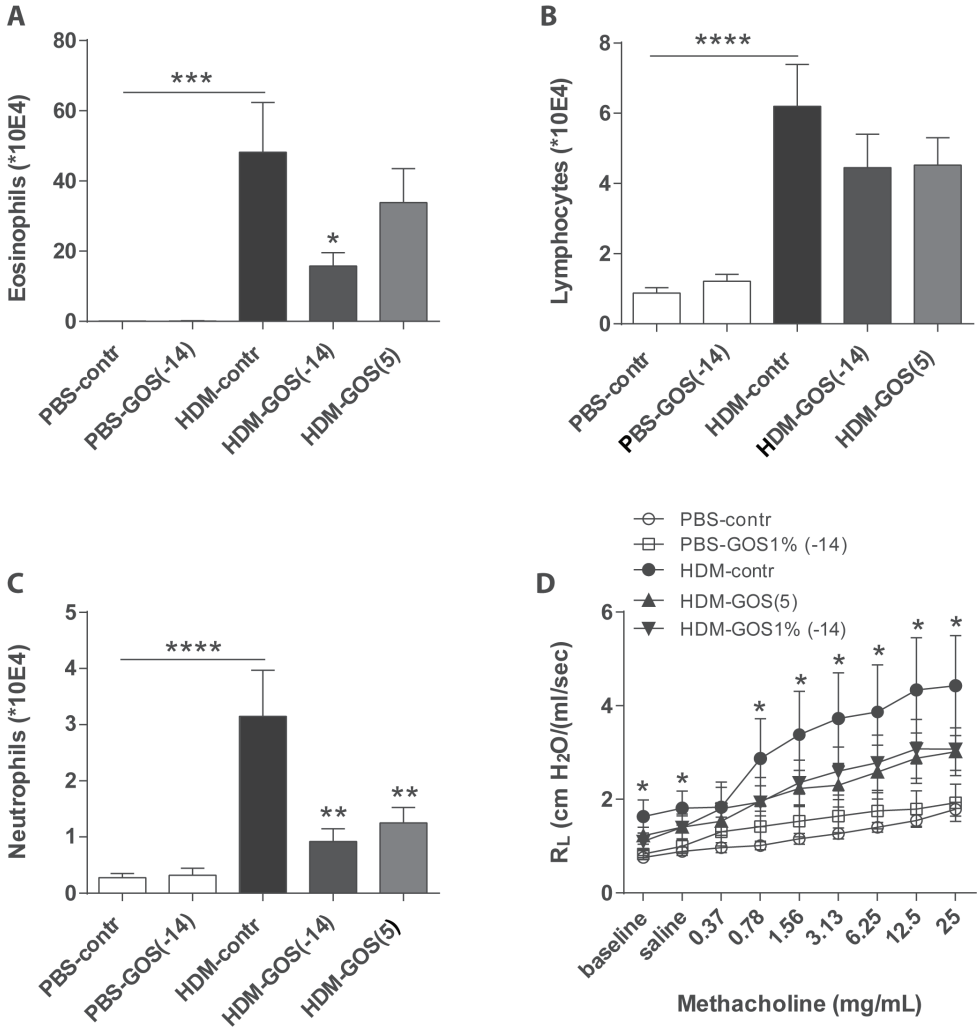

Fig. 1 Airway inflammation and hyperresponsiveness after treatment with GOS.

Infiltration of inflammatory cells in the BALF of house dust mite allergic mice. PBS: PBS-sensitized and challenged mice, HDM: HDM -sensitized and challenged mice. Contr: control diet, GOS(-14): 1% GOS diet from day -14 to day 14, GOS(5): 1% GOS diet from day 5 after sensitization to day 14. Absolute number of eosinophils (**A**), lymphocytes (**B**) and neutrophils (**C**). Airway resistance (R_L) in response to increasing doses of methacholine on day 14 (**D**). Results are shown as mean ± SEM. Statistical significance of differences was tested using post hoc Bonferroni's multiple comparisons test after One-Way ANOVA. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, n = 7-9 mice/group.

Epithelial cells

Airway epithelial cells are the main cells that form a barrier against allergens and therefore are the first cells to come in contact with the allergen [36]. The epithelial cells can be stimulated by allergen and then release different cytokines (interleukin (IL)-33) and chemokines (Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF), Chemokine Ligand (CCL)5, CCL20) [37]. An important cytokine is IL-33, which is a member of the IL-1 cytokine family and signals through the T1/ST2 receptor on T cells, mast cells and macrophages [38,39]. From clinical studies it is known that IL-33 protein of mRNA levels are increased in serum, mucosal biopsies or the airways of asthmatic patients [40,41]. In a murine OVA-induced allergic asthma model, IL-33 was necessary for severe allergic inflammation in the lungs and IL-33 transgenic mice spontaneously develop goblet cell hyperplasia and eosinophilic inflammation in the lung [42,43]. Like in OVA-induced allergic asthma also HDM-induced allergic mice have increased IL-33 concentrations in lung homogenates (**Chapter Four, Five, Six, Eight and Nine**).

Whereas GOS was capable of decreasing IL-33 - although the window of effect differed between experiments (**Chapter Five, Six, Eight and Nine**) - other dietary interventions such as GF*BB* and FF*BB* were not able to modulate the HDM-induced increase in pulmonary IL-33 concentration (**Chapter Four**). In addition, budesonide also did not affect the IL-33 concentrations in the lungs of HDM-allergic mice (**Chapter Five and Six**). The latter is in agreement with human studies in which asthmatic patients treated with budesonide showed no effect on pulmonary IL-33 concentrations [44]. However, when budesonide treatment was combined with dietary intervention with GOS this resulted in a significant decrease in IL-33 concentrations in the lungs of HDM-induced allergic mice (**Chapter Six**).

Beyond IL-33 several other mediators such as GM-CSF, a member of the hemopoietic cytokine family, is produced by epithelial cells [45,46]. Enhanced production of GM-CSF is observed when epithelial cells of asthmatics are cultured [47]. When GM-CSF is overexpressed via adenovirus exposure in the lungs of mice it spontaneously induces Th2 sensitization to the inhaled OVA allergen, mainly via dendritic cell (DC) activation [48,49]. Although GM-CSF concentrations were not significantly increased in HDM-allergic mice, dietary intervention with GOS resulted in reduced GM-CSF concentrations in lungs. Although budesonide treatment alone did not reduce GM-CSF, budesonide treatment in mice fed the GOS diet did further reduce GM-CSF in HDM-allergic lungs (**Chapter Six**). This effect was also seen in PBS-control mice fed the GOS diet and treated with budesonide**(Chapter Six)**.

CCL5, also known as RANTES (regulated on activation, normal T cell expressed and secreted), is expressed in the BALF and lungs of asthmatic patients and induces eosinophil recruitment via chemokine receptor (CCR)3 [50,51]. Not only dietary GOS was capable of reducing CCL5 concentrations in the lungs of HDM-allergic mice, also dietary intervention with FF inhibited the development of high concentrations of CCL5 in lungs of HDM-allergic mice. Although CCL5 was not increased in HDM-allergic mice compared to PBS control mice (**Chapter Three**).

The final important chemokine secreted by the airway epithelium that was examined was CCL20, also known as MIP3α (macrophage inflammatory protein 3α) or LARC (liver activated regulated chemokine) [52,53]. CCL20 attracts immature DC to the lung, which in case of allergic sensitization are locally activated by Th2 driving mediators [54]. Increased levels of CCL20 were observed in asthmatic patients and in the BALF of corticosteroid resistant asthmatics [55,56]. In HDM-allergic mice increased concentrations of CCL20 were observed in supernatants of lung homogenates. Although dietary interventions with GOS, GF*BB* or FF*BB* were not able to reduce the HDM-induced increase in pulmonary CCL20 concentrations (**Chapter Four and Six**), the dietary intervention with GOS in combination with budesonide treatment resulted in a reduction of CCL20 (**Chapter Six and Seven**). In PBS control mice CCL20 concentrations were also decreased after dietary intervention with GOS and treatment with budesonide **(Chapter Six)**, although this effect was not observed in **Chapter Seven**. In **Chapter Seven** all doses of budesonide were already capable of decreasing CCL20 concentrations in the lungs of HDM-allergic mice in absence of dietary intervention with GOS. However, the levels of CCL20 were lower than those measured in experiments described in **Chapter Six,** which may explain this discrepancy.

Taken together, all above described epithelial mediators are important in the activation of innate immune cells such as innate lymphoid cells (ILC2) and DC, which are important cells for the initiation of the adaptive and allergen-specific immune response. Reducing these cytokines and chemokines by dietary interventions with NDO either or not in combination with budesonide treatment may result in decreased Th2 related allergic sensitization or symptoms. Since combined budesonide treatment and preventive dietary intervention with GOS were already capable of decreasing these mediators in PBS control mice, this may indicate that GOS sensitizes the epithelial cells for the effect of budesonide treatment which in turn may increase the efficacy of this drug treatment.

Dendritic cell

The above described epithelial-derived cytokines and chemokines have been shown to be involved in the attraction of DC to the lung after which they migrate loaded with antigen towards the draining mediastinal lymph node via CCR7 up-regulation [57]. Therefore, we investigated the inflammatory DC and their related chemokines (CCL17 and CCL22) in the lungs of the murine model of HDM-induced allergic asthma.

Increased numbers of intra-epithelial DC, pDC and mDC are observed in the airways and BALF of asthmatic patients [58,59]. In addition, in different murine models of asthma increased numbers of DC have been observed in the pulmonary tissue [60,61]. We observed an increased frequency of inflammatory DC ($CD11c^{high}CD11b^{high}MHCII^{+}Ly6C^{+}$) in the lungs of HDM-allergic mice after challenge as well (**Chapter Seven**). High dose treatment with budesonide effectively abrogated the inflammatory DC influx in HDM-allergic mice (**Chapter Seven**), which is in agreement with clinical studies where DC numbers were found to be reduced after treatment with corticosteroids [58]. However, dietary intervention with GOS did not affect the increased DC numbers in lungs of HDM-allergic mice and the GOS diet did not enhance the effectiveness of budesonide on the HDM-induced inflammatory DC response (**Chapter Seven**). Hence, concerning this aspect, dietary intervention with GOS is not as effective as a budesonide treatment under the conditions tested in this HDM-allergic mice model.

Activated DC secrete CCL17, chemokine ligand 17 (TARC; thymus and activation regulated chemokine) and CCL22, chemokine ligand 22 (MDC; macrophage derived chemokine). Both chemokines are ligands for CCR4 and important in allergic responses as CCR4 is expressed by allergen-induced Th2 lymphocytes [62-67]. When CCL22 was neutralized with specific antibodies in an OVA-induced murine asthma model, AHR was prevented and the induction of eosinophils was reduced. Similar effects were found when a specific antibody against CCL17 was used [62,68]. Increased levels of CCL17 and CCL22 were found in sputum of adult asthmatics and in plasma of children with asthma treated with corticosteroids compared to controls. Asthmatics not treated with corticosteroids demonstrated even pronounced increased levels of CCL17 in sputum, serum and plasma [69,70]. Human asthmatic patients showed a reduced CCL17 expression in bronchial epithelium after treatment with inhaled budesonide. Also in the serum of steroid-treated asthmatic children compared with non-steroid-treated asthmatic children reduced CCL22 levels were observed [71,72]. Hence, these Th2 polarizing chemokines are important determinants in the pathophysiology of human asthma. In lung homogenates of HDM-allergic mice fed the control diet compared to control mice, an increase in CCL17 and CCL22 concentrations was observed (**Chapter Three - Seven**). The latter is in agreement with studies in which CCL17 and CCL22 concentrations were increased in the lungs of HDM-induced allergic mice [73,74]. Alike dietary intervention with GOS, also GF or FF

either or not combined with *B. breve* did not significantly affect CCL17 and CCL22 concentrations in the lungs of HDM-allergic mice (**Chapter Three and Four**). By contrast, high dose treatment with budesonide significantly reduced the HDM-induced CCL17 and CCL22 concentrations, whereas lower dose treatment did not show an effect (**Chapter Five and Seven**). Although dietary intervention with GOS tended to decrease CCL17 and CCL22 concentrations, the combination of the GOS diet and budesonide treatment was most effective in inhibiting these chemokines in HDM-allergic mice (**Chapter Six**). The results indicate that GOS is able to improve the effect of budesonide treatment to further decrease DC-related allergy driving chemokines. This might occur via altering the levels of SCFA produced by the gut microbiome. SCFA can suppress airway inflammation and are able to increase the hematopoiesis of DC precursors in the bone marrow leading to lung-resident DC, which suppress the activation of Th2 effector cells in the lung [75].

T helper 2 (Th2)

After activation, DC prime naïve T cells in the local lymph nodes to develop into Th2 cells which are the main T helper cells in asthma. These Th2 cells migrate to the lung and like ILC2 produce IL-5 and -13, resulting in eosinophilic inflammation and AHR [54,76,77]. Interleukin (IL)-4, -5 and -13 were the first Th2 derived cytokines demonstrated to be important in allergy and asthma. Both IL-4 and IL-13 have similar capacities, like promoting B-cell IgE-isotype switching [78], promoting inflammation [79], development of AHR and goblet metaplasia [80]. IL-5 is critical for B cell survival, eosinophil growth, maturation and activation and inhibits eosinophil apoptosis [81-83] and enhances basophil development [84]. From animal studies it is known that IL-4, -5 and -13 play important roles in allergic asthma. Specific blockade of IL-4 with anti-IL-4 antibodies in mice inhibits eosinophilic inflammation and AHR in an OVA-model of asthma [85,86]. Moreover, IL-4 deficient mice are protected from developing allergic asthma [87]. IL-13 is capable of mimicking most of the functions of IL-4 since they bind the same receptor [88,89]. Indeed, also anti-IL-13 antibodies decreases mucus production and AHR [88,90]. Moreover, IL-13 deficient mice did not develop mucus production and AHR after antigen challenge in a OVA-allergic asthma model [91]. IL-4 and IL-13 levels are increased in the airways, BALF and sputum obtained from asthmatic patients, and IL-4 levels were also found to be enhanced in the serum [92-94]. When mild asthmatic patients were nebulized with IL-4 a significant increase in AHR was observed concomitantly with elevation of eosinophil numbers in the sputum [95]. Both in the airways and the peripheral circulation high levels of IL-5 are observed in asthmatic patients [96-98]. Alike in asthmatic patients in the murine model of HDM-allergic asthma, IL-4, -5 and -13 concentrations in supernatants of lung homogenates were increased compared to the control mice (**Chapter Three - Seven**). Dietary interventions with GF or FF were not able to decrease HDM-induced IL-5 and -13 concentrations whereas a combination of FF*BB* decreased IL-4 concentrations

(**Chapter Three - Four**). On the other hand, dietary intervention with GOS reduced IL-13 concentrations in lung homogenates, but this effect was lost after restimulation of the lung cells with HDM, although not observed in all studies (**Chapter Five – Seven**). Although the dietary intervention with GOS already reduced IL-13 concentration when combined with budesonide treatment, also IL-4 and -5 concentrations were decreased in lung tissue of HDM-allergic mice (**Chapter Six - Seven**). Budesonide treatment alone also did have an inhibitory effect on HDM-induced increase in IL-4 and -13 concentrations (**Chapter Five and Seven)**, although this was not observed in **Chapter Six,** but did not affect IL-5 concentrations **(Chapter Five - Six)**. Originally, Th2 cells were thought to be the only cells to predominate in producing these cytokines [97]. Indeed, we did found an increase in the frequency of Th2 cells in HDM-allergic mice which was decreased after dietary intervention with GOS either or not combined with budesonide treatment (**Chapter Six - Seven**). However, IL-4, -5, and -13, are also produced by other inflammatory cell such as ILC2, mast cells, basophils and eosinophils [99-102]. **Chapter Five, Six, Eight and Nine** show that GOS is capable of reducing IL-33 concentrations which might lead to less activation of ILC2. ILC2 are innate lymphoid cells that are not antigen specific and are activated by different mediators (e.g IL-33, TSLP, IL-25). These ILC2 express the transcription factor GATA3 and have the ability to produce type 2 cytokines, such as IL-4, -5 and -13 [103-105]. Hence, whether this decrease in IL-4, -5 and -13 in HDM-allergic mice fed with GOS is related to the reduced Th2 frequency and/or maybe the suppression of other cells needs to be further elucidated.

Regulatory T cells (Treg)

The role and function of Treg cells in asthma is not fully understood. Several studies have been conducted in adults and children to elucidate the role of Treg cells in allergic asthma. However, different results have been observed between tissue and blood and between adults and children [106]. In the study described in **Chapter Nine** the effect of Treg cell depletion on HDM-allergic asthma was investigated by treating the mice with anti-CD25 antibody (anti-CD25). Anti-CD25 treatment is commonly used to deplete Treg cells *in vivo*, although CD25 is not a specific marker for Treg cells but is expressed at a very high levels on these cells. Other cells that express CD25 are monocytes, DC and activated B and T cells [107]. After anti-CD25 treatment $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells were significantly decreased in control and HDM-allergic mice, both in lung cell suspensions and splenocytes **(Chapter Nine)**. Since Treg cells are probably capable of down-regulating the development of allergic asthma [108], an increase in Th2 cells was observed in both spleen and lung cells of HDM-allergic mice after Treg cell depletion. This increase in pulmonary Th2 cells was associated with enhanced concentrations of the Th2 cytokines IL-4 and IL-13 in the supernatant of HDM-restimulated lung cells obtained from HDM-allergic mice. Despite the increase in Th2 after Treg depletion, no or a modest effect

on eosinophilia was observed in HDM-allergic mice which is in line with findings reported by others [109,110].

Next we investigated the role of Treg cells in the protective effect of GOS in HDM-induced asthma. Dietary intervention with GOS significantly decreased the percentage of eosinophils in HDM-allergic mice. After treatment with anti-CD25 this protective effect of the GOS diet was lost and similar patterns were observed for neutrophils and lymphocytes in BALF of HDM-allergic mice. IL-33 and CCL5 concentrations were increased in supernatants of lung homogenates of HDM-allergic mice and anti-CD25 treatment did not affect this response. GOS decreased the concentrations of IL-33 and CCL5 in lungs from HDM-allergic mice and these effects were abolished after anti-CD25 treatment. CCL20 concentrations were increased in lung homogenates of HDM-allergic mice and even further increased after anti-CD25 treatment. Dietary intervention with GOS did not decrease CCL20 concentrations in HDM-mice, either or not treated with anti-CD25. These data suggest that the suppression of airway eosinophilia and pulmonary levels of IL-33 and CCL5 in HDM-allergic mice induced by GOS is mediated via Treg cells. We hypothesize that GOS is able to instruct functional Treg to suppress epithelial IL-33 and CCL5 production, and either via this route or directly contribute to suppression of pulmonary inflammation (see **Fig. 5** in **Chapter 9**). Indeed, in different murine models for food allergy NDO have been shown to inhibit allergic symptoms via induction of functional Treg cells, since also in these models after Treg cell depletion the protective effects of the NDO were abrogated [111,112]. Taken together, these results indicate that Treg cells play a role in the suppression of allergic asthma and that dietary intervention with GOS is capable of enhancing the suppressive function of Treg. This may contribute to the capacity of dietary GOS to reduce pulmonary IL-33 and CCL5 concentrations. Whether GOS affects the Treg directly needs to be further elucidated.

Future directions

Currently, inhaled corticosteroids with or without long-acting β-agonists are mostly used to treat asthma [113]. As these drugs have considerable side effects, new approaches are needed to improve asthmatic symptoms [114]. Dietary NDO either or not combined with beneficial bacteria may provide a new strategy for the prevention and treatment of allergic asthma. As shown in this thesis NDO may have an effect on eosinophils, Th2 cells and related cytokines and Treg cells. From literature it is known that HDM contains the endotoxin (LPS), which is a Toll Like Receptor (TLR)4 ligand and in low dose promotes Th2 immunity [115,116]. As described by Hammad *et al.*, TLR4 expression is critical for the development of Th2 cells, DC and allergic inflammation to HDM-allergens [61]. In i*n vitro* studies using intestinal epithelial cells, it was observed that NDO may act as TLR4 ligands. The effect of LPS was largely inhibited after pre-treatment with NDO. [117]. Therefore, *in vitro* studies with lung epithelial cells and *in vivo* studies may provide insight in the role of NDO on the effect of the TLR4 response in allergic asthma. Since TLR4 expression is necessary for immune responses by DC, more investigations concerning the effects of dietary interventions on subsets of DC are needed. Ruane *et al.*, observed that lung DC after intranasal immunization are able to migrate to T cells in the gastrointestinal tract and have protective capacities against intestinal pathogens. Whether this is also possible vice versa, hence, DC migration from the intestine to T cells in the lungs and contributing to the protective effect of oligosaccharides needs to be further investigated [118].

Originally Th2 cells were thought to be the most important cells in asthma, but recently a new type of cell has been discovered in the airways: the ILC2. ILC2 undergo proliferation in response to TSLP, IL-25 and IL-33 and produce high amounts of Th2 cytokines (IL-5 and -13) [104,119,120]. We have shown that NDO are capable of reducing IL-5 and -13 concentrations in lungs (**Chapter Three - Six**) therefore, it is of importance to investigate whether this decrease is caused by oligosaccharide-induced inhibition of ILC2 activation in parallel with the suppression of Th2 cell responses. This would give insight in the mechanism by which specific NDO diets can decrease cytokines and inflammatory cell influx (such as eosinophils) as well as the development of AHR in allergic asthma.

Animal studies have shown that the gut microbiota has an effect on systemic immune functions [121-124]. Also asthmatic children have different compositions of the gut microbiota, a high prevalence of *Clostridium difficile* and low prevalence of *Bifidobacterium* [6,7]. Infants provided with GOS/lcFOS showed higher amounts of *Bifidobacterium* and *Lactobacillus* species [3-5]. Future studies in the HDM-induced asthma model assessing the composition of the gut microbiota may provide insight in changes in the microbiota that can be linked to the beneficial effect of the NDO asthmatic features. Not only the gut contains a microbiota, also the lungs, which were previously thought to be sterile in health, are now known to contain various bacteria [125]. In human studies the lung

microbiome of asthmatic patients was compared to healthy controls. Asthmatics showed an increase in the frequency of *Proteobacteriae* and a decrease in the frequency of *Bacteroidetes* in the lung [126,127]. These results may indicate that affecting the lung microbiome, possibly via modulation of the gut microbiome by dietary intervention with NDO either or not combined with beneficial bacteria, may have beneficial effects on the development and/or treatment of asthma. So far, no studies have been conducted to examine whether dietary NDO can have an influence on the lung microbiome.

As we have observed positive effects of GOS on allergic features, clinical studies would be a next approach to evaluate these findings. For example, in infants at high risk of developing asthma specific NDO may be supplemented to the diet, and these infants can be followed in time to examine whether asthmatic features will occur. Besides infants, also adult patients already treated with glucocorticosteroids may be supplemented with GOS or other NDO, like FF or FF*BB*, to investigate if the effectiveness of the anti-inflammatory drug therapy can be improved. When testing GOS in adults, we need to keep in mind that in South East Asia a few very rare cases of GOS-related allergy have been reported in atopic subjects [128]. But despite the fact that GOS has been consumed by millions of consumers in the past decades, there have never been any reports of GOS-related allergy in infants and the incidence in children and adults has been found to be very low, indicating that the risk of GOS-related allergy is very limited.

To conclude

The findings in this thesis demonstrate that NDO have preventive effects on the development of HDM-induced allergic asthma. Immune modulation using dietary interventions with GF, FF either or not combined with B. *breve*, GS and in particular the GOS diet is demonstrated in our studies. This response involves modification of epithelial-derived cytokines and chemokines, DC-related chemokines, Th2 and possibly ILC2-related cytokines and reduced inflammatory cell (such as eosinophils) numbers in the lung in association with improvement of the lung function. Together, the results in this thesis have given more insight in the possible mechanisms of action of in particular GOS in the prevention of asthma. GOS was capable of suppressing airway eosinophilia and this effect was lost after Treg cell depletion, suggesting Treg cells to be involved in the protective effect of GOS. However, more research is needed to further unravel the complete mechanism of action.

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APPENDICES

Nederlandse samenvatting

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Nederlandse samenvatting

Allergische astma is een steeds meer voorkomende chronische aandoening. Volgens de Wereld Gezondheid Organisatie lijden ruim 235 miljoen mensen wereldwijd aan deze ziekte. Astma wordt onder andere gekenmerkt door aanvallen van kortademigheid, piepende ademhaling en/of hoesten. Wanneer astma-patiënten een stof (allergeen) inhaleren waarvoor ze allergisch zijn, zoals huisstofmijt, pollen, schimmels of zelfs medicijnen (bv. aspirine), wordt een allergische reactie uitgelokt. Ook lichamelijke inspanning of het inademen van koude of vochtige lucht kan leiden tot symptomen in astma-patiënten.

Wanneer allergenen in de luchtwegen terechtkomen worden epitheelcellen in de long geactiveerd, waarbij verschillende mediatoren (cytokines en chemokines) vrijkomen. Hierdoor worden verschillende typen cellen geactiveerd, waaronder dendritische cellen, T-helper 2 cellen (Th2) en type 2 lymphoïde cellen (ILC2). Dit leidt tot productie van nog meer cytokines en chemokines die ervoor zorgen dat antigeen-specifieke B cellen differentiëren in immunoglobuline E (IgE)-producerende plasmacellen. IgE bindt onder andere aan de mestcellen in de long. IgE kan vervolgens binden aan het specifieke allergeen waardoor de mestcellen geactiveerd worden en zullen degranuleren. Verschillende mediatoren worden geproduceerd en komen vrij uit de mestcel. Deze veroorzaken overmatige slijmproductie, het aantrekken van ontstekingscellen en zelfs luchtwegobstructie, waardoor de patiënt minder goed kan ademen. (zie **Figuur 1**, **Hoofdstuk Eén**).

Tegenwoordig wordt astma voornamelijk behandeld met luchtwegverwijderaars en ontstekingsremmers, zoals respectievelijk langwerkende bèta-agonisten (vb. salmeterol) en glucocorticosteroïden (vb. budesonide). Deze medicijnen kunnen de symptomen van astma (o.a. kortademigheid) en de allergische ontsteking verminderen, maar behandelen niet de oorzaak (dit zijn dus echte symptoombestrijders). Helaas hebben de ontstekingsremmers zoals glucocorticosteroïden verschillende bijwerkingen, zoals gewichtsverlies, verminderde groei bij kinderen en verminderde spierkracht.

Niet alleen allergenen en medicijnen kunnen de ontwikkeling van astma beïnvloeden, ook omgevingsfactoren zoals luchtverontreiniging en verandering in het voedingspatroon kunnen hieraan bijdragen. Het huidige "Westerse" dieet bevat weinig vezels en relatief veel verzadigde vetten en omega-6 meervoudig onverzadigde vetzuren, wat de samenstelling van het microbioom in de darm kan veranderen. Uit verschillende studies is gebleken dat het microbioom in de darm van astmapatiënten verschilt van die van gezonde personen. Door het microbioom in de darm te optimaliseren, kan mogelijk de gevoeligheid voor het ontwikkelen van allergieën verminderd worden. Voedingssupplementen zoals niet-verteerbare oligosacchariden (suikers), ook wel prebiotica genoemd, en gezondheid bevorderende bacteriën (probiotica) kunnen het microbioom mogelijk op een positieve manier beïnvloeden. Dit kan vervolgens bijdragen aan het ondersteunen van een gezond/gebalanceerd immuunsysteem.

Niet-verteerbare oligosacchariden met o.a. prebiotische eigenschappen zijn ook aanwezig in moedermelk. In moedermelk zijn ongeveer 130 verschillende soorten oligosacchariden geïdentificeerd. Op basis van deze structuren zijn prebiotica ontwikkeld, zoals galacto-oligosacchariden (GOS) gemaakt uit lactose en fructo-oligosacchariden (FOS) geïsoleerd uit onder andere witlof. Deze prebiotica worden toegevoegd aan producten zoals babymelkpoeder, omdat in koemelk veel minder oligosacchariden voorkomen dan in moedermelk. Van deze prebiotica is aangetoond dat ze selectief de groei van commensale *Bifidobacterium* en *Lactobacillus* bacteriën kunnen stimuleren.
Probiotica zijn micro-organismen, die indien in gepaste hoeveelheden toegediend, een positief effect kunnen hebben op de gezondheid. Voorbeelden zijn *Bifidobacterium* en *Lactobacillus* bacteriën.

In dit proefschrift is gebruik gemaakt van een huisstofmijt geïnduceerd allergisch astma model in muizen. Door huisstofmijt eiwitten in de luchtwegen toe te dienen, worden de muizen gevoelig gemaakt voor het allergeen. De muizen kregen een dieet gesupplementeerd met verschillende prebiotica: GOS, een combinatie van GOS met sialyllactose (GS), een combinatie van GOS en FOS (GF) of een combinatie van twee verschillende soorten FOS (FF). GF en FF werden ook gecombineerd met het probioticum, *Bifidobacterium breve* (GF*BB*, FF*BB*).

In **Hoofdstuk Twee** zijn er verschillende methodes vergeleken om luchtwegreactiviteit te meten. Met behulp van de luchtwegreactiviteitsmeting kun je bepalen hoe benauwd een muis wordt. Voor deze studie zijn twee muizenmodellen gebruikt die karakteristiek zijn voor milde en ernstige luchtwegontsteking. In het ernstige model werd een verhoogd aantal ontstekingscellen waargenomen ten opzichte van het milde model. In dit experiment werden twee verschillende methoden om luchtwegreactiviteit te meten met elkaar vergeleken: 1. de Penh meting, hierbij zijn de muizen bij bewustzijn en kunnen ze vrij rond bewegen en 2. de weerstandmeting, hierbij zijn de muizen onder narcose en worden kunstmatig beademd. Het beste 'window of effect' werd gevonden bij de weerstandmeting en daarom hebben we deze methode gebruikt in onze daarop volgende experimenten.

Bij het bepalen van de methode die het meest optimaal is om luchtwegreactiviteit te meten in een allergisch astmamodel werden de muizen allergisch gemaakt voor ovalbumine. Ovalbumine is een eiwit dat voorkomt in het kippenei. Het is veel gebruikt voor astma onderzoek echter het is geen natuurlijk allergeen waarvoor mensen na inhalatie allergische astma kunnen ontwikkelen. Daarom is in het volgende experiment huisstofmijt, als meer relevant allergeen, toegediend om de muizen allergisch te maken. Huisstofmijt is een allergeen wat bij tenminste 85% van de astmapatiënten de klachten veroorzaakt.

In **Hoofdstuk Drie** werd na toevoeging van FF of GF aan het dieet van muizen, een beschermend effect gevonden op de allergische ontsteking. In **Hoofdstuk Vier** zijn deze prebiotica gecombineerd met *Bifidobacterium breve*, een probiotische bacterie. De synbiotische mix FF*BB* verlaagde het aantal ontstekingscellen in de long, met name het aantal eosinofielen en lymfocyten. Tevens werden IL-4 en IL-5 verlaagd in de long; dit zijn belangrijke cytokines in de ontwikkeling van allergische astma. Voornamelijk het FF*BB* dieet bleek beschermend; dit in tegenstelling tot het GF*BB* dieet in dit model.

De experimenten uit **Hoofdstuk Drie** toonden tevens aan dat een dieet met GOS niet alleen de allergische ontstekingsreactie verminderde, maar ook positieve effecten had op allergische symptomen (de verhoogde luchtwegreactiviteit) in muizen. Om te bestuderen hoe sterk de effecten van een GOS dieet zijn, is in **Hoofdstuk Vijf** een vergelijking gemaakt tussen de effectiviteit van een preventief dieet met GOS en een behandeling met een inhalatie medicijn dat bij de mens gebruikt wordt om astmatische symptomen te verlichten, het glucocorticosteroïd budesonide. Budesonide werd vooraf aan de herhaalde blootstellingen aan huisstofmijt toegediend in de luchtwegen van de muis via de neus; dit omdat muizen ademhalen via de neus. Het GOS dieet verlaagde het aantal ontstekingscellen (eosinofielen) en verschillende Th2 cytokines en chemokines net zo effectief als budesonide. Tevens werd in deze studie aangetoond dat het GOS dieet de verhoogde luchtwegreactiviteit in allergische muizen verminderde, terwijl dit na budesonide behandeling niet het geval was. Hieruit kunnen we concluderen dat GOS mogelijk de ontwikkeling van allergische astma tegengaat.

Zowel het GOS dieet als de behandeling met budesonide bleken de allergische luchtwegontsteking sterk te onderdrukken in het door huisstofmijt geïnduceerd astma model in muizen (**Hoofdstuk Vijf**). In **Hoofdstuk Zes** is daarom bestudeerd of de toevoeging van GOS aan het dieet de effectiviteit van behandeling met budesonide kan verbeteren. Met het GOS dieet werd twee weken voor de eerste blootstelling aan het allergeen (sensibilisatie) gestart en ook gedurende de budesonide behandelingen kregen de muizen GOS in hun voer. Combinatie van een GOS dieet en behandeling met budesonide zorgde voor een verlaging in het aantal eosinofielen in de long, zodat dit aantal bijna gelijk was aan dat van niet-astmatische muizen. De combinatie was tevens het meest effectief in het verlagen van chemokines, cytokines en het percentage Th2 cellen in de long. Een 1% GOS dieet werd vergeleken met een 2.5% GOS dieet: hiertussen werden geen significante verschillen waargenomen en beide doseringen waren effectief. Ook in niet allergische muizen werd de basale hoeveelheid van een aantal chemokines verlaagd door budesonide in muizen die met GOS werden gevoerd. De muizen leken gevoeliger te worden voor de werking van budesonide door het GOS dieet. GOS kan er dus mogelijk voor zorgen dat de effectiviteit van budesonide wordt versterkt in de behandeling van astma.

In **Hoofdstuk Zes** werd gebruikt gemaakt van één dosis budesonide (500 µg/kg), maar dit is een relatief hoge dosis. Het verlagen van de effectieve budesonide dosering is wenselijk, omdat dan de bijwerkingen mogelijk verminderen. In **Hoofdstuk Zeven** werd de effectiviteit van een verlaging van de dosis (20, 100 en 500 µg/kg) budesonide bestudeerd, al dan niet gecombineerd met een GOS dieet. Behandeling met budesonide verminderde de verhoogde luchtwegreactiviteit, de inflammatoire dendritische cel en de Th2 type ontsteking en het aantal eosinofielen. GOS verlaagde ook het aantal eosinofielen en het percentage Th2 cellen in de long van huisstofmijt allergische muizen. Echter in deze studie werd geen verbetering gevonden van de behandeling met budesonide door de toevoeging van GOS in het dieet.

IL-33, een cytokine dat uitgescheiden wordt door het epitheel en in de long bijdraagt aan het ontwikkelen en in stand houden van huisstofmijtallergie, was verlaagd in astmatische muizen die gevoerd werden met het GOS dieet (**Hoofdstuk Vijf**). IL-33 is niet alleen in astma een belangrijk cytokine, maar kan ook in de darm bijdragen aan het tot stand komen van een ontstekingsreactie en schade aan het darmslijmvlies. In **Hoofdstuk Acht** zijn de effecten van GOS naast het door huisstofmijt geïnduceerde astma model ook bestudeerd in een muismodel waarbij de darmbarrière werd verstoord. Om de darmbarrière te verstoren werden de muizen met het mycotoxine deoxynivalenol (DON) behandeld middels orale toediening. DON is een toxine van schimmels die op bepaalde soorten granen aanwezig kan zijn. DON verhoogt IL-33 expressie in de darm, maar wanneer de muizen tijdens de behandeling met DON een GOS dieet kregen was de IL-33 concentratie verlaagd in de darm. Door de DON geïnduceerde verhoging van IL-33 in de darm of de huisstofmijt geïnduceerde verhoging van IL-33 in de long tegen te gaan, kan GOS mogelijk het ontstaan van allergieën en ontstekingen die geassocieerd worden met hoge IL-33 expressie verminderen.

Hoewel astma voornamelijk een Th2 type allergie is, zijn ook andere T-cellen vaak veranderd in de long, waaronder regulatoire T cellen (Treg). In **Hoofdstuk Negen** zijn selectief Treg cellen van de muizen weggehaald (gedepleteerd) door middel van een injectie met een specifiek antilichaam (anti-CD25). Dit antilichaam zorgt ervoor dat de Treg cellen door andere immuuncellen worden verwijderd. Na controle van long- en miltcellen bleek inderdaad dat nagenoeg geen Treg cellen meer in het weefsel aanwezig waren en dit zorgde voor een sterkere Th2 reactie. Uit deze resultaten kan geconcludeerd worden dat de Treg cel een beschermend effect heeft op de ontwikkeling van astma. Ook werd bestudeerd of Treg cellen betrokken waren bij het beschermende effect van een GOS dieet. Huisstofmijt allergische muizen die een GOS dieet kregen, hadden een verlaagd percentage eosinofielen en lagere IL-33 en CCL5 concentraties in de longen. Echter als de Treg cellen verwijderd werden voor de dieet interventie met GOS, was het beschermende effect van GOS weg. Uit deze resultaten blijkt dus dat GOS een positief effect heeft op de preventie van astma, mogelijk door het ondersteunen van de functie van Treg cellen in de long.

Naast de preventie van huisstofmijt allergische astma is ook bestudeerd of GOS kan worden ingezet ter behandeling van astma. De muizen kregen in deze studie het GOS dieet nadat ze al een keer in contact gekomen waren met huisstofmijt (sensibilisatie), maar voordat deze blootstelling werd herhaald (allergeen challenge). Uit de resultaten bleek dat bij gebruik van het GOS dieet als behandelmethode het aantal eosinofielen en lymfocyten niet verlaagd werd, maar het aantal neutrofielen wel. Naast de Th2 geïnduceerde astma bestaat er ook een vorm van astma waarin de neutrofielen een belangrijke rol spelen, zogenaamd neutrofiele astma. Hierbij zou GOS mogelijk een beschermend effect kunnen hebben als het wordt ingezet als behandeling.

De onderzoeksresultaten beschreven in dit proefschrift laten zien dat prebiotica muizen kunnen beschermen tegen het ontwikkelen van allergische astma. Diëten met GF, FF, GF*BB*, en vooral FF*BB* of GOS kunnen hierbij het immuunsysteem in gunstige zin beïnvloeden. Ontstekingscellen (eosinofielen), cytokines, chemokines, het percentage Th2 cellen en/of de verhoogde luchtwegreactiviteit waren verlaagd na een dieet met GOS of FF*BB*. De resultaten geven vooral meer inzicht in het mogelijke werkingsmechanisme van GOS in de preventie van astma. GOS verlaagde het aantal eosinofielen en dit effect ging verloren na verwijdering van Treg cellen. Mogelijk spelen de Treg cellen een belangrijke rol in het beschermende effect van GOS.

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En vergeet niet: 'Elk einde is een nieuw begin'.

Curriculum Vitae

Kim Verheijden was born on the 1st of September 1981 in Hulst, the Netherlands. In 1997, she graduated from her secondary school at the Reynaertcollege in Hulst and started and successfully finished the intermediate vocational education, pathology at the Baronie College in Breda, the Netherlands. In 2002 she started the bachelor program Biology & Medical Laboratory Research, at the Institute for Life Sciences & Chemistry of the University of Applied Sciences Utrecht, the Netherlands. During this period, she performed two internships at the Utrecht University, division of Pharmacology supervised by Prof. Dr. G. Folkerts. In the first internship she investigated a rheumatoid arthritis model in two different mouse strains (C57BL/6 versus DBA/1J). In her second internship she set up a smoke-induced lung emphysema model using C57BL/6 and BALB/c mice. After graduation in September 2005 she joined the contract research organization Curax BV, Utrecht, the Netherlands as a research technician. After working as a research technician for 6½ years she started her PhD study at the Utrecht University, Utrecht Institute for Pharmaceutical Science, Division of Pharmacology supervised by Prof. Dr. J. Garssen, Prof. Dr. G. Folkerts, Dr. A.D. Kraneveld and Dr. L.E.M. Willemsen. She investigated whether and how oligosaccharides suppress allergic asthma, described in this thesis. She was trained in the Drug Innovation PhD program of graduate school of Life Sciences, Utrecht University. Kim conducted her PhD research project within the framework of the Carbohydrate Competence Center (CCC-WP25, Immunomodulating properties of oligosaccharides from various sources).

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