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Regulatory T Cell Depletion Abolishes the Protective Effect of Dietary Galacto-Oligosaccharides on Eosinophilic Airway Inflammation in House Dust Mite–Induced Asthma in Mice[1,2]

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Abstract

Background: In a murine model for house dust mite (HDM)–induced asthma, dietary galacto-oligosaccharides have been shown to suppress allergic symptoms. Previously, CD25$^+$ regulatory T cells (Tregs) induced by nondigestible oligosaccharides were found to protect against allergy development.

Objective: The aim of the current study was to examine the effect of anti–CD25-induced Treg depletion in a murine HDM-induced asthma model and to study the contribution of Tregs in the protective effect of dietary intervention with galacto-oligosaccharides.

Methods: Male BALB/c mice (aged 6–8 wk) were intranasally sensitized and challenged with phosphate-buffered saline (PBS) or HDM. Two weeks before sensitization and throughout the whole experiment, mice were fed a control or 1% w/w galacto-oligosaccharide diet. Tregs were depleted by anti-mouse CD25 antibody (intraperitoneally injected). On day 14, T helper cell subtypes in lung and spleen were analyzed and cytokines were measured. Leukocyte subtypes were analyzed in the bronchoalveolar lavage fluid, and interleukin (IL)–33 and chemokines were measured in lung homogenate supernatants.

Results: Anti-CD25 treatment depleted CD25$^+$ Forkhead box P3$^+$ Tregs in the lung and spleen of control and HDM-allergic mice ($P < 0.0001$) by >70% while increasing the percentage of activated T helper cells ($P < 0.05$) and type 2 T helper cells ($P < 0.05$). This was associated with increased IL-10, IL-4, and IL-13 concentrations in supernatants of ex vivo restimulated lung cells ($P < 0.01$). Bronchoalveolar lavage fluid leukocyte numbers and percentages of eosinophils and lymphocytes were greater in HDM-allergic mice compared with PBS mice ($P < 0.01$) but remained unaffected by the anti-CD25 treatment. Galacto-oligosaccharides decreased airway eosinophilia compared with HDM-allergic mice fed the control diet (from 47.8% ± 6.7% to 26.6% ± 8.5%, $P < 0.01$). This protective effect was lost in anti–CD25-treated mice ($P < 0.05$). In lung homogenates of HDM-allergic mice, IL-33 was increased compared with PBS mice (from 2.8 ± 0.3 to 5.4 ± 0.6 ng protein/mg, $P < 0.01$). Galacto-oligosaccharides abrogated the increase in IL-33 compared with HDM-allergic mice fed the control diet (3.0 ± 0.6 ng protein/mg, $P < 0.05$), which was abolished by the anti-CD25 treatment ($P < 0.01$).

Conclusions: Treg depletion enhances pulmonary type 2 T helper cell frequency and cytokine release in HDM-induced asthma in mice. Galacto-oligosaccharides decreased airway eosinophilia and IL-33 concentrations in the lung, which was abrogated by Treg depletion. This indicates that galacto-oligosaccharides have a beneficial effect in the prevention of HDM-induced allergic asthma by supporting pulmonary Treg function. *J Nutr* doi: 10.3945/jn.115.224402.

Keywords: allergy, asthma, galacto-oligosaccharides, regulatory T cell, anti-CD25

Introduction

Allergic asthma is a chronic inflammatory disease occurring as a result of type 2 T helper (Th2)[7] immune responses. High pulmonary IL-4, IL-5, and IL-13 responses; airway hyperresponsiveness; and eosinophilic inflammation are major characteristics of allergic asthma (1). Around 300 million people worldwide have asthma, and the incidence continues to rise in developed countries (2). Type 1 T helper (Th1) cells were first assumed to be able to downregulate Th2 cells, but research has shown that regulatory T cells (Tregs) also contribute to downregulate the development of

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allergic diseases and asthma (3). Natural or inducible Tregs express both Forkhead box P3 (Foxp3) and CD25 and represent up to 10% of $CD4^+$ T cells (4). Previous animal studies have shown that $CD4^+CD25^+$ Tregs can control allergic airway diseases (5–7). Several studies have been conducted to investigate the role of Tregs in asthmatic patients. However, results differ between adults and children and between airway tissue and blood (8). Children with moderate to severe asthma showed more Tregs than pediatric patients with mild asthma, and both had higher mRNA expression of Foxp3 than control subjects (9). In adults, $CD4^+CD25^+$ lymphocyte levels were higher in the blood of those with mild asthma compared with those with severe asthma and with healthy controls (10). In contrast, Smyth et al. (11) reported that airway Treg numbers increase with more severe disease in the bronchoalveolar lavage fluid (BALF) of patients with asthma. This could counterbalance 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 nondigestible oligosaccharides (NDOs) was able to reduce allergic symptoms. Treg depletion abrogated the protective effect of the NDO diet, indicating that NDOs may act via the induction of functional Tregs (12, 13).

Therefore, there is an increased interest in using NDOs to prevent the development or reduce symptoms in allergic diseases. Galacto-oligosaccharides are specific NDOs, which are selectively fermentable in the intestine, with beneficial effects on the growth and/or the activity of lactobacilli and bifidobacteria (14–16). NDOs, either in combination with or without beneficial bacteria, reduce allergic symptoms not only in murine models of food allergy but also in asthma (17–19). In a house dust mite (HDM)–induced allergic asthma model, we found that galacto-oligosaccharides were capable of reducing allergic symptoms 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, Th2 cytokine production was decreased upon treatment of adult patients with asthma (22). Because galacto-oligosaccharides have a protective effect on allergic symptoms in an HDM-induced asthma model, we examined the contribution of $CD25^+$ Tregs in the preventive effect of dietary galacto-oligosaccharides on asthma-associated airway inflammation by using in vivo anti-CD25 depletion in a murine model for HDM-allergic asthma.

Methods

Mice. Six- to 8-wk-old male BALB/c mice (Charles River) were housed under biocontained sterile conditions using HEPA-filtered isocages (Tecniplast). 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 galacto-oligosaccharides (Vivinal galacto-oligosaccharides syrup; FrieslandCampina Domo), as described by Akbari et al. (23). The diets were provided 2 wk before sensitization and continued during the whole experiment, from day –14 to day 14. Mice were intranasally sensitized with PBS in the presence or absence of 1 μg HDM (Greer Laboratories) on day 0 and challenged intranasally with PBS or 10 μg HDM on days 7–11, while under isoflurane anesthesia, as described in a previous study (20, 24). One day before sensitization (day –1), control or 1% galacto-oligosaccharide–fed mice were not treated or were intraperitoneally injected with 200 μL (1 mg/mL) rat anti-mouse CD25 (anti-CD25, a monoclonal antibody from a rat PC61 hybridoma cell line; EPIRUS Biopharmaceuticals Netherlands BV) for in vivo depletion of $CD25^+$ Tregs. A second treatment was carried out 1 d before challenge (day 6) (13). Mice were killed on day 14 (20, 24) using an overdose of euthasate, intraperitoneal injection. (**Figure 1**).

Bronchoalveolar lavage. Lungs were lavaged 4 times with 1 mL pyrogen-free saline (0.9% NaCl, 37°C). The BALF cells were centrifuged (400 × *g*, 5 min at 4°C), and total numbers of BALF cells were counted with the use of a Bürker-Türk chamber (magnification 100×). For differential BALF cell counts, cytospin preparations were stained with Diff-Quik (Merz & Dade A.G.). Numbers of eosinophils, lymphocytes, and neutrophils were scored with light microscopy (25).

Preparation of lung homogenates. A Precellys 24 tissue homogenizer (Bertin Technologies) was used to homogenize lung samples in 1% Triton X-100 (Sigma-Aldrich)/PBS-containing protease inhibitor (Complete Mini; Roche Diagnostics). Homogenates were centrifuged at 24,000 × *g*, 10 min at 4°C, and supernatants were collected and stored at −20°C until further use. Protein concentration was measured with the use of the Pierce BCA protein assay kit standardized to BSA (Thermo Fisher Scientific). Homogenates were diluted to a final concentration of 1 mg protein/mL before cytokine or chemokine measurements (26, 27).

Ex vivo lung restimulation with HDMs. After a 30-min enzymatic digestion of the lungs using digestion buffer that contained DNase I and Collagenase A (Roche Diagnostics), lung cell suspensions were prepared. Digestion was stopped by adding fetal calf serum (HyClone Laboratories). Lung cells were washed and resuspended in Roswell Park Memorial Institute 1640 culture medium (Lonza) supplemented with 10% heat-inactivated fetal calf serum and 0.1% penicillin-streptomycin solution (Sigma-Aldrich). Lung cells (4×10^5 cells/well) were cultured in medium with or without 50 μg/mL HDM (Greer Laboratories), and supernatant was harvested after 4 d of culture at 37°C in 5% CO_2 and stored at −20°C until further analysis (28).

Lung T cell subsets assessed by flow cytometry. PBS blocking buffer containing 1% BSA and 5% fetal calf serum was used for 30 min to block aspecific background. Per well, 5×10^5 cells were plated and incubated at 4°C for 30 min with different antibodies against CD25–Alexa F488, Foxp3-allophycocyanin, CD4–peridinin chlorophyll Cy5, CD69–fluorescein isothiocyanate conjugate, trans-acting T cell–specific transcription factor (GATA3)–phycoerythrin, T-box transcription factor (Tbet)–eFLUOR660 (eBioscience), or matching isotype controls. Cells were permeabilized for intracellular staining by using the Foxp3 staining buffer set, according to the manufacturer's protocol (eBioscience). Flow cytometry was conducted by using a fluorescence-activated cell sorter (FACS Canto II; BD) and analyzed with Flowlogic Software (Inivai Technologies) (29).

Measurement of cytokines and chemokines. Chemokine ligand 5 (CCL5), IL-33, and chemokine ligand 20 (CCL20) were measured with a DuoSet ELISA (R&D Systems) and IL-13 with a Ready-SET-Go! ELISA (eBioscience), all according to the manufacturers' protocol. Cytokine concentrations in supernatants of lung cell restimulation were determined

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[7] Abbreviations used: BALF, bronchoalveolar lavage; CCL5, chemokine ligand 5; CCL20, chemokine ligand 20; Foxp3, Forkhead box P3; HDM, house dust mite; NDO, nondigestible oligosaccharide; Th1, type 1 T helper; Th2, type 2 T helper; Treg, regulatory T cell.

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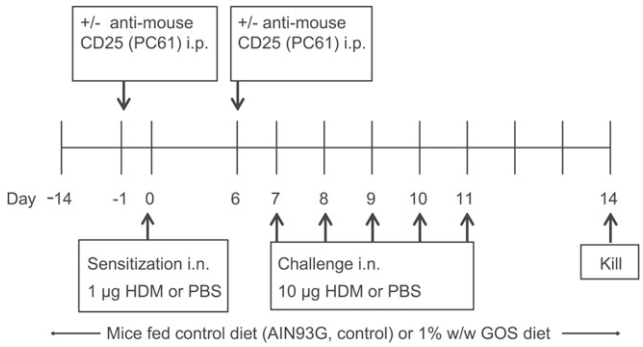

FIGURE 1 Protocol of the study. Male BALB/c (6- to 8-wk-old) mice were i.n. sensitized with PBS or HDM on day 0 and i.n. challenged from days 7 to 11. On day −1 and day 6, mice were i.p. injected with 200 μL (1 mg/mL) rat anti-mouse CD25 (PC61) monoclonal antibody. Mice were fed the control diet (AIN93G, control) or a 1% w/w GOS diet 2 wk before sensitization that was continued during the entire experiment. Mice were killed on day 14. GOS, galacto-oligosaccharide; HDM, house dust mite; i.n., intranasally.

by a standard IL-13 flex set and Th1/Th2/Th17 kit (BD Biosciences). The concentrations of these mediators were expressed as pg/mg protein in supernatants of lung homogenates and pg/mL in restimulation supernatants.

Statistical analysis. Results are expressed as means ± SEMs. Flow cytometry and ex vivo restimulation data were obtained from 6 mice, BALF cells from 7–9 mice, and cytokines and chemokine in lung homogenates from 5–6 mice. Data were statistically analyzed with the use of a 1-factor ANOVA (flow cytometry, BALF cells, and chemokines and cytokines in lung homogenates) or 2-factor ANOVA (ex vivo restimulation), followed by Bonferroni's post hoc testing. Results were considered statistically significant if $P < 0.05$. For the 2-factor ANOVA P value for ex vivo restimulation, sensitization and ex vivo restimulation × sensitization interaction are reported. Analyses were performed with the use of GraphPad Prism (version 6.0; GraphPad Software). BALF cell data were square root transformed, and ex vivo restimulation data were log transformed to normalize data distribution before analysis.

Results

Anti-CD25 antibody treatment diminishes the frequency of Tregs and enhances activated T helper and Th2 cells in HDM-allergic mice. Anti-CD25 treatment depleted Tregs in the spleen and lung of PBS mice ($P < 0.0001$). The Treg depletion was associated with an increased frequency of activated T helper cells ($P < 0.05$) and Th2 cells ($P < 0.0001$) in the spleen but not in the lung. In the spleen of HDM-allergic mice, these findings were similar (**Figure 2**A–D). In the lung, a significant increase in $CD25^+Foxp3^+CD4^+$ Tregs was observed in HDM-allergic mice compared with PBS mice ($P < 0.0001$), which was not observed in the spleen (Figure 2A, E), and anti-CD25 treatment decreased the percentage of Tregs by >70% in lung cells of HDM-allergic mice (Figure 2E). Also, the frequency of activated T helper cells was significantly greater in lungs of HDM-allergic mice compared with PBS mice ($P < 0.01$) and was further enhanced after Treg depletion ($P < 0.01$) (Figure 2F). Although the frequency of $GATA3^+CD4^+$ Th2 cells was not enhanced in the lungs of HDM-allergic mice, it was significantly greater after Treg depletion ($P < 0.05$) (Figure 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 (Figure 2D, H).

Pulmonary IL-10, IL-4, and IL-13 increase after Treg depletion. In PBS mice, no cytokine release was observed after ex vivo restimulation with medium or HDM, whereas the HDM-allergic mice showed an HDM-specific increase in IL-10, IL-4, and IL-13 release ($P < 0.0001$) (Figure 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 as well as HDM-allergic mice (Figure 2I–K). Typically, anti-CD25 treatment increased IFN-γ concentrations after HDM restimulation in both PBS ($P < 0.0001$) and HDM-allergic mice ($P < 0.01$) (Figure 2L).

Treg depletion abolishes the suppressive effect of dietary galacto-oligosaccharides on airway eosinophilia. The total number of BALF cells was significantly greater in HDM-allergic mice than in PBS mice fed the control diet ($P < 0.001$). Anti-CD25 treatment did not affect the number of total BALF cells in either PBS or HDM-allergic mice (**Figure 3**A). Dietary intervention with galacto-oligosaccharides did not show an effect on total BALF cells (Figure 3A).

In HDM-allergic mice, the percentage of eosinophils was significantly greater than in the PBS mice and remained unaffected upon anti-CD25 treatment ($P < 0.0001$) (Figure 3B). Dietary intervention with galacto-oligosaccharides significantly reduced the percentage of eosinophils in HDM-allergic mice ($P < 0.01$), and this effect was lost after treatment with anti-CD25 antibody ($P < 0.05$). Anti-CD25 treatment in galacto-oligosaccharide–fed HDM-allergic mice resulted in a significantly greater percentage of eosinophils compared with their corresponding PBS group ($P < 0.0001$) (Figure 3B).

The percentage of lymphocytes was greater in HDM-allergic mice than in PBS mice fed the control diet ($P < 0.01$), and Treg depletion did not affect this in either PBS or HDM-allergic mice (Figure 3C). Treatment with anti-CD25 significantly increased the percentage of lymphocytes in PBS mice fed the galacto-oligosaccharide diet compared with galacto-oligosaccharide–fed PBS mice not injected with anti-CD25 ($P < 0.0001$). In HDM-allergic, galacto-oligosaccharide–fed mice, injection with anti-CD25 tended to increase the percentage of lymphocytes ($P = 0.10$).

The percentage of neutrophils tended to be greater in the HDM-allergic mice than in PBS mice fed the control diet ($P = 0.10$), and the anti-CD25 treatment did not affect this (Figure 3D). However, in the PBS as well as HDM-allergic mice fed the galacto-oligosaccharide diet, the anti-CD25 treatment enhanced the percentage of neutrophils compared with galacto-oligosaccharide–fed mice not treated with anti-CD25 ($P < 0.01$).

Treg depletion abrogates the suppressive effect of dietary galacto-oligosaccharides on IL-33 concentrations in lung homogenates. In lung homogenate supernatants of HDM-allergic mice fed the control diet, IL-33 concentrations were greater ($P < 0.01$) and CCL5 concentrations tended to be greater ($P = 0.09$) than in PBS mice fed the control diet. Anti-CD25 treatment did not affect this in either PBS or HDM-allergic mice (**Figure 4**A, B). Dietary intervention with galacto-oligosaccharides reduced the IL-33 concentration ($P < 0.05$), and the same tendency was observed for CCL5 ($P = 0.06$), but these protective effects were lost upon Treg depletion ($P < 0.0001$ and $P < 0.01$, respectively) (Figure 4A, B). CCL20 concentrations were greater in HDM-allergic mice fed the control diet than in PBS mice ($P < 0.05$) and further increased upon anti-CD25 treatment ($P < 0.05$) (Figure 4C). Dietary intervention with galacto-oligosaccharides did not show an effect on CCL20 concentrations in HDM-allergic mice (Figure 4C).

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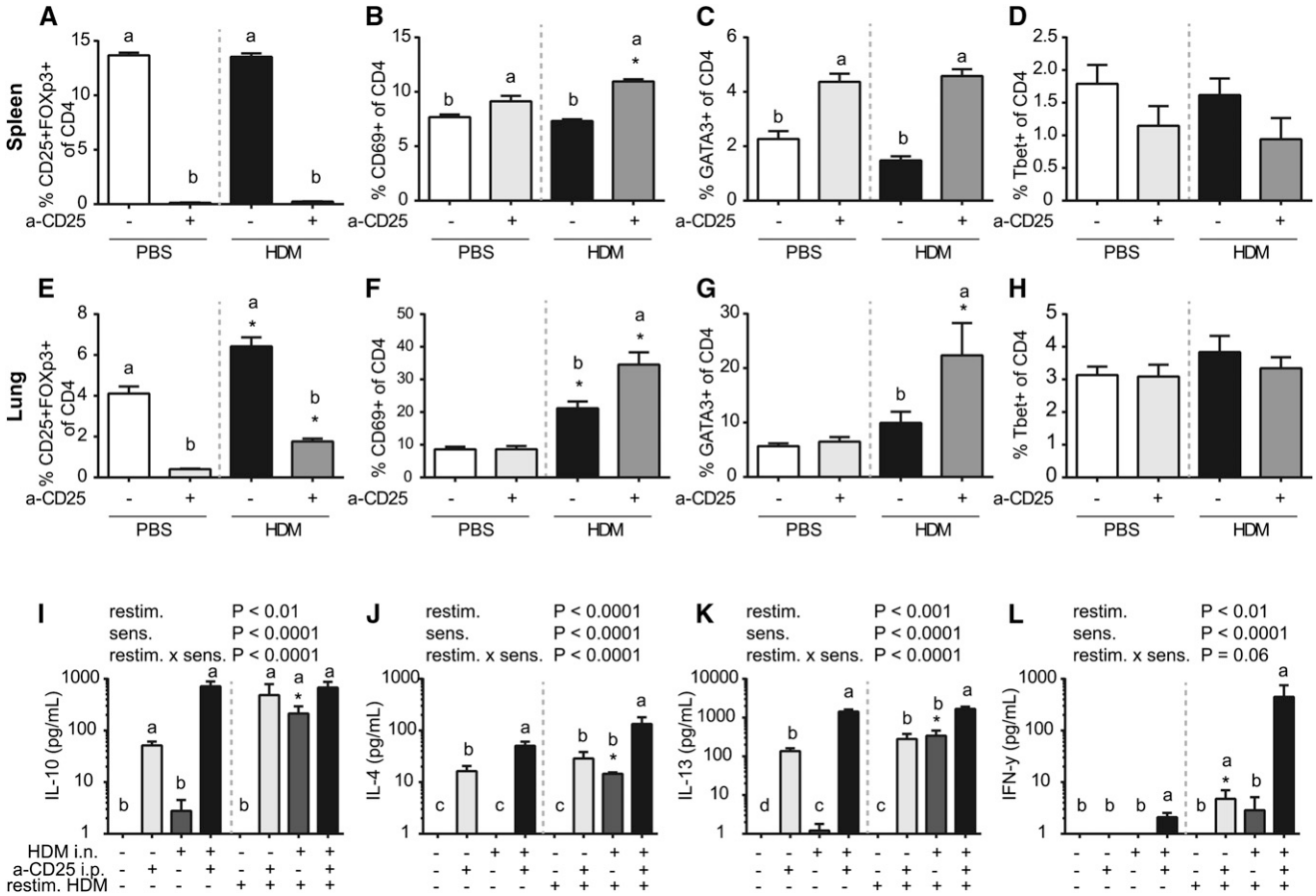

FIGURE 2 Percentages of various CD4 cells from spleen (A–D) and lung (E–H) and cytokine concentrations in supernatants of lung cell suspensions (I–L) in mice sensitized and challenged with PBS or HDM and either not treated or injected with rat anti-mouse CD25 to deplete Tregs. Spleen and lung T cell phenotypes were determined and lung cells were restimulated ex vivo. Values are means ± SEMs, n = 6 mice/group. Different from corresponding PBS mice, $^*P < 0.05$. Within PBS or HDM treatment (A–H) or within no stimulation or stimulation with HDM ex vivo (I–L); labeled means without a common letter differ, $P < 0.05$. One-factor ANOVA followed by Bonferroni's multiple comparison test (A–H) and 2-factor ANOVA followed by Bonferroni's multiple comparison test (I–L). a-CD25, anti-mouse CD25; Foxp3, Forkhead box P3; GATA3, trans-acting T cell–specific transcription factor; HDM, house dust mite; i.n., intranasally; restim., restimulation; sens., sensitization; Tbet, T-box transcription factor; Treg, regulatory T cell.

Discussion

This study was conducted to examine the effect of anti-CD25 Treg depletion on allergic responses after dietary intervention with galacto-oligosaccharides in a murine model for HDM-allergic asthma. Although CD25 is not a specific marker for Tregs, anti-CD25 treatment is commonly used to deplete Tregs in vivo (30). To confirm whether the depletion of Tregs occurred after anti-CD25 treatment, we analyzed lung and spleen cells and found a significant reduction in $CD25^+Foxp3^+CD4^+$ Tregs in control as well as HDM-allergic mice. The latter is in agreement with different animal studies in which anti-CD25 treatment was able to reduce the number of Tregs (6, 31). From human studies, it is known that Tregs can suppress Th2 responses (32, 33). Because Tregs 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, whereas in PBS mice, these effects were observed only in the spleen. Th2 cells are known to produce different cytokines such as IL-4 and IL-13 (34, 35), and HDM-allergic mice showed an HDM-specific increase of the cytokines in restimulated lung cell supernatants. However, increased IL-4 and IL-13 concentrations were also measured in restimulated lung cell supernatants after anti-CD25 treatment in both control as well as HDM-allergic mice independent of the allergen. This indicates that Tregs 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 $CD25^+CD4^+$ Tregs are not the only secretors of IL-10; many other cells (e.g., dendritic cells, lymphocytes, and macrophages) are also capable of secreting this cytokine (36, 37). These cellular sources other than Tregs may explain the significant increase in IL-10 release by lung cells of anti–CD25-treated mice. In contrast to the increase in Th2 cell frequency, no differences were observed in Th1 frequency in anti–CD25-treated mice.

However, anti-CD25 treatment resulted in an increase in Th1-type IFN-γ in PBS mice ex vivo restimulated with HDM and medium-exposed HDM-allergic mice, but the increase in IFN-γ was most pronounced in anti–CD25-treated HDM-allergic mice ex vivo exposed to HDM. This may be a result of loss of suppression, rendering the Th1 cells more active, or a compensatory mechanism for the loss of Tregs since Th1-type cytokines are known to suppress Th2 polarization.

Taking these results together, we can conclude that anti-CD25 treatment before sensitization and challenge is capable of selective depletion of Tregs in control and HDM-allergic mice, showing a functional role of these cells in the suppression of T cell responses in the lung.

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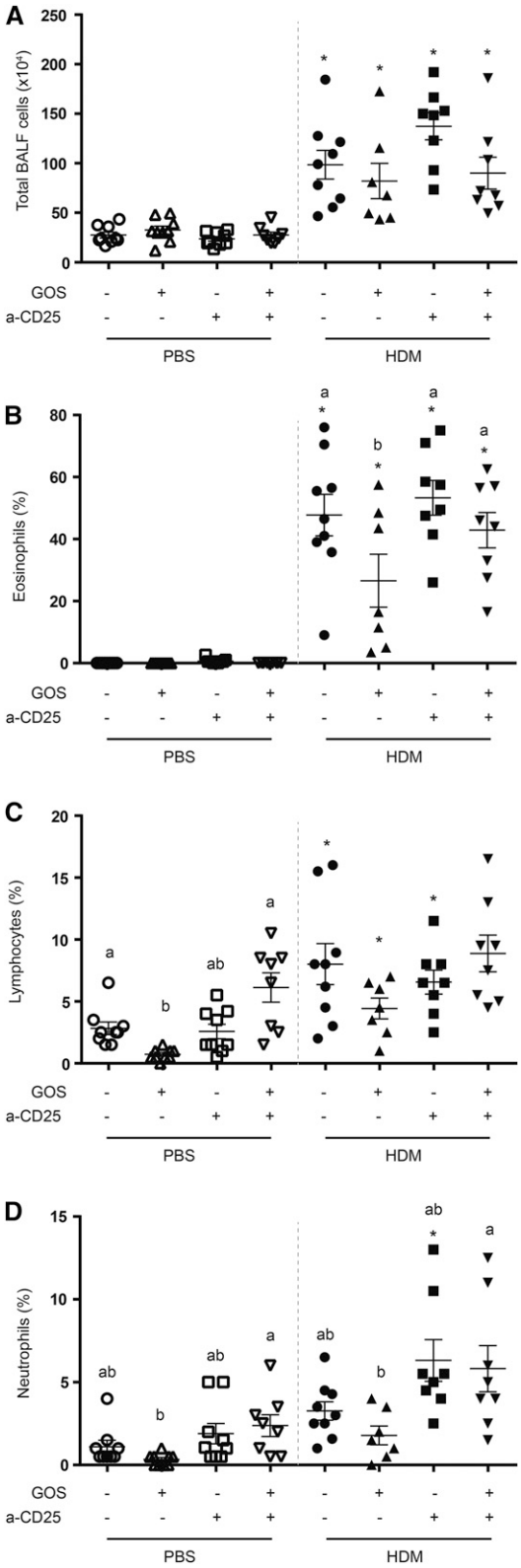

FIGURE 3 The total number of BALF cells (A) and percentages of eosinophils (B), lymphocytes (C), and neutrophils (D) in mice fed control or 1% w/w GOS diet and sensitized and challenged with PBS or HDM and either not treated or injected with rat anti-mouse CD25 to deplete Tregs. Values are means ± SEM, n = 7–9 mice/group. Different from corresponding PBS mice, $^*P < 0.05$. Within PBS or HDM treatment, labeled means without a common letter differ, $P < 0.05$. One-factor ANOVA followed by Bonferroni's multiple comparison test. a-CD25, anti-mouse CD25; BALF, bronchoalveolar lavage fluid; GOS, galacto-oligosaccharide; HDM, house dust mite; Treg, regulatory T cell.

Next, we examined the effect of dietary intervention with galacto-oligosaccharides on pulmonary inflammation and studied the contribution of Tregs in the protective effect of this diet because in murine models for food allergy, it was shown that Tregs were functionally involved in the protective effect of an oligosaccharide diet (12, 13).

Total BALF cells and the frequency of eosinophils and lymphocytes were increased in HDM-allergic mice compared with PBS 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 inflammatory cell infiltration because the total BALF cell number and percentage of eosinophils, lymphocytes, and neutrophils were not increased compared with HDM-allergic mice not treated with anti-CD25. Also, in the PBS mice fed the control diet, Treg depletion did not result in airway inflammatory cell influx. These data are supported by previous mice studies that showed no effect or a modest increase in pulmonary eosinophil numbers after Treg depletion (7, 38).

Dietary intervention with galacto-oligosaccharides 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 galacto-oligosaccharides are able to reduce airway eosinophilia in HDM-allergic mice is mediated via Tregs. The mice were fed the galacto-oligosaccharide diet 2 wk before the first sensitization and during the whole study protocol, and the anti-CD25 treatment was performed before sensitization and the challenge phase, resulting in Treg depletion in the spleen and lung. This implies that both thymus-derived natural Tregs and peripherally induced Tregs could be targeted by galacto-oligosaccharides and removed by the anti-CD25 treatment. Although in the current model, it is not clear which Tregs are affected by the galacto-oligosaccharide diet, in previous studies that used a murine model for cow milk allergy, an NDO diet induced protective Tregs only when mice were fed the NDO during sensitization with cow milk proteins. Only splenocytes from these mice and not those from sham sensitized mice protected naive recipient mice from developing cow milk allergy upon sensitization, and this effect was lost upon Treg depletion (12, 13). This suggests that dietary NDOs are capable of improving the function of induced Tregs and not that of natural Tregs. However, this remains to be further explored for the HDM asthma model.

Although in HDM-allergic mice, galacto-oligosaccharides did not significantly suppress the percentage of lymphocytes in the BALF, in the PBS mice, the galacto-oligosaccharide diet did reduce this number, which was abrogated by Treg depletion. Hence, dietary galacto-oligosaccharides may be capable of suppressing the basal percentage of lung lymphocytes via a mechanism mediated by Tregs. Currently, it remains to be elucidated how this is being established. HDM is known to induce IL-33 and CCL5 release by airway epithelial cells in vitro and in asthmatic patients (35, 39–41). Indeed, in HDM-allergic mice, IL-33 was enhanced compared with PBS mice, and CCL5 showed the same tendency. In HDM-allergic mice, dietary intervention with galacto-oligosaccharides decreased IL-33 and tended to decrease CCL5. The latter is in agreement with previous studies, in which we showed a decrease in IL-33 and CCL5 concentrations in supernatants of lung homogenates of HDM-allergic mice after dietary intervention with galacto-oligosaccharides (20).

Tregs may exert their protective effect via suppression of dendritic cell activation and/or via dampening T cell–induced inflammatory responses, eosinophils, and mast cell activation and even via targeting structural cells either indirectly or directly via cell-cell contact or secretion of regulatory cytokines (42).

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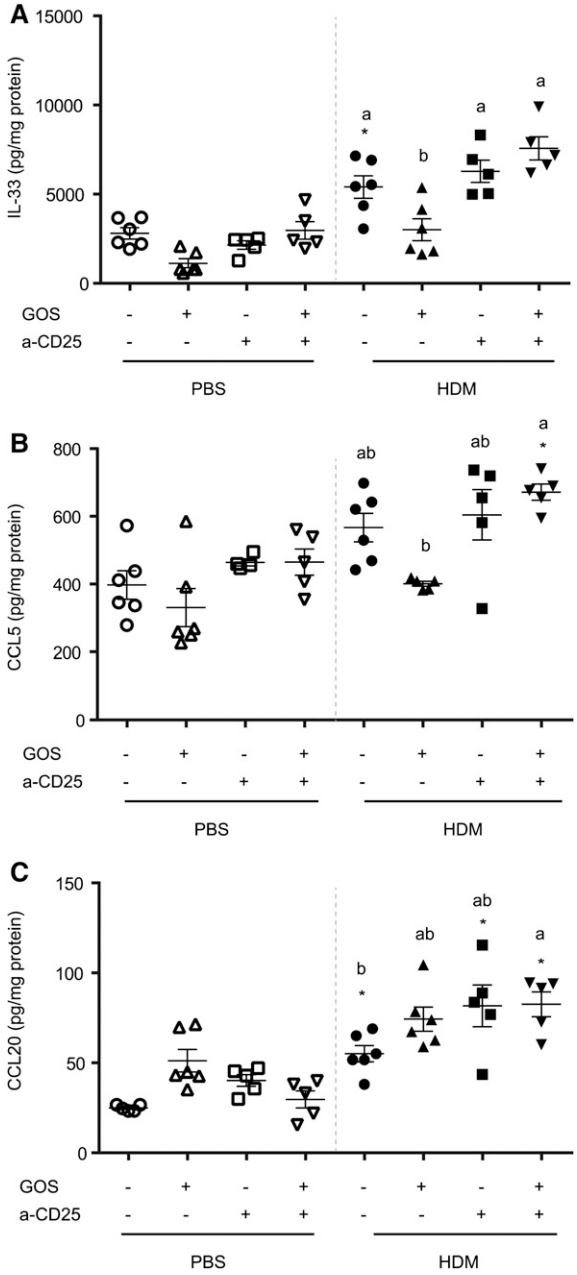

FIGURE 4 IL-33 (A), CCL5 (B), and CCL20 (C) concentrations in the supernatants of lung homogenates of mice fed control or 1% w/w GOS diet and sensitized and challenged with PBS or HDM and either not treated or injected with rat anti-mouse CD25 to deplete Tregs. Values are means ± SEM, n = 5–6 mice/group. Different from corresponding PBS, $*P < 0.05$. Within PBS or HDM treatment, labeled means without a common letter differ, $P < 0.05$. One-factor ANOVA followed by Bonferroni's multiple comparison test. a-CD25, anti-mouse CD25; CCL, chemokine ligand; GOS, galacto-oligosaccharide; HDM, house dust mite; Treg, regulatory T cell.

Currently, it is unknown if Tregs can dampen airway epithelial cell activation. In this study, anti-CD25 treatment did not influence IL-33 and CCL5 concentrations of control or HDM-allergic mice but, more important, did abolish the suppressive effect of a dietary intervention with galacto-oligosaccharides on IL-33 and CCL5. Hence, Tregs facilitate the galacto-oligosaccharide–induced suppression of these mediators in HDM-allergic mice. CCL5 is produced by several cell types (e.g., epithelial cells, macrophages, mast cells, basophils). CCL5 is also known to be produced by and attracts eosinophils to the airways of patients with asthma (41, 43), and eosinophils are activated by IL-33 (44). IL-33, among others, is produced by HDM-exposed airway epithelial cells and is upstream in the inflammatory cascade induced by HDM during sensitization and challenge (35). Therefore, our studies suggest that Tregs induced by dietary galacto-oligosaccharides may dampen IL-33 and CCL5 release, for example, by epithelial cells and thereby may suppress HDM-induced sensitization and ameliorate pulmonary inflammation.

Hence, the suppression of airway eosinophilia by dietary galacto-oligosaccharides may be caused by a Treg-mediated dampening of IL-33 and CCL5 production in the HDM-allergic mice.

The suppression of IL-33 by galacto-oligosaccharides was rather selective because galacto-oligosaccharides did not reduce CCL20 concentrations in the lung of HDM-allergic mice. The chemokine CCL20 is also produced by airway epithelial cells and increased in concentration in asthmatic patients (45–47). 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 increased upon anti-CD25 treatment in HDM-allergic mice fed the control diet. Hence, the loss of Tregs 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 galacto-oligosaccharides did not affect CCL20 concentrations in HDM-allergic mice injected or not treated with anti-CD25. Furthermore, HDM-induced IL-33 release may involve a different pathway than that for CCL20, with the latter being less sensitive for suppressive effects of galacto-oligosaccharides in HDM-allergic mice.

In conclusion, these results indicate that Tregs 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, Tregs as such are not capable of suppressing airway eosinophilia and IL-33 and CCL5 release. By contrast, dietary intervention with galacto-oligosaccharides suppresses airway eosinophilia and IL-33 concentrations in supernatants of lung homogenates via a mechanism mediated by Tregs. This indicates that dietary intervention with galacto-oligosaccharides has a beneficial effect on the prevention of HDM-induced allergic asthma by supporting pulmonary Treg function.

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References

1. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol 2008;8:183–92.
2. Lambrecht BN, Hammad H. The immunology of asthma. Nat Immunol 2015;16:45–56.
3. Schmidt-Weber CB. Th17 and treg cells innovate the TH1/TH2 concept and allergy research. Chem Immunol Allergy 2008;94:1.
4. Shevach EM, DiPaolo RA, Andersson J, Zhao D, Stephens GL, Thornton AM. The lifestyle of naturally occurring CD4 CD25 Foxp3 regulatory T cells. Immunol Rev 2006;212:60–73.
5. Kearley J, Buckland KF, Mathie SA, Lloyd CM. Resolution of allergic inflammation and airway hyperreactivity is dependent upon disruption of the T1/ST2-IL-33 pathway. Am J Respir Crit Care Med 2009;179:772–81.
6. Leech MD, Benson RA, De Vries A, Fitch PM, Howie SE. Resolution of Der p1-induced allergic airway inflammation is dependent on CD4 +CD25+Foxp3+ regulatory cells. J Immunol 2007;179:7050–8.

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7. Lewkowich IP, Herman NS, Schleifer KW, Dance MP, Chen BL, Dienger KM, Sproles AA, Shah JS, Kohl J, Belkaid Y, et al. CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function. J Exp Med 2005;202:1549–61.
8. Langier S, Sade K, Kivity S. Regulatory T cells in allergic asthma. Isr Med Assoc J 2012;14:180–3.
9. Lee JH, Yu HH, Wang LC, Yang YH, Lin YT, Chiang BL. The levels of CD4+CD25+ regulatory T cells in paediatric patients with allergic rhinitis and bronchial asthma. Clin Exp Immunol 2007;148:53–63.
10. Abdulamir AS, Kadhim HS, Hafidh RR, Ali MA, Faik I, Abubakar F, Abbas KA. Severity of asthma: the role of CD25+, CD30+, NF-kappaB, and apoptotic markers. J Investig Allergol Clin Immunol 2009;19:218–24.
11. Smyth LJ, Eustace A, Kolsum U, Blaikely J, Singh D. Increased airway T regulatory cells in asthmatic subjects. Chest 2010;138:905–12.
12. Schouten B, van Esch BC, Hofman GA, Boon L, Knippels LM, Willemsen LE, Garssen J. Oligosaccharide-induced whey-specific CD25(+) regulatory T-cells are involved in the suppression of cow milk allergy in mice. J Nutr 2010;140:835–41.
13. Schouten B, van Esch BC, Hofman GA, de Kivit S, Boon L, Knippels LM, Garssen J, Willemsen LE. A potential role for CD25 regulatory T-cells in the protection against casein allergy by dietary non-digestible carbohydrates. Br J Nutr 2012;107:96–105.
14. Gibson GR, Roberfroid MB. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr 1995;125:1401–12.
15. Gourbeyre P, Denery S, Bodinier M. Probiotics, prebiotics, and synbiotics: impact on the gut immune system and allergic reactions. J Leukoc Biol 2011;89:685–95.
16. Slavin J. Fiber and prebiotics: mechanisms and health benefits. Nutrients 2013;5:1417–35.
17. Vos AP, Haarman M, van Ginkel JW, Knol J, Garssen J, Stahl B, Boehm G, M'Rabet L. Dietary supplementation of neutral and acidic oligosaccharides enhances Th1-dependent vaccination responses in mice. Pediatr Allergy Immunol 2007;18:304–12.
18. Sagar S, Vos AP, Morgan ME, Garssen J, Georgiou NA, Boon L, Kraneveld AD, Folkerts G. The combination of *Bifidobacterium breve* with non-digestible oligosaccharides suppresses airway inflammation in a murine model for chronic asthma. Biochim Biophys Acta 2014;1842:573–83.
19. Sonoyama K, Watanabe H, Watanabe J, Yamaguchi N, Yamashita A, Hashimoto H, Kishino E, Fujita K, Okada M, Mori S, et al. Allergic airway eosinophilia is suppressed in ovalbumin-sensitized Brown Norway rats fed raffinose and alpha-linked galactooligosaccharide. J Nutr 2005;135:538–43.
20. Verheijden KA, Willemsen LE, Braber S, Leusink-Muis T, Delsing DJ, Garssen J, Kraneveld AD, Folkerts G. Dietary galacto-oligosaccharides prevent airway eosinophilia and hyperresponsiveness in a murine house dust mite-induced asthma model. Respir Res 2015;16:17.
21. Arslanoglu S, Moro GE, Boehm G, Wienz F, Stahl B, Bertino E. Early neutral prebiotic oligosaccharide supplementation reduces the incidence of some allergic manifestations in the first 5 years of life. J Biol Regul Homeost Agents 2012;26:49–59.
22. van de Pol MA, Lutter R, Smids BS, Weersink EJ, van der Zee JS. Synbiotics reduce allergen-induced T-helper 2 response and improve peak expiratory flow in allergic asthmatics. Allergy 2011;66:39–47.
23. Akbari P, Braber S, Alizadeh A, Verheijden KA, Schoterman MH, Kraneveld AD, Garssen J, Fink-Gremmels J. Galacto-oligosaccharides protect the intestinal barrier by maintaining the tight junction network and modulating the inflammatory responses after a challenge with the mycotoxin deoxynivalenol in human Caco-2 cell monolayers and B6C3F1 mice. J Nutr 2015;145:1604–13.
24. Verheijden KA, Willemsen LE, Braber S, Leusink-Muis T, Jeurink PV, Garssen J, Kraneveld AD, Folkerts G. 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. Eur J Nutr 2015 May 24 (Epub ahead of print; DOI: 10.1007/s00394-015-0928-8).
25. Sagar S, Verheijden KA, Georgiou NA, Garssen J, Kraneveld AD, Vos AP, Folkerts G. Differential regulation of inflammation and immunity in mild and severe experimental asthma. Mediators Inflamm. 2013;2013:808470.
26. Braber S, Koelink PJ, Henricks PA, Jackson PL, Nijkamp FP, Garssen J, Kraneveld AD, Blalock JE, Folkerts G. Cigarette smoke-induced lung emphysema in mice is associated with prolyl endopeptidase, an enzyme involved in collagen breakdown. Am J Physiol Lung Cell Mol Physiol 2011;300:L255–65.
27. Koelink PJ, Overbeek SA, Braber S, Morgan ME, Henricks PA, Abdul Roda M, Verspaget HW, Wolfkamp SC, te Velde AA, Jones CW, et al. Collagen degradation and neutrophilic infiltration: a vicious circle in inflammatory bowel disease. Gut 2014;63:578–87.
28. Sagar S, Morgan ME, Chen S, Vos AP, Garssen J, van Bergenhenegouwen J, Boon L, Georgiou NA, Kraneveld AD, Folkerts G. *Bifidobacterium breve* and *Lactobacillus rhamnosus* treatment is as effective as budesonide at reducing inflammation in a murine model for chronic asthma. Respir Res 2014;15:46.
29. van den Elsen LW, van Esch BC, Hofman GA, Kant J, van de Heijning BJ, Garssen J, Willemsen LE. Dietary long chain n–3 polyunsaturated fatty acids prevent allergic sensitization to cow's milk protein in mice. Clin Exp Allergy 2013;43:798–810.
30. Couper KN, Lanthier PA, Perona-Wright G, Kummer LW, Chen W, Smiley ST, Mohrs M, Johnson LL. Anti-CD25 antibody-mediated depletion of effector T cell populations enhances susceptibility of mice to acute but not chronic *Toxoplasma gondii* infection. J Immunol 2009;182:3985–94.
31. Kwon Y, Sohn S, Lee G, Kim Y, Lee H, Shin M, Bae H. Electro-acupuncture attenuates ovalbumin-induced allergic asthma via modulating CD4+CD25+ regulatory T cells. Evid Based Complement Alternat Med 2012:May 10;2012.
32. Ling EM, Smith T, Nguyen XD, Pridgeon C, Dallman M, Arbery J, Carr VA, Robinson DS. Relation of CD4 CD25 regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet 2004;363:608–15.
33. Akdis M, Verhagen J, Taylor A, Karamloo F, Karagiannidis C, Crameri R, Thunberg S, Deniz G, Valenta R, Fiebig H, et al. Immune responses in healthy and allergic individuals are characterized by a fine balance between allergen-specific T regulatory 1 and T helper 2 cells. J Exp Med 2004;199:1567–75.
34. Kudo M, Ishigatsubo Y, Aoki I. Pathology of asthma. Front Microbiol 2013;4:263.
35. Salazar F, Ghaemmaghami AM. Allergen recognition by innate immune cells: critical role of dendritic and epithelial cells. Front Immunol 2013;4:356.
36. Trifunović J, Miller L, Debeljak Ž, Horvat V. Pathologic patterns of interleukin 10 expression—a review. Biochem Med (Zagreb) 2015;25:36–48.
37. Hawrylowicz CM. Regulatory T cells and IL-10 in allergic inflammation. J Exp Med 2005;202:1459–63.
38. Suto A, Nakajima H, Kagami S, Suzuki K, Saito Y, Iwamoto I. Role of CD4 CD25 regulatory T cells in T helper 2 cell–mediated allergic inflammation in the airways. Am J Respir Crit Care Med 2001;164:680–7.
39. Prefontaine D, Lajoie-Kadoch S, Foley S, Audusseau S, Olivenstein R, Halayko AJ, Lemiere C, Martin JG, Hamid Q. Increased expression of IL-33 in severe asthma: evidence of expression by airway smooth muscle cells. J Immunol 2009;183:5094–103.
40. Préfontaine D, Nadigel J, Chouiali F, Audusseau S, Semlali A, Chakir J, Martin JG, Hamid Q. Increased IL-33 expression by epithelial cells in bronchial asthma. J Allergy Clin Immunol 2010;125:752–4.
41. Lukacs NW. Role of chemokines in the pathogenesis of asthma. Nat Rev Immunol 2001;1:108–16.
42. Palomares O, Yaman G, Azkur AK, Akkoc T, Akdis M, Akdis CA. Role of Treg in immune regulation of allergic diseases. Eur J Immunol 2010;40:1232–40.
43. Marques RE, Guabiraba R, Russo RC, Teixeira MM. Targeting CCL5 in inflammation. Expert Opin Ther Targets 2013;17:1439–60.
44. Saluja R, Ketelaar ME, Hawro T, Church MK, Maurer M, Nawijn MC. The role of the IL-33/IL-1RL1 axis in mast cell and basophil activation in allergic disorders. Mol Immunol 2015;63:80–5.
45. Zijlstra GJ, Fattahi F, Rozeveld D, Jonker MR, Kliphuis NM, van den Berge M, Hylkema MN, ten Hacken NH, van Oosterhout AJ, Heijink IH. Glucocorticoids induce the production of the chemoattractant CCL20 in airway epithelium. Eur Respir J 2014;44:361–70.
46. Vroman H, van den Blink B, Kool M. Mode of dendritic cell activation: the decisive hand in Th2/Th17 cell differentiation. Implications in asthma severity? Immunobiology 2015;220:254–61.
47. Pichavant M, Charbonnier AS, Taront S, Brichet A, Wallaert B, Pestel J, Tonnel AB, Gosset P. Asthmatic bronchial epithelium activated by the proteolytic allergen Der p 1 increases selective dendritic cell recruitment. J Allergy Clin Immunol 2005;115:771–8.

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