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ORIGINAL ARTICLE

Th2 related markers in milk allergic inflammatory mice model, versus OVA

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Abstract Experimental studies on allergic asthma are limited by the high cost of the administrated allergens. In this study we tested the allergic potency of low fat milk as a cheap substitute to the widely used standard allergen, ovalbumin (OVA). BALB/c female mice (4 weeks old) were sensitized intraperitoneally with low fat milk/or OVA followed by intranasal challenge with the two allergens on days 28 and 29. At day 31, serum, bronchoalveolar lavage fluid (BALF), and lungs were harvested. Mice of the low fat milk model showed infiltration of eosinophils, macrophages, lymphocytes, and neutrophils in BALF comparable to that of the OVA model. Both allergic protocols led to the production of similar numbers of Th2 cells and induced comparable expression of Th2 cytokine (IL-13) as evident by real time PCR for IL-13 and GATA3 (Th2 transcription factor) and confirmed by immunofluorescence for Th2 surface markers (T1/ST2). In addition, both mouse models had similar elevated levels of allergen specific antibody, IgG1 and IgE. Notably, HE, PAS, and LUNA stained lung sections from low fat milk treated mice had higher average pathological scores as compared to OVA treated mice. In conclusion, this study suggests that the low fat milk-induced inflammation showed hallmarks of allergic airway inflammatory model such as eosinophilic influx in BALF, increased numbers of Th2 cells, augmented expression of IL-13, elevated levels of circulatory IgG1 and IgE, signs of robust pulmonary inflammation, and most importantly it is a cheap and promising model for studying acute allergic airway inflammation and acute asthma.

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1. Introduction

Asthma is a chronic disease that affects people of all ages, in particular children. World health organization (WHO) estimates that 235 million people currently suffer from asthma. The critical symptoms of asthma are reversible airway

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obstruction, inflammation, and airway hyperactivity (AHR) [1]. Allergic asthma is the commonest form of asthma affecting 90% of asthmatic children and 50% of asthmatic adults [2]. Patients with allergic asthma are hypersensitive to allergens to which they have become sensitized. It is characterized by increased Th2 cytokines (e.g., interleukin [IL] 4, IL-5, and IL-13) [3], serum immunoglobulin (Ig) E as well as eosinophilic infiltration in the lung [4].

There are multiple experimental models of allergic asthma based on the kind of allergens, animal species, and the protocol followed for sensitization and challenge [5,6]. Murine models are commonly used in investigating the allergy-driven inflammatory reactions since they are cheap, available and their immune system is well studied. BALB/c mice are the most preferable mouse strain in experimental asthma because their immune response is shifted toward Th2 response [7].

Many allergens are shown to have a clinical relevance such as chicken egg ovalbumin (OVA), house dust mite and cockroach extracts with OVA being the standard allergen that is widely used in experimental models of allergic asthma [8,9]. Nevertheless, the applicability of OVA models is limited by the high cost and the induction of modest pulmonary inflammation and mild AHR.

Although the current experimental models of allergic asthma recapitulate the human clinical manifestation in some features such as increased IgE, airway inflammation, goblet cell hyperplasia, and epithelial hypertrophy; they differ from asthmatic patients in major respects. Examples for the latter, the differences in the pattern and distribution of pulmonary inflammation [10], the absence of the chronic inflammation of the airway wall and airway remodeling changes as well as their airways cure inflammation and AHR in short period after the final allergen challenge [11]. Consequently, it is of great importance to establish new experimental allergic models that closely mimic the human model.

Milk protein is a well-known cause of allergy in children through the induction of Th2-related cytokines and IgE [12]. Patients with milk allergy are more susceptible to develop allergic asthma [13,14]. In a study made by Husain and his group [15] to investigate the gene expression analysis in mesenteric lymph nodes of different groups of mice i.p. sensitized by OVA, β-lactoglobulins from cow milk and peanut agglutinin, 150 differentially expressed genes in the same directions were overlapped between the three groups. These overlapped differentially expressed genes were further analyzed by gene ontology (GO), revealing 46 GO biological process, 22 out of them were related to either autoimmune or hypersensitivity processes.

Therefore, in the present study we sought to investigate the efficacy of adjuvant free low fat milk as an effective, cheap alternate to OVA in inducing allergic airway inflammation in BALB/c mice. In this animal model, we are aiming also to challenge the mice in a simple intranasal route to minimize mice handling. This new model will help in the future to facilitate the fast screening of new effective agents against airway allergy. In this new animal model for allergic airway inflammation, we hope to prove the allergic and the inflammatory responses in BALF and lung parenchyma.

2. Materials and methods

2.1. Milk and OVA-induced acute allergic airway inflammatory models

2.1.1. Animals

BALB/c (4 weeks old) female mice were purchased from animal house, Theodor Bilharz Research Institute, Giza, Egypt) and maintained under specific-pathogen-free conditions. Animal experiments meet the relevant guidelines for the care and use of laboratory animals. The animal protocol was approved by the Institutional Animal Care and Use Committee established at Neuron Med & Beauty, Vienna, Austria.

2.1.2. Milk and ovalbumin challenge

Briefly, mice were sensitized intraperitoneally with 10 μg low fat (Baby) milk or ovalbumin (OVA, Sigma-Aldrich, St. Louis, MO, USA) in a total volume of 200 μl (1% PBS (wt/vol)) on days 0, and 21. At days 28 and 29, mice were challenged intranasally with 50 μg Milk/or OVA in 50 μl 1% PBS, b.i.d. On day 31 (48 h after the last challenge) serum, bronchoalveolar lavage fluid (BALF), and lungs were harvested. We tested different types of the available low fat milk (powder) in the Austrian market. All of them induced a strong allergic asthma response in mice compared to OVA. In this experiment we used Aptamil Junior 2+ milk (Milupa GmbH, Puch, Austria).

2.2. Total and differential analysis of the cellular contents of BALF

BALF was collected as described previously [16]. Briefly, trachea was cannulated and lung was lavaged 3 times with 0.4 ml cold sterile PBS. Freshly prepared BALF cells were washed, mixed with Trypan blue, counted for determining the cell viability and the total cells/ml. BALF cells were spun onto microscope slides using a cytospin 2 centrifuge (Shandon; Thermo, Waltham, MA) with 1×10^5 cells fixed on each slide. Cytospin slides were stained with *May-Grünwald-Giemsa* to determine differential cell counts (the total numbers of neutrophils, eosinophils, macrophages, and lymphocytes) and to be expressed as the absolute number from the total cell counts.

2.3. ELISA

Milk and OVA-specific IgG1 and IgE were detected by ELISA. For determination of IgE, sera were diluted 1:20 and IgE level was determined using BD Biosciences ELISA kit (NJ, USA) following the manufacturer's instructions. For determination of IgG1 level, ELISA plates (Corning, Lowell, MA, USA, 3590) were coated with 50 μl low fat milk or OVA (Sigma Aldrich, A7641) solution (10 μg/ml), and incubated overnight at 4 °C. Then, the plates were washed 3 times with 300 μL washing buffer (PBS/Tween20), blocked with 1% BSA/PBS/Tween20 for 2 h at room temperature, and incubated with serially diluted sera (within a range of 1:500 to 1:7,812,500) in blocking buffer overnight at 4 °C. The plates were incubated with biotin labelled goat anti mouse IgG1 (Southern Biotech, Birmingham, AL, USA, 1030-05) for 2 h at 4 °C followed by Streptavidin-HRP and TMB (tetramethylbenzidine), (Southern Biotech, Birmingham, AL, USA, 1030-05). Optical density was

measured in Spectra Max M5 max spectrophotometer at 450 nm.

2.4. Histopathology

The right lungs were flushed and fixed immediately after BALF collection in 4% paraformaldehyde solution for 2 days. Then, lungs were dehydrated and embedded in paraffin. The paraffin embedded tissues were cut into 3 μm sections and stained with hematoxylin and eosin (H&E) [16,17] for morphological evaluation and analysis of cell infiltrates around central and peripheral airways and in the parenchyma (central and peripheral); periodic-acid Schiff (PAS) reagent [16,17] for detection the mucus production by respiratory epithelial goblet cells in central and peripheral airways; and Luna [18] stains for grading and counting the eosinophilic infiltration in central and peripheral lung parenchyma, around airway and blood vessels. The H&E stained sections were graded according to the severity of the gross of the histological changes into 3 grades, grade 1 is the least severe and grade 3 is the most severe. The goblet cells distribution in the central and peripheral airways was graded as following; grade 0, no goblet cells; grade 1, 20%; grade 2, 20–40%; grade 3, 41–60%; grade 4, 61–80%; and grade 5; 80% goblet cells infiltration of the airways [16].

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from the lower lobe of left lungs using the RNeasy kit (QIAGEN, Valencia, CA) according to manufacturer's protocols. Thermo Scientific NanoDrop™ Spectrophotometer was used to determine the RNA quantity and purity. Total RNA was reverse transcribed and SYBR green-based qRT-PCR was performed on the synthesized cDNA using an Applied Biosystems 7900HT thermal cycler. Mouse HPRT was used for normalization. The following set of primers were used; HPRT-forward primer (5′-3′) TTGC TCGAGATGTGATGAAGGA, HPRT-reverse AAAGTTG AGAGATCATCTCCACCAA, GATA3-forward CTCGGCC ATTCGTACATGGAA, GATA3-reverse GGATACCTCTG-CACCGTAGC, IL13 forward GCAACATCACACAAGAC-CAGA, and IL13 reverse GTCAGGGAATCCAGGGCTAC. Relative alteration in gene expression was estimated by the ΔΔCT method.

2.6. Immunofluorescence staining

Immediately after collection, the upper lobe of left lungs were embedded in Tissue Tek Optimum Cutting Temperature compound (Sakura FineTek, Torrance, CA), snap frozen in liquid nitrogen and kept at −80 °C until ready to use. Lungs were sectioned at 3 μm thickness and placed on positively charged microscope slides (Superfrost/Plus, Fisher Scientific, Waltham, MA), then kept at -20 °C. Lung sections were air-dried for 15 min, then fixed for 10 min in ice-cold acetone, and blocked with 10% horse serum for 1 h at room temperature. Thereafter, lung sections were stained with FITC-labeled T1/ST2 rat anti-mouse monoclonal antibody (R&D systems, Minneapolis, MN, USA) for 60 min at 37 °C, followed by 3 times wash with PBS for 3 min each. Slides were stained with DAPI for 5 min in the dark and cover slipped with Gold Prolong anti-fade mounting media (Molecular Probes). Stained lung sections were examined using an Olympus Provis AX 70 fluorescent microscope.

2.7. Statistical analysis

Data were presented as the mean and standard error and were analyzed using Prism software (GraphPad, La Jolla, CA). The parametric unpaired student t test or the non-parametric Mann Whitney was used to compare the mean of different groups based on the normal distribution of the data, difference between variances, and the sample size. Statistically significant difference between groups was considered if $p \leq 0.05$.

3. Results

3.1. Low fat milk increases airway inflammatory response in intranasal challenged mice.

In order to test the capacity of low fat milk to induce eosinophilic acute airway inflammation, mice were challenged with low fat milk, analyzed for inflammatory cells in BALF (2 days after the last intranasal challenge) and compared to that of OVA-sensitized and intranasally challenged mice. Both low fat milk and OVA intranasally challenged mice had significantly higher total number of eosinophils (Fig. 1, $p = 0.0002$ in both groups), lymphocytes (Fig. 1, $p = 0.0002$ in both groups), and macrophages (Fig. 1, $p = 0.0002$ in both groups) in the two groups of mice when compared to the control counterparts. However, neutrophils showed reduced total numbers (Fig. 1, $p = 0.0001$ and $p < 0.0001$ for OVA and milk treated mice; respectively). Here we show that the total numbers of the

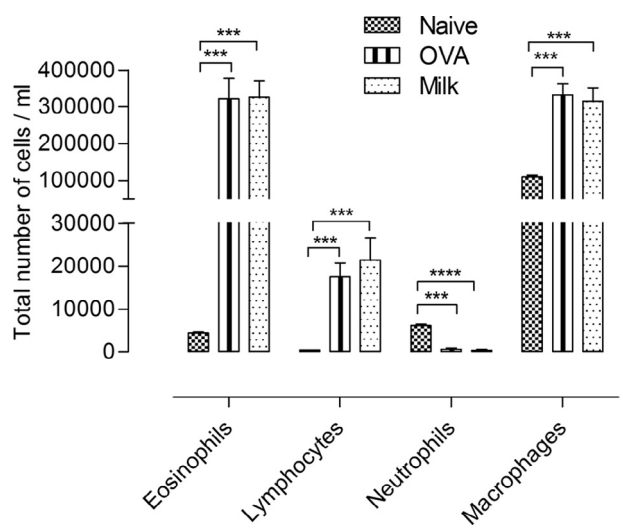

Fig. 1 Intranasal challenge with low fat milk induces infiltration of inflammatory cells in BALF. Differential counting of May Grünwald-Giemsa-stained cells was used to determine the total numbers of inflammatory cells (eosinophils, lymphocytes, neutrophils, and macrophages) in BALF of naïve control group, OVA-treated mice, and low fat milk treated mice. The data are shown as mean and standard error of mean from one representative experiment ($n = 10$ per group). This experiment was repeated 3 times. ($^{***}p < 0.001$; $^{***}p < 0.0001$).

BALF differential count in milk treated mice are as high as OVA model. The total numbers of eosinophils, lymphocytes, neutrophils, and macrophage did not show any statistically significant differences between OVA and milk treated mice (Fig. 1). Together, these data reveal that low fat milk is as potent as OVA in inducing allergic airway inflammatory response evident by the infiltration of comparable numbers of inflammation-driving cells in the airway.

3.2. Low fat milk intranasal challenge induces dramatic cellular inflammation in the lung of acute mice model

To further evaluate the effect of low fat milk intranasal challenge on airway inflammatory response, lung histopathology was evaluated. Histopathological analysis of lung tissue (as shown by H&E-staining; Fig. 2A) revealed significant infiltration of inflammatory cells in lungs of low fat milk and OVA sensitized mice when compared to PBS-treated group. Staging of lung tissue infiltration was based on the extent of the invasion of the inflammatory cells all over the lung tissue. The extension of the inflammation in the lung is detected in central lung parenchyma (CL), peripheral lung parenchyma (PL), around central airways (CA), and around peripheral airways (PA), by using a scale of 3 grades (0: no inflammation, 1: mild, 2: moderate, and 3: severe) [16]. Low fat milk challenged group of acute allergic airway inflammatory mice showed increased infiltration of the lung tissue by the inflammatory cells, and showed a significant result compared to the OVA challenged mice group. Noticeable mucus hyper-production was detected by PAS staining (Fig. 2B) in both low fat milk and OVA groups. Grading of mucus hyper-production was done by estimation of the percentage of mucus producing goblet cells to the whole airway lumen [16]. The distribution of goblet cells in the central and peripheral airways was graded into 5 grades. Grade 0, free of goblet cells; Grade 1, $<20\%$; Grade 2, >20–40%; Grade 3, >40–60; Grade 4, >60–80%; Grade 5, >80–100%. Mucus cell production in both low fat milk and OVA were significantly higher than of the PBS control group, with significant increase in low fat milk compared to OVA groups. Parenchymal lung eosinophils infiltration (LUNA staining, Fig. 2C) was graded. Quantification of the eosinophils in central (CL) and peripheral lung parenchyma (PL), around airways (AW) and blood vessels (BV) with total of 10 fields with 100× [18], showed significant increase of eosinophils in low fat milk group compared to OVA groups. Both low fat and OVA groups showed significant results compared to PBS-treated mice ($p < 0.0001$ for HE, PAS, and LUNA staining in milk and OVA treatment, Fig. 2D). Interestingly, H&E, PAS, and LUNA-stained lung sections from low fat milk challenged mice had higher average pathological scores compared to OVA-treated mice (Fig. 2A–D). Lung pathology showed significantly higher allergic inflammatory responses in low fat milk group compared to OVA group, which was not detected in total BALF and differential cell counts.

3.3. Low fat milk leads to increased pulmonary expression of Th2-related markers

In this study we aimed to compare our allergic inflammatory airway response to the well-studied OVA-induced allergic asthma model. Allergic asthma is characterized by increased numbers of Th2 cells and elevated levels of Th2 related cytokines [3]. IL-13 is one of the main Th2 cytokines upregulated in OVA-induced asthma models. To assess the influence of low fat milk on Th2 cell subsets, lungs were analyzed for GATA-3 (Th2 transcription factor) and IL-13 mRNA, 2 days after the last milk challenge and compared to OVA and PBS-treated mice. Analysis of the GATA-3 and IL-13 transcription profile showed that mice challenged with low fat milk and OVA had comparable expression of both transcripts, which was significantly higher than that of the mock-challenged mice (Fig. 3A, $p = 0.04$ and 0.009 for GATA-3 of OVA and milk-treated mice; respectively and Fig. 3B, $p = 0.02$ and 0.0003 for IL-13 of OVA and milk-treated mice; respectively). The findings of the up regulation of Th2-related transcripts in the lung of low fat milk-sensitized mice were confirmed by T1/ST2 immunofluorescence (Fig. 4A). Similar to what we found with the qRT-PCR data, lung cryosections stained for the surface markers T1/ST2 revealed the presence of comparable numbers of $T1/ST2^{+}$ cells between low fat milk and OVA-treated mice, which were distinctly higher than that of the control counterparts (Fig. 4A). T1/ST2 expression in a microenvironment with a significant high expression of GATA-3, suggests the presence of Th2 response. We scored the percentage of T1/ST2 positive cells in the different groups in 10 lung fields with 1000× magnification, in a total area of 15×10^4 μm^2 based on dFOV (diameter of field of view), compared to the DAPI positive cells in the same area. We found comparable percentage of T1/ST2 positive cells between OVA and milk treated mice, which both were higher than naïve mice (Fig. 4B). Collectively, these data clearly reveal that, similar to OVA, low fat milk intranasal challenge has a pronounced impact on Th2 cell numbers and induction of asthma-related Th2 cytokines.

3.4. Low fat milk challenge resulted in elevated levels of specific IgG1 and IgE in serum

To further test the efficacy of low fat milk as allergen in allergic inflammatory model, Low fat milk and OVA-specific IgG1 levels and IgE were measured by ELISA in sera collected before the start of the experiment (set as naive) or collected at the end of the sensitization protocol (day 31). Allergen-specific IgG1 was induced only in milk and OVA-challenged mice, while the levels of OVA-specific IgG1 (data not shown) or milk-specific IgG1 (Fig. 5A, $p = 0.0001$ at dilution 1/500) were close to background levels in sera taken prior to the sensitization experiment. Low fat milk challenge greatly induced the humoral immune response resulting in elevated levels of specific IgG1 in serum but in a lower level than induced upon OVA challenge. The significant increase in level of specific IgG1 in serum of low milk challenge group compared to the OVA challenged group was detected at dilution 1/500 (Fig. 5A, $p = 0.0001$). Interestingly, milk treated mice showed higher levels of IgE than that produced in response to OVA treatment (Fig. 5B, $p = 0.009$).

4. Discussion

Experimental models of asthma are very important in order to study the underlying disease mechanism and to test the novel therapeutic regimes. Although OVA mouse models of allergic asthma are well-defined and closely resemble the clinical

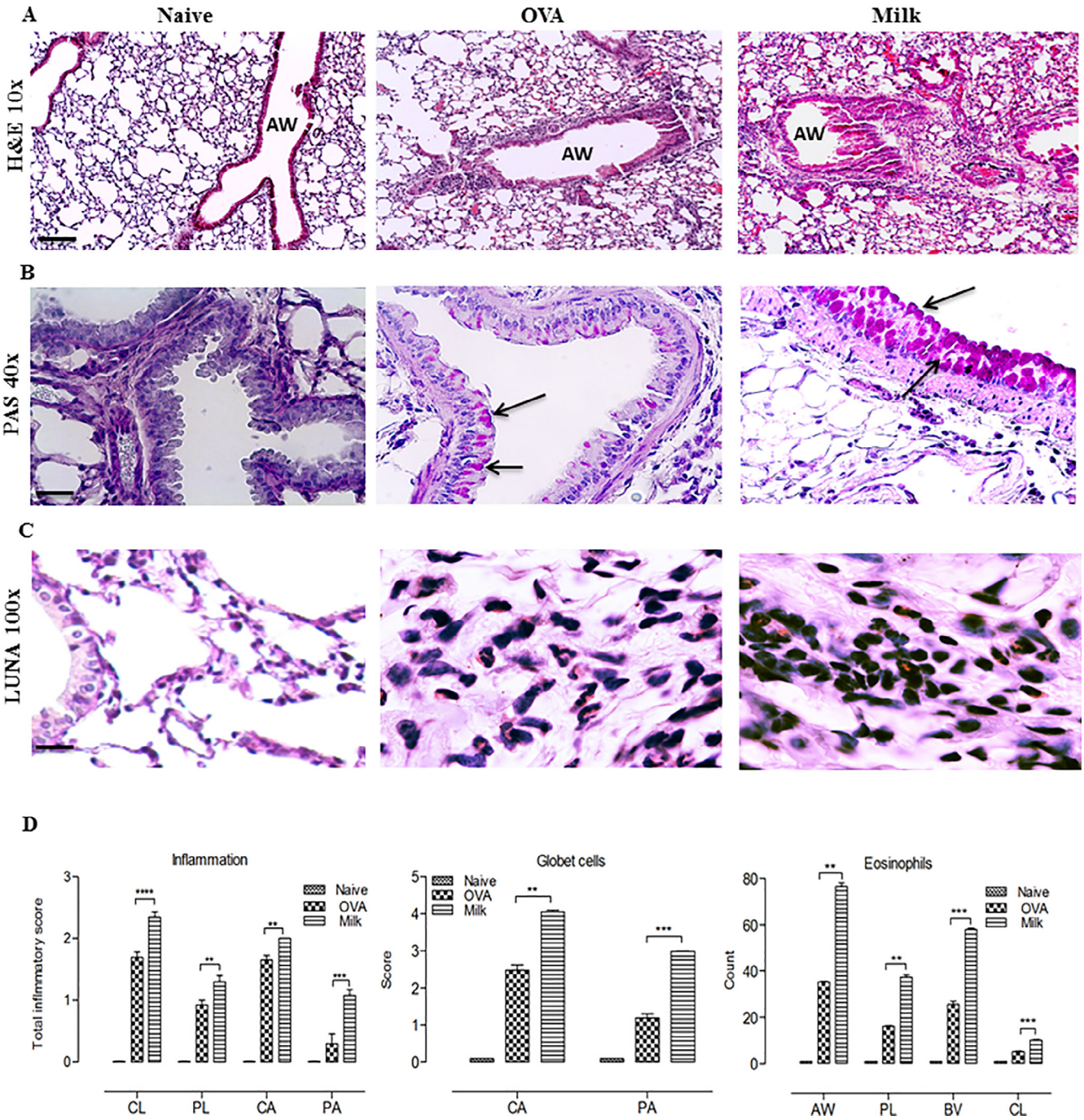

Fig. 2 Intranasal challenge with low fat milk induces vigorous inflammation in lung. Representative sections of (A) H&E-(scale bar 200 μm; original magnification 10×), (B) Periodic-acid Schiff reagent (PAS)-(scale bar 40 μm; original magnification 40×), and (C) Luna-(scale bar 20 μm; original magnification 100×) stained lung tissues from naïve, OVA and low fat milk-challenged mice. (D) Represents the estimated score for the total inflammation in HE-stained sections at specific areas of lung parenchyma (left), the estimated number of goblet cells in the central and peripheral airways of PAS-stained sections (middle), and the estimated numbers of eosinophil at determined areas of lung parenchyma of LUNA-stained sections (right). Photomicrographs (and related scoring) are from a single lung tissue section representative of groups of 6 mice. ($^{**}p < 0.01$; $^{***}p < 0.001$). AW, airways; BV, blood vessel; CA, central airways; CL, central lung; PA, peripheral airways; PL, peripheral lung.

manifestations seen in human, their wide application is restricted by the high cost. In this study we established a novel murine model of allergic airway inflammation using low fat milk as an allergen combined with an easy intranasal challenge mimicking the milk aspiration in children. Intranasal challenging is considered a suitable route to study allergic airway after the exposure to allergens. Our milk model is advantageous because it recapitulates the complex features of allergic airway

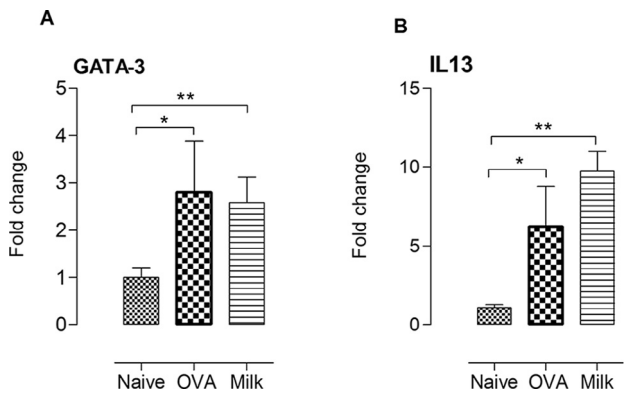

Fig. 3 Intranasal challenge with low fat milk leads to increased expression of Th2 related markers in lung. The relative mRNA expression of GATA-3 (A) and IL13 (B) was measured by qRT-PCR in lungs of OVA- and low fat milk treated mice (n = 6 per group). The data of GATA-3 and IL13 were normalized to the expression of the house keeping gene HPRT. Data are presented as the fold change relative to the mean of naïve control group (n = 6). This experiment was repeated 3 times ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

inflammation in mice similar to the models of OVA-induced allergic airway inflammation but, when compared to the existing OVA allergy models, our milk model induces more robust pulmonary inflammation and humoral immune response with affordable cost and handling.

Multiple protocols for allergen sensitization and challenge have been developed in mice using the standard allergen, OVA [5]. However, these models are irrelevant to human allergic airway inflammation in major aspects. For example, OVA is not a naturally inhaled allergen, the route of sensitization is not often by inhalation through the airways, and the airway response is temporary and results in an acute form of the disease [10,19]. Recently, many attempts have been achieved to improve the existing protocols either by using new inhaled allergens with clinical relevance such as dust mite, pollen and moulds [8,20–22] or by modulating the chronic OVA protocols [11]. However, the cost of the allergen will remain the main obstacle for the applicability of these models and it thereby highlights the need for finding less expensive alternative allergens.

Cow milk is the leading cause of food allergy in children [14], which is associated in the majority of cases with onset allergic asthma later in life [23,24]. Milk aspiration by infants and young children causes airway inflammation and obstruction [25]. Albeit allergic reaction against cow milk develops mainly against milk proteins such as casein or beta-lactoglobulin [26], many studies showed that fat ingredients within cow milk play a protective role against developing asthma [27,28], and it is likely thereby that low fat milk can induce asthma. Additionally, milk has reasonable price when compared to the present allergens. Hence in the present study we compared the potency of low fat milk to that of OVA with regard to the induction of allergic airway inflammation like symptoms in Balb/c mice.

Asthma complicated features are attributed mainly to the infiltration of eosinophils and lymphocytes into the airway wall causing mucus hypersecretion [3,29]. In the present study, we focused more on the allergic inflammatory response in the airway, which is an important factor in allergic asthma disease. Therefore, percentage and total cell counts of eosinophils and lymphocytes were calculated as major biomarkers for allergic asthma. Our data indicated that low fat milk increased effectively both cell subsets in BALF comparably to OVA induced allergic airway inflammation. Consistent with the previous reports [5,30], the pulmonary histological examination showed that OVA treated mice had various degrees of inflammation. Surprisingly, the milk challenged mice presented with more serious pulmonary inflammation as evident by increased eosinophil influx in lung and mucus hypersecretion. Therefore, both inflammatory cell counts in BALF and pulmonary histological assay present confirmatory evidence that low fat milk could significantly induce eosinophilic airway inflammation and thus it is a promising candidate allergen in allergic airway inflammatory models.

Human and experimental studies of allergic asthma have explored shifting of the adaptive immunity toward Th2 rather than Th1 allergen-specific response with higher levels of Th2 mediators, IL4, IL5, and IL13 [31–33]. Similar to human and OVA-induced asthma, we did observe a significant increase in the total numbers of Th2 and elevated level of Th2 related cytokine, IL13, in low fat milk challenged mice. The significant increase in the total inflammatory cells with special concern to the high eosinophilic BALF and lung infiltration in addition to significant hyperplasia of airway goblet cells in the milk model, directed us to continue our study to the direction of Th2 allergic airway inflammatory response.

The induction of allergen specific IgG1 is one of the hallmarks of allergic Th2 response that contributes to increased airway inflammation, eosinophilic infiltration and Th2 cytokine production [34,35]. Our low fat milk model successfully induced the humoral immune response as OVA model did as shown by the elevated levels of milk specific IgG1. The significant increase in the response of IgG1 to milk was found to be higher in 1/500 dilution compared to OVA with lower response of the other high dilution. This profile of the relationship between milk and OVA IgG1 response was repeated in other experiments (data not shown). The controversial results of IgG1 in allergic asthma model mice [36] are not applicable in our model. The elevated levels of IgG1, in response to our new milk sensitization and challenge of the allergic inflammatory model, exclude the inhibitory effect of Fcγ RIIB on our model. Stimulation of Fcγ RIIB with increase in the allergic airway inflammation suggests that Th2 inflammation in lung had occurred. Not only the increased level of the IgG1 to milk but also the total IgE increase is supporting the hypothesis of the induction of the allergic inflammatory response in our milk model compared to the well-established OVA model [36].

The multiple phenotypic expression of IL33 signal through its receptor T1/ST2 depends mainly on the cytokine profile in the microenvironment that contributes to Th1 or Th2 mediated immune response [37]. It was found that IL 33 receptor (T1/ST2) regulates the early production of IL 13 in an allergic airway model of fungus inflammation which is controlled by GATA3 Th cells. This GATA3/IL13 and/ IL33 R complex are the key factors in the production of goblet cells, eosinophils, and macrophages in BALF and lung tissue [38].

In our new milk allergic inflammatory model, the histopathological study of the central and peripheral lung, revealed that the extension of the invasion of the total inflam-

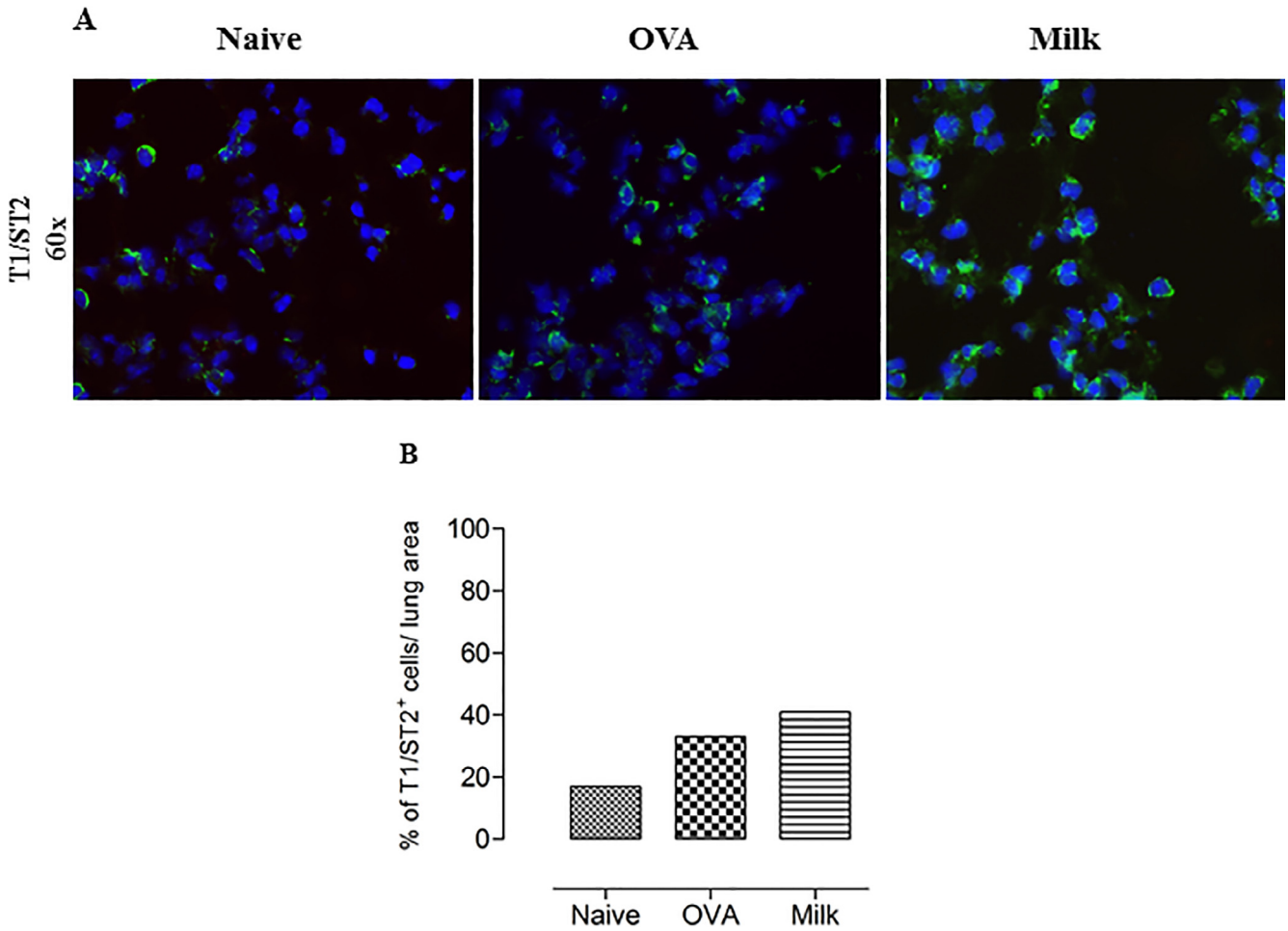

Fig. 4 Intranasal challenge with low fat milk increases the numbers of T1/ST2$^+$ cells in lung. (A) T1/ST2$^+$ cells (FITC-green) was detected using immunofluorescence microscopy on consecutive frozen lung sections from OVA, low fat milk treated mice and naïve control mice (n = 6 per group). The blue color demonstrates the nuclear staining by DAPI dye. Magnification is 60×. (B) Immunofluorescence analysis for quantification of T1/ST2$^+$ cells relative to the area of lung. We scored the percentage of T1/ST2 positive cells in the different groups compared to the DAPI positive cells in the same area. We calculated the number of the expressed T1/ST2 cells in 10 lung fields with 1000× magnification, in a total area of 15×10^4 μm^2, based on dFOV (diameter of field of view).

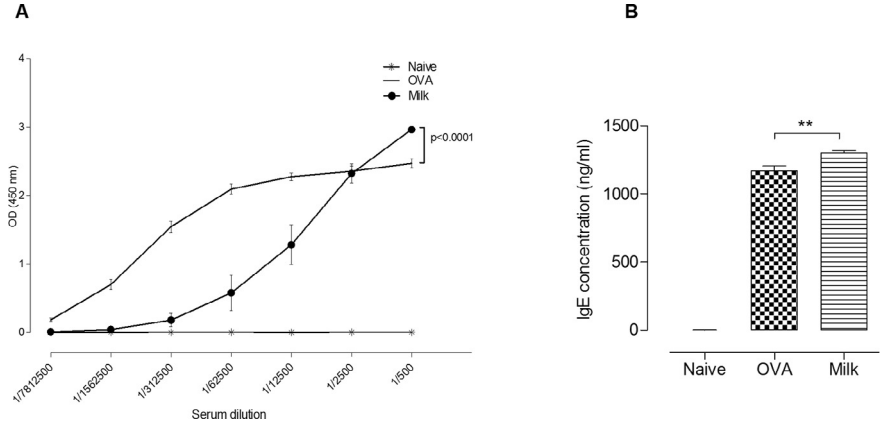

Fig. 5 Intranasal challenge with low fat milk induces augmented levels of allergen specific IgG1 and IgE in serum. ELISA was used to determine the titers of low fat milk, OVA-specific IgG1 (A) and IgE (B) in sera collected before starting the sensitization experiment (naive) and after the last intranasal challenge at day 31. The data are shown as mean and standard error of mean from one representative experiment (n = 6 per group). This experiment was repeated 3 times.

matory cells (H&E), eosinophils (Luna), and mucus hyperproduction by goblet cells (PAS) to the peripheral lung compartment matches the result of Zoltowska et al. [39]. Zoltowska and his group found that IL33 and its receptor are playing an important role in promoting the extension of the inflammatory and goblet cells to the distal peripheral compartment of the lung in asthma model mice induced by house dust mite.

In our allergic inflammatory milk mice model, we could prove that GATA3, IL13 and/ IL33 R (T1/ST2) are significantly expressed in the lung tissue which are accompanied by significant increase in total inflammatory cells, eosinophils in BALF and lung tissue. The significant hyperplasia of the air way goblet cells in the milk allergic airway inflammatory mice model goes hand in hand with the high level of IL 13 and GATA3 expressions in lung tissue.

5. Conclusion

In summary, herein we present a novel model for acute allergic airway inflammation. Our milk allergen-induced model exhibits the common characteristics of allergic airway inflammatory responses such as airway and pulmonary inflammation, Th2 biased immune response and production of allergen specific IgG1 and IgE. Most importantly, this model is much cheaper than the available murine models of allergic airway inflammation. Added to the availability of the low fat milk as allergens, intranasal challenge proved to be a potent route to induce remarkable airway hypersensitivity. We highly recommend this protocol to rapid, efficient, cheap and accessible model to study further pathophysiological changes during food allergy induced asthma attacks. Indeed, it is a promising model to open the horizon to test new antiallergic/anti-inflammatory agents. However, further studies are warranted to investigate other hallmarks of allergic asthma such as AHR, Th1, Th2, Th17 and other biomarkers using this model.

Competing interests

The authors declare no competing financial interests exist.

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References

[1] P.W. Finn, T.D. Bigby, Innate immunity and asthma, Proc Am Thorac Soc 6 (3) (2009) 260–265.
[2] Expert Panel Report 3 (EPR-3): guidelines for the diagnosis and management of asthma-summary report 2007. J Allergy Clin Immunol 2007;120(5 Suppl):S94–138.
[3] J.D. de Boer et al, Asthma and coagulation, Blood 119 (14) (2012) 3236–3244.
[4] L. McKinley et al, TH17 cells mediate steroid-resistant airway inflammation and airway hyperresponsiveness in mice, J Immunol 181 (6) (2008) 4089–4097.
[5] R.K. Kumar, C. Herbert, P.S. Foster, The "classical" ovalbumin challenge model of asthma in mice, Curr. Drug Targets 9 (6) (2008) 485–494.
[6] G.R. Zosky, P.D. Sly, Animal models of asthma, Clin Exp Allergy 37 (7) (2007) 973–988.
[7] J.A. Boyce, K.F. Austen, No audible wheezing: nuggets and conundrums from mouse asthma models, J Exp Med 201 (12) (2005) 1869–1873.
[8] J.R. Johnson et al, Continuous exposure to house dust mite elicits chronic airway inflammation and structural remodeling, Am J Respir Crit Care Med 169 (3) (2004) 378–385.
[9] S.B. Sarpong, L.Y. Zhang, S.R. Kleeberger, A novel mouse model of experimental asthma, Int Arch Allergy Immunol 132 (4) (2003) 346–354.
[10] R.K. Kumar, P.S. Foster, Modeling allergic asthma in mice: pitfalls and opportunities, Am J Respir Cell Mol Biol 27 (3) (2002) 267–272.
[11] S.J. McMillan, C.M. Lloyd, Prolonged allergen challenge in mice leads to persistent airway remodelling, Clin Exp Allergy 34 (3) (2004) 497–507.
[12] S. Sommanus et al, Cow's milk protein allergy: immunological response in children with cow's milk protein tolerance, Asian Pac J Allergy Immunol 32 (2) (2014) 171–177.
[13] A.B. Sprikkelman, H.S. Heymans, W.M. Van Aalderen, Development of allergic disorders in children with cow's milk protein allergy or intolerance in infancy, Clin Exp Allergy 30 (10) (2000) 1358–1363.
[14] G. Sampaio et al, Transient vs persistent cow's milk allergy and development of other allergic diseases, Allergy 60 (3) (2005) 411–412.
[15] M. Husain, H.J. Boermans, N.A. Karrow, Mesenteric lymph node transcriptome profiles in BALB/c mice sensitized to three common food allergens, BMC Genomics 12 (2011) 12.
[16] R. Grausenburger et al, Conditional deletion of histone deacetylase 1 in T cells leads to enhanced airway inflammation and increased Th2 cytokine production, J Immunol 185 (6) (2010) 3489–3497.
[17] J. Wallmann et al, Mimotope vaccination for therapy of allergic asthma: anti-inflammatory effects in a mouse model, Clin Exp Allergy 40 (4) (2010) 650–658.
[18] Luna L.G. Manual of histologic staining methods of the armed forces institute of pathology. New York: McGraw-Hill; 1968, p. 2.
[19] B. Fuchs, A. Braun, Improved mouse models of allergy and allergic asthma–chances beyond ovalbumin, Curr Drug Targets 9 (6) (2008) 495–502.
[20] Y. Wang et al, Effective treatment of experimental ragweed-induced asthma with STAT-6-IP, a topically delivered cell-penetrating peptide, Clin Exp Allergy 41 (11) (2011) 1622–1630.
[21] N. Goplen et al, Combined sensitization of mice to extracts of dust mite, ragweed, and Aspergillus species breaks through tolerance and establishes chronic features of asthma, J Allergy Clin Immunol 123 (4) (2009), p. 925-32 e11.
[22] J. Kerzerho et al, Programmed cell death ligand 2 regulates TH9 differentiation and induction of chronic airway hyperreactivity, J Allergy Clin Immunol 131 (4) (2013), p. 1048–57, 1057 e1-2.
[23] D.J. Hill et al, Cow milk allergy within the spectrum of atopic disorders, Clin Exp Allergy 24 (12) (1994) 1137–1143.
[24] A. Host et al, Clinical course of cow's milk protein allergy/intolerance and atopic diseases in childhood, Pediatr Allergy Immunol 13 (Suppl 15) (2002) 23–28.
[25] I.A. Janahi et al, Recurrent milk aspiration produces changes in airway mechanics, lung eosinophilia, and goblet cell hyperplasia in a murine model, Pediatr Res 48 (6) (2000) 776–781.
[26] J. Jo et al, Role of cellular immunity in cow's milk allergy: pathogenesis, tolerance induction, and beyond, Mediators Inflamm 2014 (2014) 249784.

[27] A.H. Wijga et al, Association of consumption of products containing milk fat with reduced asthma risk in pre-school children: the PIAMA birth cohort study, Thorax 58 (7) (2003) 567–572.
[28] E. Maslova et al, Low-fat yoghurt intake in pregnancy associated with increased child asthma and allergic rhinitis risk: a prospective cohort study, J Nutr Sci 1 (2012).
[29] B.S. Bochner, W.W. Busse, Advances in mechanisms of allergy, J Allergy Clin Immunol 113 (5) (2004) 868–875.
[30] J. Grundstrom et al, Development of a mouse model for chronic cat allergen-induced asthma, Int Arch Allergy Immunol 165 (3) (2014) 195–205.
[31] M. Larche, D.S. Robinson, A.B. Kay, The role of T lymphocytes in the pathogenesis of asthma, J Allergy Clin Immunol 111 (3) (2003) 450–463, quiz 464.
[32] L.R. Bisset, P. Schmid-Grendelmeier, Chemokines and their receptors in the pathogenesis of allergic asthma: progress and perspective, Curr Opin Pulm Med 11 (1) (2005) 35–42.
[33] T. Agrawal, G.K. Gupta, D.K. Agrawal, Vitamin D supplementation reduces airway hyperresponsiveness and allergic airway inflammation in a murine model, Clin Exp Allergy 43 (6) (2013) 672–683.
[34] M.K. Pandey, Molecular basis for downregulation of C5a-mediated inflammation by IgG1 immune complexes in allergy and asthma, Curr Allergy Asthma Rep 13 (6) (2013) 596–606.
[35] N.W. Lukacs, M.M. Glovsky, P.A. Ward, Complement-dependent immune complex-induced bronchial inflammation and hyperreactivity, Am J Physiol Lung Cell Mol Physiol 280 (3) (2001), p. L512-8.
[36] J.W. Williams, M.Y. Tjota, A.I. Sperling, The contribution of allergen-specific IgG to the development of th2-mediated airway inflammation, J Allergy (Cairo) 2012 (2012) 236075.
[37] M. Komai-Koma et al, Interleukin-33 promoting Th1 lymphocyte differentiation dependents on IL-12, Immunobiology 221 (3) (2016) 412–417.
[38] D. Piehler et al, The IL-33 receptor (ST2) regulates early IL-13 production in fungus-induced allergic airway inflammation, Mucosal Immunol 9 (4) (2016) 937–949.
[39] A.M. Zoltowska et al, The interleukin-33 receptor ST2 is important for the development of peripheral airway hyperresponsiveness and inflammation in a house dust mite mouse model of asthma, Clin Exp Allergy 46 (3) (2016) 479–490.