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Experimental protocol for development of adjuvant-free murine chronic model of allergic asthma

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ABSTRACT

Background: Mouse models of allergic asthma play a crucial role in exploring of asthma pathogenesis and testing of novel anti-inflammatory drugs. Widely used acute asthma models usually developed with adjuvant (aluminum hydroxide (alum)) do not reproduce one of the main asthma feature - airway remodeling while chronic asthma model mimic the pathophysiology of human disease. Moreover, the use of alum causes distress in experimental animals and impedes the test of adjuvant-containing drugs. In this study, we aimed to develop a chronic adjuvant-free asthma model with pronounced asthmatic phenotype.

Methods: Female BALB/c mice were divided into 3 groups. The first group was sensitized with intraperitoneal injections of ovalbumin (OVA) emulsified in aluminum hydroxide on days 0, 14, 28 followed by two stages of intranasally challenge with OVA on days 41–43 and 62–64. The second group was subcutaneously sensitized with the same dose of OVA without adjuvant and challenged on the same days. The third group (negative control) included mice which did not received any kind of treatment (i.e. sensitization and challenge). Serum levels of OVA-specific IgE, IgG2a and IgG1 antibodies were detected by ELISA. Airway hyper-responsiveness was measured by non-invasive plethysmography on days 44 and 65. Bronchoalveolar lavage fluids (BALF) sampled in all groups on days 45 and 66 were analyzed by light microscopy. The left lung was removed for histological analysis. The IL-4 and IFNγ mRNA expression in BALF cells was evaluated by RT-PCR.

Results: The OVA-specific IgE antibody response was two-fold increased in mice from adjuvant-free group compared to the adjuvant group that reflects reorientation of immune response towards Th2 phenotype. At the same time, the level of OVA-specific IgG1 and IgG2a antibodies was increased in the adjuvant group. Airway hyperresponsiveness to methacholine in mice of both experimental groups was two-fold higher than in control. Analysis of cell composition in BAL has shown a significant increase in eosinophil count in both experimental groups that indicate the development of allergic inflammation. Lung histology revealed airway remodeling in both experimental groups including goblet cell hyperplasia/metaplasia, thickening of airway walls, collagen deposition in the wall of distal airways. Additionally, the tendency to develop hypertrophy of bronchial smooth muscle layer was observed. Study of gene expression in BAL cells revealed the increase of IL-4 level in both adjuvant and adjuvant-free groups while IFNγ expression in both experimental groups was similar to control group.

Conclusion: We have developed a chronic adjuvant-free mouse asthma model which possesses all necessary features of the disease including airway remodeling and is more suitable for pre-clinical evaluation of novel therapeutic approaches including adjuvant-containing drugs.

1. Introduction

Allergic asthma (AA) is a chronic inflammatory disease of the lower respiratory tract characterized by chronic eosinophilic airway inflammation, airway hyperresponsiveness (AHR) and airway remodeling (Bousquet et al., 2000). Airway remodeling defined as some structural changes of the airways, which include goblet cell hyperplasia/metaplasia, airway wall fibrosis, increased vascularity and smooth muscle thickness. These changes are the result of persistent allergen exposure that contribute significantly to asthma symptoms. Most likely airway

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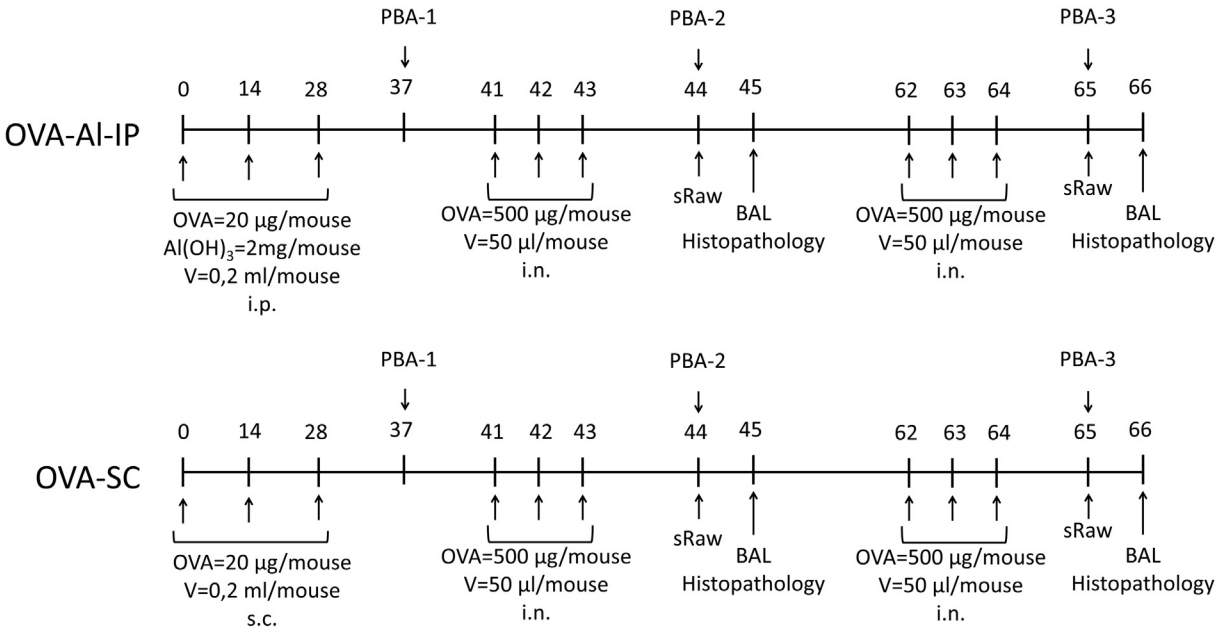

Fig. 1. Scheme of experimental protocols for developing of the chronic allergic asthma.
AHR – measurement of specific airways resistance, OVA – ovalbumin, BALF - bronchoalveolar lavage fluid, PBC – peripheral blood collection on days 37, 45, 66.

remodeling can be induced by growth factors released by cells which infiltrate the lungs during inflammation, but the precise molecular mechanism is still poorly understood (Bergeron et al., 2010; Lloyd and Robinson, 2007).

Current anti-inflammatory therapy of asthma is mainly based on the use of inhaled corticosteroids. Although these drugs are highly effective in preventing of life threatening consequences of asthma, their effect is limited in modulation of airway remodeling (Bårnes et al., 2015; Thomson, 2013). Allergen immunotherapy (AIT) is the only pathogenesis-based approach for asthma treatment using allergy vaccines (Ring and Gutermuth, 2011), which often contain adjuvants (Moingeon, 2012; Moingeon et al., 2011). Alternative approaches for asthma treatment are cytokine inhibitors such as monoclonal antibodies, soluble receptors, muteins (Boyman et al., 2015; Hansbro et al., 2011; Shilovskiy et al., 2017) and antisense technologies (Khaitov et al., 2014; Popescu and Popescu, 2007).

Identification of potential biological targets and testing of novel drugs for asthma treatment is one of the relevant tasks of modern biomedicine. Mouse models provide a powerful instrument for the revealing of molecular asthma mechanisms and studying of novel therapies (Shin et al., 2009; Zosky, 2007). The most common asthma models are short-term acute models, they reproduce asthma features such as allergen-specific IgE production, eosinophilic infiltration to the lungs, AHR and are easy to develop during short period of time. However short-term acute models do not reproduce one of the main characteristic of the human disease - airway remodeling (Nials and Uddin, 2008; Pabst, 2002; Shilovskiy et al., 2015). In addition, listed asthma features seem to resolve within a few weeks after the final antigen provocation in the animal, while in human asthma inflammation persists (Aun et al., 2017). Therefore, acute models don't allow to investigate the molecular mechanisms of airway remodeling process. On the contrary, some models of chronic asthma in animals more closely reproduce the pathophysiology of human asthma disease because AHR and pulmonary inflammation persist after the last allergen challenge (McMillan and Lloyd, 2004). Up to date almost all chronic asthma models in mice described in the literature were developed using adjuvant aluminum hydroxide (alum), which enhanced allergic response to allergen and eventually forms a pronounced signs of asthma (Kurucz and Szelenyi, 2006; Mullane and Williams, 2014; Zosky, 2007). The alum is non-physiological substance, which induce distress in experimental animals (Conrad et al., 2009), and its utilization may interfere the study of the adjuvant-containing drugs especially allergy vaccines for AIT. Therefore, in this study we aimed to develop the protocol of chronic alum-free asthma model in mice.

2. Materials and methods

2.1. Animals

Female BALB/c mice aged 6–8 weeks (18–20 g) were purchased from the animal breeding facility of the Russian Academy of Medical Sciences “Stolbovaya” (Moscow region, Russian Federation). The mice were quarantined for 5 days prior to the experimental manipulation and were fed standard laboratory rat chow (OVA free) and had unlimited access to water. Animal experiments were conducted in accordance with the principles of EU Directive 2010/63/EU “Legislation for the protection of animals used for scientific purposes” and were approved by the Ethical Committee of the National Research Center - Institute of Immunology of Federal Medico-Biology Agency of Russia, Moscow, Russian Federation.

2.2. Experimental protocol

Mice were divided into three groups (16 mice per group). Animals of the first group (OVA-Al-IP) were immunized intraperitoneally (i.p.) with OVA (20 µg/mouse) three times in a two-week interval together with $Al(OH)_3$ (alum) in dose 2 mg/mouse on days 0, 14, 28 (Fig. 1). The sensitized mice were intranasally (i.n.) challenged with OVA (50 µl/mouse of 10 mg/ml solution) on days 41–43 and 62–64. In order to reproduce the adjuvant free allergic asthma phenotype, the second group of mice (OVA-SC) was immunized subcutaneously (s.c.) with the same doses of OVA without alum on the same days. Challenge with OVA was conducted in similar manner. The third group of intact mice were neither sensitized nor challenged.

2.3. Nasal hyperreactivity

After the last challenge (on day 66) frequencies of sneezing and nasal rubbing for 5 min were counted in accordance with the procedure described earlier (Hosoya et al., 2011).

2.4. Airway hyperresponsiveness (AHR) measurement

Twenty-four hours after last OVA exposure (on days 44, 65) AHR to increased doses of methacholine (6.25, 12.5, 25 mg/ml) was assessed as specific resistance of the airways (sRaw) by whole-body plethysmography (Fine Pointe NAM, Buxco). The evaluation of sRaw was described in details previously (Khaitov et al., 2014).

2.5. ELISA

Nine days after the last immunization, 48 h after the first (day 45) and second (day 66) challenges peripheral blood was collected from the retro-orbital sinus of mice. Individual sera samples were prepared and stored at −70 °C until analysis. Serum levels of OVA-specific IgE, IgG1 and IgG2a antibodies were measured by ELISA (BD Biosciences).

2.6. Collection of bronchoalveolar lavage fluid (BALF)

All mice after blood collection on days 45 and 66 were sacrificed and tracheas were cannulated. The lungs were lavaged two times with 0.5 ml chilled RPMI 1640 (with 20% of fetal calf serum and 0.6 mM EDTA). Each BALF sample was centrifuged for 10 min at 400*g*. Cell pellets were resuspended in 0.2 ml PBS. Then BALF cell smears were stained with azur and eosin. To evaluate number of cells in BALF at least 200 cells per slide at 400× magnification were counted.

2.7. TaqMan quantitative real-time PCR (RT-PCR)

The right lobe of the lungs from each mouse was excised and stored at −70 °C until analysis by quantitative real-time PCR as described earlier (Khaitov et al., 2014). Total RNA was isolated from lung tissue using RNeasy Mini kit (Qiagen) and thereafter reverse-transcribed into cDNA using random hexamers and RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas). The cDNAs were amplified via RT-PCR using iCycler iQ5 Real-time PCR Detection System (Bio-Rad Laboratories) Table 1.

2.8. Histopathology

The left lung removed and fixed in 4% paraformaldehyde and then embedded in paraffin. Lung sections were cut and stained with hematoxylin–eosin (H&E) (for identification of the eosinophils, neutrophils and lymphocytes), with Alcian blue (for visualization of the mucin positive goblet cells) and according to Van Gieson method (for identification of fibrosis). The inflammatory changes were analyzed by light microscopy. Smooth muscle hypertrophy was graded according to modified semi-quantitative scoring system in a “blind manner” as: none (0), mild (1.0), moderate (2.0), or severe (3.0) (Ennis et al., 2005). Cell infiltration around bronchioles was counted as absolute number of cells (eosinophils, neutrophils and lymphocytes) at 400× magnification; three fields were analyzed in every lung section followed by calculation of the average cell number. Goblet cells were quantified as the percentage of total cells of bronchial epithelium at 400× magnification; three bronchi were analyzed for every lung section. To assess collagen deposition around bronchi the average thickness of collagen layer was measured using Altami Studio software (Altami, Russia); three bronchi were analyzed in every lung section; analysis of all bronchi was performed at 400× magnification.

Table 1
The following primers and probes were used.

Primers and probes	Sequence 5′–3′
mIL4-F	AGAGAGTGAGCTCGTCTGTAG
mIL4-R	GGTGCAGCTTATCGATG
mIL4-Z	(FAM) CTCTGCAGCTCCATGAGAACACTAGAG (BHQ1)
mIFNg-F	AAATCCTGCAGAGCCAGATTAT
mIFNg –R	GCTGTTGCTGAAGAAGGTAGTA
mIFNg –Z	(FAM)ACGCTTATGTTGTTGCTGATGGCC(RTQ1)
mHPRTf	GAAGAGCTACTGTAATGATCAGTCA
mHPRTr	GTATCCAACACTTCGAGAGGTC
mHPRTz	(FAM)GCTTTCCCTGGTTAAGCAGTACAGCC(RTQ1)

2.9. Statistical analysis

Data were expressed as mean ± SE (standard error) except for scoring hypertrophy of bronchial smooth muscle layer data, which were presented as median ± inter-quartile range. Data were analyzed by one-way ANOVA followed by Bonferroni's multiple comparison posttest using GraphPad Prism 7. Semi-quantitative scoring data were analyzed according to Kruskall Wallis test using GraphPad Prism 7. Data were accepted as significantly different when $p < 0.05$.

3. Results

3.1. Effects of OVA sensitization with or without adjuvant on serum levels of the OVA-specific antibodies

Increased levels of OVA-specific IgE antibodies were observed in sera samples from “OVA-Al-IP” and “OVA-SC” groups of mice at all stages of the experiment (Fig. 2A). However, three s.c. OVA immunization without adjuvant $Al(OH)_3$ followed by two stages of OVA intranasal application (group OVA-SC) results in more significant OVA-specific IgE level compared to adjuvant protocol applied to group “OVA-Al-IP”. Despite the increased level of OVA-specific IgE group “OVA-SC” demonstrated lower levels of serum OVA-specific IgG1 (Fig. 2B) and IgG2a (Fig. 2C).

3.2. Effects of OVA immunization without adjuvant on AHR

AHR is a prominent feature of asthma and we assessed AHR to increased doses of methacholine in mice from each group twice: after the first (day 49) and after the second (day 69) of i.n. OVA. Despite increased level of serum IgE after the first OVA challenge (on day 44) mice of “OVA-SC” group, demonstrated lower (but not statistically significant) level of sRaw (at methacholine concentration 12.5 mg/ml) than mice from group “OVA-Al-IP” (Fig. 3A). However, after the second OVA challenge (on day 65) sRaw parameters were similar in both groups (Fig. 3B).

3.3. Nasal hyperreactivity

Nasal hyperreactivity in response to intranasal administration of OVA solution (10 mg/ml) was measured as described earlier (Hosoya et al., 2011). We have shown that both frequency of sneezing and nasal rubbing provoked within 5 min were equally elevated in experimental groups compared to intact mice (Fig. 4). Sneezing and nasal rubbing are immediate-type response induced by mast cell mediators, including histamine. The degranulation of mast cells in nasal mucosa is affected by allergen specific IgE antibodies that were significantly upregulated in both experimental groups (Fig. 2A).

Frequences of sneezing (A) and nasal rubbing (B) within 5 min.
× – significant difference vs “OVA-Al-IP” group.
* – significant difference vs “Intact” group.

3.4. Cell infiltration in BALF

Effect of the adjuvant and adjuvant-free sensitization on cell infiltration in BALF was determined 48 h after the first (on day 45) and the second (on day 66) challenge with OVA. After the first OVA challenge, significant increase of total cells in BALF up to 350,000 and 250,000 cells/ml in both “OVA-Al-IP” and “OVA-SC” groups compared

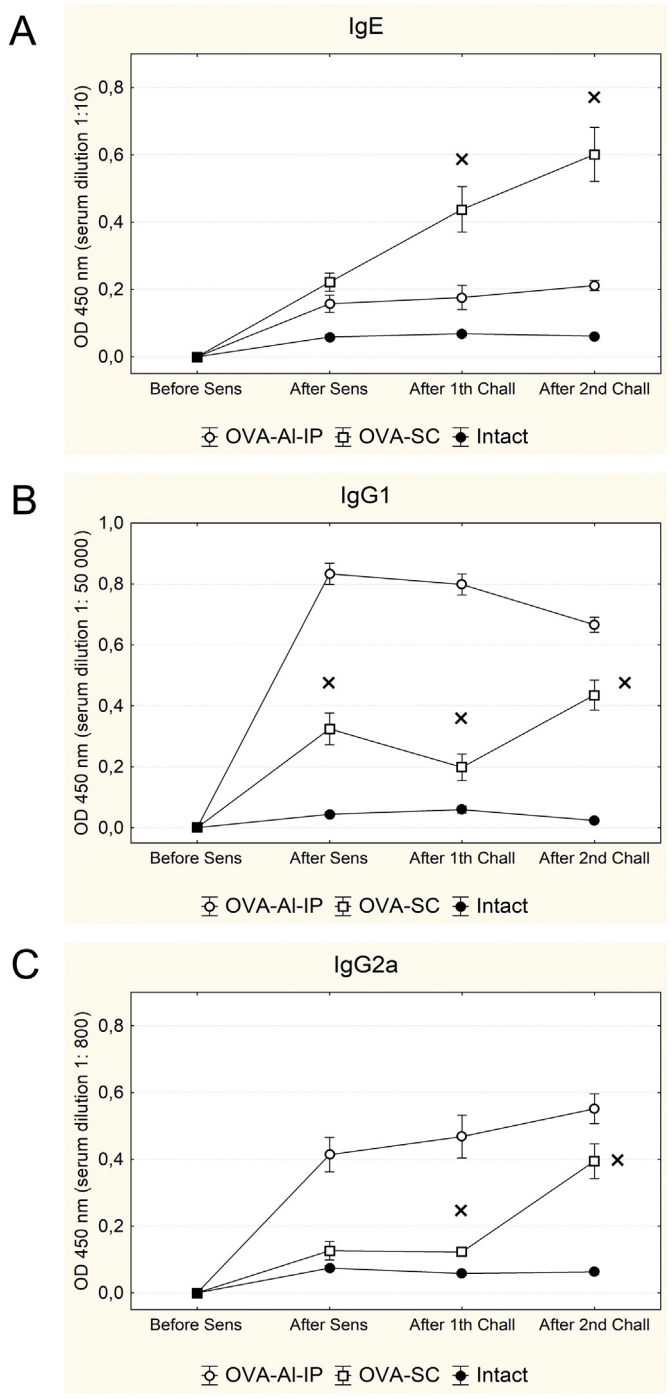

Fig. 2. Serum levels of OVA-specific IgE (A), IgG1 (B) and IgG2a antibodies (C).
× – significant difference vs "OVA-Al-IP" group.

to intact mice (100,000 cells/ml) was observed (Fig. 5A). After the second OVA challenge total cells count in BALF of groups "OVA-Al-IP" and "OVA-SC" was increased up to 400,000 cells/ml (Fig. 5B). Analysis of the cell composition in BALF samples revealed eosinophil and lymphocyte infiltration after the first and second challenge with OVA. Eosinophils as markers of allergic inflammation infiltrated in BALF equally in both "OVA-Al-IP" and "OVA-SC" groups but were more abundant than other cell types. Moreover, the number of eosinophils were increased in both groups after the second OVA challenge.

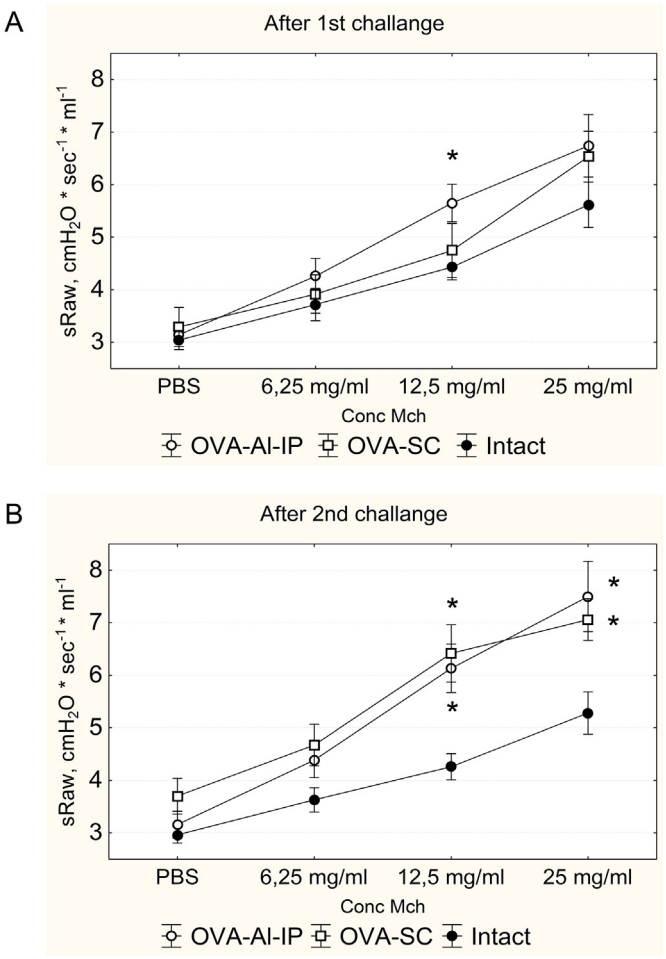

Fig. 3. Airway hyperresponsiveness to increased concentrations of methacholine evalueted after the first (A) and the second (B) challenges with OVA.
× – significant difference vs "OVA-Al-IP" group.
* – significant difference vs "Intact" group.

3.5. Histological alteration in the lungs

In general, histological analysis of hematoxylin–eosin stained lung tissues confirmed the increased eosinophilia in the lung of mice with modeled asthma observed in BALF. Thus, predominant cell types infiltrated into lungs after the first and the second OVA challenge in both experimental groups ("OVA-Al-IP" and "OVA-SC") were eosinophils, whereas more modest infiltration was noted for lymphocytes and neutrophils (Fig. 6 AB). In addition, adjuvant-free protocol was characterized by similar level of eosinophil infiltration after both allergen challenges (Fig. 6 AB). The hematoxylin–eosin staining allowed to estimate not only inflammatory cell infiltrates into the lungs, but also features of airway remodeling such as smooth muscle hypertrophy. This feature was not observed after the first OVA challenge (Fig. 6C), but was insignificantly expressed in the lungs of both experimental groups ("OVA-Al-IP" and "OVA-SC") after the second OVA challenge (Fig. 6C),

Other important features of airway remodeling are airway well thickness and fibrosis of lung tissue, which is the result of collagen deposition process. In order to quantify airway wall thickening and collagen deposition lung sections staining was performed according to the standard Van Gison protocol and thickness of bronchial walls and collagen layers around bronchi was measured by image analysis (see Section 2.8). Airway wall thickening was less pronounced in BALB/c mice immunized with aluminum hydroxide compared to intact animals (there were no significant changes). At the same time the thickening of bronchial walls was significantly increased by ~30% after repeated

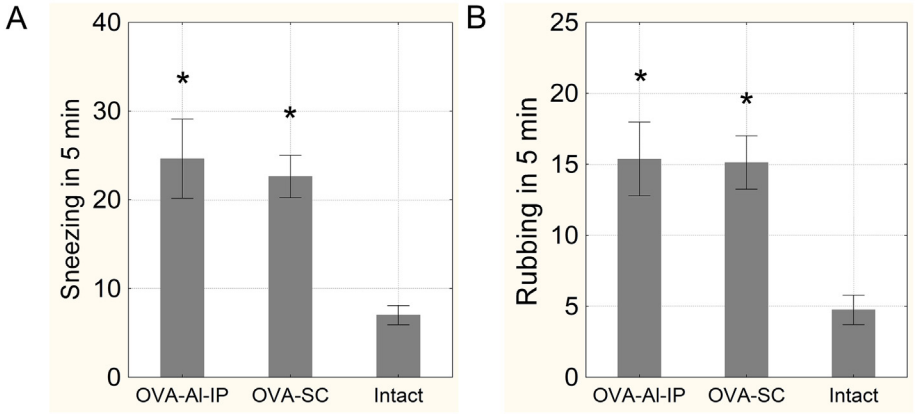

Fig. 4. Nasal hyperreactivity.

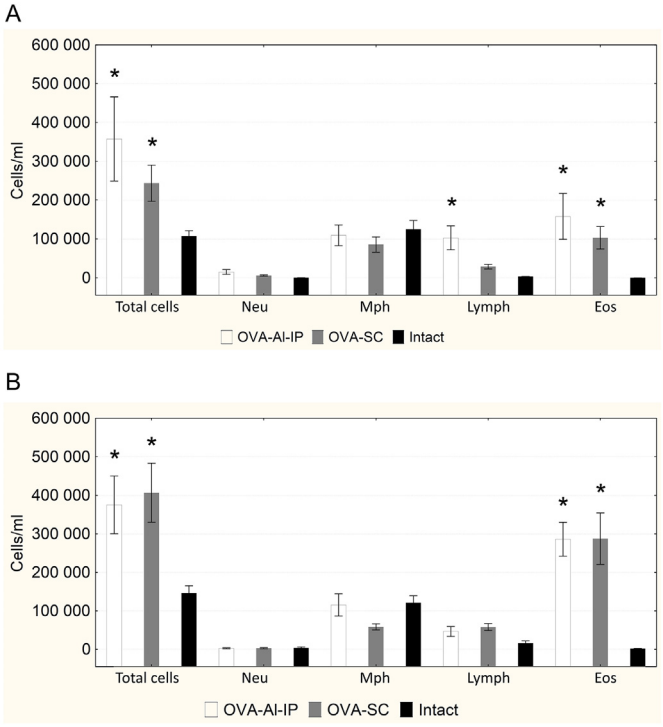

Fig. 5. Differential cell count in BALF after the first (A) and the second (B) OVA challenge.
× – significant difference vs "OVA-Al-IP" group.
* – significant difference vs "Intact" group.

OVA exposures of adjuvant-free immunized mice (Fig. 7A). Significant 1.5-fold increase in the average thickness of collagen layer in mice from both experimental groups after the second OVA challenge compared to intact mice was observed (Fig. 7 BC).

Goblet cell hyperplasia was assessed in sections of lung tissue stained with Alcian Blue. Goblet cells were not detected in the bronchial epithelium of the intact mice. At the same time mucous secreted cells were rarely presented in the epithelium of animals from both experimental groups received repeated allergen exposures (the number of goblet cells estimated from 25% to 75% of total epithelial cells). Subcutaneous immunization without adjuvant followed by challenge with the allergen resulted in increased percentage of the cells stained positive for mucins compared to animals immunized with adjuvant; 72 ± 7% vs. 32 ± 6% ($p < 0.05$) (Fig. 8 AB).

C. Semiquantitative score (0 - absent; 1 - mild; 2 - moderate; 3 - severe) was utilised to evaluete the smooth muscle hypertrophy in the lung airways. D. The microphotographs illustrate the representative morphological changes in transverse sections of bronchioles.
× – significant difference vs "OVA-Al-IP" group.
* – significant difference vs "Intact" group.

3.6. Inflammatory cytokine expression in BALF cells

Study of gene expression in cells purified from BALF revealed the increase of IL-4 level after the second OVA challenge in both adjuvant and adjuvant-free groups ("OVA-Al-IP" and "OVA-SC"). Subcutaneous OVA immunization without adjuvant demonstrated higher, but not statistically significant level of the IL-4 mRNA expression (Fig. 9A). At the same time, the IFNγ expression in both experimental groups was similar to control group (Fig. 9B).

4. Discussion

Animal models of allergic airway disease provide important tools to study mechanisms of pathogenesis and to test novel treatment approaches. There are several different models of allergic asthma employing varying species and antigens that have been developed. Mouse models of allergic airway disease provide several potential advantages compared to other animal models (Zosky, 2007). IgE is the primary allergic antibody in mice, making this species suitable for investigation of the role of humoral immune factors in the development of allergic airway disease. There are several well-characterized inbred strains of mice as well as immunological reagents such as antibodies for cytokines detection, growth factors, and cell surface markers available, that give opportunities to explore the mechanisms of allergic reactions in detail. Accordingly, major advances in the understanding of the disease concept, "asthma as Th2-dominant disease", emerged from studies in mice models (Shin et al., 2009).

As short-term acute asthma models in mice do not exhibit spontaneous AHR and smooth muscle hypertrophy they do not mimic all aspects of human asthma (Shin et al., 2009; Zosky et al., 2008). In order to achieve stable asthma phenotype in mice aluminum hydroxide as adjuvant is widely used. However, as mention above, adjuvant asthma models don't allow to test the adjuvant-containing drugs like allergy vaccines because it is difficult to separate the effect of the studied adjuvant from adjuvant used for the induction asthma pathology in mice.

In our study, we confirmed that the major features of the chronic asthma were reproduced without aluminum hydroxide. We have shown that like in human asthma, elevated levels of allergen specific IgE antibodies were detected in sera of mice immunized and challenged with OVA (Fig. 2A). It is interesting to note that the level of the OVA-specific IgE was significantly higher in group "OVA-SC", immunized without adjuvant than in group "OVA-Al-IP" received alum as adjuvant (Fig. 2A). At the same time, the levels of allergen-specific IgG1 and

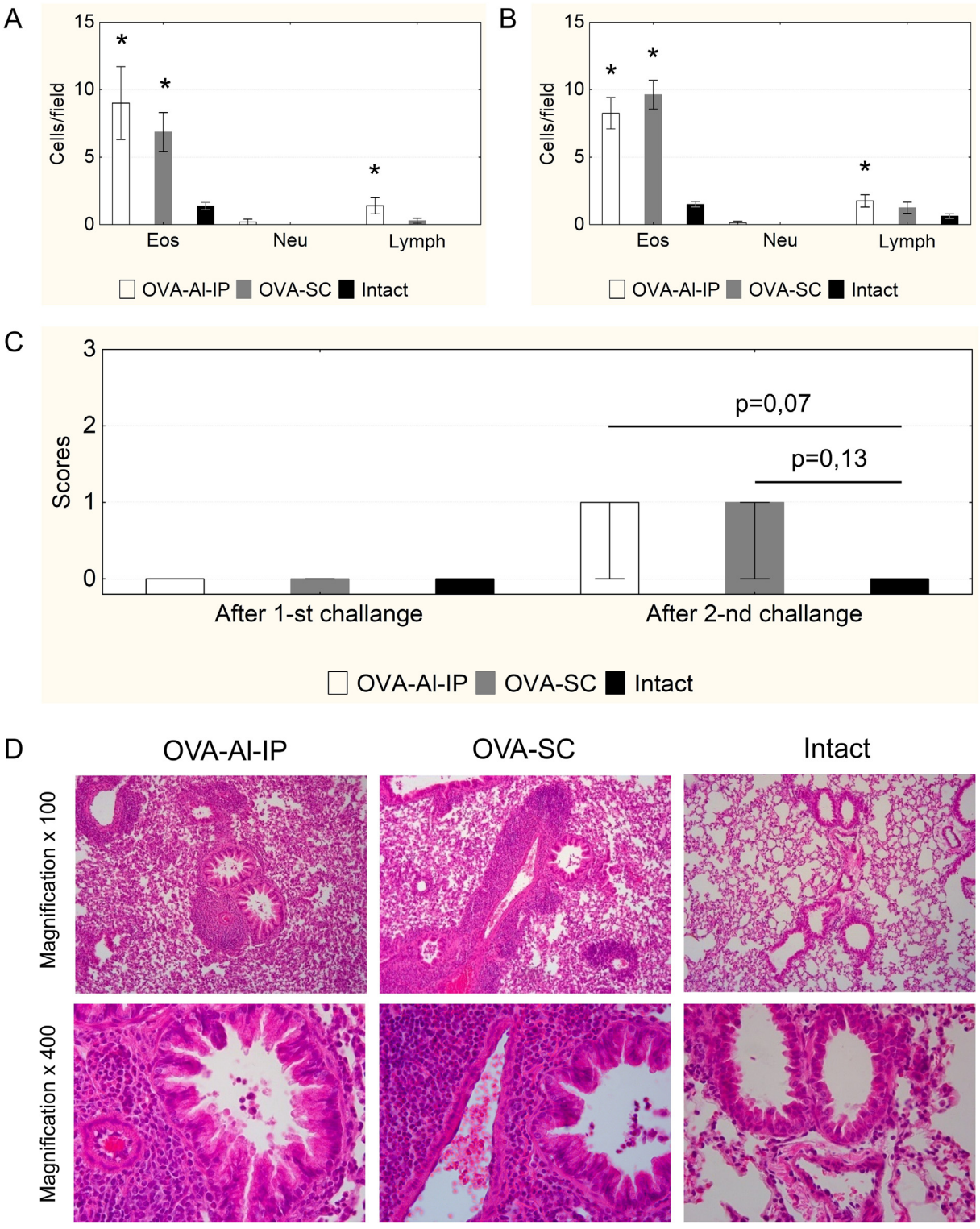

Fig. 6. Histological assessment of airway pathology.
Lung sections were stained with H&E followed by cell count in the infiltrates around bronchi after the first (A) and second (B) challenges; the number of eosinophils (Eos), neutrophils (Neu) and lymphocytes (Lymph) were counted in three fields for every mouse lung at 400× magnification; data presented as mean ± sem.

IgG2a antibodies were higher in group "OVA-Al-IP" (Fig. 2 BC). Increase of OVA-specific IgE and decrease of OVA-specific IgG2a antibodies after subcutaneous immunization without adjuvant demonstrate that this protocol provides more substantial Th2-immune response to allergen compared to intraperitoneal immunization with OVA using alum as adjuvant. Similar results were obtained in study of Conrad et al. (Conrad et al., 2009) where s.c. adjuvant-free and adjuvant i.p. immunizations of mice with OVA followed by one step challenge with the same allergen was compared. One of the difficulties in developing of chronic asthma models is induction of tolerance after repeated OVA challenges that leads to decreased level of allergen-specific antibodies in sera. In our study we have performed two-step OVA challenge and have shown continuous elevation of specific IgE, IgG1 and IgG2a antibodies in group "OVA-SC" (Fig. 2 ABC), while in adjuvant group

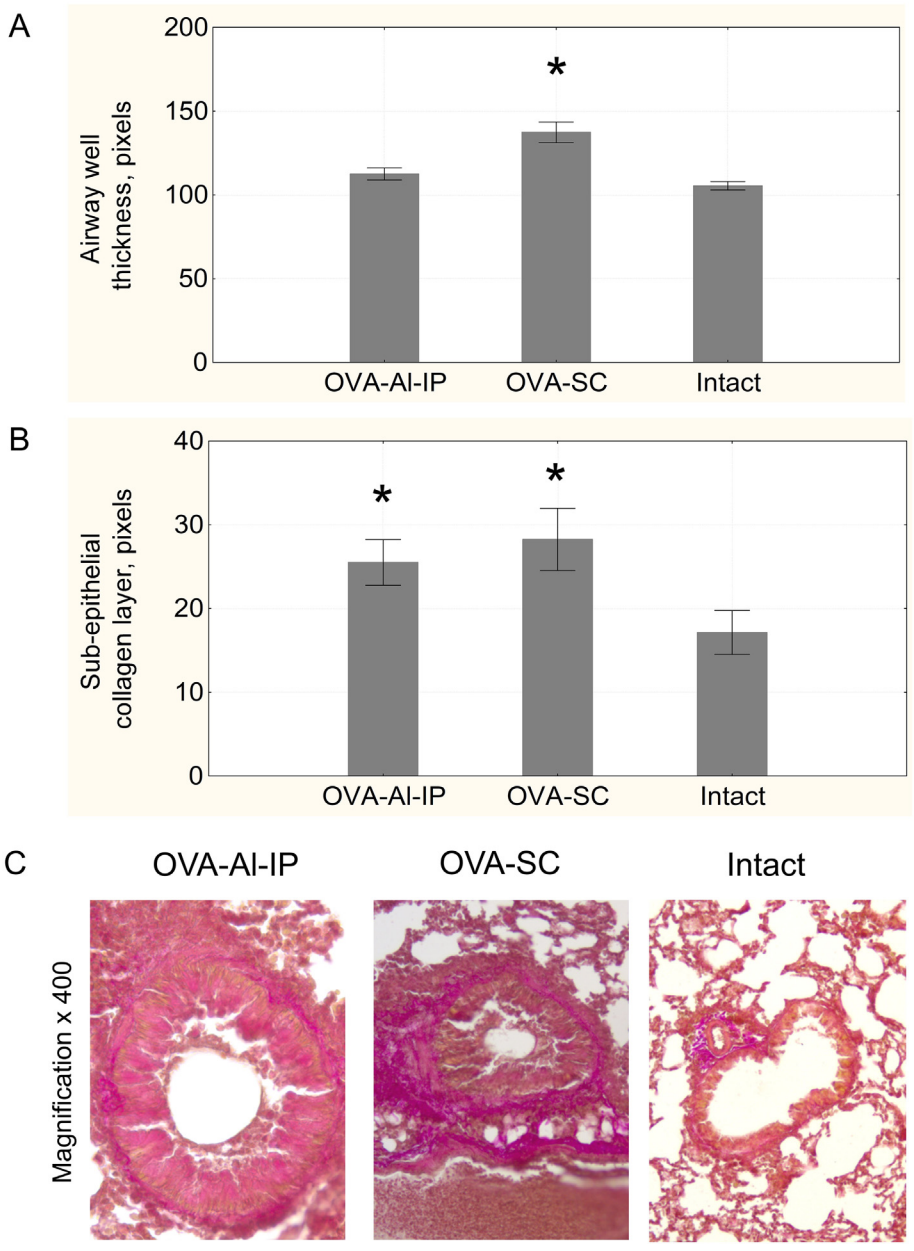

Fig. 7. Increased thickness of aiway walls, sub-epithelial collagen deposition in the airways of ovalbumin sensitized and challenged mice.
Lung sections staining was performed according to the standard Van Gison protocol. Airway well thickness (A) and thickness of collagen layer (B) was measured using Altami Studio software (Altami, Russia); three bronchi were analyzed for every mouse lung; analysis of all bronchi was performed at 400× magnification; data expressed as pixels. C. The microphotographs illustrate the sub-epithelial collagen deposition in the transverse sections of bronchioles.
× – significant difference vs "OVA-Al-IP" group.
* – significant difference vs "Intact" group.

"OVA-Al-IP" the levels of specific IgE and IgG2a reached plateau after the first OVA challenge (Fig. 2 AC) and decreased level of specific IgG1 in this group was observed (Fig. 2B).

Airway hyperresponsiveness is one of the main asthma features. In our study the method of noninvasive plethysmography was used to assess AHR. We demonstrated a similar enhances of AHR in mice with experimental asthma in both protocols of immunizations (Fig. 3). Similar results were demonstrated by Wegmann et al. who have shown the increase of airway resistance in a mouse model of chronic asthma which lasted up to 12 weeks after the challenge (Wegmann et al., 2005). Despite the higher OVA-specific IgE level in mice from "OVA-SC" group compared to "OVA-Al-IP" we did not observe increased AHR that was probably due to IgE-independent mechanisms of AHR development. There are data indicated that AHR have been developed in IgE-deficient mice (Oettgen et al., 1994). In this case AHR can be established through IgG1 antibodies (Miyajima et al., 1997). In our study mice immunized with alum had lower level of OVA-specific IgE compared to group "OVA-SC" demonstrating that the higher level of specific IgG1 could be a reason of potent AHR in this group (Fig. 2B). Together with the increased AHR we have shown the development of nasal hyperreactivity, that expressed as increased number of sneezing and nasal rubbing (Fig. 4). The nasal hyperreactivity is an important feature of experimental allergic rhinitis (Hosoya et al., 2011; Shin et al., 2014; Son et al., 2015). However, the increased levels of sneezing and nasal rubbing could be explained by the similar pathophysiological mechanisms developed in the lower and upper respiratory tracts. Indeed it is well known that allergic asthma has common pathophysiology and comorbidity to allergic rhinitis; up to 80% patients with allergic asthma suffer from allergic rhinitis (Khan, 2014).

Another prominent feature of clinical asthma is eosinophilic inflammation of airways and/or lungs with inflammatory cells. Similar to study of Conrad et al. (Conrad et al., 2009) we also found that the number of eosinophils in the BALF was equally increased in both experimental groups after the first OVA challenge (Fig. 5A). After the second challenge eosinophil numbers in BALF of both experimental groups were similarly increased (Fig. 5B) that confirmed the development of allergic inflammation in the airways without adjuvant usage. Histological analysis of hematoxylin-eosin stained lung sections

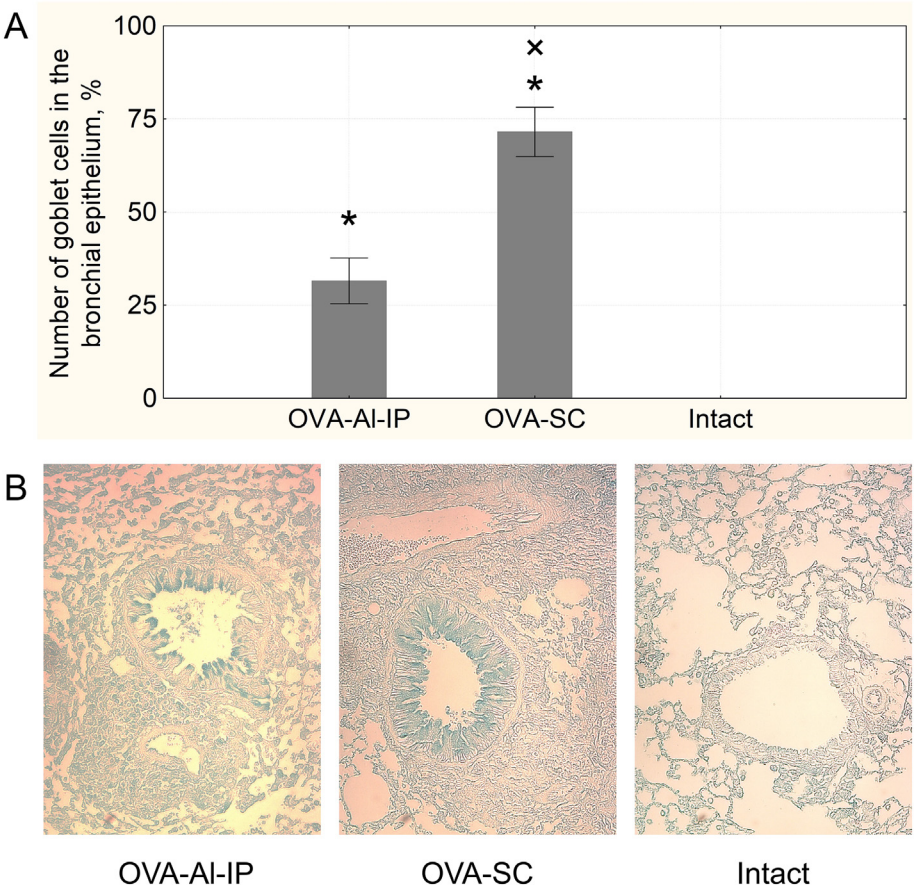

Fig. 8. Goblet cell hyperplasia of bronchial epithelium.
The lung sections were stained with Alcian Blue. A. Quantification of mucin positive goblet cells; three bronchi were analyzed for every mouse lung; analysis of all bronchi was performed at 400× magnification; data expressed as percentage of goblet cells in the bronchial epithelium. B. The microphotographs illustrate the sub-epithelial Gobler cell hyperplasia of bronchial epithelium;
× – significant difference vs "OVA-Al-IP" group.
* – significant difference vs "Intact" group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

confirmed an eosinophilic infiltration of the lung tissues (Fig. 6 AB).

Despite the increased level of OVA-specific IgE and infiltration of lungs with inflammatory cells, Conrad et al. did not observed the airway remodeling signs after the first allergen challenge (Conrad et al., 2009). At the same time in our study we observed goblet cell metaplasia/hyperplasia (Fig. 8), tendency to develop smooth muscle hypertrophy (Fig. 6C) and collagen deposition (Fig. 7) in the wall of distal airways after the second OVA challenge that indicated the development of airway remodeling. Similar data were obtained by Wegmann et al. During modeling of chronic asthma with adjuvant authors observed increase of collagen content in the subepithelial layer indicating the bronchial remodeling process (Wegmann et al., 2005). by Quantitative digital image analysis performed by Reinhardt A.K. et al. has shown increased deposition of sub-epithelial collagen in the group of mice with induced asthma compared to the control group (Reinhardt et al., 2005). Stumm C.L. et al. were first who established a model of chronic asthma with OVA-immunized mice treated with six OVA challenges. Collagen deposition around bronchi and a-SMA as markers of fibrosis as well as mucus secretion, were significantly increased in the listed asthma model (Stumm et al., 2014).

Temelkovski et al., have attempted to develop aluminum hydroxide-free chronic mouse model of allergic asthma. IgE high responder BALB/c mice were challenged for 8 weeks with low concentrations of aerosolized OVA (for 30 min/day within three days/week with 2.5% ovalbumin solution) without prior systemic immunization. The authors revealed low levels of allergen-specific IgE antibodies in sera of treated mice. At the same time preliminary systemic immunization of animals with the mixture of OVA and $Al(OH)_3$ resulted in significantly higher IgE titers (geometric mean titer 1:40 vs 1:3630). Other asthma features were also less pronounced after exposure of non-immunized mice to aerosolized OVA; authors did not observe significant thickness of sub-epithelial layer of collagen or bronchial epithelium. At the same time chronic allergen exposure of systemically immunized mice (with aluminum hydroxide) exhibited the above mentioned respiratory tract lesions (Temelkovski et al., 1998). This study indicates that lack of the systemic OVA immunization does not allow to setup the most important features of the chronic allergic asthma. Here we have described the adjuvant-free protocol with prior systemic subcutaneous sensitization, that allows to induce one of the main allergic asthma features: high level of allergen-specific IgE in sera (Fig. 2A), increased AHR (Fig. 3), substantial eosinophil recruitment to the lungs (Figs. 5, 6 AB), tendency to develop smooth muscle hypertrophy (Fig. 6C) and thickening of airway walls and sub-epithelial fibrosis (Fig. 7).

Shinagawa and Kojima described similar adjuvant-free model of allergic asthma in different strains of mice (BALB/c, A/J, C57BL/6, C3H/HeJ). In this study mice were intranasally treated with 50 µl of OVA solution (1 mg/ml) 3 days per week for 12 weeks without prior systemic immunization. This protocol allowed to induce strongly pronounced asthma features in A/J and BALB/c mice: typical airway remodeling (airway wall thickening and increased collagen deposition), persistent AHR, IgE-production and eosinophilic inflammation. However, in C57BL/6 and C3H/HeJ mice eosinophilic inflammation, airway wall thickening and AHR were not observed, except slightly increased collagen deposition. Despite both strains (A/J and BALB/c) showed similar level of serum IgE, A/J mice had almost 4-fold higher number of eosinophils in BAL, increased by 40% of hydroxyproline (a marker of collagen deposition), increased by 50% and 65% thickness of smooth-muscle layer and mucous layer, respectively. These results are explained by the tolerance induced in BALB/c in response to repeated OVA introduction, while A/J strain was less prone to this kind of tolerance. Authors summarized that A/J is the most suitable strain to develop chronic murine asthma model by intranasal antigen introduction (Shinagawa and Kojima, 2003). In the current study we have used BALB/c strain of mice, however unlike in the study described above, the

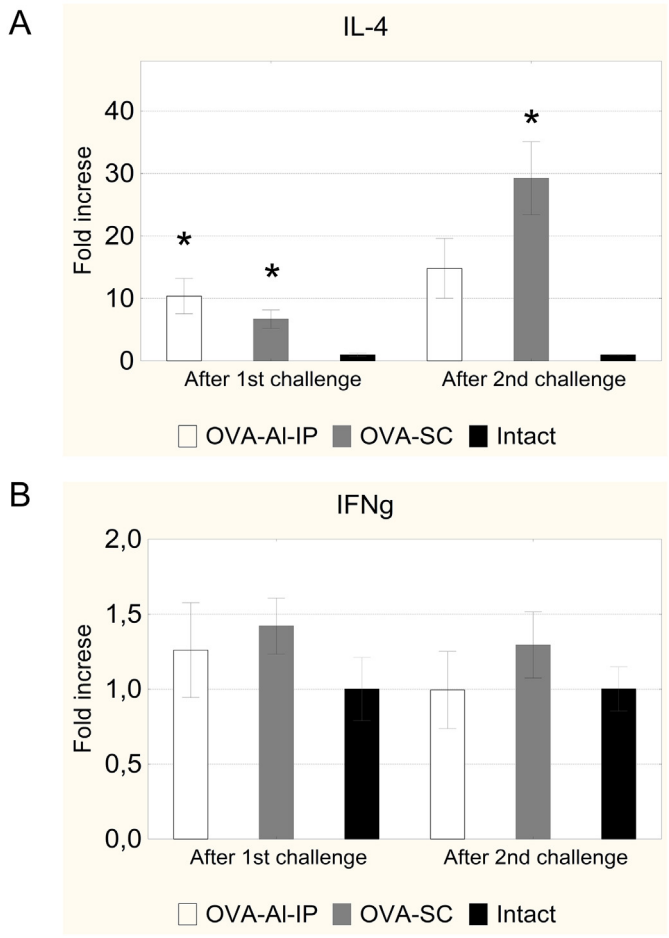

Fig. 9. mRNA expression of IL-4 (A) and IFNγ (B) in BALF cells.
The data presented as the fold increase over the intact mice. Gene expression in intact mice was accepted as 1.
× – significant difference vs "OVA-Al-IP" group.
* – significant difference vs "Intact" group.

animals were previously systemically sensitized by three subcutaneous injections. Developed protocol allows to achieve asthma features for 9.5 weeks without induction of the tolerance. We have shown that number of eosinophils in BAL is increased after the second challenge (from 30% to 75% of the total cell number) (Fig. 5 AB), it is comparable with eosinophil cell number in BAL, observed in A/J mice (about 75% of the total cell number) (Shinagawa and Kojima, 2003).

Similar study was published by Johnson et al. in 2004. In this study, BALB/c mice were exposed to HDM extract or OVA intranasally (25 μg/mouse for 5 days/week for up to 7 consecutive weeks) without previous systemic immunization using adjuvant. It was demonstrated that continuous exposure to HDM, unlike OVA, elicited severe and persistent eosinophilic airway inflammation, increased AHR, elevated level of allergen-specific IgE in sera and structural remodeling of airways (goblet cell hyperplasia, subepithelial collagen deposition and smooth muscle hypertrophy). Failure to setup allergic asthma features using described protocol is probably due to induced tolerance in response to continuous exposure to OVA (Johnson et al., 2004). In our study we have used prior stage of systemic OVA immunization without adjuvant, that allowed to induce pronounced asthma features avoiding tolerance. In addition, current protocol of OVA application resulted in even more substantial eosinophilic inflammation (up to 75% of the total cell number) (Fig. 5 AB) compared to the continuous exposure to HDM from the Johnson's study (about 30% of the total cell number) (Johnson et al., 2004).

Th2-cytokines are involved in the induction and maintenance of allergic asthma (Chatila, 2004; Steinke and Borish, 2001). Experimental evidence of the key role of Th2-cytokine - IL-4 in the development of allergic reactions, including allergic asthma exists. IL-4 switches antibody production by B-cells towards IgE, induces eosinophil recruitment to the lungs and AHR (Perkins et al., 2006). The IFNγ, produced by Th1-cells is the antagonist of IL-4 (Perkins et al., 2006). To elucidate whether the development of asthma phenotype is associated with Th2-response to allergen in our experiments we studied the expression levels of IL-4 and IFNγ genes in the cells infiltrated to BAL fluid. The increase the of IL-4 mRNA expression in both experimental groups ("OVA-Al-IP" and "OVA-SC") compared to intact mice was shown (Fig. 6A). Expression of IFNγ remained unchanged in all groups during the experiment (Fig. 6B). Taken together, these findings suggest that described adjuvant-free protocol leads to the development of the allergic asthma phenotype in mice by Th2-dependent mechanism.

5. Conclusion

In the current study, we compared adjuvant-free and traditional (using aluminum hydroxide as adjuvant) models of chronic asthma in mice. Although aluminum hydroxide is commonly used to setup adequate asthma features, we have shown that subcutaneous allergen immunization allowed to achieve asthma phenotype without adjuvant. The proposed adjuvant-free protocol possesses all necessary features of classical asthma model obtained with adjuvant (alum) including allergen-specific IgE production, AHR, allergic inflammation and remodeling of the airways. Described adjuvant-free chronic asthma model is suitable for pre-clinical studies of novel anti-asthmatic therapeutic agents including adjuvant-containing drugs.

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