



The mixture of siRNAs targeted to IL-4 and IL-13 genes effectively reduces the airway hyperreactivity and allergic inflammation in a mouse model of asthma

Shilovskiy IP^{a,*}, Sundukova MS^a, Korneev AV^a, Nikolskii AA^a, Barvinskaya ED^a, Kovchina VI^a, Vishniakova LI^a, Turenko VN^a, Yumashev KV^a, Kaganova MM^a, Brylina VE^b, Sergeev I^a, Maerle A^a, Kudlay DA^{a,c}, Petukhova O^a, Khaitov M.R.^{a,d}

^a National Research Center - Institute of Immunology of Federal Medico-Biological Agency, 115478, 24, Kashirskoye shosse, Moscow, Russian Federation

^b Federal State Budgetary Educational Institution of Higher Education «Moscow state Academy of Veterinary Medicine and Biotechnology - MVA by K.I. Skryabin» of the Ministry of Agriculture of the Russian Federation, 109472, Moscow, Russian Federation

^c Federal State Autonomous Educational Institution of Higher Education I.M. Sechenov First Moscow State Medical University of the Ministry of Health of the Russian Federation (Sechenovskiy University), 119991, Moscow, Russian Federation

^d Federal State Autonomous Educational Institution of Higher Education «N.I. Pirogov Russian National Research Medical University» of the Ministry of Health of the Russian Federation, 117997, Moscow, Russian Federation

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ABSTRACT

Bronchial asthma (BA) is one of the most common chronic inflammatory disease of airways. There are huge experimental data indicating that Th2-cytokines IL-4 and IL-13 play a key role in BA pathogenesis. They are implicated in the IgE synthesis, eosinophil infiltration to the lungs and in the development of airway hyperreactivity (AHR), that makes these cytokines the promising targets. Neutralization of IL-4 and IL-13 or its common receptor chain (IL-4Rα) by monoclonal antibodies substantially reduce asthma symptoms. RNA interference provides a novel method for regulation of gene expression by siRNA molecules. In this study we evaluated whether the siRNA targeted to IL-4 and IL-13 reduce BA symptoms in mice model.

Experimental BA was induced in BALB/c mice by sensitization to ovalbumin allergen followed by intranasal challenge. The intranasal delivery of siRNAs targeted to IL-4 and IL-13 inhibited the lung expression of these cytokines by more than 50% that led to the attenuation of AHR and pulmonary inflammation; the quantity of eosinophils in lungs which are one of the major inflammatory cells involved in allergic asthma pathogenesis decreased by more than 50% after siRNA treatment. These data support the possibility of a dual IL-4 and IL-13 inhibition by locally delivered siRNAs which in turn leads to the suppression of allergen-induced pulmonary inflammation and AHR.

1. Introduction

Bronchial asthma (BA) is a chronic inflammatory disease of the airways, which affects more than 300 million people worldwide [1]. The global mortality rate of the disease is 13 and 9 per 100 000 for men and women, respectively [2]. BA provides significant economic issues; Europe Union spends about €17.7 billion annually to treat the disease, annual cost of BA in the United States is about \$18 billion [3]. In Russian Federation expenses for BA treatment reach 14 billion rubles a year [4]. The majority of BA cases around the world (up to 80%) are allergic and

associated with the sensitization to the allergens [1,5]. Allergic asthma is characterized by common clinical symptoms (mucus hypersecretion, pulmonary inflammation, AHR) and by the presence of allergen-specific IgE-antibodies in the serum, increase in the proportion of eosinophils in the blood and BAL [5].

According to clinical and animal studies, it has been found that allergic asthma is developed through Th2-dependant mechanisms [6,7]. Activated Th2-lymphocytes induce, via the production of cytokines (IL-4, IL-5 and IL-13), an inflammatory cascade comprised of eosinophil action, IgE production, and mast-cell activation, all of which are

* Corresponding author at: 115522, Russian Federation, Moscow, Kashirskaya Shosse 24.

E-mail address: ip.shilovskiy@nrcii.ru (S. IP).

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necessary mediators causing AHR [6,7]. Cytokines IL-4 and IL-13 are the most important in the development of Th2-mediated allergic inflammation. IL-4 and IL-13 have a common receptor (chains IL-4R α and IL-13R α 1) because of this, they have similar biological functions: induce IgE isotype switching, stimulate VCAM-1 expression, which is necessary to recruit eosinophils to the lung. This heterodimeric receptor (IL-4R α /IL-13R α 1) is expressed on many immune and non-immune cells. Binding of IL-4 and IL-13 to the receptor leads to activation of tyrosine kinases of the Janus family (Jak1–3 and Tyk2), which phosphorylate transcription factor STAT6. STAT6 dimers translocate to the nucleus, where upregulate the transcription of IL-4/IL-13-responsive genes involved in Th2 differentiation, isotype class switching of B cells to IgE synthesis, AHR development, and mucus production [8,9].

Allergen-specific immunotherapy and the use of glucocorticoids and bronchodilators are the most common ways of BA treatment [10]. However, some patients, especially those with severe asthma are often resistant to conventional therapies [11–13]. To treat these severe forms of the disease, inhibitors of the IL-4R α /STAT6 pathway have been developed [14,15]. Dupilumab is a novel drug, based on monoclonal antibodies targeted to IL-4R α (common receptor chain for both IL-4 and IL-13), which was recently approved to treat BA [16,17], however its broad use is restricted by high cost.

At the same time, the new ways to regulate the gene expression are developing. RNA interference is a technology of negative post-transcriptional regulation of gene expression by small interfering RNA molecules (siRNA) [18,19]. In this study we used intranasally delivered siRNAs targeted to both IL-4 and IL-13 to reduce allergic inflammation in a mouse model of BA. Our results indicate that suppression of these cytokines decreases the allergen-induced inflammatory response and suggests that inhaled anticytokine siRNAs could be an effective anti-inflammatory approach for respiratory diseases.

2. Materials and methods

2.1. Construction of plasmids carrying mouse IL-13 gene

To clone mouse IL-13, the coding sequence of corresponding gene was amplified by PCR from concanavalin A stimulated mouse splenocyte cDNA, verified by sequencing, and cloned into the expression vector pUCHR IRES GFP [20]. Expression of the *gfp* gene, located downstream of the internal ribosome entry site (IRES), was used as an indicator of the level of IL-13 expression.

2.2. Design of siRNAs

To select the optimal sequences of siRNA molecules that can effectively suppress the target gene, we used the specialized OligoWalk software, which calculates the thermodynamic parameters of hybridization of RNA oligonucleotides, predicts their free energy of binding to the mRNA target (http://rna.urmc.rochester.edu/cgi-bin/server_exe/oligowalk/oligowalk_form.cgi). The method of selection of effective siRNA variants is described in article [21].

2.3. Cell cultures and cotransfection with plasmids and siRNAs

The human embryonic kidney HEK293T cell line was cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) containing 10% fetal calf serum (FCS), Gibco gentamicin (80 mg/L; Life Technologies, Grand Island, NY), and 25 mM HEPES. Subconfluent cultures were split with trypsin–ethylenediaminetetraacetic acid (trypsin-EDTA 0.25%, PanEco, Moscow, Russia). All culture media and reagents, unless otherwise stated, were obtained from PanEco (Moscow, Russia).

For transfection with expression plasmid carrying IL-13 and siRNAs, cells were seeded in 24-well culture plates at 1×10^5 cells per well in appropriate medium and cultured (37 °C and 5% of CO₂) for 24 hr before

transfection. In all cases, adherent cells were cotransfected with 0.5 μ g of plasmids (carrying IL-13) and 1 μ g of siRNAs, using Lipofectamine 2000 (Life Technologies) as recommended by the manufacturer. We chose the current plasmid/siRNA ratio based on our previous *in vitro* experiments made for evaluation of activity of siRNAs against IL-4 [22]. The siRNA targeting respiratory syncytial virus genome (siRSV) and siRNA targeting GFP (siGFP) were used as negative and positive controls, respectively. Twenty-four hours after transfection cell supernatants and cell lysates were harvested. The IL-13 secretion was determined by ELISA (ELISA set; BD Biosciences, San Jose, CA). The level of mRNA for IL-13 was measured by quantitative RT-PCR as described below.

2.4. Animals

Female BALB/c mice (18–20 g) aged 6–8 weeks were purchased from “Stolbovaia” Scientific Center of Biomedical Technologies, Russian Academy of Sciences and housed as ten animals per cage with food and water available *ad libitum*. All experimental procedures were approved by the local animal ethics committee and met Russian and international guidelines.

2.5. Induction of BA phenotype in mice and siRNA treatment

Female BALB/c mice (6–8-week-old, 18–20 g of body weight) were divided into 6 groups. In order to induce BA phenotype five groups of mice were sensitized with intraperitoneal (i.p.) injections of 20 μ g ovalbumin (OVA) emulsified in 2 mg of Al(OH)₃ in 0.2 ml of PBS on days 0 and 14. On day 28 mice were additionally immunized with 10 μ g of OVA emulsified in 2 mg of Al(OH)₃ in 0.2 ml of PBS. Then animals were challenged by intranasal applications with 50 μ l of 10% OVA solution in PBS on three consecutive days from 42 to 44 [23,24]. The first group did not treat with any siRNAs. The second group received non-specific siRNA targeted to GFP gene as a negative control. siRNA was i.n. delivered on four consecutive days 41–44 in a total dose of 120 μ g/mouse (daily dose was 30 μ g in 50 μ l of RNase-free water (QIAGEN)). The third group received intranasal applications with siRNAs targeted to IL-4 (siIL4) in the same regiment and dose. The fourth group was treated with siRNAs targeted to IL-13 (siIL13). The fifth group received the mixture of siIL4 and siIL13 in total dose 120 μ g (60 μ g of siIL4 and 60 μ g of siIL13). Group 6 included intact mice that were not exposed to either ovalbumin or siRNAs (Fig. 1).

2.6. Measurement of airway hyperreactivity (AHR)

Twenty-four hours after last OVA exposure (on day 44) 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 (FinePointe NAM, Buxco). sRaw measurement was described in details in previous article [25].

2.7. Elisa

Serum levels of OVA-specific IgE, IgG2a and IgG1 antibodies and level of IL-13 secretion to cell supernatants were measured by commercial ELISA sets (BD) according to the manufacturer's instructions.

2.8. Collection of bronchoalveolar lavage (BAL)

On day 45 mouse lungs were cannulated through the trachea and lavaged with 1 ml of RPMI1640 media supplemented with 10% fetal bovine sera. Total cell count in BAL samples was measured by a cytometer. After that BAL samples were centrifuged at 300 g for 10 min and cell pellets were suspended and fixed on glass slides. Cells were stained using azure–eosin (Bio-Optica, Italy) staining followed by differential cell count by light microscopy method (at least 200 cells per slide) in a blinded manner.

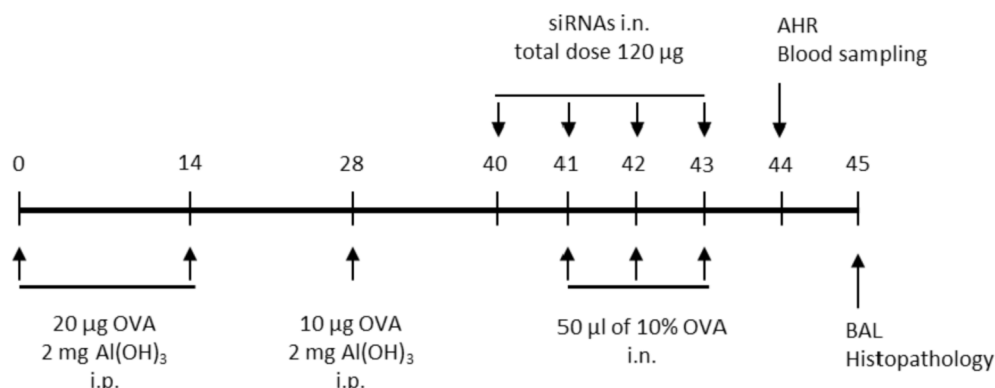


Fig. 1. Experimental protocol for induction of the BA phenotype in mice and siRNA treatment. OVA – ovalbumin, i.p. – intraperitoneally, i.n. – intranasally, PBA – analysis of peripheral blood, BAL – bronchoalveolar lavage.

2.9. 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 [22]. 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 (Thermo Fisher). The cDNAs were used for quantification of IL-4 and IL-13 gene expression via specific primer pairs and probes (Table 1) by iCycler iQ Real-time PCR Detection System (Bio-Rad Laboratories). The expression IL-4, IL-5, IL-13 and IFN γ was normalized on HPRT gene expression.

2.10. Histopathology

The left lung tissue was excised, fixed in 4% paraformaldehyde, and then embedded in paraffin. Lung sections were cut in 4 μm sections and stained with hematoxylin–eosin (H&E) (Bio-Optica, Italy). The inflammatory changes and mucus secretion were analyzed by light microscopy. The histopathological changes (cell infiltration around bronchioles and arteries, veins, proliferation of goblet cells in bronchial epithelium) were graded according to a modified semi-quantitative scoring system in a “blind manner” as: none (0), mild (1.0), moderate (2.0), or severe (3.0) [26].

2.11. Statistics

Data were expressed as mean $\pm$ SE (standard error) except for scoring data, which were presented as median $\pm$ inter-quartile range. Data were analyzed by Mann-Whitney U test using STATISTICA 8.0 (StatSoft Inc.). Semi-quantitative scoring data were analyzed according

to Kruskal-Wallis test. Data were accepted as significantly different when $p \leq 0.05$.

3. Results

3.1. Design siRNAs and evaluation of its activity in vitro

siRNAs targeted to mouse IL-4 was developed in our previous study [22]. The most potent gene silencing provided variant siIL4-408 [22]. To design siRNAs against mouse IL-13 gene OligoWalk software was used [21]. In total 47 different variants of siRNAs were predicted. Five of them (named as siIL13-95, siIL13-195, siIL13-230, siIL13-280 and siIL13-371) were chosen for synthesis and evaluation of silencing activity in *in vitro* experiments. Selected variants matched following criteria: 1) the predicted silencing activity no less than 0.75; 2) the identity to the other genes should be less than 84% to avoid off-target effects (Table 2).

To evaluate silencing activity of synthesized siRNAs in *in vitro* experiments we constructed the plasmid (pUCHR-IL-13-IRES-GFP) expressed mouse IL-13 and reporter GFP genes under control of CMV-promoter. In this plasmid between IL and 13 and GFP region IRES was inserted. As a result, a single bicistronic mRNA carrying both IL-13 and GFP is transcribed that allows to evaluate the expression of IL-13 on mRNA and protein levels in mammalian cells.

HEK293T cells were individually transfected with the mixture of pUCHR-IL-13-IRES-GFP and synthetic siRNAs using Lipofectamine 2000. siRNA against GFP which is able to target the whole bicistronic mRNA was used as a positive control. Non-specific siRNA against respiratory syncytial virus (siRSV) was used as a negative control. Two variants of siRNAs against IL-13 (siIL13-95, siIL13-195) significantly inhibited expression of mouse IL-13, based on data obtained by RT-PCR and ELISA. These siRNAs decreased the level of IL-13 mRNA by 70% and

Table 1
Sequences of primers and probes used for quantitative RT-PCR analysis.

Primers and probes	Sequence 5'-3'
mIL4-F	AGAGAGTGTGCTCGTCTGTAG
mIL4-R	GGTGCAGCTTATCGATG
mIL4-Z	(FAM) CTCTGCAGCTCCATGAGAAGACTAGAG (BHQ1)
mIL5-F	TACTGTGGAATGCTATTCCA
mIL5-R	GTAATCCAGGAAGTGCCTC
mIL5-Z	(ROX) CCGCCAAAAGAGAGAGTGTGGC (RTQ2)
mIL13-F	TCTGTGTAGCCCTGGATTC
mIL13-R	CACTACGGTCTCCAGCCT
mIL13-Z	(FAM) TTGCAATGCCATCTACAGGACCCAGA (RTQ1)
mIFN γ -F	AAATCCTGCAGAGCCAGATTAT
mIFN γ -R	GCTGTTGCTGAAGAAGGTAGTA
mIFN γ -Z	(ROX)ACGCTTATGTTGTTGCTGATGGCC(RTQ2)
mHPRTf	GAAGAGCTACTGTAATGATCAGTCA
mHPRTtr	GTATCCAACACTTCGAGAGGTC
mHPRTz	(FAM)GCTTTCCCTGGTTAAGCAGTACAGCC(RTQ1)

Table 2
Variants of siRNAs targeted to mouse IL-13.

siRNA	Sequence of antisense chain (5'-to -3')	Theoretical silencing activity predicted by OligoWalk	Sequence identity with other mouse genes, %
siIL13-95	UCAAUAAGCUCCUUAAGGGtt	0.88	79
siIL13-195	AUCCAGGGCUACAGAACTt	0.75	84
siIL13-230	UAGAUGGCAUUGCAAUUGGtt	0.86	84
siIL13-280	CGUAGUGGGGGCCUUGCGGtt	0.89	79
siIL13-371	AGGGGCGGUGGCGAAACAGtt	0.87	84

79%, respectively (compared with unspecific siRSV) (Fig. 2A). The level of IL-13 protein secreted into the medium from HEK293T cells, measured by ELISA, was downregulated by 75% and 62%, respectively (Fig. 2B). Despite siIL13-95 and siIL13-195 demonstrated similar silencing activity, for future *in vivo* studies siIL13-195 was chosen because it showed more stable reproducibility of gene silencing in the set of *in vitro* experiments.

3.2. Inflammatory cytokine expression in lung tissue

We used a mouse OVA-induced bronchial asthma to verify siRNA activity *in vivo*. Sensitized mice were challenged with OVA, and treated intranasally with siRNAs (siIL4, siIL13 and mix of siIL4/siIL13, or siGFP as negative control) as described in materials and methods section (Fig. 1). Evaluation of pulmonary gene expression revealed 1.9-fold and 2.7-fold decrease in IL-4 expression in groups receiving individual siRNA against IL-4 and IL-13, compared to mice treated with non-specific siGFP (Fig. 3A). At the same time, the lung expression of IL-13 did not change after intranasal application of siIL4-408, whereas siIL13-195 resulted in 2.2-fold downregulation of this cytokine, compared to siGFP treated animals (Fig. 3B). Intranasal delivery of the mixture of siIL4-408 and siIL13-195 reduced both IL-4 and IL-13 lung expression in 2.3 and 2.2 times, compared to non-specific siGFP (Fig. 4AB). In addition, the levels of IL-5 mRNA expression in lungs also showed significant decrease after treatment with siIL13-195 or with combination of siIL4-408 and siIL13-195, compared to non-specific siGFP. The level of IL-5 reduction reached 56% in mice received siIL13-195 and 59% in animals treated with the mixture of siRNAs, while siIL4-408 alone demonstrated only tendency to decrease in the expression of this cytokine by 31% ($p = 0.08$) (Fig. 3C). At the same time, the level of IFN γ mRNA expression in lungs showed no difference among the various groups (Fig. 3D).

3.3. Effects of siRNAs on serum allergen-specific antibodies and AHR

The levels of OVA-specific IgE, IgG2a and IgG1 in sera did not changed after the treatment with individual siRNAs against IL-4 and IL-13 or with the mixture of siIL4-408 and siIL13-195 compared to siGFP treated mice and animals received no siRNAs (Fig. 4ABC). The AHR was measured as sRaw revealed 30% improvement of lung function in mice received the mixture of siRNAs targeted both IL-4 and IL-13 compared to siGFP treated mice (Fig. 5D). Mice treated with siRNA against IL-13 alone demonstrated the trend toward 20% decrease in AHR ($p = 0.08$). At the same time, suppression of IL-4 by individual siRNA did not

affect the AHR (Fig. 4D).

3.4. Effects of siRNAs on pulmonary inflammation

Local inflammation in the lungs after intranasal treatment with siRNAs was assessed by the calculation of cell composition of BAL and by histological analysis. Challenge with OVA significantly increased total cell number in BAL samples, while treatment with siRNA against IL-13 alone or against both IL-4 and IL-13 resulted in 2-fold reduction of total cell number, compared to mice received non-specific siGFP. Differential cell count revealed that intranasal delivery of siIL13-195 or mixture of siIL4-408 and siIL13-195 substantially reduced the number of macrophages (in 2.3 and 2.0 times, respectively), neutrophils (in 6.1 and 3.3 times, respectively), eosinophils (in 2.7 and 2.6 times, respectively) and lymphocytes (in 2.2 and 2.7 times, respectively). At the same time, suppression of IL-4 alone with siIL4-408 did not influence significantly on the number of these cells (Fig. 5).

The histological changes in the lung tissue were graded according to a modified semi-quantitative scoring system in a blinded manner [26]. Performed analysis in general confirmed the decrease in eosinophil and lymphocyte infiltration of the lungs after treatment with siIL13-195 alone or with the mixture of siIL4-408 and siIL13-195, compared to siGFP treated mice. The number of mucous secreting goblet cells in the bronchial epithelium and score of total inflammation were also significantly decreased in these groups compared to animals received non-specific siGFP (Fig. 6).

4. Discussion

As mentioned above, asthma - is a common chronic inflammatory disease of the airways. Despite the availability of a broad-spectrum anti-inflammatory drugs the question of uncontrolled or poorly controlled BA cases still exists, that drives the development of new therapeutic approaches. Recent advances in studies of molecular mechanisms of asthma pathogenesis revealed that Th2-cytokines (especially IL-4 and IL-13) take important part in the initiation and maintenance of this pathology. For example, IL-4 and IL-13 are involved in the production of immunoglobulin E (IgE), development of smooth muscle contractility, mucus hyperproduction, and recruitment of immune cells into sites of inflammation [27–29]. These discoveries led to the emergence of anti-cytokine therapy as a distinguish direction of asthma treatment [14,15]. The role of IL-4 and IL-13 in BA pathogenesis was established in studies on knockout mice, and to date these cytokines are considered to

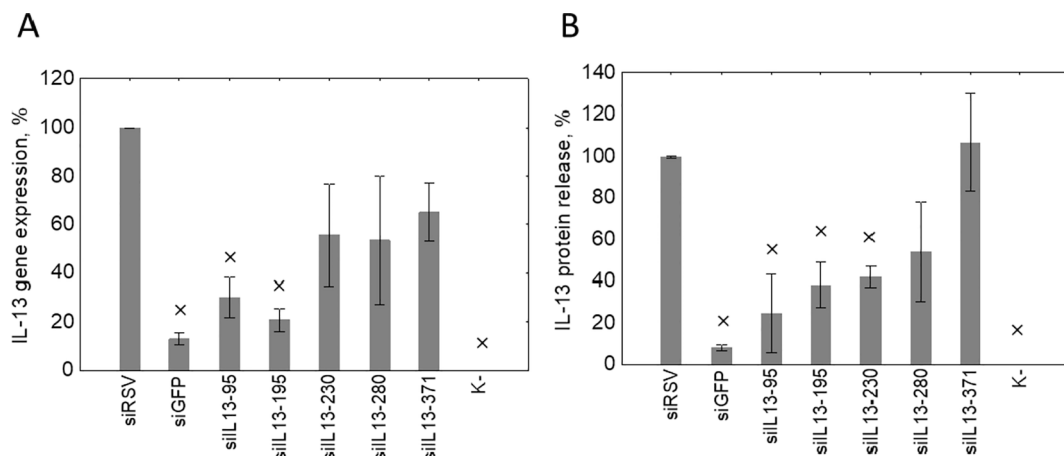


Fig. 2. Silencing activity of siRNAs against IL-13 *in vitro*. mRNA and protein expression of IL-13 in HEK293T cells was assessed by RT-PCR (A) and ELISA (B). Normalized residual activity of IL-13 in HEK293T cells transfected with 0.5 μ g pUCHR-IRES-IL-13-GFP and 1 μ g of the siRNAs is shown. Data are presented as the percentage of IL-13 residual activity compared to cells transfected with unspecific siRSV. Data were normalized by running housekeeping gene human beta-actin (for RT-PCR) and for the 100,000 cells tested (for ELISA). Data are presented as mean $\pm$ standard error. $\times$ - statistically significant vs "siRSV" according to Mann-Whitney U test. $N = 5$.

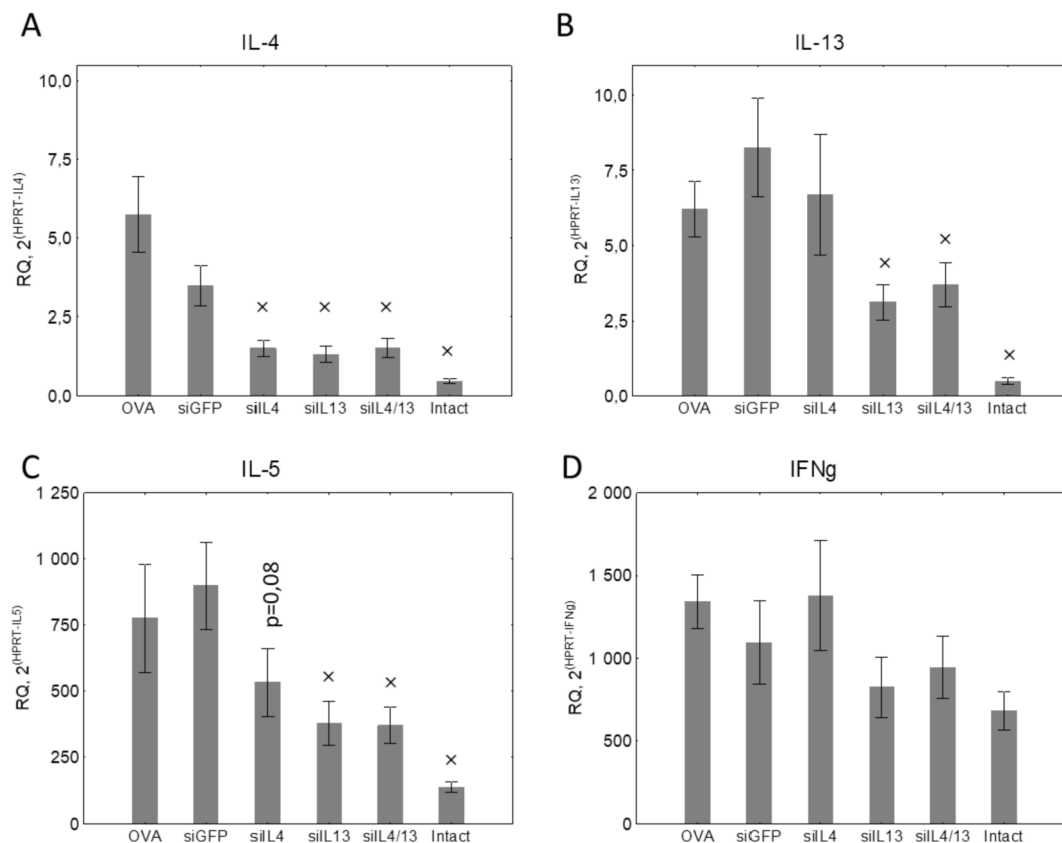


Fig. 3. Pulmonary cytokine mRNA expression in the mouse lungs. Quantification of IL-4 (A), IL-13 (B), IL-5 (C) and IFN γ (D) levels were evaluated in lung homogenates by quantitative RT-PCR. Data were normalized by running housekeeping gene mouse HPRT. Data are presented as mean relative quantity (RQ) $\pm$ standard error. x - statistically significant vs “siRSV” according to Mann-Whitney *U* test. N = 10.

be well-studied [30,31]. Therefore IL-4 and IL-13 were first targets for the development of new anti-cytokine drugs, based in monoclonal antibodies [14,15]. Recent results showed that more potent therapeutic effect achieved when both cytokines (IL-4 and IL-13) were down-regulated simultaneously. This can be explained by that these cytokines are involved in the allergic response though similar molecular pathways; receptor complex of IL-4 and IL-13 includes common chain IL-4 α . The concept of dual inactivation of IL-4 and IL-13 was successfully realized in Dupilumab – the drug based on monoclonal antibody against common receptor chain IL-4 α [16,32].

The discovery of RNA interference as a mechanism of post-transcriptional gene regulation provided new opportunities for the development anticytokine drugs [33]. Lee and colleagues published a study in which mice undergone induction of BA phenotype with OVA followed by intratracheal injection of lentiviral particles expressing short hairpin RNAs (shRNA) against IL-4 and IL-13. Individual or combined inhibition of both cytokines in lungs resulted in the reduction of allergic inflammation (eosinophilia) and AHR. It's useful to note that dual suppression IL-4 and IL-13 demonstrated more pronounced decrease in AHR compared to individual downregulation either IL-4 or IL-13 [34].

In this study we characterize the effect of local delivery of synthetic siRNA molecules targeted to IL-4 and IL-13 on the severity of BA phenotype in mice. It is worth to note that Lee et al. suppressed IL-4 and IL-13 genes using lentiviral vectors that carry genes encoding shRNA [34]. In the current study we used a different approach – synthetic siRNA molecules were used to suppress the expression of these cytokines. Synthetic molecules are more convenient for following implementation into clinical practice compared to transgenic lentiviruses in terms of standardization procedure (the purity and authenticity of synthetic siRNA can be confirmed using conventional pharmacological

methods) and safety (lentiviral vectors reach the nuclei and insert own genome randomly in the chromosomes of target cells). Preliminary *in vitro* experiments identified the most potent siRNAs against IL-4 [22] and IL-13 (Fig. 2) out of more than 40 *in silico* designed variants. Molecules siIL4-408 and siIL13-195 provided about 5-fold silencing of IL-4 and IL-13 expression, respectively.

In *in vivo* experiments developed siRNAs were intranasally delivered (on four consecutive days in total dose 120 μ g) to mice with induced BA phenotype. We chose the dose of siRNA based on our previous experiments [22] and *in vivo* studies performed by other authors in which so called “naked” siRNAs used [35,36]. As a result, application of siRNA against IL-13 alone led to a marked reduction in the expression of IL-4, IL-5, and IL-13, indicating cross regulation of these cytokines. Similar results were obtained by Lee et al. [34] and McKenzie et al [37]. Authors reported that Th2 cells from IL-13-deficient mice produced significantly reduced levels of Th2-cytokines (IL-4 and IL-5) compared to Th2-cells derived from wild type mice [37]. In vivo experiments confirmed the impaired Th2 response in IL-13-deficient mice, while exogenous IL-13 increased Th2-like cytokine response [37]. Authors hypothesize that IL-13 may regulate IL-4 and IL-5 expression though indirect or direct influence of T-cells. It is known that under IL-13-deficiency macrophages upregulate IL-12 production [38]; therefore it is possible that upregulated IL-12 production enhance polarization of IFN γ -producing Th1-cells, resulting in suppression of IL-4 and IL-5 expression. In our experiments we did not observe any changes in IFN γ expression after silencing of IL-13 (Fig. 3) that makes unlikely indirect way of regulation of IL-4 and IL-5 expression by IL-13. However, IL-13 can affect directly on T-cells through activation of JAK/STAT6 signaling pathway; phosphorylated STAT6 translocates to the nucleus where activates IL-4 and IL-5 promoters [37]. Considering this it is possible that silencing of IL-13 by siRNA may downregulate other Th2-like cytokines (IL-4 and IL-5) as

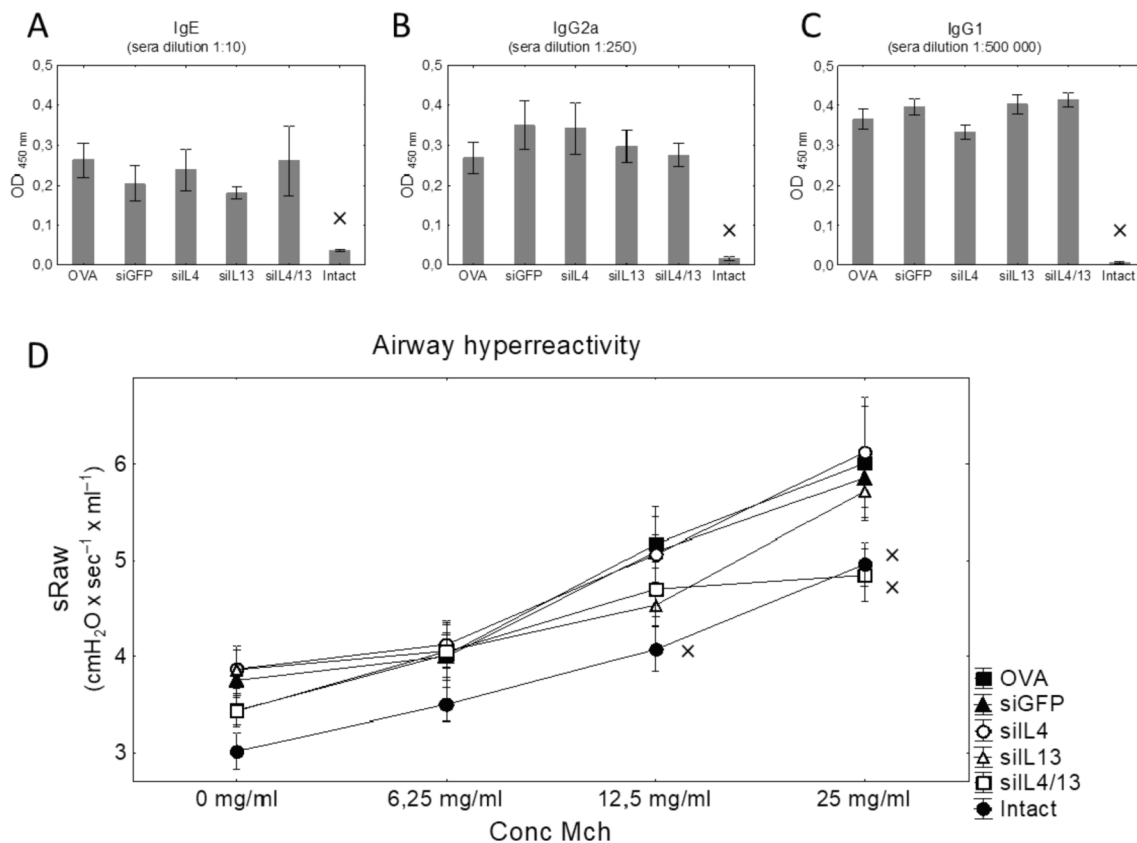


Fig. 4. Effect of siRNAs against IL-4 and IL-13 on the level of allergen-specific immunoglobulins and AHR. Serum levels of OVA-specific IgE (A), IgG2a (B) and IgG1 (C) antibodies. Data are presented as mean optical density (OD) at wavelength 450 nm $\pm$ standard error. AHR in response to aerosolized methacholine (Mch) measured as specific resistance of airways (sRaw) on the next day after the last challenge. Data are presented as mean sRaw $\pm$ standard error. $\times$ - statistically significant vs "siRSV" according to Mann-Witney U test. N = 10.

was observed in our experiment (Fig. 3).

However, siRNA against IL-4 substantially reduced lung expression IL-4, but not IL-13, while combination of both siRNAs reduced IL-4 and IL-13 mRNA expression in the lungs (Fig. 3). Our data not completely coincide with the results of Lee and colleagues [34], as they revealed that suppression of IL-4 resulted in IL-13 downregulation. We hypothesize that high pulmonary IL-13 expression could be maintained by the other cells despite the suppression of Th2-response by siRNA against IL-4. It is well known that CD4⁺Th2-cells, basophils, eosinophils, and mast cells constitutively express both IL-4 and IL-13 mRNA transcripts and represent important sources of Th2 cytokines during allergic inflammation [30,39]. However recently discovered type 2 innate lymphoid cells (ILC2) produce high amounts of IL-5 and IL-13, but not IL-4 in response to epithelial cell derived cytokines (IL-25, IL-33 and TSLP) [40,41]. Most likely activated ILC2s maintain high level of IL-13 pulmonary expression despite downregulation of IL-4 by siRNA.

Dual suppression of IL-4 and IL-13 by siRNAs resulted in downregulation of another Th2-cytokine – IL-5 which is involved in eosinophilic pulmonary inflammation [42]. Despite the suppression of Th2-response siRNAs against IL-4 and IL-13 did not affect the Th1-cytokine IFN γ in lungs (Fig. 3). Our study showed that intranasal delivery of IL-4 and IL-13 siRNAs indeed reduced the Th2- but not Th1-response, confirming the efficacy of IL-4 and IL-13 mRNA blockade by RNAi. The IL-5 level in mice that received siRNAs against IL-4 and IL-13 was also significantly blocked. This was not surprising, because IL-5 production by lymphocytes requires IL-4 and IL-13.

It is known that IL-4 and IL-13 regulate IgE synthesis. However, we did not observe the inhibition of OVA-specific IgE after treatment with IL-4 and IL-13 siRNAs (Fig. 4), that can be explained by local, rather than systemic effect of intranasally delivered siRNAs. Other authors observed

similar findings after intratracheal anti-IL-4/IL-13 treatment with short hairpin RNAs [34]. IL-13 is one of the main cytokine to cause AHR through increased contractility of airway smooth muscle cells [43,44]. In our study, we found that decrease in IL-13 by individual siRNA resulted in 20% reduction of AHR (but not statistically significant, $p = 0.08$), while dual suppression of IL-4 and IL-13 by the mixture of siRNAs significantly improves lung function (Fig. 5). These data coincide with studies conducted by Lee et al. [34] in which combined suppression of both cytokines resulted in higher inhibition of AHR compared to individual suppression of IL-4 either IL-13.

Analysis of BAL cell composition and lung sections showed that number of cells (macrophages, neutrophils, eosinophils and lymphocytes) infiltrating to the lungs were substantially decreased (Fig. 6) after treatment with single siIL13-195 or with combination of siIL4-408 and siIL13-195, but not with siIL4-408 alone. Interestingly that suppression of IL-4 by siIL4-408 alone did not reduce pulmonary eosinophilia despite downregulation of IL-5, it could be explained by insufficient suppression of IL-5, as a major cytokine providing eosinophil migration and activation. It was previously described that IL-13 mediates pulmonary infiltration of inflammatory cells including eosinophils, macrophages, and lymphocytes. The mechanisms of IL-13 elicited lung inflammation include induction of chemokines (CCL2, MCP-2, MCP-3, and CCL11), which provide chemotaxis of macrophages/monocytes to the lungs [31,45] and upregulation of adhesion molecules VCAM-1 and ICAM-1, which allow macrophages, neutrophils and lymphocytes recruit from the bloodstream into the airways [31,45]. These facts can explain why single siRNA against IL-4 failed to reduce lung inflammation; despite the suppression of this cytokine high level of IL-13 maintain the pulmonary inflammation.

It is known that IL-13 induces mucus hypersecretion by bronchial

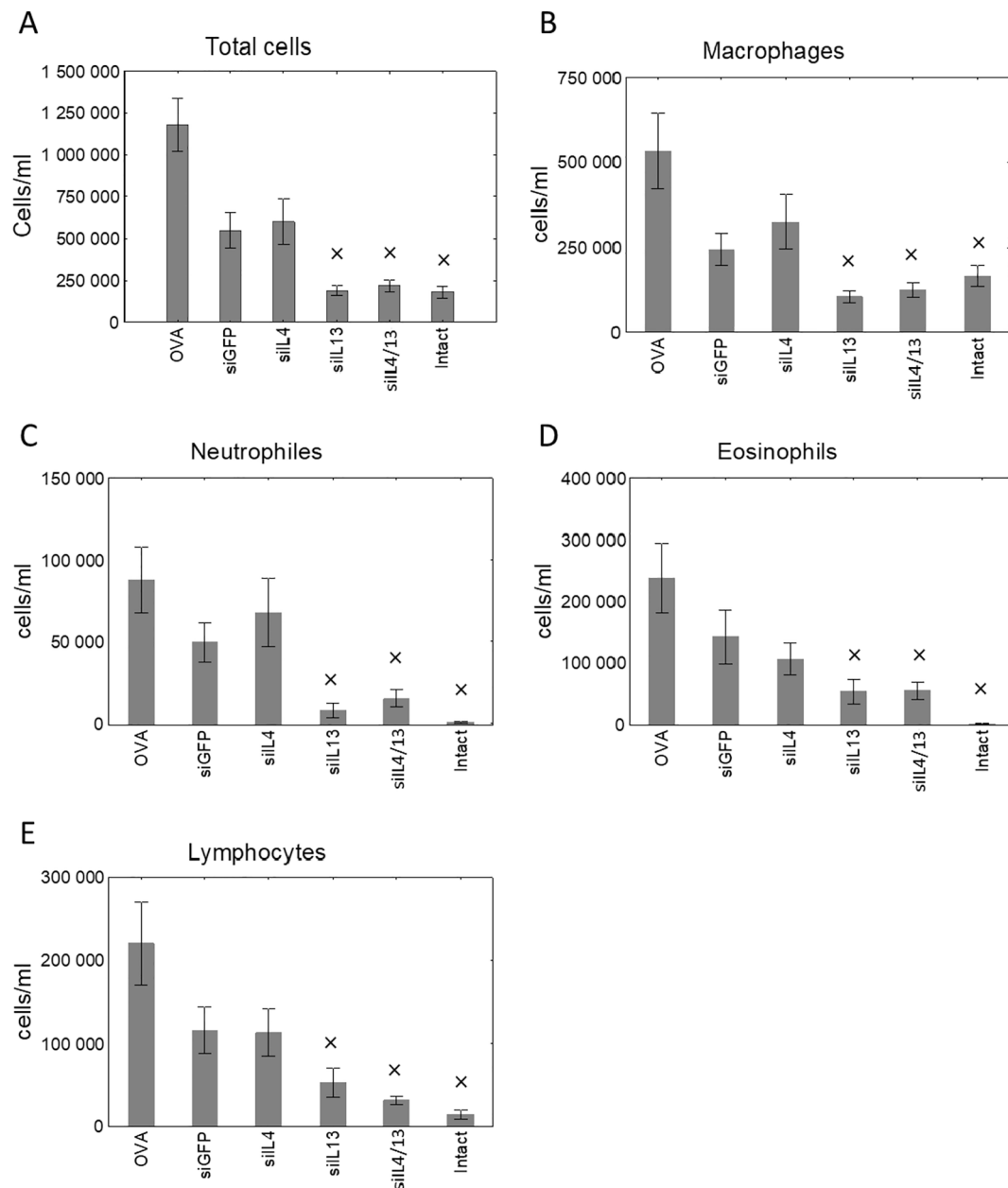


Fig. 5. Effect of siRNAs targeted to IL-4 and IL-13 on BAL cell composition. Two ways after the last allergen challenge lungs were lavaged and total cell number was determined (A). Differential cell count of macrophages (B), neutrophils (C), eosinophils (D) and lymphocytes (E) in BAL samples was performed. Data are presented as mean of cells per 1 ml $\pm$ standard error. X - statistically significant vs “siRSV” according to Mann-Witney U test. N = 10.

epithelium during BA development [45]. Performed histopathological analysis showed decrease in the proportion of mucus secreting goblet cells in the respiratory epithelium and total inflammatory score after suppression of IL-13 by siIL13-195 alone or by the mixture of siIL4-408 and siIL13-195 (Fig. 6).

The described results show the lack of beneficial effects of single IL-4 suppression by siRNA on BA phenotype. At the same time, application of single siRNA targeted to IL-13 demonstrated more substantial reduction in severity of BA features. Although both Th2-cytokines (IL-4 and IL-13) contribute to BA pathogenesis, a substantial body evidences to crucial role of IL-13 in the pathology are published (see reviews [31,45]). IL-13 rather than IL-4 is sufficient to induce many BA features independently of other Th2 cytokines: IgE synthesis, airway inflammation, sub-epithelial fibrosis, mucus hypersecretion and AHR. Despite beneficial effect of single siIL13-195 on BA features, dual application of both siRNAs (siIL4-408 and siIL13-195) showed a greater inhibitory effect in

AHR, that coincides to the data obtained by Lee and colleagues [34].

The most important bottleneck in the development of effective siRNA-based therapeutics is effective delivery. In this study we delivered so called “naked” siRNAs to mouse lung intranasally. The systemic delivery of naked siRNA generally fails to produce a significant gene-silencing effect, but local delivery to the lung has already been reported to be successful (see review [46]). The mechanisms by which naked siRNA crosses the cell membrane remains to be answered, although some explanation could be hypothesized: 1) the reduced nuclease activity in the lung provides more long-term siRNA half-life [47]; 2) lung mucosa (especially in sites of inflammation) is enriched with cationic peptides and proteins which may interact with negatively charged siRNA and form nanocomplexes, that could be taken up by cells through endocytosis.

The question of what cell types are taking up naked siRNA in the lung remains to be answered. In the current study we did not determine the

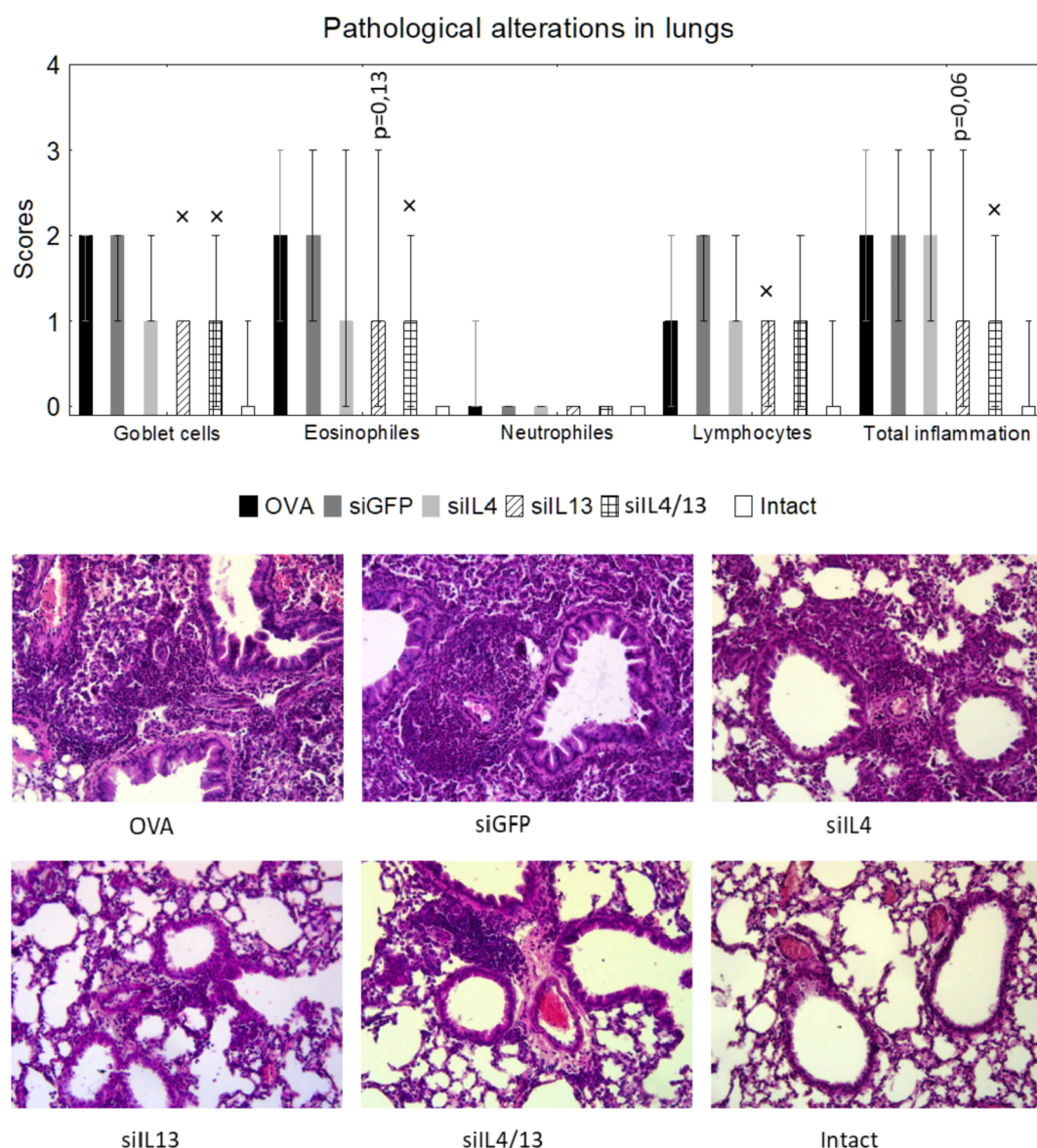


Fig. 6. Histological alterations in lung tissues. A. Scores obtained for histological analysis of pathological alterations in lungs: eosinophil, lymphocyte and neutrophil infiltration around the bronchi, number of goblet cells in the bronchial epithelium, and total inflammation. The severity of pathological features were graded according to semi-quantitative method [26]: 0 – non, 1 – mild, 2 – moderate, 3 – severe. Data are presented as median $\pm$ interquartile range. $\times$ – statistically significant vs “siRSV” according to Kruskal Wallis test. B. Photomicrographs of alveoli and surrounding tissue after hematoxylin and eosin staining of lungs.

location of siRNA. However, Karras and colleagues have shown that naked oligonucleotides specifically inhibited IL-4Ra protein expression in lung eosinophils, macrophages, dendritic cells, and airway epithelium after inhalation delivery [48]. As the suppression of IL-4 and IL-13 occurred it could be hypothesized that siRNA delivered to the Th2-cells as main producers of these cytokines.

RNAi technology can be a basis for development of anticytokine therapeutic approach to treat inflammatory diseases including BA. One the important advantage of siRNAs as pharmacological substances is high specificity of suppression of target genes. The potent gene silencing (up to 90%) is achieved by 100% identity between siRNA and target gene sequences. One nucleotide mismatch out of 19 nucleotides of siRNA almost completely abolishes silencing effect, two nucleotide mismatches make siRNA unspecific for desired gene [49,50]. Human genome sequences deposited in data bases allow to avoid “off-target” effects of designed siRNAs. Additionally, enzymes existing inside of cells eliminate siRNAs that makes possible unwanted effects temporal.

In summary, we show that local administration of siRNAs targeted to

IL-4 and IL-13 reduces the lung expression of these cytokines, AHR and pulmonary inflammation. Collectively these findings suggest that dual blockage of IL-4 and IL-13 by siRNA could be a novel approach for the therapy of inflammatory respiratory diseases including bronchial asthma.

CRediT authorship contribution statement

Shilovskiy IP: Conceptualization, Methodology, Validation, Data curation, Writing – original draft, Writing – review & editing, Project administration. **Sundukova MS:** Methodology, Formal analysis, Investigation, Writing – original draft, Visualization. **Korneev AV:** Investigation, Writing – original draft, Writing – review & editing. **Nikolskii AA:** Investigation, Funding acquisition. **Barvinskaya ED:** Investigation. **Kovchina VI:** Investigation. **Vishnakova LI:** Investigation. **Turenko VN:** Investigation. **Yumashev KV:** Investigation. **Kaganova MM:** Investigation. **Brylina VE:** Data curation. **Sergeev I:** Data curation. **Maerle A:** Data curation. **Kudlay DA:** Data curation. **Petukhova O:**

Data curation. **Khaitov M.R.**: Conceptualization, Methodology, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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