

Respiratory syncytial virus upregulates IL-33 expression in mouse model of virus-induced inflammation exacerbation in OVA-sensitized mice and in asthmatic subjects

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ABSTRACT

Background: Bronchial asthma (BA) is a chronic disease of the airways. The great majority of BA exacerbations are associated with respiratory viral infections. Recent findings point out a possible role of proinflammatory cytokine interleukin-33 (IL-33) in the development of atopic diseases. Although, little is known about the role of IL-33 in virus-induced BA exacerbations.

Methods: We used mouse models of RSV (respiratory syncytial virus)-induced inflammation exacerbation in OVA-sensitized mice and RSV infection alone in adult animals to characterize expression of *il33* in the mouse lungs. Moreover, we studied the influence of *il33* knockdown with intranasally administrated siRNA on the development of RSV-induced inflammation exacerbation. In addition, we evaluated the expression of *IL33* in the *ex vivo* stimulated PBMCs from allergic asthma patients and healthy subjects with and without confirmed acute respiratory viral infection.

Results: Using mouse models, we found that infection with RSV drives enhanced *il33* mRNA expression in the mouse lung. Treatment with anti-*il33* siRNA diminishes airway inflammation in the lungs (we found a decrease in the number of inflammatory cells in the lungs and in the severity of histopathological alterations) of mice with RSV-induced inflammation exacerbation, but do not influence viral load. Elevated level of the *IL33* mRNA was detected in *ex vivo* stimulated blood lymphocytes of allergic asthmatics infected with respiratory viruses. RSV and rhinovirus were the most detected viruses in volunteers with symptoms of respiratory infection.

Conclusion: The present study provides additional evidence of the crucial role of the IL-33 in pathogenesis of RSV infection and virus-induced allergic bronchial asthma exacerbations.

1. Introduction

Bronchial asthma (BA) is one of the most common chronic inflammatory disorders of respiratory tract. Episodic dyspnea, wheezing, bronchial spasm and breathing difficulty are among the most typical manifestations of BA. In addition, structural changes in the airways including subepithelial and airway wall fibrosis, goblet cell

hyperplasia/metaplasia, smooth muscle thickening and increased vascularity are observed. Allergic BA (~70–80% of total BA cases) is characterized by the elevated amount of total and allergen specific IgE antibodies in serum and high eosinophil count in blood, airway mucosal layers and bronchoalveolar lavage (BAL) fluid [1,2].

Respiratory viral infections cause a greater level of morbidity in asthmatic subjects than in the healthy population [3]. Highly

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pathogenic strains of respiratory viruses trigger severe damage of the lungs, and consequently tremendous amounts of proinflammatory cytokines and chemokines are produced by the infected cells, that can cause mild to severe exacerbations of BA [4]. Respiratory viruses such as rhinoviruses and RSV (respiratory syncytial virus or Human orthopneumovirus) are the main trigger of BA exacerbations [5].

Currently, β_2 – agonists, antileukotriene agents and topical corticosteroids are widely used for BA treatment. These drugs reduce exacerbation by suppressing the symptoms of asthma, but they are not associated with the pathogenesis of the disease [6]. In the therapy of allergic BA, the allergen-specific immunotherapy (AIT) is particularly important. However, AIT is available only for patients with a defined cause of allergy development. In addition, the application of AIT is limited for patients sensitized to several allergens simultaneously [7]. At the same time, alternative options for BA treatment (such as monoclonal antibodies, soluble receptors, protein inhibitors, etc.) are actively developed [8]. Recent studies indicate the determining role of Th2-cytokines (IL-4, IL-5 and IL-13) in the BA pathogenesis, which led to the development of the anti-cytokine therapy of BA. Despite that, an active search for new genes involved in the pathogenesis of BA is ongoing. Consequently, modern therapies of severe BA have limited efficacy [9,10]. Thus, search for novel therapeutics and identification of new targets for drug development is a high priority goal.

Recent findings point out a possible crucial role of proinflammatory cytokine IL-33 in the development of atopic diseases [11,12]. IL-33 is a novel member of the IL-1 cytokine family. IL-33 exerts biological effects by binding to the specific primary receptor ST2 and the coreceptor IL-1 receptor accessory protein (IL-1RAcP) [13]. Soluble IL-33 is known to increase the secretion of Th2 cytokines (IL-4, IL-5, and IL-13) and to act as a chemoattractant for Th2 lymphocytes both *in vivo* and *in vitro*. IL-33 is involved in many immunopathologies such as arthritis, anaphylactic shock, and autoimmune diseases [14]. Associations between IL-33 and allergic BA were found in animal model studies. In particular, using ST-2 deficient mice or animals treated with either the soluble IL-33 receptor or with anti-IL-33, anti-T1/ST2 antibodies, reduction of different features of asthma have been shown [15–18].

Although, little is known about the role of IL-33 in virus-induced allergic BA exacerbations. In the current study we aimed to investigate the role of IL-33 in the pathogenesis of virus-induced allergic BA exacerbations and to evaluate the potential of IL-33 as a target for the development of novel anti-asthmatic drugs.

2. Materials and Methods

2.1. Animals and viral strain

Experiments with animals were carried out in accordance with the EU Directive 2010/63/EU for animal experiments. Female 6- to 8-week-old BALB/c mice (Stolbovaya, Moscow, Russia) were kept on an ovalbumin (OVA)-free diet under pathogen-free conditions. The RSV A2 strain was propagated in Hep-2 cells (human epithelial carcinoma derived cells) and purified by sucrose gradient centrifugation [19]. The final preparations had titers in the range of 10^8 TCID₅₀ (median tissue culture infective dose)/mL.

2.2. Allergic asthma exacerbation model

A schematic presentation of treatment protocols is shown in Fig. 1. In experiments with RSV-induced inflammation exacerbation in OVA-sensitized mice (Fig. 1A) four groups of animals were formed: Group OVA (mouse model of allergic BA), Group OVA + RSV (mouse model of RSV-induced inflammation exacerbation in OVA-sensitized mice), Group RSV (mice infected with RSV alone), Group Intact (control, treatment with PBS).

To develop mouse model of allergic BA, mice were sensitized with 20 µg ovalbumin (OVA) from chicken eggs, emulsified in 2 mg of

aluminum hydroxide, intraperitoneally on days 1, 14, and 28. Then mice were intranasally challenged with aerosolized 1% OVA solution on three consecutive days (40–42) to induce allergic airway inflammation. Animals from Groups 3 and 4 were treated with PBS on indicated time points. On day 38 mice from Groups 2 and 3 were intranasally infected with 50 µL of RSV A2 strain (5×10^6 TCID₅₀/mouse).

In experiments with siRNA (Fig. 1B) using model of RSV-induced inflammation exacerbation in OVA-sensitized mice three groups of animals were formed: Group OVA/RSV + siGFP (treated with non-specific siRNA against GFP), Group OVA/RSV + siIL33 (treated with specific siRNA against *il33*), Group OVA/RSV (treated with PBS) and Group Intact (did not receive any treatment and served as a negative control). First two groups were intranasally treated with siRNA on days 38–42 in total dose of 120 µg/mouse. We utilized “naked” siRNA without any carrier.

In both experiments, we assessed lung function on day 5 after infection and mice were sacrificed on day 6 after infection (Fig. 1A, B) for end-point analyses. Airway hyperresponsiveness (AHR) was performed as described previously [20].

For histopathological analysis, the left lobe of each lung was fixed in 4% formalin and stained with hematoxylin and eosin. Slides were rehydrated through a series of decreasing ethanol washes and stained with a periodic acid–Schiff kit (Sigma-Aldrich) to visualize goblet cells. The histopathological changes were graded according to a modified semiquantitative scoring system in a blinded manner (none, 0; mild, 1.0; moderate, 2.0; or severe, 3.0).

For BAL fluid differential cell counts cellular fractions were lavaged from mouse lungs and processed for differential azure–eosin staining by cytopinning. Cell counting was performed by light microscopy (at least 200 cells per slide) in a blinded manner.

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

We excised the right lobe of the lung from each mouse and analyzed by quantitative real-time PCR. Total RNA was isolated from the 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 (ThermoFisher, USA). The reverse transcription reaction product was amplified via RT-PCR using an iCyclerIQ Real-time PCR Detection System (Bio-Rad Laboratories). Primers and probes used in this study are described in Table 1. Relative quantification of RT-qPCR was used to detect changes in expression of the target genes relative to a reference gene, which is housekeeping *hprt* in mice (Fig. 2B–G, 3B–E) and human β -actin gene in human samples (Fig. 4F). Quantitative PCR results for mRNA expression at the Fig. 2 B–G are presented as Δ Ct values, calculated by the formula: ratio (reference/target) = $2^{Ct(hprt)-Ct(gene\ of\ interest)}$, and as the $2^{-\Delta\Delta Ct}$ [21] for the results presented at Fig. 3B–E and 4F (the results are presented as fold change relative to the group of intact mice and healthy subjects in mouse and human study, respectively). The number of copies for viral RNA quantification was calculated using a standard curve generated by amplification of plasmid DNA. The amplification cycle consisted of 95 °C for 10 min and 45 cycles of 95 °C for 15 s, 60 °C for 15 s.

2.4. Flow cytometry

Cells purified from the lung tissue were used for flow cytometry analysis. Cell suspension was stained with fluorophore labeled antibodies to determine expression of CD45 + leukocytes, CD3 + T cells; CD19 + B-cells; Ly6G + neutrophils; SiglecF + eosinophils; CD324 + epithelial cells by flow cytometry (Fig. S1). All antibodies are from eBioscience. Flow cytometry was performed using a BD FACS-Canto™ II (Becton Dickinson). The results were analyzed with FlowJo software version 7.6.5 (Tree Star). The CountBright™ (Thermo Fisher Scientific) absolute counting beads were mixed with the cell sample and assayed via flow cytometry. Results are reported as absolute number of

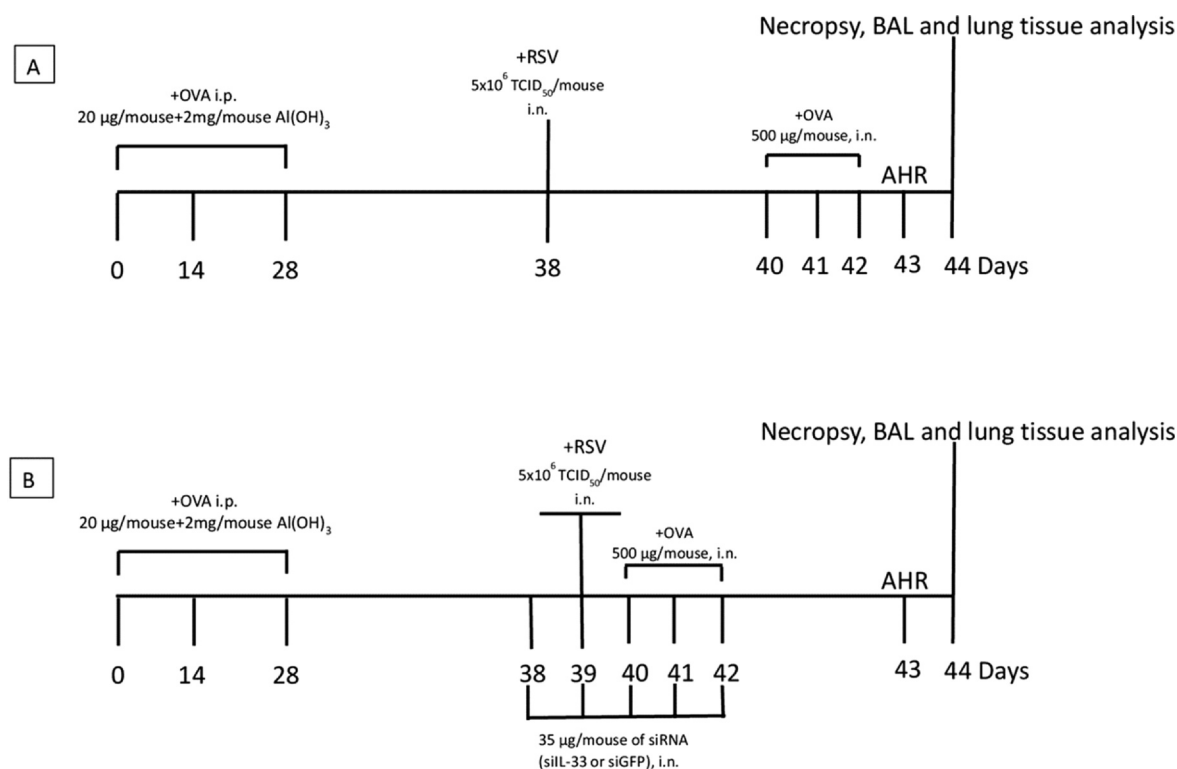


Fig. 1. Experimental protocols of RSV-induced inflammation exacerbation in OVA-sensitized mice (A) and siRNA treatment (B). OVA - ovalbumin from chicken eggs; RSV- respiratory syncytial virus (A2 strain); AHR - airway hyperresponsiveness; BAL - bronchoalveolar lavage; i.n. - intranasal; i.p. - intraperitoneal; siGFP, siIL-33 - siRNAs against GFP and mouse IL-33, respectively.

Table 1

Primers and probes for qRT-PCR (5'-3').

RSV-F	GAAGCATTAATGACCAATGA
RSV-R	CATCTGATGTGCTTTTGC
RSV-Z	TGAGACACATCATGCTGGACACCTCTA
mHPRT-F	TATACCTAATCATTATGCCGAGGAT
mHPRT-R	CTTTAATGTAATCCAGCAGGTCAG
mHPRT-Z	TCCTCATGGACTGATTATGGACAGGACT
mIL4-F	AGAGAGTGAGCTCGTCTGTAG
mIL4-R	GGTGCAGCTTATCGATG
mIL4-Z	CTCTGCAGCTCCATGAGAACTAGAG
mIFNγ-F	AAATCCTGCAGAGCCAGATTAT
mIFNγ-R	GCTGTTGCTGAAGAAGGTAGTA
mIFNγ-Z	ACGCTTATGTTGTTGCTGATGGCC
mil33-F	GGAGAATGGATGTTATGTGATC
mil33-R	TGTTCCACCATCAGCTTCTTC
mil33-Z	CCAAGAGCAAGACAGGTGCTACTAC
mTbeta-F	GTTCCCATTCCTGTCTCTC
mTbeta-R	GGTGGACATATAAGCGGTT
mTbeta-Z	CAGCACCCTGGCGGTACCAGAG
hB-Act-F	CATCACCATTGGCAATGAG
hB-Act-R	CACACTTCATGATGGAGTTGAAG
hB-Act-Z	CTTCTCTCTGGGATGGAGTCTGTG
hIL33-F	GGTGTTACTGAGTTACTATGAGTCTCAA
hIL33-R	CAGGGTTACCATTAACATCTTACCA
hIL33-Z	CCCTCAAATGAATCAGGTGACGGTGT

cells per 1 mL.

2.5. Elisa

To determine the level of OVA-specific antibodies of IgE, IgG1 and IgG2a classes in mice serum, commercial ELISA (BD) kits were used in accordance with the manufacturer's recommendations. IL-33 Mouse ELISA Kit (Invitrogen) was used to evaluate IL-33 level in mouse BAL fluid.

2.6. Clinical studies

During 2016–2017 the biomaterial from volunteers and patients hospitalized to the bronchial asthma department of NRC Institute of Immunology FMBA were collected. Participation in the research was voluntary. Clinical studies were carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). According to the Helsinki Declaration, patients gave their oral and written consent before being included in the study.

The volunteers (N = 96) were divided into following groups:

1. Group “BA” - patients with moderate to severe allergic bronchial asthma that meet the inclusion/exclusion criteria, N = 34;
2. Group “BA + ARVI” - patients suffering from moderate to severe allergic bronchial asthma, who have had symptoms of acute respiratory viral infection (ARVI) for no more than 4 days, corresponding to the inclusion/exclusion criteria, N = 18;
3. Group “Healthy + ARVI” – healthy volunteers with ARVI symptoms for no more than 4 days, without any signs of allergic bronchial asthma or atopic diseases that meet the criteria for inclusion/exclusion, N = 21;
4. Group “Healthy” - healthy volunteers who meet the inclusion/exclusion criteria, N = 23.

Demographic characteristics of the study participants are presented in [Table 2](#). [Table 3](#).

Criteria for inclusion/exclusion of volunteers in the study are presented in [Table 2](#).

After signing an informed consent to participate in the study, all volunteers were screened. Sampling of biomaterial and performance of instrumental examination of the patient was carried out once ([Table 4](#)).

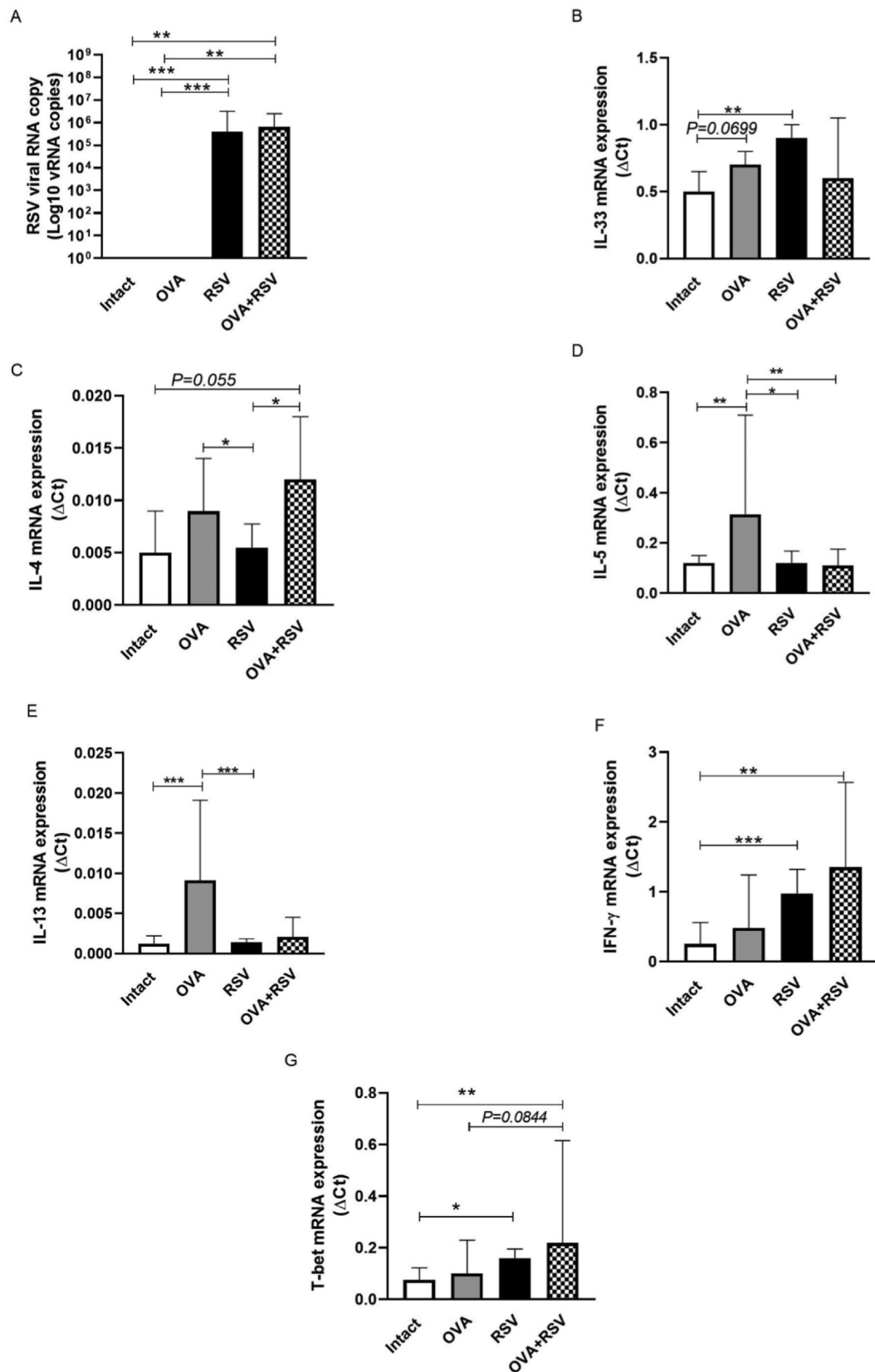


Fig. 2. Characteristics of RSV-induced inflammation exacerbation in OVA-sensitized mice. Mice were OVA –sensitized, infected with RSV A2 and challenged with OVA. On day six after infection mice were sacrificed for end-point analyses. qRT-PCR analysis of lung tissue of mice: A-viral load; mRNA expression of *il33* (B); *il4*(C); *il5*(D); *il13*(E); *ifn-γ* (F); *t-bet* (G). Data are from one experiment representative of two independent experiments with ten mice/group. Differences between multiple groups were estimated using a Kruskal–Wallis test followed by post-hoc testing (if the Kruskal–Wallis was significant) using un-paired Mann–Whitney U tests. Data show medians + interquartile range. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

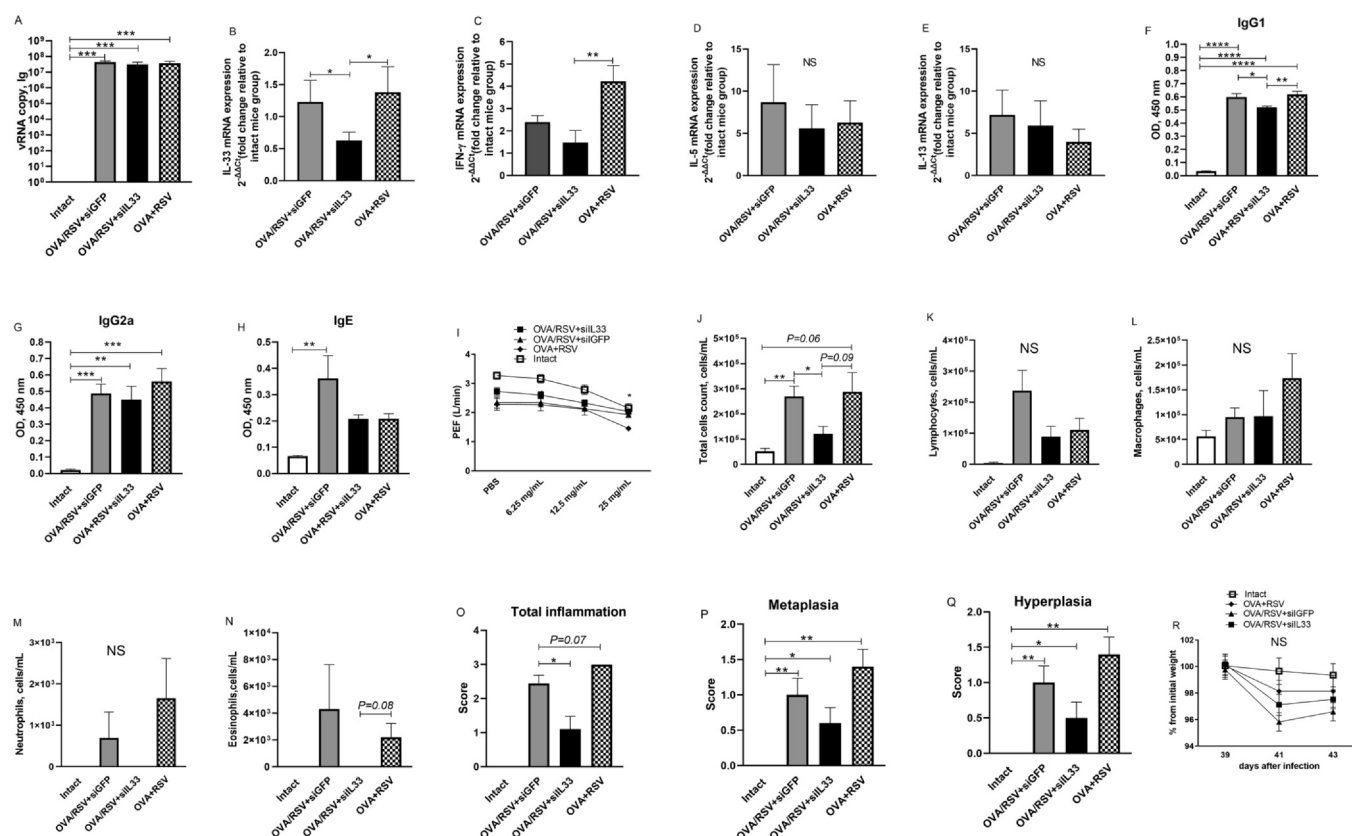


Fig. 3. Intranasal treatment of mice with siRNA against IL33 does not influence viral load but influences cell count in BAL, pathologic alterations in the lung and IgG1 OVA specific antibodies. Mice were OVA-sensitized, infected with RSV A2, challenged with OVA and treated with specific anti IL33 siRNA or control siRNA against GFP. Viral load (A) and *il33* (B), *ifn γ* (C); *il5* (D); *il13* (E); mRNA expression was measured in the lung tissue by qRT-PCR. F, G and H – OVA-specific IgG1, IgG2a and IgE, respectively, measured by ELISA in mice blood. I-lung function PEF (the peak expiratory flow). Cells count in BAL of mice was evaluated under light microscopy, cells/mL: J- total cell count; K-lymphocytes; L- macrophages; M- neutrophils; N-eosinophils. Reduced pathologic alterations in the lung of mice treated with specific IL33 siRNA: O- total inflammation; P-lung metaplasia; Q-lung hyperplasia. R- change of the mice weight (% from initial). Data are from one experiment representative of two independent experiments with ten mice/group. Data show means + s.e.m. Differences between multiple groups were analyzed using one-way ANOVA with Tukey's post hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = ANOVA not significant.

2.7. Statistical analysis

The Shapiro-Wilk test for normality was performed. Data were presented as means + s.e.m for parametric analyses and as medians + interquartile range for nonparametric analyses, as indicated in the figure legends. Statistical analyses were performed using Prism 8 (GraphPad Software). For normally distributed data we performed ordinary one-way ANOVA with (if the ANOVA was significant) Tukey's post hoc tests for multiple comparisons to determine differences between groups, or if not normally distributed the Kruskal–Wallis test followed by post-hoc testing (if the Kruskal–Wallis was significant) using un-paired Mann-Whitney U tests was performed, as mentioned in the respective figure legends. To determine the impact of multiple factors, multiple linear regression or multiple logistic regression was conducted. Data were accepted as significantly different when $P < 0.05$.

3. Results

3.1. IL-33 expression is induced by RSV infection in mice

To evaluate the role of IL-33 in virus-induced allergic BA exacerbation we developed models of RSV infection and RSV-induced inflammation exacerbation in OVA-sensitized mice. To induce allergic inflammation, we sensitized mice with OVA prior infection (Fig. 1). The main characteristics of the models are presented in Supplementary (Fig. S2, S3).

To evaluate cell infiltration into the lung we studied the cell composition of BAL by light microscopy (Fig. S2A-E) and lung tissue by flow cytometry (Fig. S2F-L). The total cell count in BAL was 1.5–3-fold higher in experimental animals than in intact mice (Fig. S2A). In addition, we observed an increased number of eosinophils (Fig. S2B) in BAL of OVA group, while lymphocytes were predominantly detected in RSV-infected mice (Fig. S2C). Macrophages were predominantly detected in OVA and RSV groups (Fig. S2D), while neutrophils were in OVA group (Fig. S2E).

In the lung tissue, we found significantly increased total cells amount, number of CD45 + leucocytes, CD3 + T-cells and CD19 + B-cells ($p < 0.05$, $p < 0.01$) in the RSV and OVA + RSV groups (Fig. S2F-I). Number of SingletF8 + eosinophils was significantly higher in OVA and OVA + RSV groups ($p < 0.05$, $p < 0.01$) (Fig. S2J). While number of Ly6G + neutrophils was elevated in infected groups of animal RSV and OVA + RSV ($p < 0.01$) (Fig. S2K). We found no significant difference in the amount of CD324 + epithelial cells (Fig. S2L).

Animals from groups OVA and OVA + RSV were characterized by deterioration of lung function (Fig. S3A). In RSV infected mice, significant body weight loss (Fig. S3B) was observed. Up to 9 and 15% in OVA + RSV and RSV groups, respectively, compared with the intact mice.

Both groups of mice OVA and OVA + RSV showed elevated levels of OVA-specific IgG1, IgG2a and IgE antibodies ($p < 0.001$) confirming development of allergic immune response in OVA sensitized animals (Fig. S3C-E).

Histological examination of lung sections showed moderate

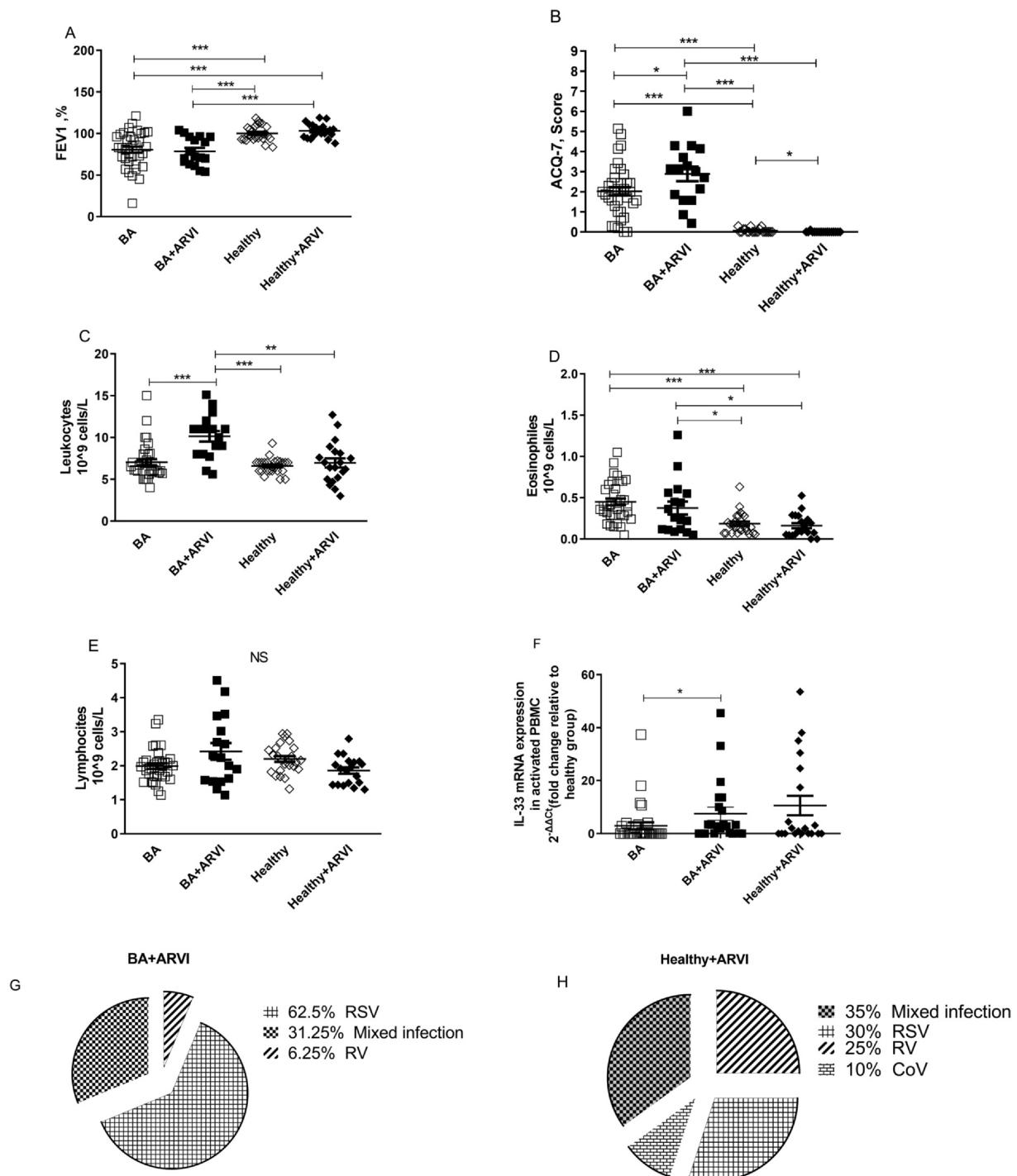


Fig. 4. Volunteers' screening results. Study participants ($N = 96$) were divided into the following groups: "BA" - patients with moderate to severe allergic bronchial asthma (BA), $N = 34$; "BA + ARVI" - patients suffering from moderate to severe allergic BA, who have had symptoms of acute respiratory viral infection (ARVI) for no more than 4 days, $N = 18$; "Healthy + ARVI" - healthy volunteers with ARVI symptoms for no more than 4 days, without any signs of BA and atopic diseases, $N = 21$; "Healthy" - healthy volunteers, $N = 23$. Spirometry was performed to evaluate FEV-1 (forced expiratory volume) (A). All participants filled out the Asthma Control Questionnaire (ACQ-7) (B). Blood samples were collected for analysis. A white blood cell count was performed to evaluate leukocytes (C), eosinophils (D) and lymphocytes (E) amount. (F) IL33 mRNA expression in PHA activated PBMC from different group of patients. Viruses detected in nasal swabs of BA + ARVI (G) and Healthy + ARVI (H) groups. Nasal swabs from patients with symptoms of acute respiratory infection were collected during 2016-2017 and analyzed by RT-PCR for the presence of nucleic acids of rhinoviruses (Rv), respiratory syncytial virus (RSV), metapneumovirus (Mpv), adenoviruses (Adv), bocaviruses (Bov), parainfluenza viruses 1-4 (PIV 1-4), coronaviruses (CoV), and Influenza A virus (InfA). Data represented as percentage of positive samples. Mixed infection (nucleic acids of more than one virus was detected). Only samples from groups "BA + ARVI" and "Healthy + ARVI" were analyzed. Differences between multiple groups were estimated using a Kruskal-Wallis test followed by post-hoc testing (if the Kruskal-Wallis was significant) using un-paired Mann-Whitney U tests. Data show medians + interquartile range. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, NS = Kruskal-Wallis not significant.

Table 2
Demographic characteristic of the study participants.

Groups	Gender		Average age
	Male	Female	
BA	14	20	40.6
BA + ARVI	7	11	50.2
Healthy	8	13	28.8
Healthy + ARVI	9	14	28.7

inflammation for all experimental groups compared to intact mice (Fig. S3F). In addition, histological analysis of the lungs revealed changes in the functional state of the bronchial epithelium - hyperplasia and metaplasia were observed (Fig. S3G, H).

RSV RNA was detected in infected groups of animals (RSV and OVA + RSV) (Fig. 2A) by quantitative RT-PCR. To evaluate whether the viral and/or allergic environment upregulates expression of *il33*, *il4*, *il5*, *il13*, *ifn-γ* and *t-bet* we calculated qRT-PCR data presented at Fig. 2B-G by Δ Ct method and compared experimental groups to the intact group and then experimental groups between each other. Significant upregulation of *il33* mRNA expression ($p < 0.01$) was observed in RSV infected group only. We found a trend towards increased level of *il33* expression in OVA group ($p < 0.0699$) (Fig. 2B). Significant elevation of *il4* mRNA expression was detected in OVA and OVA + RSV group ($p < 0.05$), compared to RSV group (Fig. 2C). Expression of *il5* and *il13* (Fig. 2D, E) was significantly increased in OVA group compared to the intact, RSV and OVA + RSV groups ($p < 0.01$, $p < 0.05$ for *il5*) and to the intact and RSV groups ($p < 0.001$ for *il13*). The *ifn-γ* and *t-bet* (Fig. 2F, G) mRNA expression was upregulated in OVA + RSV and RSV groups ($p < 0.01$, $p < 0.001$ and $p < 0.01$, $p < 0.05$, respectively). The level of IL-33 in BAL fluid was undetectable by ELISA (data not shown).

3.2. Anti-IL-33 siRNAs reduce IL-33 expression, level of IgG1 antibodies in serum, total cell count and inflammation in lung, but do not influence viral load

We utilized a mouse model of RSV-induced inflammation exacerbation in OVA-sensitized mice to analyze the effects of knockdown of IL-33 activity by siRNA *in vivo*. OVA-sensitized mice were infected with the RSV A2 strain and treated intranasally with “naked” siRNAs (siIL33 or siGFP [as negative controls]) as described in Materials and Methods and Fig. 1B. We analyzed the expression levels of RSV viral RNA (vRNA) and *il33* mRNA (Fig. 3A and B, respectively) in the lung tissue by RT-PCR. The intranasal application of siRNA against IL-33 to sensitized/challenged and infected animals reduced *il33* mRNA expression by 50% compared with negative control but did not influence the level of RSV vRNA. Moreover, we detected a decreased expression of *ifn-γ* mRNA in mice treated with siIL33 compared to OVA + RSV group $p < 0.01$ (Fig. 3C). We found no significant difference in *il5* and *il13* expression between experimental groups (Fig. 3D, E).

In addition, we found a significant ($p < 0.05$, $p < 0.01$) decrease

in level of Th2 –dependent OVA specific IgG1 antibodies in serum of mice treated with specific anti-IL33 siRNA (Fig. 3F) compared to control groups. At the same time, we found no significant difference in the level of OVA-specific IgG2a, IgE and AHR (Fig. 3G, H, I).

Treatment with siRNA against IL-33 resulted in a significant decrease of total cells count (Fig. 3J). In addition, we observed a tendency to decreased number of lymphocytes, macrophages, neutrophils, and eosinophils (Fig. 4K, L, M, N) in BAL samples of experimental animals. Histological analysis revealed significantly ($p < 0.05$) decreased total inflammation in siIL33 treated mice (Fig. 3O) and a trend towards reduced lung metaplasia and hyperplasia in the same group (Fig. 4P, Q). Finally, we found no significant difference in body weight between the groups (Fig. 4R).

3.3. IL33 mRNA expression is increased in asthma patients with acute respiratory viral infections predominantly caused by RSV and RV

To investigate the role of IL-33 in allergic BA pathogenesis both in the context of co-occurring viral infection as well as in uninfected populations we performed a clinical study in humans. Using clinical and experimental tools, we analyzed lung function, blood count and *IL33* mRNA level in *ex vivo* stimulated PBMCs.

Using the standard protocols in all groups of subjects, we determined lung function by evaluating the peak expiratory flow FEV1 (Fig. 4A). FEV1 was significantly decreased in allergic BA and BA + ARVI (87.2 and 74.9%) groups compared to Healthy and Healthy + ARVI (100.8 and 104.7%) groups. Additionally, the ACQ-score obtained using the asthma control questionnaire was significantly increased in allergic BA and BA + ARVI (2.43 and 3.41) groups compared to control Healthy and Healthy + ARVI groups (0.00 and 0.03) (Fig. 4B).

To evaluate inflammation status in volunteers we analyzed blood counts (Fig. 4C-E). Total leucocyte counts in peripheral blood revealed increased cell numbers in the BA + ARVI group compared to all groups (Fig. 4C). In addition, we detected increased eosinophil levels in allergic BA and BA + ARVI groups compared to Healthy and Healthy + ARVI groups (Fig. 4D). Finally, peripheral lymphocytes counts did not show any significant difference between any groups (Fig. 4E).

Next, we measured mRNA of *IL33* in human activated PBMC (Fig. 4F). We observed that *IL33* in the context of preexisting asthma pathology was promoted by viral infection in BA + ARVI group compared to allergic BA group ($p < 0.05$).

To determine which virus is the most common in patients with allergic BA diagnosis and acute respiratory infection symptoms, we analyzed nasal swabs from all the participants (Fig. 4G,H). Using qRT-PCR we detected nucleic acids of multiple viruses such as rhinoviruses (Rv), respiratory syncytial virus (RSV), metapneumovirus (Mpv), adenoviruses (Adv), bocaviruses (Bov), parainfluenza viruses 1–4 (PIV 1–4), coronaviruses (Cor), and Influenza A virus (InfA). Thus, we found out that RSV (62.5%), mixed infection (31.25%) and Rv (6.25%) prevailed in BA + ARVI group. Out of five samples with detected mixed infection two were identified as combination of RSV and Rv, one – RSV,

Table 3
The inclusion and exclusion criteria for volunteers of the study.

Inclusion criteria	Exclusion criteria
• Signed informed consent • Age between 18 and 75 years • Patients with moderate and severe allergic asthma for at least two years • Volunteers with symptoms of ARVI for the last four days and detected respiratory viruses (RT-PCR in nasal swabs) • For control groups (without asthma and healthy) - absence of ARVI symptoms during last thirty days • Ability to cooperate adequately in the research process	• Pregnancy, lactation • Symptoms of other respiratory diseases • Acute conditions that can significantly affect the results of the study • Pulmonary tuberculosis (active and inactive forms) • Cancer • Parasitic invasion during the last six months • Systemic steroid therapy • Therapy with antileukotrienes • Unwillingness of the patient to take further part in the study

Table 4
Screening of volunteers included in the study.

Medical history	Registration of subjective health complaints, medical history (previous and concomitant systemic diseases; drug therapy at the time of inclusion in the study (all systemic and topical drugs); data for the presence of drug allergy); demographic data (gender, age, height, weight, etc.)
Filling out the questionnaire	All volunteers filled out the Asthma Control Questionnaire (ACQ-7).
Instrumental examination	Spirometry (FEV ₁) was performed using a spirometer (MasterScreen IOS spirometer, CareFusion, Germany)
Sampling of biomaterial (blood sample and nasal swab were collected from every participant)	Blood tests: a white blood cell count was performed to evaluate leukocytes amount using Beckman Coulter LH500 Hematology Analyzer (USA, CA). Nasal swabs were tested for the presence of the viral nucleic acids of common respiratory pathogens using kit for RT-PCR “AmpliSens® ARVI-screen-FRT” according to the manufacturer's instructions (ILS, Russia). For the study of <i>il-33</i> gene expression in leukocytes of peripheral venous blood PBMCs were isolated via Ficoll density gradient centrifugation, seeded in 12-well plates in concentration of 5×10^6 cells/mL and stimulated with 20 µg/mL of phytohaemagglutinin (PHA). In 24 h cells were lysed in RLT buffer and proceeded for further analysis by qRT-PCR. Unstimulated cells were used as control.

Mpv and Cor, one -Rv and Cor, one -PIV1 and Cor. In Healthy + ARVI group mixed infection was detected in 35% of volunteers, RSV in 30%, Rv-25% and Cor in 10%. Out of seven samples with detected mixed infection three were identified as combination of Rv and Cor, one – RSV and Cor, one -Rv and PIV3, one -PIV3 and InfA, one- RSV and InfA. Thus, RSV was prevalent in mixed infections of BA-ARVI group (60%), while Cor and Rv (57% and 43%, respectively) in Healthy + ARVI group. In general, RSV and RV were the most detected pathogens in both groups of volunteers (58% and 33%, respectively).

We found no significant correlations between *IL33* expression and lung function and different cell types and virus types in clinical study (data not shown).

4. Discussion

IL-33, a novel IL-1 family member but unlike other IL-1 family members, IL-33 has been associated with the promotion and amplification of both systemic and localized T helper (Th) 2 cell responses [22]. IL-33 might contribute to the pathogenesis and progression of lung pathologies such as asthma [12,23] and COPD [24]. Viral infections are important triggers of exacerbations of allergic asthma [25]. The role of IL-33 during virus-induced allergic BA exacerbation have been studied not sufficiently, especially during RSV infection. In this study, we used mouse models of RSV-induced inflammation exacerbation in OVA-sensitized mice and RSV infection alone in adult animals to characterize expression of *il33* in the mouse lungs. Moreover, we studied influence of *il33* knockdown with intranasally administrated siRNA on the development of RSV-induced inflammation exacerbation in OVA-sensitized mice. In addition, we aimed to evaluate expression of *IL33* in the *ex vivo* stimulated PBMCs from allergic asthma patients and healthy subjects with and without confirmed acute respiratory viral infection.

To reproduce RSV-enhanced allergic inflammation in OVA-sensitized animals we infected mice in one and two days before challenge with OVA in model-development and siRNA-treatment experiments, respectively (Fig. 1). This protocol has been chosen in accordance with the study of Barends et al. [26] suggesting that RSV inoculation shortly before or during challenge with OVA has not completely abolished OVA-induced expression of Th2 cytokines and histopathologic alteration in the lungs. Moreover, Rameix-Welti et al. [27] demonstrated that the peak of RSV replication in mouse lungs is detected on days 4 and 5 after the infection. Therefore, we challenged mice with OVA during the peak of viral replication.

Developed mouse models of RSV A2 infection and RSV-induced inflammation exacerbation in OVA-sensitized mice (OVA sensitization followed by RSV A2 strain infection) were characterized by efficient virus replication in the lungs, significant weight loss of the infected animals, impaired lung function, increased number of infiltrated cells into the lungs. We found differences between neutrophils count in BAL and lung homogenate evaluated by light microscopy and FACS, respectively (Fig. S2E, K). The highest number of neutrophils in BAL was

observed in OVA group. This is in accordance with study of Taube et al. demonstrating that a single intranasal OVA challenge in OVA sensitized mice cause FcγRIII-mediated and OVA IgG1 - dependent early (up to 24 h) neutrophils influx [28]. In our study neutrophils infiltration into the BAL was still elevated at 48 h after the third challenge. Probably this is due to an increased number of OVA challenges. Neutrophil recruitment into the lung compartment is a hallmark of RSV infection of both mouse and man [29]. In our model we also found an increased number of neutrophils in lung homogenates of the infected animals.

The IL-33 in BAL fluid was not detected in our mouse model, while markedly increased *il33* mRNA level was revealed in lung homogenates from mice infected with RSV. Using developed mouse models, we found that infection with RSV drives enhanced *il33* mRNA expression in the mouse lungs (Fig. 2B). It had been previously shown that IL-33 is characterized by both constitutive and inducible expression in the lung tissue [30]. IL-33 is released from necrotic cells in response to inflammation/infection [31]. Moreover, IL-33 is involved in the response to viral infection. Marked increase in the production of IL-33 was detected in adult mice infected with RSV [32] and in RSV-infected neonatal mice [33]. So, there is no doubt that IL-33 is a part of complex events involved in virus (including RSV) pathogenesis and our study also confirms this. The pathogenic mechanisms responsible for RSV induced immunopathophysiology remain unclear. However, growing evidence suggests involvement of group 2 innate lymphoid cells (ILC2s) in this process [34,35]. It has been shown that RSV induced rapid IL-33 expression and an increase in ILC2 numbers in the lungs of neonatal, but not in adult mice [33].

With increased expression of *il33* in RSV-infected animals we found an increased expression of *ifn-γ* mRNA (Fig. 2F). It seems that interaction between IL and 33 and IFN-γ remains a subject of controversy. Different studies have reported a down-regulating [36,37], a null [38], or an up-regulating [39,40] effect of IL-33 on IFN-γ levels. Moreover, the study of Lynch J. P. et al. [41] demonstrating that IL-33 as a potent suppressor of innate antiviral immunity acts via downregulation of viperin and interferon regulatory factor 7 gene expression. However, in this study the authors used Pneumonia virus of mice and exposure to low-dose of the cockroach extract.

In addition, we have evaluated expression of the Th1-associated transcription factors T-bet and found a significant increase in *t-bet* mRNA expression in infected animals (Fig. 2G). As has been shown by Baumann et al. T-bet and STAT4 driven ST2 is induced on Th1 CD4 + cells *in vitro* and *in vivo* during lymphocytic choriomeningitis virus infection. This data suggest that IL-33 may act directly on Th1 effector cells during infection to enhance antiviral Th1 cell responses [42].

In our study the development of type 2 inflammation was characterized by an increased expression of type 2 cytokines *il4*, *il5* and *il13* (Fig. 2C, D, E) and associated with eosinophilia in OVA sensitized animals (Fig. S2B, J). An increased expression of *il33* was accompanied by a decreased amount of *il4*, *il5* and *il13* mRNA in RSV group compared to OVA group (Fig. 2C, D, E). According to the study by D. Préfontaine at

al. IL-4 and IL-13 failed to up-regulate IL-33 gene expression in human airway smooth muscle cells and even down-regulated IL-33 gene expression in high doses [43] suggesting that IL-33 expression itself is not necessarily dependent on the Th2 cytokine microenvironment. In addition, an elevated level of *ifn- γ* and *t-bet* mRNA detected in RSV infected mice (Fig. 2F, G) may exert direct inhibitory effects on Th2 cytokines [44]. Taken together, the data indicates that IL-33 may influence both Th1- and Th2-oriented immune responses.

Growing evidence of IL-33 involvement in virus-induced BA exacerbation makes it an attractive target for drug development. It has been shown that knocking down of *il33* gene or neutralizing IL-33 with monoclonal antibodies led to less pronounced BA symptoms in experimental mice [45,46]. SAR440340 is a monoclonal anti-interleukin-33 antibody that is now under clinical trials for COPD and asthma treatment [47]. Conversely, administration of exogenous IL-33 to mice induced the disease symptoms [48].

The monoclonal antibody therapy is quite expensive and it is important to search for new cheaper evidence-based treatment options. Small interfering RNAs (siRNA) is a promising therapeutic tool for personalized treatment of various life-threatening diseases because it is able to regulate the expression of disease-causing genes in a sequence-specific manner [49]. As has been shown in the current study intranasal administration of the siRNA targeted to the *il33* gene reduce *il33* expression in lungs, level of Th2 –dependent IgG1 antibodies in serum, total cell count and inflammation in lungs (Fig. 3B, F, J, O), but do not influence the viral load and *il5*, *il13* expression (Fig. 3A, D, E). Decreased level of IgG1 indicates Th2-to-Th1 switch. It has been previously shown that treatment with IL-33 elevates levels of total serum IgA, IgE, and IgG1, but decreases IgG2a [50]. In our study we did not find significant changes in IgG2a and IgE level probably due to local intranasal route of administration of the naked siRNA which minimized the systemic effects. However, local effect from siRNA treatment in the lungs such as reduced inflammation was observed.

Interestingly, decreased *ifn- γ* mRNA expression has been found in mice treated with siRNA targeted *il33* (Fig. 3C). Similar effect was observed in the study of Yuejin Liang et al. that revealed that IL-33 deficiency led to decreased levels of IFN- γ expression in mice infected with lymphocytic choriomeningitis virus [51]. Taken together with the data from model experiment discussed above we suggest that IL-33 may play a crucial role in provoking IFN- γ production in RSV infection.

We found an increased expression of *il33* caused by RSV infection, but we did not find any changes in the amount of viral RNA after treatment with anti-*il33* siRNA. This finding is in contradiction with the study of R. Werder et al. showing that treatment of mice with anti-IL33 antibodies reduced rhinovirus replication [52]. On the other hand treatment of mice with anti-ST2 antibodies had no effect on RSV load in mice [32]. One should note that action of IL-33 in viral infection is highly virus specific, i.e. dependent on virus type [53]. For example, IL-33 reduced the viral load of coxsackievirus B in mice [54], but exacerbated influenza A virus-induced airway inflammation [55].

Finally, we evaluated *IL33* expression in *ex vivo* stimulated blood cells isolated from infected/non infected asthmatic and healthy volunteers (BA, BA + ARVI, Healthy, Healthy + ARVI groups). BA and BA + ARVI groups of patients were characterized by a decreased value of FEV1, increased ACQ score, increased blood eosinophilia (Fig. 4A, B, D). Low FEV1 levels usually correlate with BA diagnosis in clinic. An ACQ score above 1.5 typically reflects poor prognosis. Cases within this range are characterized by uncontrolled asthma progression and disease state exacerbation due to allergen exposure and/or coincident ARVI infection. No difference was observed between BA and BA + ARVI groups for eosinophil count. This indicates that BA is the main driver of eosinophil activation, regardless of ARVI infection status. Taken together these data confirm allergic BA status in volunteers. In addition, an increased number of leukocytes was detected in BA + ARVI group. The data suggest that viral infection in the context of pre-existing BA serves to exacerbate peripheral leukocyte activation to higher levels

than seen in either of these conditions (BA or ARVI infection).

Elevated level of the *IL33* mRNA was detected in blood samples of subjects from BA + ARVI group. In human increased IL-33 and type 2 cytokines were observed during experimental rhinovirus-induced asthma exacerbation [56] and elevated IL-33 and IL-13 were observed in nasal aspirates from infants hospitalized with RSV [33]. Moreover, serum levels of IL-33 were closely associated with the degree of liver injury in patients with hepatitis B and C virus infections [57,58]. The data confirm a crucial role of IL-33 in viral infection in human, especially in virus-induced asthma exacerbation. Notably IL-33 can promote allergic inflammation by the selective activation of the basophils and eosinophils [59]. In addition, we evaluated diversity of the respiratory viruses caused ARVI symptoms in volunteers (in healthy subjects and patients with BA) (Fig. 4G, H). In accordance with data from different groups RSV (58%) and RV (33%) as single infection or in combination with another viruses were the most detected viral pathogens for infected asthma patients and healthy subjects, respectively [60]. Despite the fact that RSV infection is usually associated with bronchiolitis in infants and young children there is evidence to support the role of RSV as an important cause of acute respiratory illness in adults too [61,62]. These include the elderly, subjects with cardiopulmonary diseases, and immunocompromised hosts. Therefore, people with asthma may experience asthma attacks as a result of RSV infection. For this reason, in the current study we have evaluated RSV and allergic asthma interactions using the mouse model.

In conclusion, the present study provides additional evidence of the crucial role of the IL-33 in pathogenesis of RSV infection and virus-induced bronchial asthma exacerbations. Treatment with anti-*il33* siRNA diminishes airway inflammation in the lungs of mice with RSV-induced allergic asthma exacerbation, but do not influence the viral load. Taken together the data prove that IL-33 is a promising target for asthma therapy.

Author contribution

All authors contributed to the writing of the manuscript and have approved the final version for publication. Conceptualization, Methodology: Khaitov M.R., Nikonova A.A., Shilovskiy I.P., Kurbacheva O.M., Khaitov R.M., Kudlay D.A.; Investigation: Galitskaya M.A., Sokolova A.R., Sundukova M.S., Mitin A.N., Komogorova V.V., Litvina M.M., Sharova N.I., Zhernov Y.V.; Formal analysis: Nikonova A.A., Shilovskiy I.P., Gaisina A.R., Dvornikov A.S.; Writing - Original Draft: Nikonova A.A., Dmitrieva-Posocco O., Shilovskiy I.P., Kurbacheva O.M., Khaitov M.R.; Writing - review & editing: Nikonova A.A., Galitskaya M.A., Mitin A.N., Kurbacheva O.M., Khaitov M.R.

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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Appendix A. Supplementary data

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