



Therapeutic effects of R8, a semi-synthetic analogue of Vasicine, on murine model of allergic airway inflammation via STAT6 inhibition

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ABSTRACT

This is a follow-up study of our previous work in which we screened a series of Vasicine analogues for their anti-inflammatory activity in a preventive OVA induced murine model of asthma. The study demonstrated that R8, one of the analogues, significantly suppressed the Th2 cytokine production and eosinophil recruitment to the airways. In the present study, we have been using two standard experimental murine models of asthma, where the mice were treated with R8 either during (preventive use) or after (therapeutic use) the development of asthma features. In the preventive model, R8 reduced inflammatory cell infiltration to the airways, OVA specific IgE and Th2 cytokine production. Also, the R8 treatment in the therapeutic model decreased methacholine induced AHR, Th2 cytokine release, serum IgE levels, infiltration of inflammatory cells into the airways, phosphorylation of STAT6 and expression of GATA3. Moreover, R8 not only reduced goblet cell metaplasia in asthmatic mice but also reduced IL-4 induced Muc5AC gene expression in human alveolar basal epithelial cells. Further, R8 attenuated IL-4 induced differentiation of murine splenocytes into Th2 cells in vitro. So, we may deduce that R8 treatment profoundly reduced asthma features by attenuating the differentiation of T cells into Th2 cells by interfering with the binding of IL-4 to its receptor in turn decreasing the phosphorylation of STAT6 and expression of GATA3 in murine model of asthma. These preclinical findings suggest a possible therapeutic role of R8 in allergic asthma.

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1. Introduction

Asthma is a chronic inflammatory airway disease affecting over 300 million people worldwide with an expected increase of a further 100 million by 2025 [1,2]. Despite remarkable advances in diagnosis and treatment, asthma is still a serious public health problem, particularly due to off targets and side effects of commonly available anti-asthma drugs. Asthma has shown a drastic increase in the global prevalence, morbidity, mortality and economic burden over the last 40 years, especially in children [3].

The features of asthma are mediated by dominant T helper 2 (Th2) immune response and characterized by airway hyperresponsiveness (AHR), airway inflammation, increased IgE levels and mucus hypersecretion. Stimulation of T cell receptor by antigen ligation causes the differentiation of peripheral naive CD4⁺ T cells into effector T cells that

produce increased levels of cytokines. Based on the cytokine signature, these cells are grouped into Th1, Th2, Th17 and regulatory T-cell subsets. These cells have been shown to modulate the features of allergic asthma in both animals and humans. The amount of Th2 cytokines is elevated in the airway tissues of asthmatics and animal models [4–6]. Individually or synergistically, Th2 cytokines mediate most of the asthma features. IL-5 is known to play an imperative role in eosinophil maturation, differentiation, recruitment and survival. IL-4 and IL-13 are essential in IgE (immunoglobulin E) class switching in B cells. IL-13 is however known to play the most important and central role in allergic asthma [7]. It contributes in airway fibrosis, AHR, mucus production, IgE synthesis and airway inflammation [8]. The Th2 cell differentiation is induced upon binding of interleukin 4 (IL-4) to its receptor, causing phosphorylation of signal transducer and activator of transcription protein 6 (STAT6) through the Jak/STAT cascade. The phosphorylated STAT6 forms a homodimer and translocates into the nucleus where it binds to specific DNA sequences and activates transcription of cytokine responsive genes, especially GATA3, which is the master regulator of Th2 cell differentiation [9]. GATA3 also activates the transcription of IL-5 and IL-13 genes by directly binding to their promoters [10].

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Moreover, this transcription factor is also involved in remodeling of chromatin structure and opening of IL-4 locus [11].

In this study, we evaluated the therapeutic potential of R8, a semi-synthetic analogue of Vasicine, against asthma along with its preventive potential. The R8 was administered before and after the development of asthma features in preventive and therapeutic model of asthma, respectively. It was observed that R8 significantly reduced asthma features like AHR, airway inflammation, mucus hypersecretion, IgE and Th2 cytokine production. The results suggest that R8 reduces asthma features by acting on T cells and blocking their differentiation into Th2 cells via competitive binding to the IL-4 receptor, resulting in inhibition of STAT6 phosphorylation and GATA3 expression, two prime transcription factors for Th2 cell differentiation and asthma pathogenesis.

2. Material and methods

2.1. About the test compound

R8 (5-Benzoyl-5a,6,7,8,9,10-hexahydroazepino[2,1-b]quinazoline-12(5H)-one; molecular weight 320, exact mass: 320.15) is a semi-synthetic analogue of Vasicine, an alkaloid isolated from *Adhatoda vasica* Nees. R8 was synthesized by us as described earlier [12]. The doses of R8 used in this study were selected on the basis of EC₅₀ evaluated in our previous study [12]. It was dissolved in PBS before oral administration to animals.

2.2. Animals

The animal care and handling were done according to the guidelines issued by the Indian National Science Academy, New Delhi, India, 1992 and all animal experiments were approved by the Institutional Animal Ethics Committee (IAEC, CSIR-Indian Institute of Integrative Medicine, Jammu, India), approved by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), Ministry of Environment and Forests, Government of India. Eight to ten week old male BALB/c or Swiss mice weighing between 20 and 22 g were used and were acclimatized for 7 days for the pre-clinical studies.

2.3. Toxicity assessment

R8 was evaluated for acute oral toxicity in Swiss mice as per the OECD guideline-420 [13]. One group of Swiss mice received an oral dose of 2000 mg/kg (Limit dose) of R8 and another group was kept as control. The animals were kept in Perspex chambers and monitored for any toxic symptoms for constant 4 h and then intermittently up to 24 h. Animals were further observed daily for next 13 days for mortality and observable toxicity, as described previously [14]. Finally, the number of survivors was noted. Blood was collected through cardiac puncture and the animals were sacrificed. Hematological parameters were measured using an automatic hematology analyzer (XT-1800i, Sysmex, USA) and the biochemical parameters were measured using a semi-automatic biochemical analyzer (Chem-7, Erba, Mannheim, Germany).

2.4. Grouping, sensitization, challenge and treatment of mice

There were two experimental OVA models:

- Preventive model, OVA/OVA/R8 (7.5, 15 and 30 mg/kg)
- Therapeutic model, OVA/OVA/R8 (7.5, 15 and 30 mg/kg)

Six groups of mice ($n = 5-6$ in each): SHAM/PBS/VEH (normal controls, VEH-vehicle), OVA/OVA/VEH (OVA controls, OVA, chicken egg ovalbumin, Grade V, Sigma), OVA/OVA/DEXA-0.75 mg/kg (Dexamethasone, Sigma) and OVA/OVA/R8-7.5, 15 and 30 mg/kg were used in both preventive and therapeutic models.

In both models, mice were sensitized on days 0, 7 and 14 with 50 μ g OVA adsorbed on 4 mg alum or 4 mg alum alone (SHAM control) and were challenged from Day 21 to Day 32 with 3% OVA or PBS (for SHAM control) consecutively as described earlier [15]. R8 and dexamethasone were dissolved in PBS and administered orally, twice and once a day respectively. R8 and dexamethasone were administered from day 20 to day 32 in preventive model and day 24 to day 27 in therapeutic model. These mice were euthanized on day 33 in preventive model and day 28 in therapeutic model.

2.4.1. Bronchoalveolar lavage (BAL), sera separation and cell count

On day 28 or 33, each mouse was sacrificed, BAL was performed and BAL fluids were processed to separate cell pellets and supernatants, as described earlier [15]. Briefly, the collected BAL fluid was centrifuged at 1000 rpm, for 10 min at 4 °C. The cell pellets were washed thrice and resuspended in PBS; a small portion was taken to evaluate the total cell number; and differential cell counts were estimated after staining with Leishman's stain [16]. Blood was withdrawn by cardiac puncture and serum was separated, as described previously [16].

2.4.2. Measurement of airway hyperresponsiveness

Airway resistance to methacholine (Sigma-Aldrich, USA) was evaluated, giving a measure of airway hyperresponsiveness, using invasive airway mechanics/flexiVent (Scireq, Canada), that is able to integrate the computer-controlled mouse ventilator with the respiratory mechanic measurements. Every anesthetized mouse [ketamine (20 mg/kg) i.p., and thiopental sodium (40 mg/kg) i.p.] was tracheally intubated and ventilated and airway resistance measured with increasing doses of methacholine [0 mg/mL (PBS alone), 2, 4, 8, 12, 16, 20 and 25 mg/mL] methacholine [15].

2.4.3. Measurement of IL-4, IL-5 and IL-13 in lung homogenates

Lung tissue homogenates (prepared by homogenization of 50 mg of tissue with 500 μ L of PBS and centrifugation at 10,000 g for 30 min) in duplicate were used for sandwich ELISA (R&D Systems) for IL-4, IL-5 and IL-13 detection. Results are expressed in picograms/100 μ g protein.

2.4.4. Gene expression analysis of IL-4, IL-5 and IL-13

The mRNA expressions of IL-4, IL-5, and IL-13 from the lung homogenates were done by real-time quantitative PCR. RNA was extracted from the flash-frozen mice lungs using TRIzol reagent, according to the manufacturer's protocol (Invitrogen, Canada). The quantity and integrity of RNA were quantified by using a Nanodrop spectrophotometer (Thermo Fischer, USA) and agarose gel electrophoresis. Equal quantities of RNA (3 μ g) from control as well as treated samples were used for cDNA synthesis using Revertaid First strand cDNA synthesis kit (Fermantas, USA) and oligo (dT) primers as previously described [17]. Primer sequences were designed by Primer Express software (ABI, USA) for all the genes studied, GAPDH forward: 5'-TGTGTCCGTCGTGGATCTGA-3'; GAPDH reverse: 5'-TGCTGCTTCACCACCTTCT-3'; IL-5 forward: 5'-GGAGATGGAACCAAGGCTT-3'; IL-5 reverse: 5'-GATGCAAC

Table 1A

Mean values of various biochemical parameters, measured from sera of female mice treated with R8/vehicle (single oral dose). Values are represented as mean $\pm$ SEM ($n = 5$ /group), Student's *t*-test.

Parameters	Control	Treated
Glucose (mg/dL)	101.8 $\pm$ 8.5	108.3 $\pm$ 19.4
Triglyceride (mg/dL)	158.4 $\pm$ 15.6	162.3 $\pm$ 20.2
Cholesterol (mg/dL)	85.4 $\pm$ 6.4	77.1 $\pm$ 5.6
SGOT (IU/L)	103.4 $\pm$ 18.8	109.8 $\pm$ 11.4
SGPT (IU/L)	45.3 $\pm$ 6.7	41.7 $\pm$ 5.04
Total protein (g/dL)	4.05 $\pm$ 0.11	5.22 $\pm$ 0.15
Creatinine (mg/dL)	0.71 $\pm$ 0.05	0.62 $\pm$ 0.053
Urea (mg/dL)	38.2 $\pm$ 2.5	44.19 $\pm$ 4.4
Bilirubin (mg/dL)	0.13 $\pm$ 0.06	0.11 $\pm$ 0.04
ALP (IU/L)	173.1 $\pm$ 11.3	181.5 $\pm$ 16.1

Table 1B

Mean values of various hematological parameters measured from whole blood of female mice treated with R8/vehicle (single oral dose). Values are represented as mean $\pm$ SEM ($n = 5/\text{group}$), Student's *t*-test.

Parameters	Control	Treated
WBC ($10^3/\mu\text{L}$)	10.8 $\pm$ 0.9818	11.34 $\pm$ 1.825
RBC ($10^6/\mu\text{L}$)	9.478 $\pm$ 0.2003	9.918 $\pm$ 0.4823
HGB (g/dL)	13.1 $\pm$ 0.3162	13.2 $\pm$ 0.7855
HCT (%)	41.56 $\pm$ 0.9146	40.76 $\pm$ 2.167
MCV (fL)	43.9 $\pm$ 0.9935	39.28 $\pm$ 1.76
MCH (pg)	13.84 $\pm$ 0.2676	13.28 $\pm$ 0.1855
MCHC (g/dL)	31.54 $\pm$ 0.5419	32.38 $\pm$ 0.6522
PLT ($10^3/\mu\text{L}$)	147 $\pm$ 139.8	144.1 $\pm$ 69.4
NEUT (%)	19.46 $\pm$ 3.903	19.52 $\pm$ 8.712
LYMPH (%)	74.08 $\pm$ 4.27	54.26 $\pm$ 15.18
MONO (%)	4.5 $\pm$ 0.68	5.24 $\pm$ 0.5418
EO (%)	2.1 $\pm$ 0.09487	1.8 $\pm$ 0.2345
BASO (%)	0.06 $\pm$ 0.029	0.06 $\pm$ 0.025

GAAGAGGATGAGG-3'; IL-4 forward: 5'-TCGGCATTGTAACGAGGTC-3'; IL-4 reverse: 5'-TTGCTGTGAGGACGTTGGC-3'; IL-13 forward: 5'-TTCCCTGACCAACATCTCCAA-3'; IL-13 reverse: 5'-TTGCGGTACAGAGGCCA TG-3'. Real-time PCR reactions were performed in triplicates using SYBR Premix Ex Taq (Takara, Dalian, China) in 48-well optical plates using ABI StepOne Real-time qPCR system (Applied Biosystems, Foster City, CA, USA). The PCR reaction (20 μL) included 0.2 μL cDNA template, 200 nM each of the primers and 10 μL 2 $\times$ SYBR Premix Ex Taq. The cycling parameters were 95 $^{\circ}\text{C}$ for 20 s, followed by 40 cycles of 95 $^{\circ}\text{C}$ for 15 s and 60 $^{\circ}\text{C}$ for 1 min.

2.4.5. Measurement of OVA-specific immunoglobulin E (IgE)

OVA specific IgE levels were measured from serum as per the previously described method [15], with minor modifications. Briefly, 2 μg OVA was coated in each well of the microplate before adding sera and bound IgE were detected by biotinylated anti-mouse IgE (BD Pharmingen) and streptavidin-horseradish peroxidase (HRP) conjugates (BD Pharmingen). The absorbances were converted to arbitrary units.

2.4.6. Histopathology of lungs

Lung tissues were fixed in 4% formaldehyde and then paraffin embedded. The 6 μm sections were cut, mounted on Superfrost glass slides (Fischer Scientific) and stained with hematoxylin & eosin or periodic acid-Schiff (PAS) (all from Sigma-Aldrich) to assess inflammatory changes and goblet cell metaplasia, respectively as described earlier [16,18].

2.4.7. Western blot analysis

The lungs were homogenized, using an Ultra-turrax, in a homogenizing buffer (1% NP-40, 150 mM NaCl, 50 mM Hepes, PMSF, and complete protease/phosphatase inhibitor cocktail). Protein estimation of the supernatant was done by Bradford method. For western blotting, 40–50 μg of the protein was denatured at 100 $^{\circ}\text{C}$ for 5 min in Tris–Glycine SDS sample loading buffer (63 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, 0.0025% bromophenol blue, 5% β -mercaptoethanol). Protein samples were applied to SDS gels and resolved at 70 V (300 mA) for 3 h and then electro-transferred to polyvinylidene difluoride (PVDF) membrane (BioRad, Hercules, CA USA) in transfer buffer (25 mM Tris, 192 mM Glycine and 20% methanol), using BioRad Mini Transblot

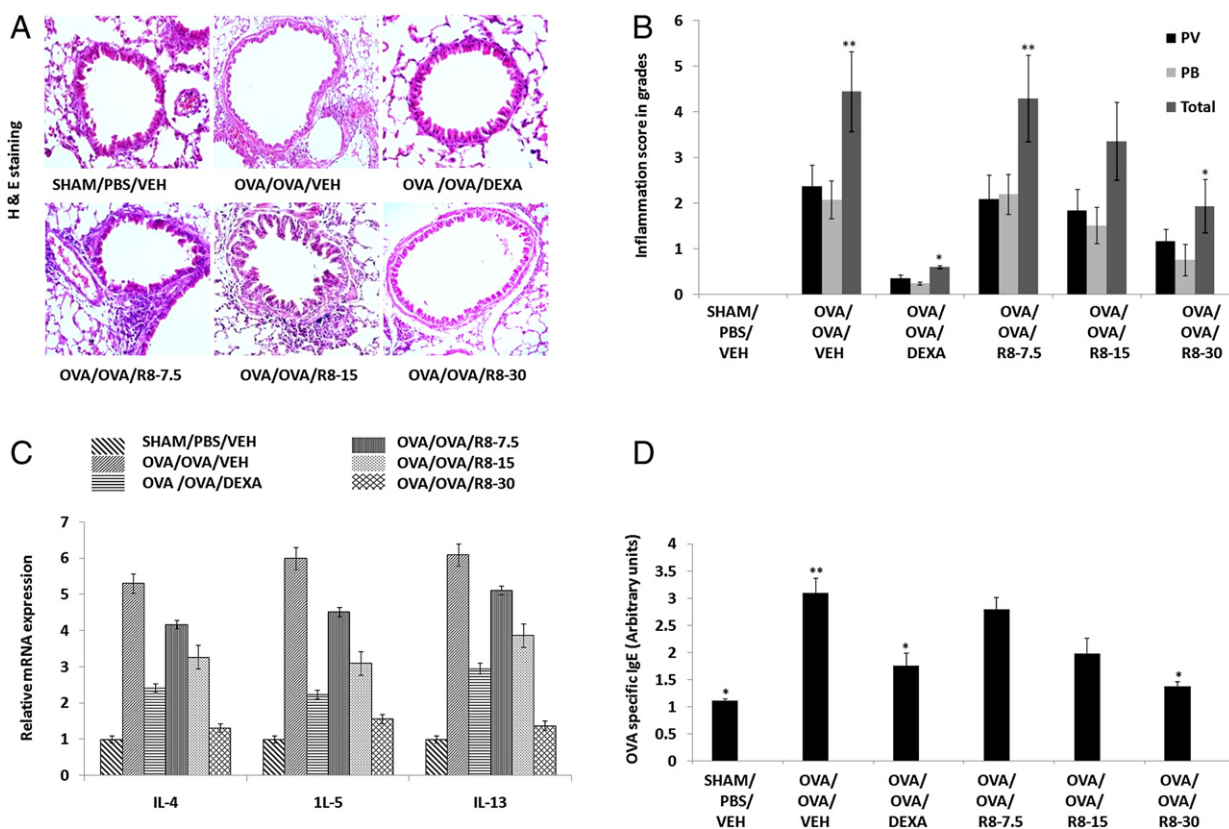


Fig. 1. (A) Preventive effect of R8 on airway inflammation evaluated using hematoxylin and eosin staining. Representative photographs of stained lung tissue sections are shown; all photographs are at $\times 40$ magnification. (B) Effects of R8 treatment on perivascular (PV), peribronchial (PB), and total lung inflammation scores. (C) Relative mRNA expression of IL-4, IL-5, and IL-13 in lung homogenates. Oral administration R8 significantly reduced the expression of these cytokines in a dose dependent manner. (D) Effects of R8 on OVA-specific IgE levels. A significant reduction of OVA-specific IgE levels in sera was observed in R8 treated animals, with a maximum decrease at 30 mg/kg. Data is represented as mean $\pm$ SEM, *denotes $p < 0.05$, as compared to OVA/OVA/VEH, and **denotes $p < 0.05$, as compared to SHAM/PBS/VEH, Student's *t*-test.

Table 2

Effects of R8 on total and differential cell count in BAL fluid in a preventive murine model. *denotes $p < 0.05$, as compared to the OVA/OVA/VEH, and **denoted $p < 0.05$, as compared to the SHAM/PBS/VEH using *t*-test. TCC, total cell count; macro, macrophage; mono, mononuclear cells (monocytes and lymphocytes); neutro, neutrophils; eosino, eosinophil.

	TCC ($\times 10^4/\text{mL}$)	Differential count (in percentage)			
		Macro	Mono	Eosino	Neutro
SHAM/PBS/VEH	$5.2 \pm 2.0^*$	39.4 ± 4.2	$52.6 \pm 7.1^*$	$1.3 \pm 0.8^*$	$3.1 \pm 1.2^*$
OVA/OVA/VEH	$56.4 \pm 16.1^{**}$	5.4 ± 8.1	$16.3 \pm 7.1^{**}$	$54.6 \pm 9.8^{**}$	18.6 ± 6.1
OVA/OVA/R8-7.5	26.5 ± 9.7	21.2 ± 5.3	26.4 ± 8.6	29.8 ± 5.1	17.7 ± 4.5
OVA/OVA/R8-15	18.1 ± 4.3	35.6 ± 7.1	28.5 ± 6.4	$17.5 \pm 4.8^*$	13.3 ± 3.1
OVA/OVA/R8-30	$17.5 \pm 6.5^*$	38.6 ± 3.2	46.2 ± 16.4	$6.4 \pm 5.0^*$	10.6 ± 4.1
OVA/OVA/DEXA	13.3 ± 7.4	41.3 ± 10.4	38.3 ± 7.9	$11.4 \pm 3.7^*$	$1.1 \pm 1.2^*$

Electrophoretic transfer Cell for 90–120 min at 150 V. Membranes were blocked in 5% fat free dry milk or 3% BSA dissolved in TBST (20 mM Tris, pH 8.0, 500 mM sodium chloride, 0.5% Tween 20) for 2 h at room temperature. Anti-STAT6, anti-p-STAT6 and anti-GATA3 antibodies were used to determine expression of corresponding proteins.

2.4.8. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptotic assay

TUNEL assay of apoptosis was performed on lung tissue sections using an in situ apoptosis detection kit (Dead End Calorimetric TUNEL system; Promega) as described earlier [19,20].

2.5. Cell culture

Human adenocarcinoma cell line A549, purchased from American Type Culture Collection (no. CCL-185; Manassas, VA) was cultured with DMEM containing 10% FBS, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin. The cells (1×10^6 cells/well) were seeded into six-well plates (Costar, Corning, NY, USA) and grown overnight in complete media. Cells were then washed and maintained in serum-free media overnight before incubation with vehicle (PBS) or R8 at the concentrations indicated in the figure legends.

2.5.1. Effect on expression of Muc5AC in human lung epithelial cells

The A549 cells (1×10^6 cells/well) were stimulated with 50 ng/mL of rIL-4 (R & D systems) and RNA was collected (as described above) for real time expression of Muc5AC gene. Primer sequences were designed by Primer Express software (ABI, USA) for Muc5AC, GAPDH forward: 5'-TGTGTCCGTCTGGATCTGA-3', GAPDH reverse: 5'-TGCTGCTTCACACCTTCT-3'; Muc5AC forward: 5'-CGTGTGTGTCACGAGAACGT-3', reverse 5'-ATCTTGATGGCCTTGAGCA-3'. Real-time PCR reactions were performed in triplicates using SYBR Premix Ex Taq (Takara, Dalian, China) in 48-well optical plates using ABI StepOne Real-time qPCR system (Applied Biosystems, Foster City, CA, USA). The PCR reaction (20 μL) included 0.2 μL cDNA template, 200 nM each of the primers, and 10 μL 2 X SYBR Premix Ex Taq. The

cycling parameters were 95 °C for 20 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.6. Th cell differentiation

Spleens were harvested aseptically from naive Balb/c mice and minced into small pieces in RPMI medium supplemented with 10% FCS, 1% penicillin/streptomycin and 50 μM β -mercaptoethanol, as described previously with some modifications [21–23]. This was followed by filtration of the suspension and centrifugation of the filtrate at 4 °C. The pellet was then treated with a hemolysis buffer to eradicate red blood cells. The splenocytes were then resuspended in RPMI medium, stimulated with concanavalin A (2.5 $\mu\text{g}/\text{mL}$) and subsequently treated with the test compound at 100 μM and cultured (3.5×10^5 cells per well). Cells were then incubated at 37 °C with 5% CO₂. After 24 h, the cells were treated with different recombinant and neutralizing antibodies, e.g. for Th1 differentiation, the cells were cultured in the presence of anti-IL-4 neutralizing antibody (10 $\mu\text{g}/\text{mL}$), recombinant mouse IFN- γ (5 ng/mL) and recombinant mouse IL-12 (5 ng/mL), with or without test compound, R8. Similarly, for Th2 differentiation, cells were cultured with recombinant mouse IL-4 (4 ng/mL), anti-IL-12 (10 $\mu\text{g}/\text{mL}$) and anti-IFN- γ (10 $\mu\text{g}/\text{mL}$) antibodies, with or without R8 and for Th17 cell differentiation, cells were cultured with anti-IFN- γ , anti-IL-12, anti-IL-4 (10 $\mu\text{g}/\text{mL}$ each), recombinant mouse IL-6 (4 ng/mL) and TGF- β (1 ng/mL) antibodies with or without R8. The cells were again incubated at 37 °C with 5% CO₂. After 48 h of treatment with antibodies, the cell supernatant was used to confirm the differentiated states of cells by performing ELISA of IFN- γ , IL-4 and IL-17.

2.7. Statistics

Data is shown as mean $\pm$ SEM and was statistically analyzed using Students Newman Keul's test or *t*-test. The $p < 0.05$ was considered to be statistically significant.



Fig. 2. Protocol for induction of allergic asthma in mice. The schematic diagram shows the protocol for inducing allergic asthma in mice and their treatment by R8 and dexamethasone (DEXA) in a therapeutic mode. Mice were sensitized and challenged using ovalbumin (OVA) as an allergen and using alum as an adjuvant. Sensitizations were done on days 0, 7 and 14. The animals were challenged using 3% OVA, from day 21 to day 27 and treated by R8/DEXA after the development of asthma i.e. from day 24 to day 27 and on day 28, the airway hyperresponsiveness was estimated.

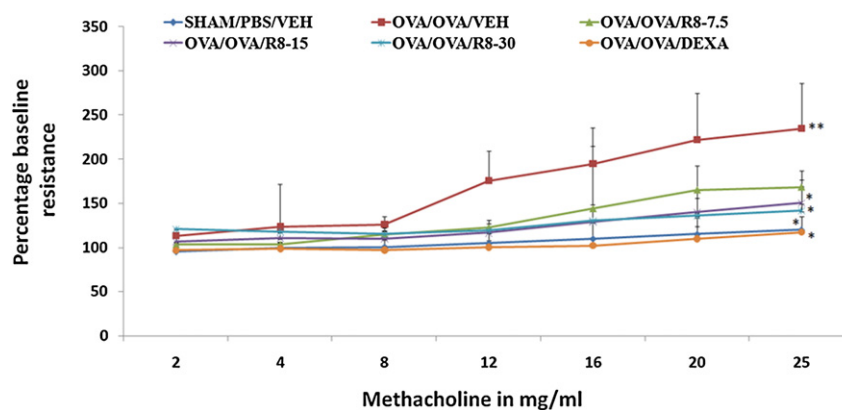


Fig. 3. Effect of R8 on airway hyperresponsiveness during allergic asthma. R8 administration reduces airway hyperresponsiveness in mice as measured by a methacholine dose responsive cure for airway resistance. Data is represented as mean $\pm$ SEM, *denotes $p < 0.05$, as compared to OVA/OVA/VEH, and **denotes $p < 0.05$, as compared to SHAM/PBS/VEH, Student's *t*-test.

3. Results

3.1. Effect of R8 on acute toxicity biochemical and hematological parameters

R8 did not show any treatment related toxic manifestations and mortality up to an oral dose of 2000 mg/kg (b/w), as compared to the vehicle control animals. The animals did not show any changes in the general appearance during the observation period of two weeks. Also, no significant change was observed in weekly body weight, feed & water consumption (data not shown) and any of the hematological (Table 1A) or biochemical parameters (Table 1B).

3.2. R8 prevents the development of allergic airway inflammation

To determine the preventive effect of R8 on airway inflammation, mice were sensitized and challenged as described in Materials and methods. As shown in Fig. 1A, PBS sensitized and challenged (SHAM/PBS/VEH) mice showed a normal bronchovascular structure without any signs of inflammation. However, OVA sensitized and challenged mice (OVA/OVA/VEH) showed accumulation of recruited inflammatory cells in the bronchovascular regions. In contrast, OVA sensitized and challenged mice treated with different concentrations of R8, showed a dose dependent reduction in the recruitment of inflammatory cells in the bronchovascular regions (Fig. 1A and B).

3.3. R8 reduces airway eosinophilia in preventive model

As there was a reduction in the recruitment of inflammatory cells by R8 treatment, we wanted to validate this by estimating the cell counts in the BAL fluid. As shown in Table 2, BAL fluid analysis revealed that there was a significant increase in the number of infiltrated cells, including eosinophils, in OVA/OVA/VEH mice as compared to SHAM/PBS/VEH. However, OVA mice administered with different concentration of R8 or Dexamethasone in the preventive model showed a reduction in total cell counts in BAL fluid, as well as absolute numbers of macrophage, neutrophil and eosinophil as compared to OVA/OVA/VEH (Table 2).

3.4. R8 reduces the levels of Th2 cytokines in preventive model

As there was a reduction in the recruitment of inflammatory cells by R8 administration, we wanted to determine the expression of Th2 cytokines such as IL-4, IL-5 and IL-13 that are closely associated with the pathogenesis of asthma. The mRNA expression of these cytokines in lung was estimated, as described in Material and methods. The SHAM/PBS/VEH mice showed lower expression of IL-4, IL-5 and IL-13, whereas they were markedly elevated in OVA/OVA/VEH mice. However, oral administration of R8 significantly reduced the expression of these cytokines in a dose dependent manner (Fig. 1C). So, the oral administration R8 in the preventive model was effective in reducing the Th2 cytokine

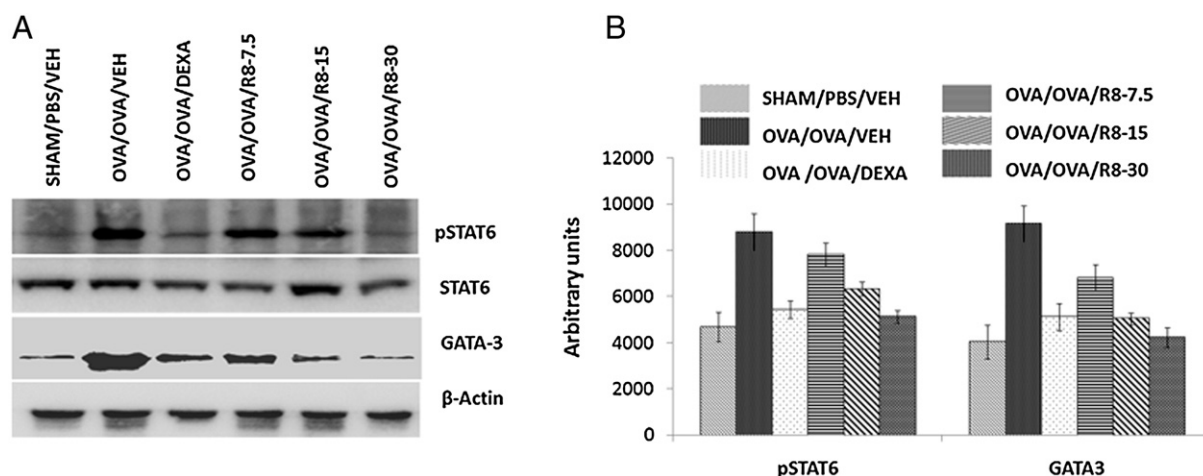


Fig. 4. Effect of R8 on expression of Th2 specific transcription factors. (A) R8 downregulated GATA3 expression and STAT6 phosphorylation in a dose depending manner, measured by western blotting. (B) Quantitative analysis of GATA3 and pSTAT6 by densitometry.

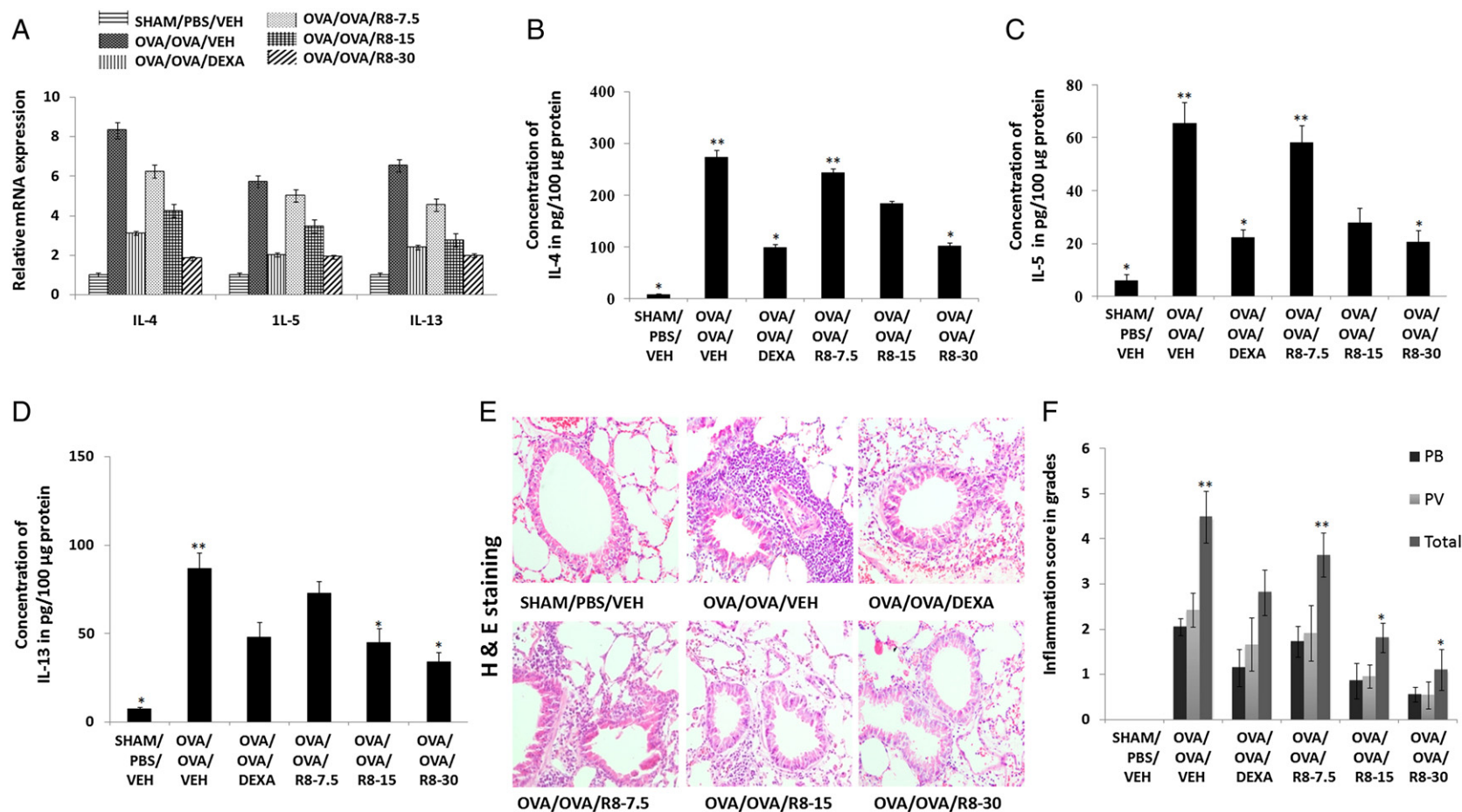


Fig. 5. (A) Relative mRNA expression of IL-4, IL-5, and IL-13 in lung homogenates, by real-time quantitative PCR measured from lung homogenates of therapeutic mouse model. Measurement of (B) IL-4, (C) IL-5, and (D) IL-13 levels from lung homogenates through ELISA. The results obtained for IL-4, IL-5 and IL-13, both at protein and gene level are in agreement with each other with the highest effect of R8 observed at 30 mg/kg. (E) Effect of R8 on airway inflammation in therapeutic mouse model evaluated using hematoxylin and eosin staining, performed in lung tissue sections. Representative photographs are shown; all photographs are at $\times 40$ magnification. (F) Effect of R8 treatment on perivascular (PV), peribronchovascular (PB), total lung inflammation scores. Data is represented as mean $\pm$ SEM, *denotes $p < 0.05$, as compared to OVA/OVA/VEH, and **denotes $p < 0.05$, as compared to SHAM/PBS/VEH, Student's *t*-test.

levels significantly and the maximum fall was observed at 30 mg/kg dose and this reduction is comparable to dexamethasone treatment.

3.5. R8 reduces OVA-specific IgE in sera

Since there was a reduction in various Th2 cytokines and IL-4 and IL-13 are required for IgE class switching, we determined the serum levels of OVA-specific IgE as described in [Materials and methods](#). The SHAM/PBS/VEH mice showed lower levels of OVA specific IgE whereas significantly increased levels were produced in OVA groups ([Fig. 1D](#)). The levels of OVA specific IgE were dose dependently reduced with R8 treatment in preventive model with a maximum decrease at 30 mg/kg dose and this reduction was similar to dexamethasone treatment.

3.6. R8 reduces airway hyperresponsiveness to methacholine in therapeutic model

As demonstrated above, we observed a reduction in the features of allergic airway inflammation in the preventive murine model of asthma. However, it is always essential to understand the effect of any test drug in a therapeutic model since asthmatic patients come to the hospital after the development of the asthma symptoms. In our earlier studies, we demonstrated that after 3 consecutive OVA challenges, mice showed the features of allergic airway inflammation such as airway hyperresponsiveness, airway inflammation and mucus metaplasia. So we administered R8 from the fourth day of challenge to see its therapeutic effect ([Fig. 2](#)). It is important to note that OVA or saline challenges were continued during the phase of treatment as stoppage of challenges would lead to alleviate the features of allergic airway inflammation. On day 28, AHR was estimated in mice as described in [Materials and methods](#). As shown in [Fig. 3](#), there was an increase in airway resistance with increasing doses of methacholine in OVA/OVA/VEH group as compared to SHAM/PBS/VEH. However, this increase in airway resistance was reduced in other OVA challenged groups treated with different doses of R8 or dexamethasone.

3.7. R8 reduces expression of pSTAT6 and GATA3 in lungs

In preventive model, we found a significant fall in expression of Th2 cytokines with R8 treatment. Since, STAT6 and GATA3 are the principal transcription factors responsible for Th2 cell differentiation and the expression of Th2 cytokines is under direct transcriptional regulation of GATA3 which in turn is activated by phosphorylated STAT6, so we evaluated the effect of R8 on the expression of STAT6, phosphorylated STAT6 and GATA3 in the therapeutic mouse model through western blotting. It was observed that R8 treated mice showed a decreased expression of GATA3 and pSTAT6 in a dose dependent manner ([Fig. 4](#)).

3.8. R8 reduces the expression of Th2 cytokines in a therapeutic model of allergic airway inflammation

As R8 decreased the expression of GATA3 which is essential for the initiation of the expression of Th2 cytokines such as IL-4, IL-5 and IL-13, we wanted to measure their mRNA expression in lungs of R8 treated allergic mice. Expectedly, there was an increase in the mRNA expression and protein levels of all these cytokines in OVA/OVA/VEH mice ([Fig. 5A–D](#)). However, R8 treatment of the allergic mice showed significant reduction in the expression and production of IL-4, IL-5 and IL-13 and these reductions were comparable to dexamethasone treatment ([Fig. 5A–D](#)). So the decrease in the expression of GATA3 was corroborated with the levels of Th2 cytokines. Thus, it can be concluded that R8 decreased the production of IL-4, IL-5 and IL-13 via its inhibitory effect on GATA3 expression which occurs only after phosphorylation of STAT6 which was also reduced by R8.

3.9. R8 reduces the airway inflammation in a therapeutic asthma model

As there was a reduction in expression and production of Th2 cytokines, which are responsible for the recruitment of inflammatory cells in the airway, we determined this effect using histopathology and BAL fluid analysis. As shown in [Fig. 5E, F](#) and [Table 3](#), there was an increase in inflammation around the bronchovascular regions and more eosinophils were observed in the BAL fluid of OVA/OVA/VEH mice as compared to SHAM/PBS/VEH mice. However, R8 treatment in a therapeutic asthma model showed a significant reduction of airway inflammation and airway eosinophilia and similar results were observed in dexamethasone treated group ([Fig. 5E](#) and [Table 3](#)).

3.10. R8 reduces OVA specific IgE and Goblet cell metaplasia in a therapeutic asthma model

Since there was a reduction in the expression of IL-4 and IL-13 which are crucial for IgE class switching and goblet cell metaplasia, we wanted to evaluate these parameters in the therapeutic asthma model also. As shown in [Fig. 6](#), there was an increase in the levels of OVA specific IgE and bronchial epithelial mucus in OVA/OVA/VEH mice as compared to SHAM/PBS/VEH mice. However, both of these features were significantly reduced with R8 treatment.

Since asthma features were reduced drastically with 30 mg/kg of R8, we wanted to determine the effect of this dose on goblet cell metaplasia. As shown in [Fig. 7A](#) and [B](#), OVA/OVA/VEH mice showed more goblet cell metaplasia compared to SHAM/PBS/VEH mice. However, R8 treatment significantly reduced the goblet cell metaplasia. To evaluate the possible reason of this reduction, we determined the expression of Muc5AC in IL-4 induced A549 (human lung epithelial) cells in the presence or absence of R8. As shown in [Fig. 7C](#), the absence of R8 showed more expression of Muc5AC whereas there was a R8 concentration dependent reduction in the expression of Muc5AC whose expression is itself controlled by STAT6.

Table 3

Effects of R8 on total and differential cell count in BAL fluid in atherapeutic murine model.

	TCC ($\times 10^4$ /mL)	Differential count (in percentage)			
		Macro	Mono	Eosino	Neutro
SHAM/PBS/VEH	3.5 $\pm$ 1.0*	48.4 $\pm$ 6.5	51.6 $\pm$ 6.5	–	–
OVA/OVA/VEH	51.2 $\pm$ 12.0**	7.8 $\pm$ 2.4	13.6 $\pm$ 4.0	65.7 $\pm$ 4.9**	12.9 $\pm$ 3.3
OVA/OVA/R8-7.5	20.3 $\pm$ 9.9	26.8 $\pm$ 7.6	32.9 $\pm$ 13.0	32.3 $\pm$ 11.0	8.0 $\pm$ 3.8
OVA/OVA/R8-15	18.4 $\pm$ 10.2	38.2 $\pm$ 6.9	36.2 $\pm$ 2.3	22.2 $\pm$ 4.4	3.4 $\pm$ 1.3
OVA/OVA/R8-30	15.3 $\pm$ 7.3*	38.9 $\pm$ 3.3	42.2 $\pm$ 2.7	13.9 $\pm$ 2.5*	5.1 $\pm$ 1.4
OVA/OVA/DEXA	17.5 $\pm$ 11.2	47.0 $\pm$ 13.0	34.5 $\pm$ 9.3	15.3 $\pm$ 4.6*	3.1 $\pm$ 1.6

TCC, total cell count; macro, macrophage; mono, mononuclear cells (monocytes and lymphocytes); neutro, neutrophils; eosino, eosinophil.

* Denotes $p < 0.05$, as compared to the OVA/OVA/VEH.

** Denotes $p < 0.05$, as compared to the SHAM/PBS/VEH using *t*-test.

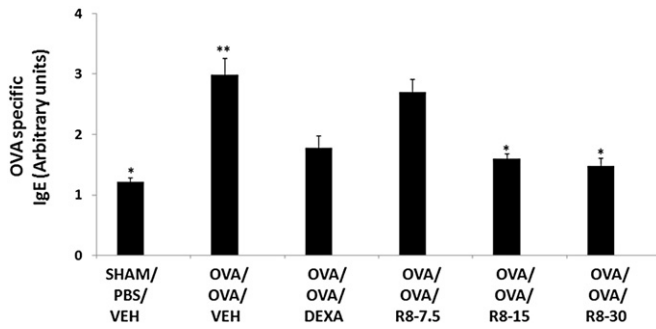


Fig. 6. Effects of R8 on OVA-specific IgE levels in therapeutic mouse model. Data is represented as mean $\pm$ SEM, *denotes $p < 0.05$, as compared to OVA/OVA/VEH, and **denotes $p < 0.05$, as compared to SHAM/PBS/VEH, Student's t -test.

3.11. R8 reduces apoptosis of bronchial epithelia in asthmatic airway

In our earlier studies, we demonstrated that asthmatic mice have mitochondrial dysfunction and apoptotic bronchial epithelia and these are dependent on IL-4. Since we found a reduction in IL-4 and subsequent asthma features, we wanted to determine the effect of R8 treatment on the apoptosis of bronchial epithelia. For this, the TUNEL apoptosis assay was performed in lung sections as described in [Materials and methods](#). As shown in [Fig. 8](#), the OVA/OVA/VEH mice showed increased apoptosis especially in bronchial epithelial cells. However, R8 treatment (OVA/OVA/R8-30 mg/kg) showed a significant reduction in the same.

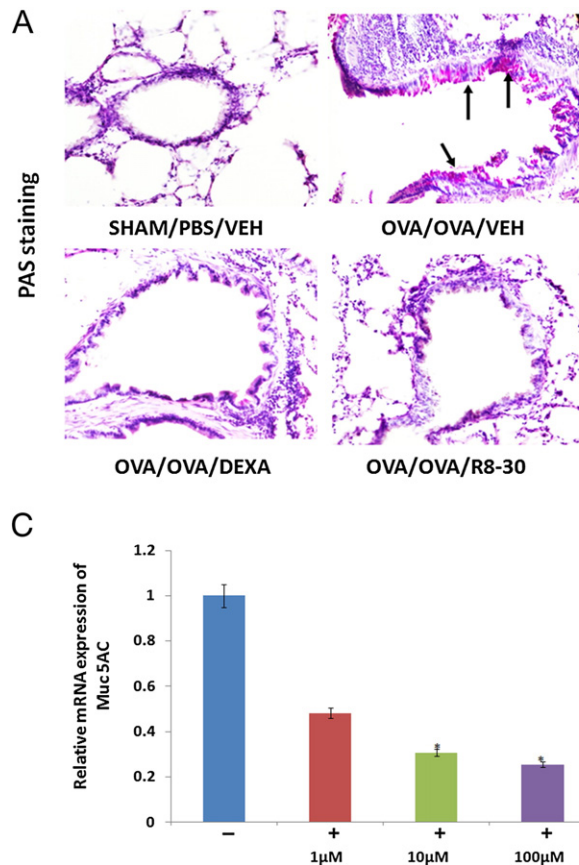


Fig. 7. Effects of R8 on goblet cell metaplasia in therapeutic mouse model and Muc5AC expression in IL-4 induced human lung epithelia. (A) Measurement of goblet cell metaplasia was estimated by PAS staining in lungs of mice treated with R8 and DEXA. Representative photographs are shown; all photographs are at $\times 40$ magnification. Arrows indicated the mucus positive goblet cells. (B) Effect of R8 on airway mucin content, estimated by quantitative morphometry. (C) Relative mRNA expression of Muc5AC gene by real-time quantitative PCR, upon exposure of A549 (human lung epithelial) cells with rIL-4 and treatment of different concentrations of R8 (1 μ M, 10 μ M, 100 μ M). Data is represented as mean $\pm$ SEM, *denotes $p < 0.05$ as compared to the untreated control, Student's t -test.

3.12. Effect of R8 on differentiation of T cell subsets

Since R8 was significantly able to suppress the key asthma features in the above described mouse model by reducing the expression of GATA3 and phosphorylation of STAT6, the two principal transcription factors responsible for Th2 cell differentiation, we evaluated its effect on the differentiation of T cell subsets. The assay was performed on murine splenocytes which were stimulated to differentiate into Th1, Th2 and Th17 cells, using different recombinant and neutralizing antibodies as described in [Material and methods](#). The differentiated status of the cells was finally confirmed by measuring the levels of IFN- γ , IL-4 and IL-17 for Th1, Th2 and Th17 cell differentiation respectively. It was observed that R8 did not interfere with the differentiation of Th1 or Th17 cells, as measured by the levels of IFN- γ ([Fig. 9A](#)) and IL-17 ([Fig. 9B](#)) respectively. However, it was observed that R8 remarkably suppressed the production of IL-4 upon receiving a Th2 stimulatory signal in the form of rIL-4 ([Fig. 9C](#)), depicting that R8 suppresses the differentiation of murine splenocytes into Th2 cells by interfering with the binding of IL-4 to its receptor, decreasing the expression of GATA3 and phosphorylation of STAT6 and hence justifying the reduction in Th2 cytokine production.

4. Discussion

In our previous study, we screened 10 semi-synthetic analogues of Vasicine, an alkaloid from *A. vasica* for their anti-asthmatic effect. Through this screening, we found that R8 possesses a significant anti-asthma potential, evaluated in a preventive murine model of asthma [12]. As a follow-up study, we demonstrate here that R8 reduces asthma

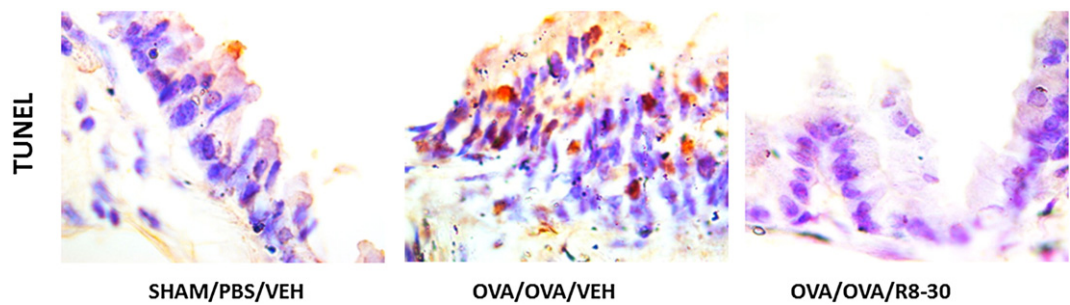


Fig. 8. TUNEL apoptosis assay in lung tissue sections. Brown color indicates the TUNEL positive apoptotic cells.

features by interfering with the binding of IL-4 to its receptor on T cells, thus modulating the phosphorylation of STAT6 and expression of GATA3 and hence blocking the differentiation of Th2 cells.

It has been strongly suggested in various studies that current focus on asthma drug development should either to improve the currently available drugs or explore new compounds or biologics targeting Th2 cytokines or Th2-specific transcription factors [24]. It is because of the critical and primary role of Th2 cytokines in mounting airway inflammation and their transcription factor that regulate their expression. The practical aspect of this approach has required blocking monoclonal antibodies, fusion proteins and inhibitors of the Th2-cell transcription factors, including GATA-binding protein 3 (GATA3) and STAT6 [25]. The GATA family of transcription factors including six members (GATA1–GATA6), have a common DNA binding domain that is

conserved among vertebrate species. The GATA3 is the most important of all the GATA family members involved in immune biology [26–28]. This transcription factor is best known to function as a principal regulator of Th2 cell differentiation. GATA3 is a Th2 cytokine responsive gene whose transcription occurs as a result of phosphorylation of STAT6, which is induced via binding of IL-4 to its receptor [29,30]. Coordinated regulation of Th2 cytokines like IL-4, IL-5 and IL-13 is important for typical allergic responses such as asthma. These cytokines play a central role in AHR development, IgE production, airway eosinophilia and mucus hypersecretion which are the foremost characteristic features of allergic asthma [31–33].

In this study, we used two standard experimental murine asthma models where male Balb/c mice were sensitized and challenged with OVA and treated with R8 (7.5,15 and 30 mg/kg) or vehicle alone, either

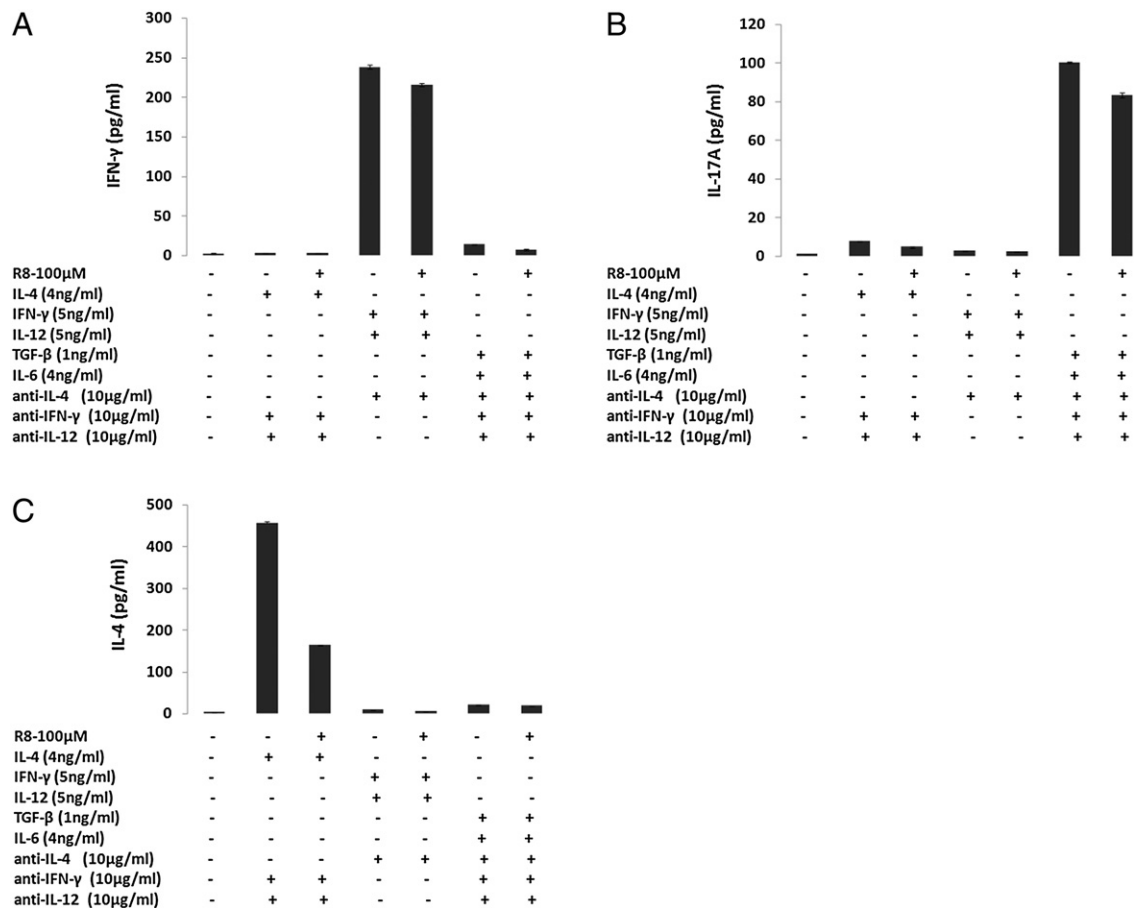


Fig. 9. Effect of R8 on Th cell differentiation. Differentiation in mouse splenocytes was induced by treating them with different recombinant and neutralizing antibodies and levels of IFN-γ (A), IL-17 (B) and IL-4 (C) measured by ELISA to evaluate the effect of R8 on differentiation of Th1, Th17 and Th2 cells respectively.

during (preventive use) or after (therapeutic use) the development of asthma features. In the preventive model, R8 was found to suppress inflammatory cell infiltration into the airways, OVA specific IgE and Th2 cytokine production. A dose dependent effect was observed with a maximum effect at 30 mg/kg. The results so obtained were in agreement with our previous study [12]. Since R8 showed anti-asthma effect in the preventive model, we wanted to test its efficacy in a therapeutic mouse model. To determine the therapeutic effect of R8 in asthma, it was administered to the mice after the development of asthma symptoms. Since allergen exposure is likely to continue during asthma therapy of human subjects, OVA challenges were continued during R8 treatment, until day 27 (Fig. 2). Our previous studies have demonstrated that OVA-sensitized and challenged mice show asthma features such as methacholine induced AHR and airway inflammation only after 3 consecutive OVA challenges. R8 treatment was therefore started after 3 days of OVA challenge, but was continued till day 27 along with R8 treatment.

In OVA-immunized asthma model, AHR is closely associated with eosinophilia, as evidenced by increased numbers of eosinophils in the BAL fluid [34]. Also, OVA specific IgE is a hallmark of allergic diseases [35]. In this therapeutic model, R8 treatment decreased methacholine induced AHR in OVA-immunized asthmatic mice (Fig. 3). It also reduced the OVA-specific IgE production (Fig. 6). Furthermore, the infiltration of inflammatory cells into the airways was lowered to the levels observed in the dexamethasone or control group (Fig. 5E, F and Table 3). This decreased accumulation of inflammatory cells was also confirmed in lung sections. We further evaluated the effect of R8 on the phosphorylation of STAT6 and expression of GATA3. The results so obtained were in concurrence with the results of cytokine production following a dose dependent pattern with the highest reduction at 30 mg/kg.

During an asthma attack, in addition to tracheal contraction, goblet cells secrete more mucus, which causes obstruction and difficulty in breathing through narrowed airways. In our study, R8 treatment reduced the goblet cell metaplasia. In addition, the effect of R8 on IL-4 induced expression of Muc5AC was also evaluated in A549 (human alveolar basal epithelial) cells induced with human recombinant IL-4. R8 (1, 10 and 100 μ M) was found to suppress the expression of Muc5AC gene in these cells significantly with maximum reduction at 100 μ M (Fig. 7C). The Muc5AC is the predominant mucin gene expressed in human airway epithelial cells and its expression is augmented in asthmatic patients [36]. A549 cell line has previously been shown to be appropriate for studies of Muc5AC mRNA and protein expression [37]. Since, R8 remarkably and dose dependently reduced the expression/production of Th2 cytokines in both the OVA murine models, we evaluated its effect on the differentiation of T cell subsets using murine splenocytes. It was observed that R8 reduced the differentiation of IL-4 stimulated murine splenocytes into Th2 cells. However, it didn't interfere with the differentiation of other cell types, Th1 and Th17. Based on the experiments performed like IL-4 induced expression of Muc5AC gene and IL-4 induced differentiation of murine splenocytes, we may presume that R8 interferes with the binding of IL-4 to its receptor.

In conclusion, we demonstrated that R8 treatment profoundly inhibited Th2 mediated asthma features like the secretion of Th2 cytokines, AHR, airway eosinophilia, OVA specific IgE levels and mucus production by acting on T cells (on IL-4 receptor) and blocking their differentiation into Th2 cells. It resulted in the inhibition of STAT6 phosphorylation and GATA3 expression which in turn blocked the expression/production of Th2 cytokines, the signature of Th2 cells, making us to presume that R8 acts by blocking the Th2 cell differentiation. These preclinical findings suggest possible therapeutic benefits of R8 in allergic asthma.

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