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Attenuated *Escherichia coli* ameliorates airway hyper responsiveness in a mouse model of ovalbumin induced asthma: Role of TNF-α, IL's, IgE and IgG

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ABSTRACT

Allergic asthma is a chronic inflammatory airway disease that is characterized by airway hyperresponsiveness (AHR), accumulation of eosinophils and goblet cell hyperplasia. The efficacy of attenuated E. coli (AEC) strain has been reported in various disease. To evaluate the effect of AEC in mice model of Ovalbumin (OVA)-induced AHR. Materials and method: AHR was induced in Female BALB/c mice (18-20 g) by intraperitoneal injection of OVA (50 µL, emulsified in 0.8 mg aluminium hydroxide) on day 7, 8, 9 and 20 followed by intranasal challenges of OVA on days 20, 23, 27, 30 and 34. OVA control mice will receive an equal volume of saline. Mice were treated with either AEC (10^8 CFU/ml) or montelukast (10 mg/kg) or vehicle on day 0, 7, 20 and 27 immediately before administration of OVA. AHR to methacholine were assessed on day 35, 24 h after the last pulmonary challenge. Treatment with AEC caused significant restoration in OVA-induced alteration in the cellular count of bronchoalveolar lavage fluid, hematological count, and pulmonary function test. Elevated levels of OVA-induced oxido-nitrosative stress was significantly decreased by AEC treatment. Total IgE, OVA-specific IgE and IgG1 in serum as well as lung TNF-α, IL-4, IL-5, IL-8 and IL-10 levels was significantly reduced by AEC. Histological aberration induced by OVA was reduced by AEC treatment. AEC inhibits OVA-induced AHR in mice via down-regulation of nitroso-oxidative stress, cytokine, and chemokine, as well as IgE and IgG, releases supporting its anti-inflammatory and bronchodilator role during the allergic response.

Keywords: Attenuated *Escherichia coli*, Airway hyperresponsiveness, TNF-α, IL's, IgE, IgG1

INTRODUCTION

Asthma is one of the most common chronic inflammatory airway disease which is characterized by airway obstruction, airway inflammation, eosinophils and mast cells infiltration, goblet cell hyperplasia and airway hyperreactivity (AHR). According to reports of World Health Organization asthma is the most common chronic disease among children and that affected around 300 million people worldwide moreover, in many industrialized countries its prevalence exceeds 10% of the population [1]. In India around 57,000 deaths were reported in 2004 which was considered as one of the leading cause of morbidity and mortality [2].

Various indoor allergens (such as pet dander and dust mites in bedding, carpets, and stuffed furniture) as well as outdoor allergens (such as pollens and molds), tobacco smoke, chemical irritants, and air pollution has been reported as trigger factors for asthma. However, development of allergic disorders such as asthma can be influenced by various infectious diseases and there is an inverse relationship between the onset of allergic disorders and the incidence of infections has been reported by an epidemiological survey [2, 3].

Generation of allergen-specific Th-2 type (T helper cells type 2) response in asthma is the most predominant mechanisms for the development of AHR. In AHR, Th2 cells produce Interleukin (IL-4, IL-5, and IL-13) that are important for development of Th-2 cells, regulation of immunoglobulin E (IgE) production and accumulation of eosinophils [4]. According to 'hygiene hypothesis' survival in cleaner environments in childhood reduced exposure to infectious organisms, however, it may increased the prevalence of allergic disorders like asthma [5].

Animal models played a vital role in the development of a potential drug for treatment of allergic disorders [6]. One of the most frequent food allergens is Ovalbumin (OVA) that cause egg-induced anaphylaxis and atopic dermatitis in childhood. OVA-induced Th2 response is well-established and reproducible animal model of AHR [7]. Intraperitoneal OVA administration followed by repeated OVA aerosol challenge leads to recruitment of eosinophils in bronchial airways that in turn caused elevated serum IgE levels resulted in airway resistance [8].

Currently available therapies asthma includes steroids, β_2-adrenergic blocker, leukotriene inhibitors, anticholinergics and mast cell stabilizers, however, they cannot completely control the symptoms of asthma, and even intensive treatment shows little effect on healthcare utilization [9]. Thus, efforts are required to identify new remedies.

It has been reported that Th1 immune responses inhibit Th2-mediated diseases in a non-antigen-specific manner thus protective effect from infectious organisms may be useful to down-regulate allergic Th2 responses [10, 11]. Thus, bacterial mediated Th1 immune response can be used as the nonspecific protective vaccines against asthmatic disorder. Previously it has been reported that administration of attenuated *Salmonella typhimurium* reduces ovalbumin-induced AHR in mice via inhibition of Th2 responses [12]. Several reports have described attempts to evaluate the efficacy of attenuated *E. coli* strain against various disease in animals. Recently, administration of live attenuated *Escherichia coli* strain has been showed effect against porcine Enterotoxigenic *E. coli* (ETEC) diarrhea [13]. Moreover, the efficacy of live attenuated *E. coli* vaccine has been proven against lipopolysaccharide (LPS) induced respiratory tract infection in broiler chickens [14, 15]. However, the efficacy of attenuated *E. coli* strain against OVA-induced AHR has not been reported yet. Hence, the aim of the present investigation was to evaluate the efficacy of attenuated *E. coli* strain against OVA-induced AHR by assessing various biochemical and histological changes in laboratory mice.

MATERIALS AND METHODS

2.1. Animals

Female BALB/c mice (18-20 g) were procured from the central animal facility of the Institute. The animals were housed in standard polypropylene cages and maintained at controlled room temperature (22 ± 2°C) and humidity (55 ± 5%) with 12:12 h light and dark cycle. All the mice were provided with commercially available mice normal pellet diet (NPD) (Pranav Agro, Baroda) and water *ad libitum*. The study was approved by the institutional animal ethics committee, and procedures were followed according to the guidelines of the committee for the purpose of control and supervision of experiments on animals (CPCSEA), Govt. of India.

2.2. Chemicals and kits

Ovalbumin (OVA, egg albumin grade II), montelukast, and aluminum hydroxide was purchased from Sigma Chemical Co. (St Louis, MO, USA). Sulphanilamides, naphthylamine diamine HCl, and phosphoric acid were obtained from Loba Chemie Pvt. Ltd., Mumbai, India. All other chemicals were of analytical grade and purchased from S.D. Fine Chemicals, Mumbai, India. The auxotrophic *Escherichia coli* (*E. Coli*) strain was procured and grown in medium at 37 °C with vigorous shaking until they reached mid-log phase (10^8 colony forming units (CFU)/ml). Albumin kits were purchased from Accurex Biomedical Pvt. Ltd., Mumbai, India. Mice TNF-α, IL-4, IL-5, IL-8, IL-10, IL-13, IgE, IgG1 and IgG2a ELISA kits were purchased from Bethyl Laboratories, Inc. (TX, United States), MyBioSource, Inc. (CA, United States) and RayBiotech, Inc. (GA, United States).

2.3. Sensitization, drug treatment, and OVA challenge

Animals will be randomly divided into following groups, each consisting of 10 animals

Group I: Normal group: Animals did not undergo any OVA allergen. They were treated with vehicle (100 µl PBS with 5% sodium bicarbonate, p.o.) on day 0, 7, 20 and 27.

Group II: OVA Control group: Animals were received intraperitoneal (50 µL) injection of OVA on day 7, 8, 9 and 20 followed by intranasal challenges of OVA on days 20, 23, 27, 30 and 34. They were treated with vehicle (100 µl PBS with 5% sodium bicarbonate, p.o.) on day 0, 7, 20 and 27 immediately before administration of OVA.

Group III: Montelukast treated group: (MLT): Animals were received intraperitoneal (50 µL) injection of OVA on day 7, 8, 9 and 20 followed by intranasal challenges of OVA on days 20, 23, 27, 30 and 34. They were treated with montelukast (10 mg/kg, p.o.) on day 0, 7, 20 and 27 immediately before administration of OVA.

Group IV: *E. coli* treated group: (AEC): Animals were received intraperitoneal (50 µL) injection of OVA on day 7, 8, 9 and 20 followed by intranasal challenges of OVA on days 20, 23, 27, 30 and 34. They were treated with attenuated *E. coli* (10^8 CFU/ml, p.o.) on day 0, 7, 20 and 27 immediately before administration of OVA.

All mice (except normal) were sensitized by four intraperitoneal (i.p) injections of 50 µg OVA which was emulsified in 0.8 mg aluminium hydroxide in 200 µl saline on days 7, 8, 9 and 20. Normal mice were injected with 0.8 mg aluminum hydroxide in 200 µl saline alone. Mice were challenged on days 20, 23, 27, 30 and 34 by inhalation of either normal saline or OVA aerosols in an exposure chamber for 20 min. Aerosols were generated by nebulizing 2% OVA solution in saline, or saline alone, with a nebulizer [9, 12]. *E. coli* treated group mice were treated with 10^8 CFU bacteria in 100 µl PBS with 5% sodium bicarbonate whereas montelukast treated group were treated with montelukast (10 mg/kg, p.o.) on days 0, 7, 20 and 27 immediately before the i.p. injection of OVA or receiving an OVA aerosol.

2.4. Measurement of methacholine hyperresponsiveness

Whole-body flow-through plethysmography (EMKA Technologies, France) was used to measure airway hyper-responsiveness (AHR) in response to aerosolized acetyl β-methylcholine (Mch, 50 mg/mL) in unrestrained and conscious mice on day 35 (24 h after the last pulmonary challenge). Either aerosolized PBS or Mch was nebulized through the inlet of the main chamber for 2 min, and the response to each dose was subsequently measured for 5 min. Recorded variables included Inspiratory time (Ti), Expiratory time (Te), Peak inspiratory flow (PIF), Peak expiratory flow (PEF), tidal volume (TV), expired volume (EV), frequency of breathing (f), enhanced pause (P_{enh}) value [16].

2.5. Measurement of peripheral blood oxygen content

To assess peripheral blood oxygen content *in-vivo*, mice were monitored for the percentage of hemoglobin saturated with oxygen (pulse Ox). On day 36, mice were anesthetized with ether and a peripheral pulse Ox sensor (ChoiceMMed, V1.0CF3, MD300CF3, China) was attached to the tail. Pulse Ox readings were taken as the animal regained consciousness [17].

2.6. Hematological analysis and serum biochemistry

On day 36, the blood was withdrawn by retro-orbital puncture for determination of hematological count and serum parameters (IgG1, IgG2a, and IgE analysis).

2.7. Bronchoalveolar Lavage Fluid (BALF) analysis

On day 36 after blood collection, the BALF was collected for total cell count by placing polyethylene catheter into the trachea. BALF was collected by washing with two separate aliquots of 1mL of Hank's balanced Salt Solution through the trachea. The first wash was centrifuged, and the BALF supernatant stored for biochemical measurements. The second wash was centrifuged, and the cell pellet from first wash were pooled with the cell pellet from the second. A total cell count was obtained by using a Coulter counter mode. For differential counting, cells were prepared in a cytospin apparatus. Prepared cytospin slides were stained with Diff-Quick and cell differentials were performed after counting 300 cells. Determination of various biochemical measurements viz. BALF total protein, Superoxide Dismutase (SOD), glutathione (GSH), malondialdehyde (MDA), Nitric Oxide (NO), Myeloperoxidase (MPO) and albumin were carried out according to earlier reported methods [9, 18-25].

2.8. Preparation of lung homogenates

After the BAL fluid had been collected, the lungs were removed and immediately homogenized in 3 mL of ice-cold PBS buffer. Right lung supernatant were utilized for subsequent measurement of SOD, GSH, MDA, NO, MPO and albumin according to earlier reported methods [9, 26-34]. Left lung supernatant were utilized for subsequent

measurement of cytokine and chemokine analysis. One lung tissue from each group was processed for histopathological examination.

2.9. Determination of Cytokine and chemokine in Lung homogenate
Lung tissue homogenate levels of TNF-α, IL-4, IL-5, IL-8, IL-10, and IL-13 were determined by using an enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions.

2.10. Determination of OVA-specific IgG1, IgG2a, IgE and total IgE
OVA-specific IgG1, IgG2a, IgE and total IgE antibody titers were determined by ELISA according to the manufacturer's instructions.

2.11. Lung tissue histopathology:
After BAL fluid had been obtained, lung tissue was fixed in 10% (v/v) neutral buffered formalin for 24 h for histopathological studies. It was processed for 12 h using isopropyl alcohol, xylene and paraffin embedded for light microscopic study (Nikon E200, Japan). Paraffin-embedded tissue sections cut at 5µm thickness were prepared and stained after deparaffination using hematoxylin and eosin stain (H & E) to verify morphological assessment. Photomicrographs were captured at a magnification of 40X.

2.12. Statistical analysis
Data are expressed as mean ± standard error mean (SEM). Data analysis was performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA). Data were analyzed by using One-way repeated analysis of variance (ANOVA) followed by Tukey's multiple comparison *post hoc* tests. A value of $p < 0.05$ was considered to be statistically significant.

RESULTS

3.1. Effect of attenuated E. coli treatment on OVA-induced alteration in body weight, lung weight, and lung injury index of mice
There was significant decreased ($p < 0.001$) in the body weight and significant increased ($p < 0.001$) in the absolute as well as relative lung weight of OVA control group as compared to normal control group. Treatment with ACE (10^8 CFU/ml) significantly inhibited ($p < 0.05$, $p < 0.05$ and $p < 0.001$) OVA-induced decreased body weight as well as increased absolute as well as relative lung weight as compared to OVA control group. When compared with OVA control group, montelukast (10 mg/kg) treatment showed significant increased ($p < 0.001$) in the body weight whereas it showed significant decreased ($p < 0.01$ and $p < 0.001$) in the absolute as well as relative lung weight (Figure 1).

3.2. Effect of attenuated E. coli treatment on OVA-induced alteration in percent oxygen saturation of mice
Intraperitoneal administration of OVA significantly decreased ($p < 0.001$) the percent oxygen saturation of OVA control group as compared to the normal group. Administration of ACE (10^8 CFU/ml) significantly increased ($p < 0.01$) percent oxygen saturation as compared to OVA control group. Whereas montelukast (10 mg/kg) treatment also showed significant increased ($p < 0.001$) in percent oxygen saturation as compared to OVA control group (Figure 1).

3.3. Effect of attenuated E. coli treatment on OVA-induced alteration in lung function test of mice
OVA control group showed significant alterations ($p < 0.001$) in the lung function test as compared to the normal group. This alteration in lung function test after OVA administration was significantly restored ($p < 0.001$) by ACE (10^8 CFU/ml) treatment as compared to OVA control group. Treatment with montelukast (10 mg/kg) also showed significant restored ($p < 0.001$) in lung function test as compared to OVA control group. When compared with ACE (10^8 CFU/ml) treated mice, montelukast (10 mg/kg) treatment showed significantly decreased ($p < 0.01$) in enhanced pause and expiratory time. (Table 1)

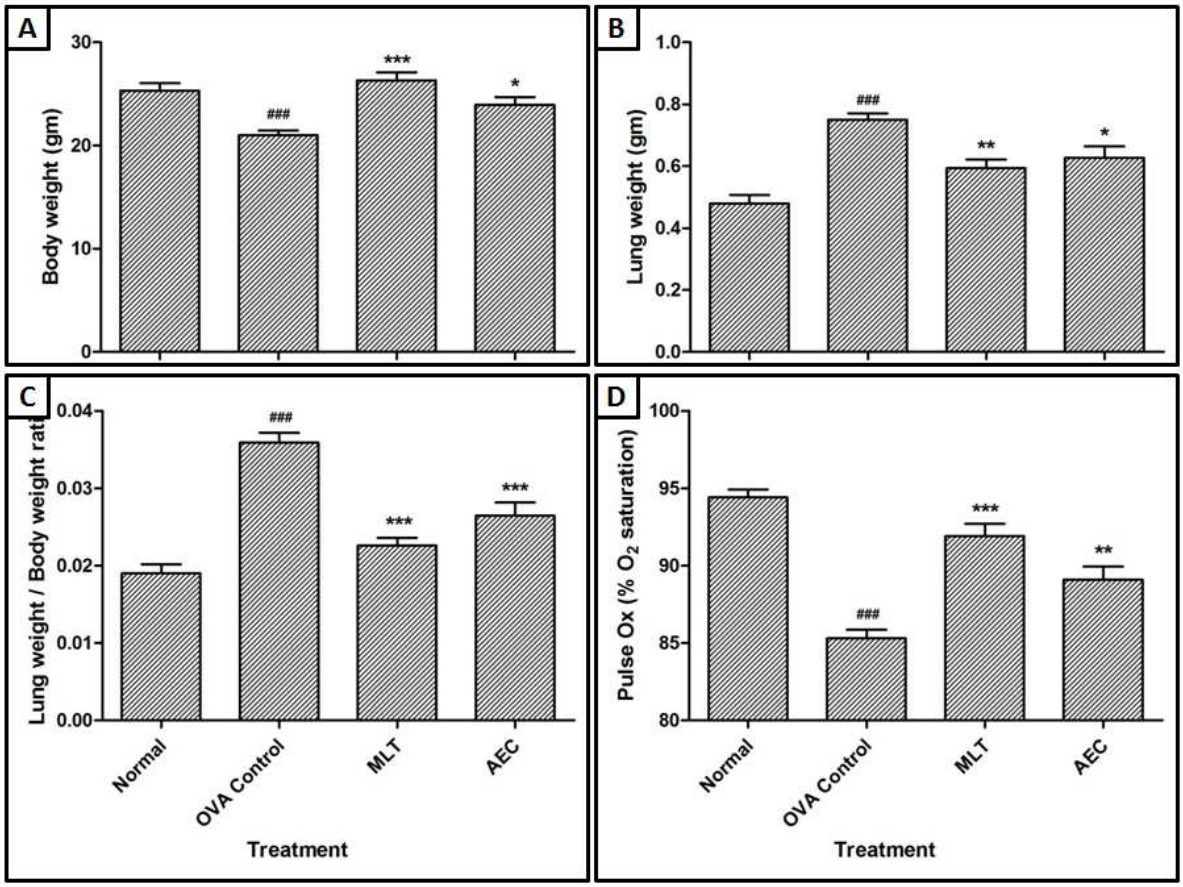

Figure 1. Effect of attenuated *E. coli* treatment on Ovalbumin induced an alteration in body weight (A), lung weight (B), lung injury index (C) and percent oxygen saturation (D) of mice

*Data was analyzed by One-Way ANOVA followed by Tukey's multiple comparison post hoc tests. $^{###}p < 0.001$ as compared with normal group and *p < 0.05, **p < 0.01, ***p < 0.001 as compared with OVA control group. OVA: Ovalbumin, MTL: montelukast, AEC: Attenuated E. coli. (n = 10).*

Table 1 Effect of attenuated *E. coli* treatment on Ovalbumin induced alteration in lung function test of mice

Parameters	Normal	OVA control	MLT	AEC
Peak inspiratory flow (PIF) (mL/s)	6.40 ± 0.22	3.82 ± 0.29$^{###}$	5.97 ± 0.23***	5.22 ± 0.28**
Peak expiratory flow (PEF) (mL/s)	7.57 ± 0.22	4.23 ± 0.27$^{###}$	6.22 ± 0.45***	5.62 ± 0.27*
Tidal volume (TV) (mL)	0.58 ± 0.02	0.44 ± 0.02$^{##}$	0.57 ± 0.02**	0.55 ± 0.02*
Frequency of Breathing (f) (bpm)	291.5 ± 9.30	444.7 ± 8.98$^{###}$	356.9 ± 6.89***	383.5 ± 12.33***
Enhanced pause (Penh)	1.83 ± 0.13	5.96 ± 0.26$^{###}$	2.95 ± 0.26***$^{\$\$}$	4.48 ± 0.45**
Inspiratory time (Ti) (s)	0.21 ± 0.02	0.06 ± 0.007$^{###}$	0.18 ± 0.01***	0.13 ± 0.01*
Expiratory time (Te) (s)	0.27 ± 0.03	0.06 ± 0.008$^{###}$	0.25 ± 0.01***$^{\$\$}$	0.16 ± 0.009**

*Data was analyzed by One-Way ANOVA followed by Tukey's multiple comparison post hoc tests. $^{##}p < 0.01$, $^{###}p < 0.001$ as compared with normal group, *p < 0.05, **p < 0.01, ***p < 0.001 as compared with OVA control group, and $^{\$\$}p < 0.01$ as compared with one another group. OVA: Ovalbumin, MTL: montelukast, AEC: Attenuated E. coli. (n = 10).*

3.4. Effect of attenuated E. coli treatment on OVA-induced alteration in hematological parameters of mice

OVA control group showed significantly decreased ($p < 0.001$) in the Hb, RBC, HCT, MCH, lymphocyte count whereas PLT, WBC, neutrophil, monocyte, eosinophil count were increased significantly ($p < 0.001$) as compared to normal group. When compared with OVA control group, the Hb, RBC, HCT, MCH, lymphocyte count were increased significantly ($p < 0.01$) whereas PLT, WBC, neutrophil, monocyte, eosinophil count were decreased significantly ($p < 0.01$) in the ACE (10^8 CFU/ml) treated group. Montelukast (10 mg/kg) treatment also showed significant inhibition ($p < 0.001$) in the OVA-induced alteration in hematological parameters as compared to OVA control group. The altered HCT and WBC count was more significantly restored ($p < 0.01$) by montelukast (10 mg/kg) treatment as compared to ACE (10^8 CFU/ml) treatment. (Table 2)

Table 2 Effect of attenuated *E. coli* treatment on Ovalbumin induced alteration in hematological parameters and BALF differential cell count of mice

Table 3

Parameters	Normal	OVA control	MLT	AEC
Hb (g/dL)	15.20 ± 0.27	12.55 ± 0.37$^{###}$	14.88 ± 0.38***	14.29 ± 0.41**
RBC (X 10^{12}/L)	10.84 ± 0.24	8.08 ± 0.29$^{###}$	10.59 ± 0.34***	9.86 ± 0.42**
HCT (%)	53.38 ± 0.61	42.99 ± 0.73$^{###}$	51.19 ± 0.79***,$}$	47.23 ± 1.02**
MCH (pg)	14.94 ± 0.69	9.85 ± 0.36$^{###}$	13.30 ± 0.57**	13.14 ± 0.67**
PLT (X 10^{9}/L)	797.5 ± 17.74	945.8 ± 34.03$^{##}$	828.3 ± 26.98*	840.6 ± 23.96*
WBC (X 10^{6}/mL)	8.22 ± 0.24	13.94 ± 0.45$^{###}$	9.72 ± 0.29***,$}$	11.71 ± 0.58**
Neutrophile (%)	30.80 ± 0.75	36.80 ± 1.37$^{##}$	31.70 ± 1.31*	31.70 ± 1.11*
Lymphocyte (%)	63.40 ± 1.04	52.10 ± 1.45$^{###}$	59.90 ± 1.70***	60.30 ± 0.84***
Monocyte (%)	2.50 ± 0.40	5.60 ± 0.45$^{###}$	4.80 ± 0.32	4.10 ± 0.37*
Eosinophile (%)	3.30 ± 0.36	5.50 ± 0.47$^{##}$	3.60 ± 0.42**	3.90 ± 0.23*
BALF Total cells (X 10^{5}/mL)	2.60 ± 0.40	30.00 ± 0.94$^{###}$	9.80 ± 1.08***,}$	14.20 ± 0.96***
BALF Neutrophile (X 10^{5}/mL)	0.60 ± 0.40	4.40 ± 0.50$^{###}$	2.20 ± 0.48*	1.80 ± 0.37**
BALF Lymphocyte (X 10^{5}/mL)	0.60 ± 0.40	3.40 ± 0.50$^{###}$	1.00 ± 0.31**	1.40 ± 0.50*
BALF Eosinophile (X 10^{5}/mL)	0.80 ± 0.20	10.20 ± 0.91$^{###}$	4.60 ± 0.67***	6.60 ± 0.40**
BALF Macrophages (X 10^{5}/mL)	0.60 ± 0.24	12.00 ± 1.78$^{###}$	2.00 ± 0.77***	4.40 ± 0.74***

*Data was analyzed by One-Way ANOVA followed by Tukey's multiple comparison post hoc tests. $^{##}p < 0.01$, $^{###}p < 0.001$ as compared with normal group, *p < 0.05, **p < 0.01, ***p < 0.001 as compared with OVA control group, and $^{$}p < 0.05$, $^{$$}p < 0.01$ as compared with one another group. OVA: Ovalbumin, MTL: montelukast, AEC: Attenuated E. coli, Hb: haemoglobin, RBC: red blood corpuscles, HCT: hematocrit, MCH: mean corpuscular haemoglobin, PLT: platelet, WBC: white blood corpuscles (n = 6-10).*

3.5. Effect of attenuated E. coli treatment on OVA-induced alteration in BALF differential cell count of mice

BALF total cells, neutrophil, lymphocyte, eosinophil, and macrophages counts were increased significantly in the OVA control group as compared to the normal group. Administration of ACE (10^8 CFU/ml) showed significantly decreased ($p < 0.01$) in the BALF total cells as well as differential cell count as compared to OVA control group. Moreover, montelukast (10 mg/kg) treatment also showed significant reduction ($p < 0.001$) in the BALF total cells as well as differential cell count as compared to OVA control group. BALF total cells count was more significantly decreased ($p < 0.05$) in the montelukast (10 mg/kg) treated group as compared to ACE (10^8 CFU/ml) treated group. (Table 2)

Table 4 Effect of attenuated *E. coli* treatment on Ovalbumin induced alteration in the levels of SOD, GSH, MDA, NO, MPO and albumin in BLAF and lung of mice

Parameters	Normal	OVA control	MLT	AEC
SOD level in BALF (U/mg of protein)	1.86 ± 0.12	0.72 ± 0.07$^{###}$	1.62 ± 0.22**	1.46 ± 0.20*
GSH level in BALF (µg/mg of protein)	1.18 ± 0.11	0.29 ± 0.07$^{###}$	0.93 ± 0.09***	0.67 ± 0.08*
MDA level in BALF (nM/ mg of protein)	2.04 ± 0.24	5.68 ± 0.22$^{###}$	3.17 ± 0.50***	3.62 ± 0.23**
NO level in BALF (µg/mL)	12.01 ± 1.04	60.40 ± 3.74$^{###}$	44.82 ± 3.62*	49.76 ± 3.28
MPO level in BALF (U/mL)	4.09 ± 0.31	13.17 ± 1.04$^{###}$	9.89 ± 0.38**	9.50 ± 0.29**
Albumin level in BALF (g/dL)	0.34 ± 0.07	0.82 ± 0.06$^{##}$	0.48 ± 0.06*	0.51 ± 0.08*
SOD level in Lung (U/mg of protein)	8.19 ± 0.53	3.34 ± 0.34$^{###}$	7.18 ± 0.54***	5.86 ± 0.60*
GSH level in Lung (µg/mg of protein)	4.37 ± 0.30	0.85 ± 0.09$^{###}$	2.75 ± 0.32***	1.84 ± 0.07*
MDA level in Lung (nM/ mg of protein)	3.54 ± 0.27	7.95 ± 0.47$^{###}$	5.67 ± 0.27**	6.20 ± 0.50*
NO level in Lung (µg/mL)	52.74 ± 7.38	205.4 ± 16.18$^{###}$	121.6 ± 15.47**	153.9 ± 14.91
MPO level in Lung (U/mL)	12.29 ± 1.32	39.86 ± 2.30$^{###}$	26.99 ± 2.77**	26.85 ± 2.17**
Albumin level in Lung (g/dL)	0.39 ± 0.09	2.38 ± 0.25$^{###}$	1.03 ± 0.14***	1.49 ± 0.17*

*Data was analyzed by One-Way ANOVA followed by Tukey's multiple comparison post hoc tests. $^{##}p < 0.01$, $^{###}p < 0.001$ as compared with normal group and *p < 0.05, **p < 0.01, ***p < 0.001 as compared with OVA control group. SOD: superoxide dismutase, GSH: glutathione, MDA: malondialdehyde, NO: nitric oxide and MPO: Myeloperoxidase, OVA: Ovalbumin, MTL: montelukast, AEC: Attenuated E. coli (n = 5).*

3.6. Effect of attenuated E. coli treatment on OVA-induced alteration in the level of SOD, GSH, MDA, NO, MPO and albumin in BLAF of mice

There was significant decreased ($p < 0.001$) in the BALF SOD and GSH level whereas of MDA, NO, MPO and albumin level in BALF were increased significantly ($p < 0.001$) in OVA control group as compared to normal group. Treatment with ACE (10^8 CFU/ml) significantly increased ($p < 0.05$) BALF SOD and GSH level as compared to OVA control mice. Administration of montelukast (10 mg/kg) also produce significant increased ($p < 0.01$ and $p < 0.001$) in BALF SOD and GSH levels as compared to OVA control mice. Administration of ACE (10^8 CFU/ml) significantly inhibited ($p < 0.01$, $p < 0.01$ and $p < 0.05$) OVA-induced increased in BALF MDA, MPO and albumin levels as compared to OVA control mice. Treatment with montelukast (10 mg/kg) also showed

significant decreased ($p < 0.001$, $p < 0.05$, $p < 0.01$ and $p < 0.05$) in BALF MDA, NO, MPO and albumin levels when compared with OVA control mice. (Table 4)

3.7. Effect of attenuated E. coli treatment on OVA-induced alteration in the level of SOD, GSH, MDA, NO, MPO and albumin in lung of mice

OVA control group showed significant decreased ($p < 0.001$) in the lung SOD and GSH levels whereas as significant increased ($p < 0.001$) in the lung MDA, NO, MPO and albumin levels as compared to normal group. ACE (10^8 CFU/ml) treatment showed a significant increase ($p < 0.05$) in the lung SOD and GSH levels as compared to OVA control mice. However, when compared with OVA control group, lung MDA, MPO and albumin levels was significantly decreased ($p < 0.05$, $p < 0.01$ and $p < 0.05$) in the ACE (10^8 CFU/ml) treated group. Montelukast (10 mg/kg) treatment also showed significant increased ($p < 0.001$) in the lung SOD and GSH levels whereas significant decreased ($p < 0.01$, $p < 0.01$, $p < 0.01$ and $p < 0.001$) in the MDA, NO, MPO and albumin levels as compared to OVA control group. (Table 4)

3.8. Effect of attenuated E. coli treatment on OVA-induced alteration in level of lung TNF-α, IL-4, IL-5, IL-8, IL-10 and IL-13 levels in mice

When compared with normal group, the lung TNF-α, IL-4, IL-5, IL-8, IL-10 and IL-13 levels of OVA control mice significantly increased ($p < 0.001$) on day 36. Administration of ACE (10^8 CFU/ml) significantly decreased ($p < 0.05$, $p < 0.01$, $p < 0.05$, $p < 0.05$ and $p < 0.01$) lung TNF-α, IL-4, IL-5, IL-8 and IL-10 levels as compared to OVA control mice. Treatment with montelukast (10 mg/kg) also showed significant reduction ($p < 0.001$) in the OVA-induced increased lung TNF-α, IL-4, IL-5, IL-8, IL-10 and IL-13 levels as compared to OVA control mice. When compared with ACE (10^8 CFU/ml) treated mice, montelukast (10 mg/kg) treatment showed more significant inhibition ($p < 0.001$) in OVA-induced increased in lung IL-10 level (Figure 2).

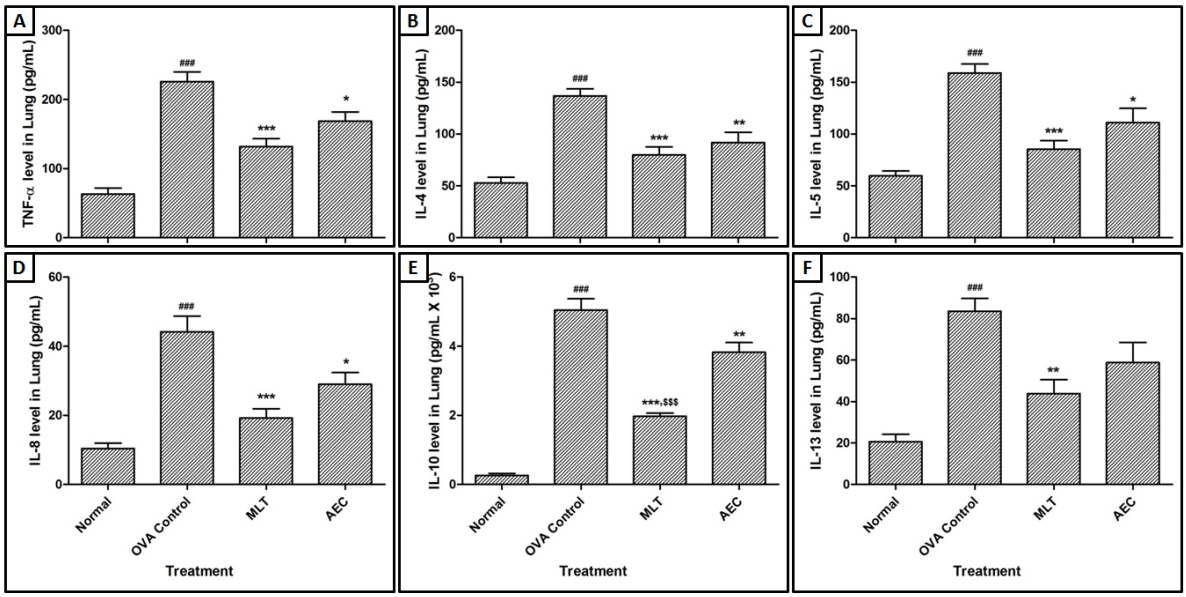

Figure 2. Effect of attenuated *E. coli* treatment on Ovalbumin induced alteration in level of lung TNF-α (A), IL-4 (B), IL-5 (C), IL-8 (D), IL-10 (E) and IL-13 (F) levels in mice

*Data was analyzed by One-Way ANOVA followed by Tukey's multiple comparison post hoc tests. ###p < 0.001 as compared with normal group, *p < 0.05, **p < 0.01, ***p < 0.001 as compared with OVA control group, and $^{\$\$\$}$p < 0.001 as compared with one another group. TNF- α: tumor necrosis factor-alpha, IL: interleukin, OVA: Ovalbumin, MTL: montelukast, AEC: Attenuated E. coli, (n = 5).*

3.9. Effect of attenuated E. coli treatment on OVA-induced alteration in the levels of serum total IgE, OVA-specific IgE, OVA-specific IgG1 and OVA-specific IgG2a of mice

OVA control group showed significant increased ($p < 0.001$) in the serum total IgE, OVA-specific IgE and IgG1 levels whereas significant decreased ($p < 0.001$) OVA-specific IgG2a level as compared to the normal group. Treatment of ACE (10^8 CFU/ml) significantly reduced ($p < 0.01$, $p < 0.05$ and $p < 0.05$) serum total IgE, OVA-specific IgE and IgG1 levels as compared with OVA control group. When compared with OVA control group,

montelukast (10 mg/kg) treatment showed significant inhibition ($p < 0.001$) in the OVA-induced alterations in serum total IgE, OVA-specific IgE, IgG1 and IgG2a levels. However when compared with ACE (10^8 CFU/ml) treated group, serum total IgE and OVA-specific IgG1 levels were more significantly decreased ($p < 0.001$) whereas OVA-specific IgG2a level was more significantly increased by montelukast (10 mg/kg) treatment (Figure 3).

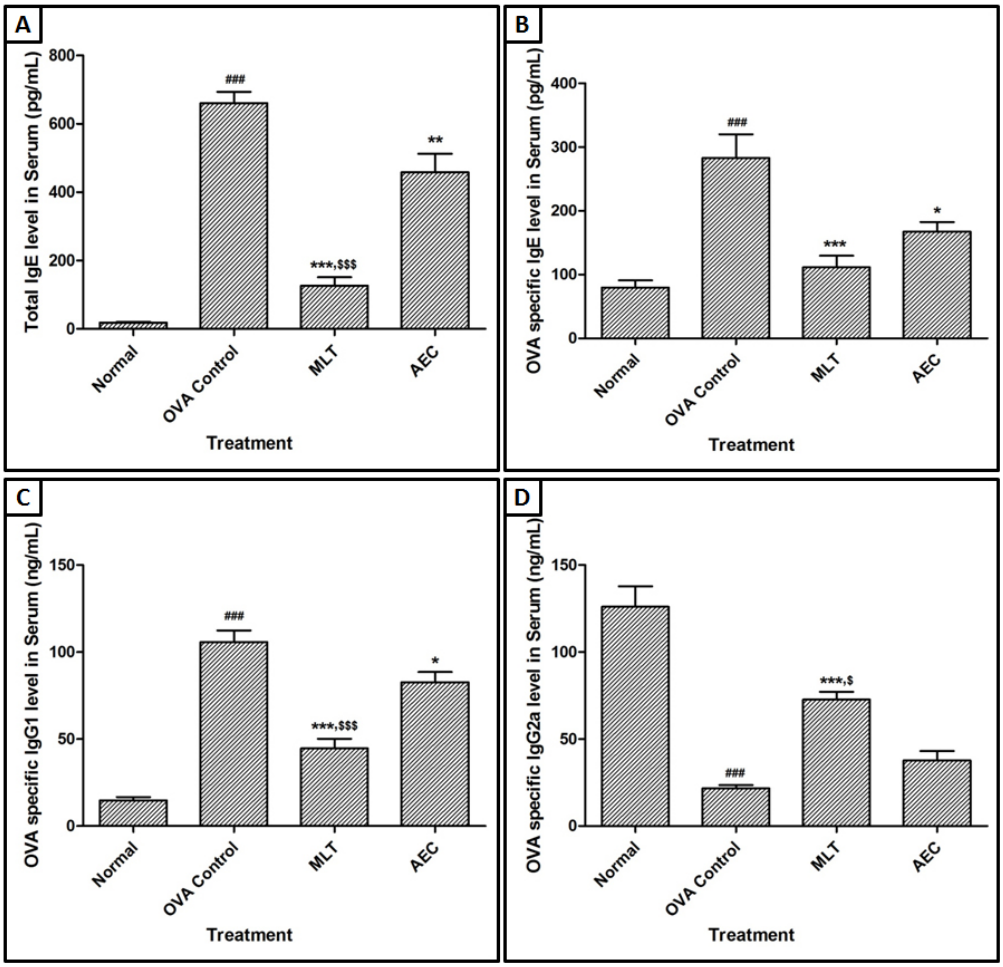

Figure 3. Effect of attenuated *E. coli* treatment on Ovalbumin induced alteration in the levels of serum total IgE (A), OVA-specific IgE (B), OVA-specific IgG1 (C) and OVA-specific IgG2a (D) of mice

Data was analyzed by One-Way ANOVA followed by Tukey's multiple comparison post hoc tests. $^{###}p < 0.001$ as compared with normal group and $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared with OVA control group and $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ as compared with one another group.

3.10. Effect of attenuated E. coli treatment on OVA-induced pathological alteration in mice lungs

Figure 4A depicted the normal architecture of lung tissue from a normal group which is devoid of any goblet cell hyperplasia, inflammatory infiltration, and hemorrhage. However, it showed the presence of mild oedema (grade 1) (black arrow). The lung histology of OVA control mice showed the presence of intense inflammatory cell infiltration in the peribronchiolar region (grade 3-4) (red arrow). The airway perimeter was increased in OVA control mice as compared to normal mice (grade 4) (yellow arrow) (Figure 4B). Montelukast (10 mg/kg) treatment attenuated the inflammatory cell infiltration in the lung tissue (grade 2) (Figure 4C) as well as increased airway perimeter (grade 2) after OVA challenge. Lung tissue from ACE (10^8 CFU/ml) showed presence of moderate infiltration of inflammatory cells (grade 3) (red arrow), oedema (grade 3) in peribronchiolar region of lung tissue (black arrow) (Figure 4D) (Table 5Table 5).

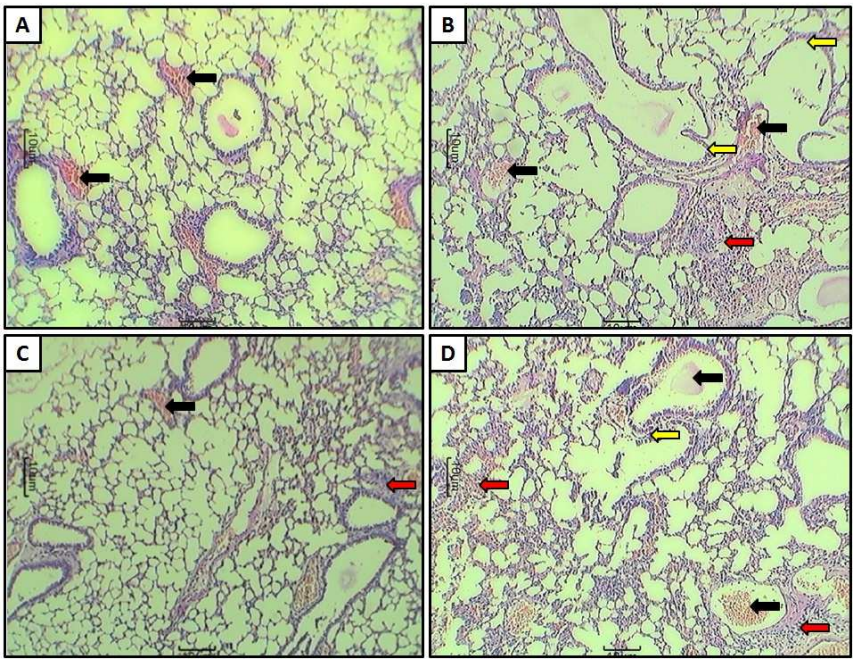

Figure 4. Effect of attenuated *E. coli* treatment on Ovalbumin induced pathological alteration in mice lungs
Photomicrograph of sections of lungs of normal (A), OVA control (B), montelukast (10 mg/kg) treated (C) and Attenuated E. coli (10^8 CFU) treated (D) mice. Lung H&E staining at 40 X.

Table 5 Effect of attenuated *E. coli* treatment on Ovalbumin induced pathological alteration in mice lungs

Parameter	Goblet cell hyperplasia	Inflammation	Eosinophils	Infiltration of leukocytes	Oedema	Hemorrhage
Normal	-	-	-	+	+	-
OVA Control	++++	+++	+++	++++	+++	+++
MTL	++	++	+	++	+	-
AEC	++	++	++	+++	++	++

OVA: Ovalbumin, MTL: montelukast, AEC: Attenuated E. coli.
Note: -: no abnormality detected, +: damage/active changes up to < 25%, ++: damage/active changes up to < 50%, +++: damage/active changes up to < 75%, ++++: damage/active changes up to > 75%

DISCUSSION

Asthma is an immune-mediated disease characterized by hyperresponsiveness of airway, accumulation of eosinophilia in lung, formation of edema, elevated mucus production, inflammation in bronchi along with increased in cytokine, chemokine as well as Immunoglobulin E (IgE) and IgG levels [35]. In the initiate and develop of asthma mast cells, T helper type-2 (TH_2) and eosinophils played a vital role [36].

It has been well documented that immune inflammatory disorder like AHR is associated with the decreased body weight and elevated relative lung weight [9]. In the present investigation, intraperitoneal administration of OVA caused significant reduction body weight whereas lung weight was increased significantly. The result of present study corroborates the findings of previous investigators [9, 37]. Administration of AEC significantly inhibited OVA-induced alterations in body weight as well as lung weight of mice.

In OVA-sensitized animals, airways hyperreactivity appeared after the 24 hr of antigen challenge and elevated levels of total and differential cell count in the BALF is the consequence of AHR [38]. This symptom of AHR is closely associated with the clinicopathological features of asthma in human. This symptom can be classified into two distinct phases viz. early or acute phase where release of histamine and leukotrienes occurred within an hour of allergen exposure resulted in bronchoconstriction which was followed by late phase response where release of mast cell, as well as inflammatory infiltration, caused elevated cytokines and chemokines levels led to airway obstruction

and inflammatory reaction [39]. Induction of allergic inflammation has been carried out by eosinophils. Migration of this eosinophils into airways caused the release of cytokines such as IL-5 and IL-13 [40, 41]. Similar findings were reported in the clinical settings where the release of eosinophils has been associated with elevated cytokines levels [42]. Neutrophils are another inflammatory cells that have been released along with eosinophils after allergen exposure [43, 44]. This is also associated with elevated airway hypersensitivity via induction and release of cytokines (IL-1β and IL-6), reactive oxygen species (ROS) and nitric oxide [45]. In the present investigation, OVA exposure caused significantly elevated in the neutrophils and eosinophils in BALF of OVA control mice. Moreover, histological findings of lung tissue from OVA control mice also showed the presence of prominent inflammatory infiltration in peribronchiolar region. However, administration of ACE showed significantly decreased in BALF total cells as well as differential cell count suggesting its role in minimizing tissue damage.

Inhalation of allergen like OVA is associated with alteration in the breathing pattern which can be assessed by determined by measuring pulmonary function tests (PFT). For a diagnosis of the nature and severity of lung injury, whole body plethysmography is very useful non-invasive technique in the determination of PFT [46]. In asthmatic condition, there was bronchoconstriction due to an elevated level of mucous production that resulted in decreased tidal volume along with increased breathing rate [47]. It is also caused significant alterations in PIF, PEF, Ti and Te. Moreover, enhanced pause (Penh) is an important hallmark of the airway obstruction, and it is strongly correlated with the bronchoconstriction in animal [48]. Treatment with ACE caused significant inhibition in the OVA-induced alteration in PFT depicting its bronchodilatory effect.

Examination of lung gas exchange provides a slight into a disease status. Determination of percent oxygen saturation with the help of fingertip pulse oxymeter is an indirect technique for quantification of oxygenated hemoglobin [46]. Its gives an idea about the status of lung gas exchange in a disease condition. Along with pulse oxy, hematological studies also helpful for determination of tissue oxygen level. The alteration in the hematological count has been reported after allergen exposure explaining the need of oxygen to repair the injury [49]. Decreased level of oxygen caused increased the turnover of collagen synthesis via increased levels of β-subunit of proplyl-4-hydroxylase [50]. Administration of AEC resulted in significantly increased the Hb and RBC count which may in turn increase blood flow and supply of oxygen to the lung tissue thus reduced hypoxia which was also further supported by the elevated level of pulse oxy.

The release of inflammatory mediators after allergen challenge is associated with the elevated level of ROS, which in turn caused depletion of intracellular GSH, a non-enzymatic biological antioxidant [26, 46, 51, 52]. GSH played a vital role in maintaining cell metabolism and integrity via detoxification of free radical species including hydrogen peroxide, superoxide, and alkoxy radicals [53-56]. Another important enzyme in the enzymatic antioxidant defense system are SOD. It is responsible for detoxification of ROS via reduction of superoxide anion to form hydrogen peroxide [57-60]. In corroboration of the previous study, administration of allergen like OVA caused significant depletion of SOD and GSH level [46]. Moreover, an elevated level of MDA is an indicator of lipoperoxidation is which caused rearrangement of the double bond in the unsaturated fatty acids leads to lipid cell membrane destruction [46, 55, 61, 62]. However, administration of AEC caused significantly increased in SOD and GSH whereas decreased in MDA level suggesting its antioxidant potential.

Furthermore, elevated ROS also associated with the release of pro-inflammatory mediators such as nitric oxide (NO) which is an unconventional intracellular messenger [63, 64]. NO reacts with ROS to gives rise vicious cycle that leads to nonspecific tissue damage [65, 66]. Clinically it has been proven that elevated production of NO has been associated with increased mucus production and infiltration of inflammatory [67, 68]. Treatment with AEC did not show any reduction in elevated nitric oxide level in either BALF or lung tissue.

It has been well documented that oxidative stress also played a significant role in damaging albumin, the susceptible amino acids of proteins. This damage caused elevated albumin and protein level in lung and BALF that indicated injury in the bronchoalveolar-capillary barrier [69]. It leads to airway obstruction and progressive respiratory insufficiency [69]. Elevated albumin levels also associated with the elevated level of b and c globulins via production of IgG and IgE. In the present investigation, OVA administration caused a significant elevation in serum albumin which is further supported by histological analysis of lung tissue where destruction of a bronchoalveolar-capillary barrier along with goblet cell hyperplasia were observed. The result of present investigation corroborates with an earlier report [4]. However, administration of AEC caused significantly decreased in albumin level reflecting its bronchoprotective potential.

In an immune-mediated disease such as asthma, T helper (Th) cells played a very crucial role in induction and maintenance of disease state. Amongst two different Th cells, Th1 responsible for secretion of interleukin (IL)-2 and interferon (IFN)-c whereas Th2 is for IL-4, IL-13, and IL-5. Th2 also responsible for switching from IgG to IgE, an important mediator of asthma and which is present abundantly on the cell surface of basophils. IgG2a has been closely associated with Th1 type response. Release of various cytokines such as IL-4 and IL-13 after Th2 response has important role in airway remodeling, inflammatory processes, AHR, goblet cell hyperplasia, eosinophil infiltration and mucus hypersecretion whereas IL-5 has role in the development, activation, migration, and survival of eosinophils. Thus, in present investigation OVA sensitization resulted in the elevated levels of cytokines and chemokines (such as TNF-α, IL-4, IL-5, IL-8, IL-10 and IL-13) in lung along with serum IgE, IgG1 and IgG2a in OVA control mice. However, administration of AEC significantly inhibited this elevated levels of TNF-α, IL-4, IL-5, IL-8, IL-10, IgE and IgG1 thus explaining its Th inhibitory potential.

Therefore, results of present study reveal that attenuated *Escherichia coli* inhibits OVA-induced AHR in mice via down-regulation of nitroso-oxidative stress, cytokine and chemokine as well as IgE and IgG release which decrease the airway resistance thus supporting its anti-inflammatory as well as bronchodilator role during the allergic response in the lung.

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