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Evaluation of potential of attenuated *E. coli* treatment in airway hyperresponsiveness in a murine asthma model induced by house dust

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ABSTRACT

House-Dust-Mites (HDMs) are a major source of indoor aeroallergens for resulted in immune inflammatory disease such as asthma. To evaluate the possible effect of attenuated Escherichia coli (AEC) in a murine model of HDM-induced asthma. Intraperitoneal (100 µL) injection of HDM on day 0 followed by intranasal challenges of 50 µL of HDM significantly induced AHR in Female BALB/c mice (18-20 g). Animals were treated with either ACE (10^8 CFU/ml) or prednisolone (10 mg/kg) or vehicle on day 0, 7, 14 and 21 immediately before administration of HDM. Various behavioral, biochemical, molecular and histological parameters were assessed. The HDM-induced alteration in lung function test, bronchoalveolar lavage cellular count, and hematological count were significantly restored by AEC treatment. Administration of AEC significantly increased SOD and GSH levels whereas elevated MDA, MPO, and NO levels were decreased significantly by AEC treatment. Elevated levels of serum HDM-specific IgE and IgG1 levels, as well as TNF-α, IL-4, IL-5 and IL-8 levels in lung, were decreased significantly by AEC. However, it failed to produce any significant down-regulation in HDM induced elevated TLR-4 mRNA expression. Administration of AEC reduced lung histological alterations induced by HDM. Thus, findings of present investigation demonstrated that oral administration of attenuated E. coli inhibited HDM-induced AHR via modulation of pro-inflammatory cytokines (TNF-α, IL-4, IL-5, and IL-8), IgE and IgG responses to improved the Th1/Th2 imbalance and attenuated lung injury.

Keywords: Attenuated *Escherichia coli*, House-Dust-Mites, TNF-α, IL's, IgE, IgG1

INTRODUCTION

Allergic asthma is a heterogeneous pulmonary inflammatory disease with characteristics features including increased airway hyperreactivity (AHR), inflammation, mucus hypersecretion, goblet cell hyperplasia as well as elevated Th2 cytokines and IgE production. Prevalence of asthma is about 8-10% of children and 3-5% of the adult population [1, 2]. In last 30 years morbidity and mortality due to asthma have increased and the reasons behind it includes increased industrialization along with increased levels of indoor allergens (dust mite, cockroach, and pet dander) [3, 4].

It has been documented that commonly inhaled antigens including pollens, cats, and house dust mite (HDM) stimulates T helper type 2 (Th2) immune responses that resulted in allergic asthma [5, 6]. Researchers have reported that toll-like receptors (TLRs) which are transmembrane proteins of the interleukin- (IL-) 1 receptor superfamily has an ability to induce innate and adaptive immunity response via production of pathogen-derived antigens (such as cytokines and chemokines) in response to naive T cells in the lymph nodes, airway dendritic cells (DCs) or macrophages [7, 8]. TLR-4 has an ability to induced activation and release of various proinflammatory cytokines

such as TNF-α, IL-1β, IL-4, and IL-8 along with inducible NO synthase (iNOS), CRP (C-reactive protein) and SAP (Serum amyloid P-component) [7].

In asthma, exposure to allergen (such as pollen and HDM) resulted in induction of inflammatory events which induces symptoms including wheezing, breathlessness, chest tightness and cough [3, 9]. Animal models played a vital role in the development of new therapeutic moieties on the basis of characteristics of the disease [10]. Allergic asthma induced by using ovalbumin (OVA) is well established animal model that resemblance with many clinicopathological characteristics of asthma, however, it is not a naturally occurring allergen in man, and repeated exposure of OVA in the absence of systemic sensitization leads to tolerance [11, 12]. Moreover, airway structural changes induced after OVA challenge also strains dependent [13, 14]. Clinically it has been proven that asthmatic patients have been associated with increased level of HDM specific IgE antibodies when exposed to an allergen. Thus, development of chronic animal models using common environmental allergens such as HDM provide better understanding of the importance of airway remodeling [15].

HDMs are a major source of indoor aeroallergens for humans, which induce asthma, rhinitis, dermatitis as well as other allergic diseases and their symptoms implicated in more than 10% of the population [15]. Fecal pellets HDM contains proteolytic enzymes that have an ability cleave epithelial tight junctions to increased epithelial permeability to have an efficient delivery of allergen [16]. In addition, they have an ability induce pro-inflammatory cytokine and IgE release in airway epithelium [17]. Furthermore, HDM also contains Lipopolysaccharides (LPS) which is a pathogen-associated molecular pattern molecule has an ability to induce innate immunity via Toll-like receptor 4 (TLR4) resulted in CD^{4+}TH2-mediated allergic inflammation. It has been reported that LPS responsible for up-regulating expression of TLR-4 in human macrophages [18].

Current treatment regimens for asthma includes glucocorticoids and bronchodilators, however, these therapies can able to produce symptomatic relief in the only fraction of patients [19]. Moreover, their side effect limits their usage. Hence, it is imperative to develop a new therapy for treatment of asthma. According to previous reports, Th2-mediated diseases can be inhibited via induction of Th1 immune responses non-antigen-specific manner [20, 21]. Hence, the various infectious organism can be utilized to inhibit allergic Th2 responses induced by different allergens. Previously attenuated *Salmonella typhimurium* has been utilized for treatment of asthma where it significantly inhibits elevated ILs and OVA-specific IgG1 levels thus reduced Th2 responses [22]. Moreover, attenuated *Escherichia coli* also showed its effectiveness against allergen-induced respiratory tract infection [23, 24]. Previous research carried in our laboratory showed attenuated *E. coli* strain has efficacy against OVA-induced AHR via inhibition of cytokine, and chemokine, as well as IgE and IgG. Hence, the aim of the present investigation was to evaluate the efficacy of attenuated *E. coli* strain against HDM-induced AHR by assessing various biochemical, histological and molecular 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

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. Household dust collection and extraction

Household dust was collected from 10 different houses in Ahmadabad. The dust samples were collected from a 1-m^2 area by using an electric vacuum cleaner with a dust collector. Sterile PBS (2 ml) was added to the dust, which was then mixed end-over-end on an orbital rotator at 4°C for 16 h. The resuspended dust was centrifuged for 10 min at 4°C, 1000 g, and the supernatant was collected for allergen measurement by an ELISA. The household dust sample used for all sensitizations and airway challenges was collected from a house that showed a high level of the cockroach allergens *Blattella germanica* 1 (Bla g1) and 2 (Bla g2). A total 5.0 g of dust was collected and resuspended in 30 ml of sterile PBS for extraction as described above. After extraction, the supernatants were aliquoted and stored at -20°C until use.

2.4. Sensitization, drug treatment, and HDM challenge

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

Group I: Normal group: Animals did not undergo any HDM allergen. They were treated with vehicle (100 µl PBS with 5% sodium bicarbonate, p.o.) on day 0, 7, 14 and 21.

Group II: HDM Control group: Animals were received intraperitoneal (100 µL) injection of HDM (mixed 1: 1 with an adjuvant) on day 0 followed by intranasal challenges of 50 µL of HDM on days 7, 14 and 21. They were treated with vehicle (100 µl PBS with 5% sodium bicarbonate, p.o.) on day 0, 7, 14 and 21 immediately before administration of HDM.

Group III: Prednisolone treated group: (Pred): Animals were received (100 µL) injection of HDM (mixed 1 : 1 with an adjuvant) on day 0 followed by intranasal challenges of 50 µL of HDM on days 7, 14 and 21. They were treated with Prednisolone (10 mg/kg, p.o.) on day 0, 7, 14 and 21 immediately before administration of HDM.

Group IV: *E. coli* treated group: (AEC): Animals were received intraperitoneal (100 µL) injection of HDM (mixed 1: 1 with an adjuvant) on day 0 followed by intranasal challenges of 50 µL of HDM on days 7, 14 and 21. They were treated with attenuated *E. coli* (10^8 CFU/ml, p.o.) on day 0, 7, 14 and 21 immediately before administration of HDM.

All mice (except normal) were sensitized by four intraperitoneal (i.p) injections of 100 µL HDM which on day 0. Normal mice were injected with 50 µl saline alone. Mice were challenged on days 7, 14 and 21 by inhalation of either normal saline or HDM aerosols (50 µL) in an exposure chamber for 20 min. Aerosols were generated by nebulizing HDM solution in saline, or saline alone, with a nebulizer [25-27]. *E. coli* treated group mice were treated with 10^8 CFU bacteria in 100 µl PBS with 5% sodium bicarbonate whereas prednisolone treated group were treated with prednisolone (10 mg/kg, p.o.) on days 0, 7, 14 and 21 immediately before the i.p. injection of HDM or receiving an HDM aerosol.

2.5. 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 23 (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 [28].

2.6. 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 23, 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 [29].

2.7. Hematological analysis and serum biochemistry

On day 23, the blood was withdrawn by retro-orbital puncture for determination of hematological count and serum parameters (IgG1, IgG2a, and IgE analysis).

2.8. Bronchoalveolar Lavage Fluid (BALF) analysis

On day 23 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 [19, 30-37].

2.9. 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 [19, 38-46]. Left lung supernatant were utilized for subsequent measurement of cytokine and chemokine analysis. Another part of tissue samples were stored at -70 ^{0}C for Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis of various markers. One lung tissue from each group was processed for histopathological examination.

2.10. 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.

2.11. Determination of HDM-specific IgG1, IgG2a, IgE and total IgE

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

2.12. Determination of TLR-4 mRNA expression by using reverse transcriptase PCR

The levels of TLR-4 mRNA were analyzed in lung tissue using a reverse transcription (RT)-PCR approach as described previously [47-50]. Briefly, single-stranded cDNA was synthesized from 5 µg of total cellular RNA using reverse transcriptase (MP Biomedicals India Private Limited, India) as described previously [47, 51]. The primer sequence for TLR-4 was (Forward: TATCCACTGTAGCATTTCTGATATACC, Reverse: TCTGCTGTTTGCTCAGGATTCGAGGC, bp: 322) and β-actin was (Forward: GTCACCCACACTGTGCCCATCT, Reverse: ACAGAGTACTTGCGCTCAGGAG, bp: 764) was provided by MP Biomedicals India Private Limited, India. Amplification of β-actin served as a control for sample loading and integrity. PCR products were detected by electrophoresis on a 1.5 % agarose gel containing ethidium bromide. The size of amplicons was confirmed using a 100-bp ladder as a standard size marker. The amplicons were visualized, and images were captured using a gel documentation system (Alpha Innotech Inc., San Leandro, CA, USA). Gene expression was assessed by generating densitometry data for band intensities in different sets of experiments, by analyzing the gel images in the Image J program (Version 1.33, Wayne Rasband, National Institutes of Health, Bethesda, MD, USA) semi-quantitatively. The band intensities were compared with constitutively expressed β-actin. The intensity of mRNAs was standardized against that of the β-actin mRNA from each sample, and the results were expressed as PCR-product/ β-actin mRNA ratio.

2.13. 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.

2.14. 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 HDM-induced alteration in body weight, lung weight, and lung injury index of mice

There was significant decreased ($p < 0.01$) in body weight and significant increased ($p < 0.01$ and $p < 0.001$) in the absolute lung weight and relative lung weight of HDM control group as compared to normal group. Treatment with AEC (10^8 CFU) significantly increased ($p < 0.05$) body weight and significantly decreased ($p < 0.05$) relative lung weight as compared to HDM control mice. Administration of Prednisolone (10 mg/kg) showed significant increased ($p < 0.01$) in body weight and significant reduction ($p < 0.05$ and $p < 0.01$) in the absolute lung weight and relative lung weight as compared to HDM control mice. (Figure 1)

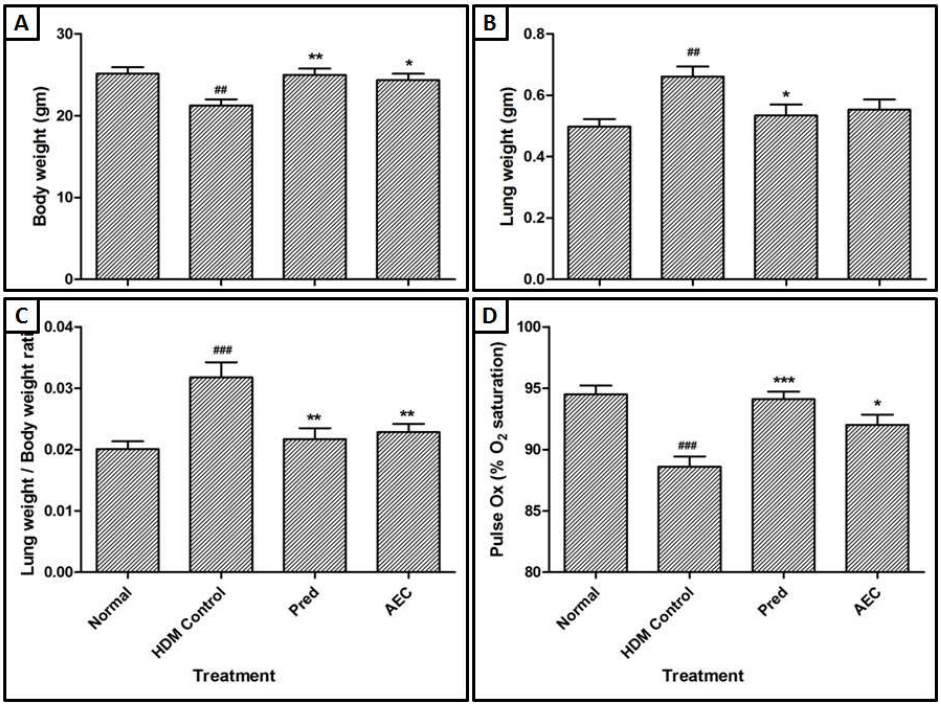

Figure 1.

Figure 2.Effect of attenuated *E. coli* treatment on HDM-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. $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ as compared with normal group and $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ as compared with HDM control group. HDM: House Dust Mite, Pred: Prednisolone, AEC: Attenuated E. coli. (n = 10).

3.2. Effect of attenuated E. coli treatment on HDM-induced alteration in percent oxygen saturation of mice

HDM control group showed significant decreased ($p < 0.001$) in the percent oxygen saturation as compared to the normal group. AEC (10^8 CFU) treatment showed a significant increase ($p < 0.05$) in the percent oxygen saturation as compared to HDM control mice. When compared with HDM control group, percent oxygen saturation was significantly increased ($p < 0.001$) in the Prednisolone (10 mg/kg) treated group. (Figure 1)

3.3. Effect of attenuated E. coli treatment on HDM-induced alteration in lung function test of mice

There was significant decreased ($p < 0.001$) in the PIF, PEF, Tidal volume, inspiratory time and expiratory time of HDM control group as compared to the normal group. Whereas, frequency of breathing and enhanced pause were increased significantly ($p < 0.001$) in HDM control group as compared to the normal group. Treatment with AEC (10^8 CFU) significantly increased ($p < 0.05$) in PIF, PEF, Tidal volume, inspiratory time and expiratory time whereas frequency of breathing and enhanced pause were increased significantly ($p < 0.05$) in AEC (10^8 CFU) treatment group as compared to HDM control mice. Treatment with Prednisolone (10 mg/kg) also showed significant restoration ($p < 0.001$) in lung function test when compared with HDM control mice. When compared

with AEC (10^8 CFU) treatment group, Prednisolone (10 mg/kg) treatment showed more significant restoration ($p < 0.05$) in PIF, PEF, and enhanced pause. (Table 1)

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

Parameters	Normal	HDM control	Pred	AEC
Peak inspiratory flow (PIF) (mL/s)	6.23 ± 0.27	3.76 ± 0.24$^{###}$	5.98 ± 0.23***$^{,\$}$	4.87 ± 0.32*
Peak expiratory flow (PEF) (mL/s)	8.51 ± 0.25	4.69 ± 0.23$^{###}$	7.44 ± 0.19***$^{,\$\$}$	6.33 ± 0.19*
Tidal volume (TV) (mL)	0.69 ± 0.04	0.45 ± 0.03$^{###}$	0.65 ± 0.04**	0.66 ± 0.03**
Frequency of Breathing (f) (bpm)	327.8 ± 10.17	440.0 ± 8.48$^{###}$	368.6 ± 14.13***	382.9 ± 12.60*
Enhanced pause (Penh)	2.42 ± 0.18	5.65 ± 0.36$^{###}$	3.27 ± 0.28***$^{,\$}$	4.45 ± 0.20*
Inspiratory time (Ti) (s)	0.22 ± 0.01	0.08 ± 0.01$^{###}$	0.19 ± 0.01***	0.15 ± 0.01*
Expiratory time (Te) (s)	0.28 ± 0.02	0.09 ± 0.01$^{###}$	0.23 ± 0.01***	0.18 ± 0.01*

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 HDM control group, and $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ as compared with one another group. HDM: House Dust Mite, Pred: Prednisolone, AEC: Attenuated *E. coli*. (n = 10).

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

When compared with normal group, the Hb, RBC, HCT, MCH and lymphocyte count were decreased significantly ($p < 0.001$) and PLT, WBC, neutrophils, monocytes and eosinophils count were increased significantly ($p < 0.001$) of HDM control mice. Administration of AEC (10^8 CFU) significantly increased ($p < 0.05$) Hb, RBC, HCT, MCH and lymphocyte count as compared to HDM control mice. Whereas, PLT, WBC, neutrophils, monocytes and eosinophils count were increased significantly ($p < 0.05$) in AEC (10^8 CFU) treatment as compared to HDM control mice. When compared with HDM control mice, Prednisolone (10 mg/kg) also showed significant inhibition ($p < 0.01$) in HDM induced alterations in hematological parameters level as compared to HDM control mice. However, Prednisolone (10 mg/kg) treatment more significantly ($p < 0.05$) restored the altered hematological parameters when compared with AEC (10^8 CFU) treated mice. (Table 2)

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

Parameters	Normal	HDM control	Pred	AEC
Hb (g/dL)	14.88 ± 0.34	11.48 ± 0.57$^{###}$	14.44 ± 0.49***	13.53 ± 0.55*
RBC (X 10^{12}/L)	11.78 ± 0.56	8.43 ± 0.56$^{###}$	11.50 ± 0.58***	11.23 ± 0.52**
HCT (%)	52.07 ± 1.43	39.86 ± 1.46$^{###}$	51.21 ± 1.12***$^{,\$}$	45.94 ± 0.88*
MCH (pg)	14.64 ± 0.41	10.02 ± 0.41$^{###}$	13.85 ± 0.46***	12.38 ± 0.72*
PLT (X 10^9/L)	824.1 ± 38.00	1164.00 ± 60.93$^{###}$	914.8 ± 49.57**	959.3 ± 46.61*
WBC (X 10^6/mL)	7.74 ± 0.31	11.64 ± 0.29$^{###}$	8.84 ± 0.35***$^{,\$}$	9.80 ± 0.38**
Neutrophils (%)	32.90 ± 1.19	43.30 ± 0.88$^{###}$	35.60 ± 0.97***	38.80 ± 0.94*
Lymphocyte (%)	62.40 ± 1.12	44.20 ± 0.57$^{###}$	57.50 ± 0.74***$^{,\$\$}$	52.70 ± 0.80***
Monocytes (%)	1.40 ± 0.26	5.80 ± 0.24$^{###}$	3.00 ± 0.25***	3.80 ± 0.20***
Eosinophile (%)	3.30 ± 0.30	6.70 ± 0.61$^{###}$	3.90 ± 0.48**	4.70 ± 0.53*
BALF Total cells (X 10^5/mL)	4.20 ± 0.58	27.20 ± 0.86$^{###}$	6.60 ± 0.81***$^{,\$\$\$}$	12.60 ± 0.74***
BALF Neutrophils (X 10^5/mL)	0.80 ± 0.37	5.00 ± 0.31$^{###}$	1.60 ± 0.24***$^{,\$}$	3.20 ± 0.37**
BALF Lymphocyte (X 10^5/mL)	0.80 ± 0.37	6.00 ± 0.44$^{###}$	1.40 ± 0.24***	2.40 ± 0.50***
BALF Eosinophile (X 10^5/mL)	0.40 ± 0.24	9.20 ± 0.37$^{###}$	1.20 ± 0.37***	2.20 ± 0.37***
BALF Macrophages (X 10^5/mL)	2.20 ± 0.73	7.00 ± 0.70$^{##}$	2.40 ± 0.67**	4.80 ± 0.86

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 HDM control group, and $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.01$ as compared with one another group. HDM: House Dust Mite, Pred: Prednisolone, 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 HDM-induced alteration in BALF differential cell count of mice

HDM control group showed significant increased ($p < 0.001$) in the BALF total and differential cell count as compared to the normal group. However, treatment of AEC (10^8 CFU) showed significant reduction ($p < 0.001$) in the HDM induced elevated BALF total and differential cell count as compared to HDM control group. When compared with HDM control group, BALF total and differential cell count was significantly decreased ($p < 0.001$) by Prednisolone (10 mg/kg) treatment as compared to HDM control group. Prednisolone (10 mg/kg) treatment showed more significant inhibition ($p < 0.001$ and $p < 0.01$) in the HDM induced increased BALF total cell count and BALF Neutrophils count as compared to AEC (10^8 CFU) treated group. (Table 2)

3.6. Effect of attenuated E. coli treatment on HDM-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 levels of the HDM control mice as compared to the normal group. Whereas, the BALF MDA, NO, MPO and albumin levels were increased significantly ($p < 0.001$) in the HDM control group as compared to the normal group. However, decreased in BALF SOD and GSH levels were significantly increased ($p < 0.01$ and $p < 0.05$) by treatment with AEC (10^8 CFU) as compared to HDM control mice. When compared with HDM control mice, BALF MDA, MPO and albumin levels were significantly decreased ($p < 0.01$, $p < 0.01$ and $p < 0.05$) in AEC (10^8 CFU) treatment group. Prednisolone (10 mg/kg) treatment showed significant inhibition ($p < 0.01$) in the HDM induced decreased BALF SOD and GSH levels as compared to HDM control group. Whereas, BALF MDA, NO, MPO and albumin levels were increased significantly ($p < 0.001$) in the Prednisolone (10 mg/kg) treatment group as compared to HDM control group. Administration of Prednisolone (10 mg/kg) showed more significant reduction ($p < 0.01$ and $p < 0.05$) in BALF NO and MPO levels as compared to AEC (10^8 CFU) treatment group. (Table 3)

Table 3 Effect of attenuated *E. coli* treatment on HDM-induced alteration in the levels of SOD, GSH, MDA, NO, MPO and albumin in BLAF and lung of mice

Parameters	Normal	HDM control	Pred	AEC
SOD level in BALF (U/mg of protein)	2.06 ± 0.19	0.85 ± 0.19$^{###}$	1.96 ± 0.15**	1.67 ± 0.15**
GSH level in BALF (µg/mg of protein)	1.23 ± 0.12	0.40 ± 0.12$^{###}$	1.16 ± 0.11**	1.07 ± 0.13*
MDA level in BALF (nM/ mg of protein)	2.37 ± 0.20	5.65 ± 0.36$^{###}$	3.83 ± 0.15***	4.06 ± 0.28**
NO level in BALF (µg/mL)	16.02 ± 2.04	63.74 ± 4.31$^{###}$	32.08 ± 4.05***,$}$	52.99 ± 4.47
MPO level in BALF (U/mL)	5.00 ± 0.44	14.52 ± 0.88$^{###}$	9.78 ± 0.54***,}$	12.81 ± 0.83**
Albumin level in BALF (g/dL)	0.34 ± 0.05	0.73 ± 0.07$^{###}$	0.41 ± 0.04**	0.48 ± 0.05*
SOD level in Lung (U/mg of protein)	8.03 ± 0.43	4.46 ± 0.33$^{###}$	7.45 ± 0.44***	6.73 ± 0.27**
GSH level in Lung (µg/mg of protein)	5.33 ± 0.387	1.79 ± 0.16$^{###}$	4.54 ± 0.50***	3.69 ± 0.31**
MDA level in Lung (nM/ mg of protein)	4.21 ± 0.35	7.86 ± 0.58$^{###}$	5.16 ± 0.37**	5.54 ± 0.49**
NO level in Lung (µg/mL)	52.74 ± 7.03	238.0 ± 21.67$^{###}$	110.0 ± 11.62***,}$	169.4 ± 10.17*
MPO level in Lung (U/mL)	12.11 ± 1.20	37.18 ± 1.47$^{###}$	18.91 ± 2.25***	23.08 ± 3.53**
Albumin level in Lung (g/dL)	0.47 ± 0.05	2.32 ± 0.26$^{###}$	0.86 ± 0.11***,}$	1.64 ± 0.15***

*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 HDM control group and $^{$}p < 0.05$, $^{$$}p < 0.01$ as compared with one another group.. SOD: superoxide dismutase, GSH: glutathione, MDA: malondialdehyde, NO: nitric oxide and MPO: Myeloperoxidase, HDM: House Dust Mite, Pred: Prednisolone, AEC: Attenuated E. coli (n = 5).*

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

There was significant decreased ($p < 0.001$) in lung SOD and GSH level in HDM control group as compared normal mice whereas HDM control group showed significant increased ($p < 0.001$) in the lung MDA, NO, MPO and albumin levels as compared to normal group. AEC (10^8 CFU) treatment significantly increased ($p < 0.01$) SOD and GSH levels, whereas there was significant decreased ($p < 0.01$) lung MDA, NO, MPO and albumin levels as compared to HDM control mice. Administration of Prednisolone (10 mg/kg) also showed significant increased ($p < 0.001$) SOD and GSH levels and significant decrease ($p < 0.001$) in lung MDA, NO, MPO and albumin levels as compared to HDM control mice. Prednisolone (10 mg/kg) treatment showed more significant decreased ($p < 0.05$) in the HDM induced increased lung NO and albumin levels as compared with AEC (10^8 CFU) group. (Table 3)

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

There was significant increased ($p < 0.001$) in the lung TNF-α, IL-4, IL-5, IL-8, IL-10 and IL-13 levels of HDM control group as compared to normal group. Treatment with AEC (10^8 CFU) significantly inhibited ($p < 0.05$) HDM induced increased lung TNF-α, IL-4, IL-5 and IL-8 level as compared to HDM control group. Prednisolone (10 mg/kg) treatment showed significant reduction ($p < 0.001$, $p < 0.001$, $p < 0.001$, $p < 0.05$ and $p < 0.01$, resp.) in lung TNF-α, IL-4, IL-8, IL-10 and IL-13 levels as compared to HDM control mice. Administration of Prednisolone (10 mg/kg) produced more significant ($p < 0.05$, $p < 0.001$ and $p < 0.01$) inhibition in HDM induced increased lung TNF-α, IL-4 and IL-8 levels as compared to AEC (10^8 CFU) treated mice. (Figure 3)

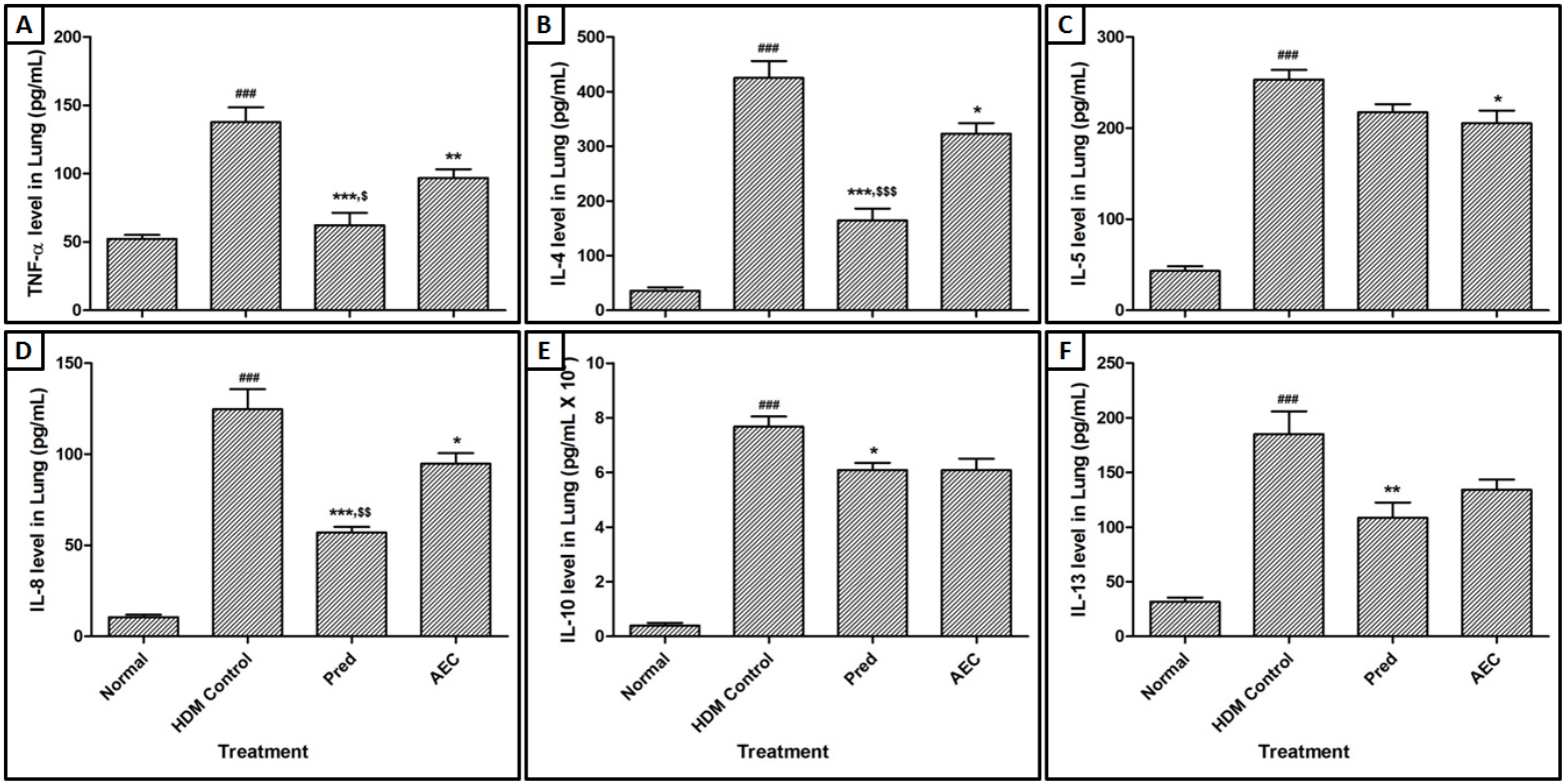

Figure 3. Effect of attenuated *E. coli* treatment on HDM-induced alteration in level of lung TNF-α (A), IL-4 (B), IL-5 (C), IL-8 (D), IL-10 (E) and IL-13 (F) levels in mice

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 HDM control group, and $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$ as compared with one another group. TNF- α: tumor necrosis factor-alpha, IL: interleukin, HDM: House Dust Mite, Pred: Prednisolone, AEC: Attenuated E. coli, (n = 5).

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

There was significant increased ($p < 0.001$) in the serum total IgE, HDM-specific IgE and HDM-specific IgG1 levels whereas HDM-specific IgG2a level was decreased significantly in HDM control group as compared to the normal group. Treatment with AEC (10^8 CFU) significantly decreased ($p < 0.01$, $p < 0.05$ and $p < 0.05$) serum total IgE, HDM-specific IgE and HDM-specific IgG1 levels as compared to HDM control group. Prednisolone (10 mg/kg) treatment also showed significant reduction ($p < 0.001$) in serum total IgE and HDM-specific IgE levels as compared to HDM control mice. (Figure 4)

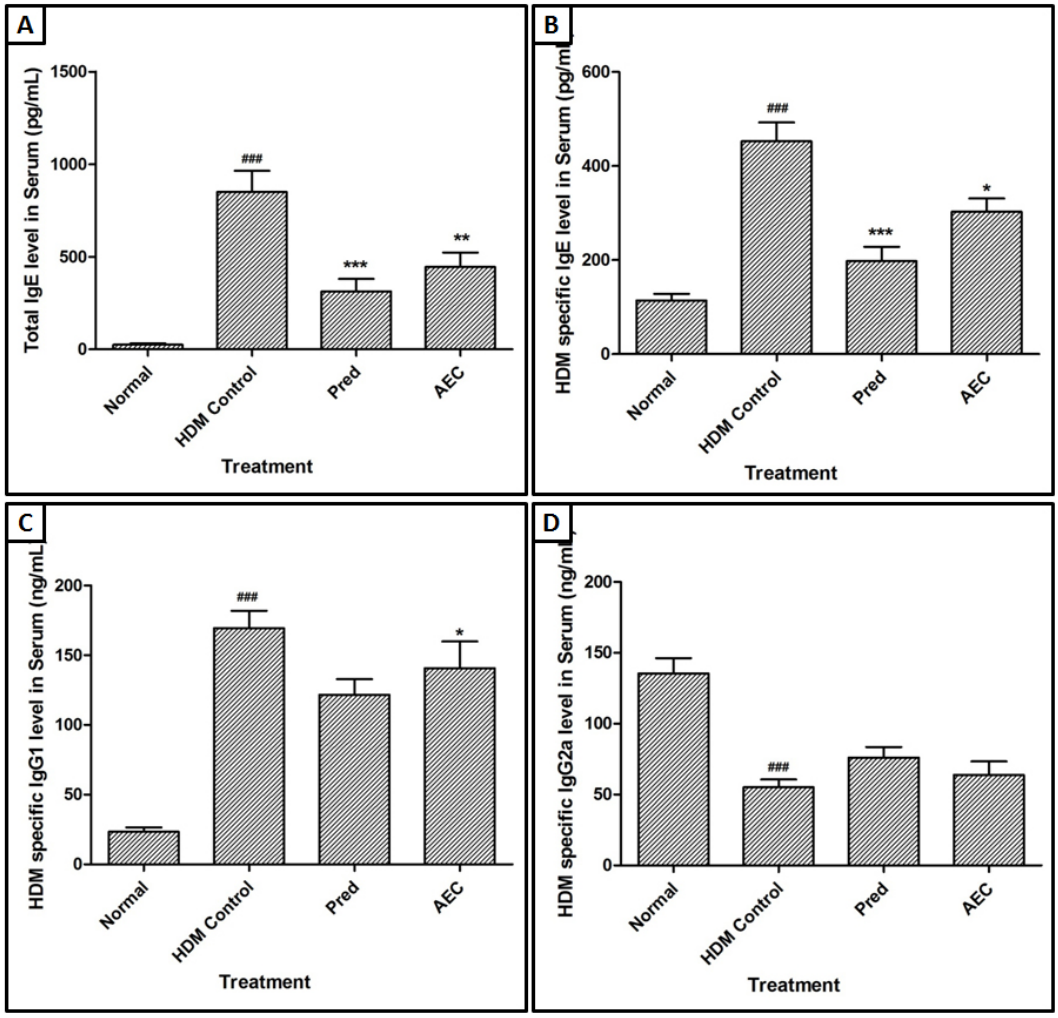

Figure 4. Effect of attenuated *E. coli* treatment on HDM-induced alteration in the levels of serum total IgE (A), HDM-specific IgE (B), HDM-specific IgG1 (C) and HDM-specific IgG2a (D) of mice

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 HDM control group and $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ as compared with one another group. HDM: House Dust Mite, Pred: Prednisolone, AEC: Attenuated E. coli, (n = 5).

3.10. Effect of attenuated E. coli treatment on HDM-induced alteration in the mRNA levels of TLR-4 of mice

HDM control group showed significant up-regulation ($p < 0.001$) in the TLR-4 m-RNA expression as compared to the normal group. AEC (10^8 CFU) and Prednisolone (10 mg/kg) treatment failed to produce any down-regulation of TLR-4 m-RNA expression as compared to HDM control mice. (Figure 5)

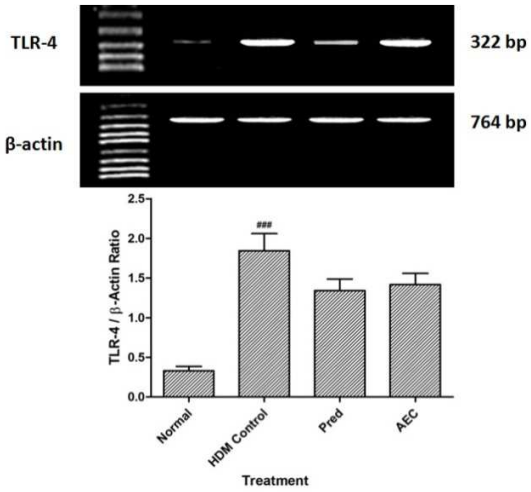

Figure 5. Effect of attenuated *E. coli* treatment on HDM-induced alteration in the TLR-4 mRNA expression of mice
Data was analyzed by One-Way ANOVA followed by Tukey's multiple comparison post hoc tests. $^{###}p < 0.001$ as compared with normal group. HDM: House Dust Mite, Pred: Prednisolone, AEC: Attenuated E. coli, (n = 4).

3.11. Effect of attenuated E. coli treatment on HDM-induced pathological alteration in mice lungs

Lungs tissue from normal group revealed normal morphologies with thin lined alveolar septa with well-organized alveolar space (Figure 6A). Administration of HDM induced severe inflammatory changes in lungs (red arrow, grade 4) and there were distorted lung morphologies (yellow arrow, grade 4). There was a severe alveolar injury with thickened intra-alveolar septa, collapsed alveolar spaces, huge inflammatory exudates and oedema (black arrow, grade 3) (Figure 6B). Treatment of Prednisolone (10 mg/kg) markedly reduced lung injury however, it showed moderate inflammatory cell infiltration (red arrow, grade 1), vascular congestion and oedema (black arrow, grade 1) (Figure 6C). Lung tissue from AEC (10^8 CFU) treated mice showed presence of moderate epithelial degeneration (yellow arrow, grade 2), inflammatory cell infiltration (red arrow, grade 3), vascular congestion and oedema (black arrow, grade 2) (Figure 6D). (Table 4)

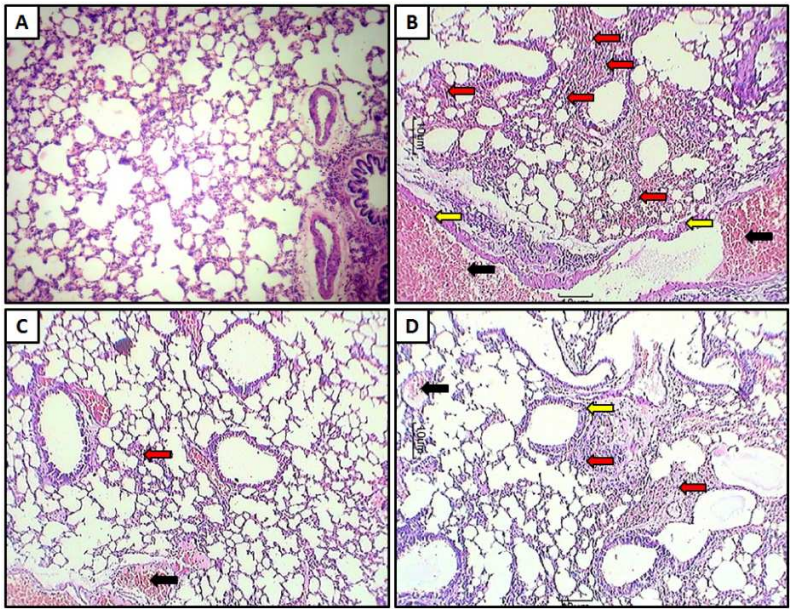

Figure 6. Effect of attenuated *E. coli* treatment on HDM-induced pathological alteration in mice lungs
Photomicrograph of sections of lungs of normal (A), HDM control (B), Prednisolone (10 mg/kg) treated (C) and Attenuated E. coli (10^8 CFU) treated (D) mice. Lung H&E staining at 40 X.

Table 4 Effect of attenuated *E. coli* treatment on HDM-induced pathological alteration in mice lungs

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

HDM: House Dust Mite, Pred: Prednisolone, 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

Allergic asthma is an immune-inflammatory disorder characterized by AHR, mucus hypersecretion, metaplasia of goblet cell along with elevated Th2 cytokines and IgE levels. HDM induced mouse model of asthma provided insight into disease status for better understanding of symptoms that clinically resemblance with human asthma thus increases confidence in the outcome of studies evaluating novel anti-asthmatic treatments. Researcher have administered corticosteroid therapy such as dexamethasone and prednisolone for treatment of allergic asthma induced by HDM extract where it modulates inflammatory cell infiltration to ameliorate HDM induced asthma [52]. In present investigation, we also evaluate the effect of administration of attenuated *Escherichia coli* (AEC) (10^8 CFU) on HDM induced asthma by evaluation its effect on release of inflammatory cell, cytokine, IgE, IgG and TLR-4.

Macrophages played a vital role in the induction of immune responses via host defense mechanism to encounter allergens [53]. A phenotype of M1 macrophages possess potential for induction and release of pro-inflammatory cytokines such as Tumor necrosis factor α (TNFα) and interferon γ (IFNγ) [54], whereas M2 macrophages played important role in tissue repair and they release in response with IL-4 or IL-13 activation [55, 56]. Eosinophils also release in response with inhaled allergens like HDM which caused activation of inflammatory dendritic cells (DCs) resulted in release of innate pro-Th2 cytokines (such as IL-25 and IL-33) thus resulted in Th2 immunity [57]. Basophils serves as antigen-presenting cells which plays vital role in amplification FcεRI+ DCs in the lung against inhaled HDM to initiate Th2 immunity [58]. Furthermore, activation of basophils has been reported to depend on activation of the innate adaptor protein MyD88 that signaling via TLR4 and the IL-33 receptor [59]. In present investigation level of macrophages and eosinophils increased in BALF indicating induction of Th2 innate immunity however, administration of AEC showed reduction of these elevated BALF total cells count.

Clinically pulse fingertip pulse oxymeter probe has been utilized routinely to monitor respiratory conditions of respiratory distress patients. The principle behind it is to determine oxygen saturation (SaO_2) of the arterial hemoglobin [60]. Pulse oxymeter provide an advantage of determination of respiratory condition in allergic infected mice without sacrificing it. In the present study, there was significant decreased in the level of percent oxygen saturation which was significantly restored by ACE treatment.

Oxidative stress has been reported to play a significant role in mediating allergic inflammatory disease including asthma [19]. Reactive oxygen species (ROS) released from activated neutrophil NADPH oxidase that causes tissue injury in the form of inflammation, ischemia, and asthma [30, 61, 62]. The mechanism behind the induction of injury includes elevated lipid peroxidation, enzymatic inactivation, glutathione depletion, an increase of vascular permeability, cellular recruitment, and DNA damage [63-66]. Furthermore, researcher has reported that excessive production in nitric oxide (NO) leads to cytotoxic and contributes to pathological alteration including excessive mucous production, inflammatory infiltration and airway remodeling [67]. Non-invasive monitoring of NO level in asthma patient correlates with the degree of bronchoconstriction as well as it serves as the markers for eosinophilic infiltration in airway epithelium [68]. There was a significant rise in the level of nitroso-oxidative stress in HDM control mice whereas administration of AEC produced significant inhibition in HDM induced an increase in nitroso-oxidative stress.

TNF-α and IL-1β are two important pro-inflammatory mediators that have been released by various cell (including mast cells, macrophages, neutrophils, eosinophils, and epithelial cells) in response to allergic inflammation. This pro-inflammatory mediator played a vital role in immunoregulation to induced bronchial hyperresponsiveness in asthmatic condition [69]. Moreover, it caused the release of neutrophils, myofibroblasts as well as eosinophils

activation and alteration vascular permeability [70-74]. Significantly elevated levels of TNF-α and IL-1β also have been documented in the airways of asthmatics patients [75]. Elevated level of TNF-α in the lung of HDM mice reflected the activation of pro-inflammatory response whereas administration of AEC inhibits this may be virtue of its anti-inflammatory potential. The results of the present investigation corroborate the findings of our previous investigation where administration of AEC inhibits the release of TNF-α in OVA-induced asthmatic mice.

Th2 cytokine, such as IL-4 played a significant role in the recruitment of mast cell that causes the release of histamine and leukotrienes [76-78]. Whereas IL-5 and IL-13 responsible for the release of eosinophils and macrophages into serum and BALF from asthmatics via terminating its differentiation within the bone marrow [79]. IL-10 is another cytokine that has produced after immune activation of a number of cells including T cells, B cells, monocytes, and macrophages. It has been reported that release of IL-4 inversely depends on the level of IFN-γ which is a negative regulator of Th2 differentiation. It is also an important mediator of IgE class switching [27]. However, in allergic condition release of IL-4 is independent of IL-13. In present study, level of both IL-4 and IL-5 were elevated in lung of asthmatics and these findings is in line with the findings of previous investigators [80, 81]. Alteration induced in lung histology after release of IL-4 included goblet cell hyperplasia, inflammatory accumulation in peribronchial region which was also confirmed in our study and it is accordance with pathological findings made by previous investigator [15]. Administration of AEC significantly reduced elevated levels of IL-4 as well as IL-5 and thus reduced the inflammatory influx in lung tissue reflected by reduced lung aberration induced by HDM.

In allergic asthma, IgE and IgG played a decisive role in allergen response. IL-4 and IL-13 promote IgE production by normal peripheral blood mononuclear cell (PBMC) [68]. The production of antigen-specific IgG2a antibodies can antagonize the allergic inflammatory cascades resulting from antigen recognition by IgE. Administration of AEC caused significant inhibition in HDM induced elevated IgE and IgG production in turn may decrease immune response.

Research carried out over past decades evidences that in various pathological conditions such as sepsis and inflammation, Toll-like receptors (TLR) play a significant role in host cell responses to microbial pathogens and their products [82, 83]. TLR-4 serves as a crucial sensor in activation of macrophages by lipopolysaccharide (LPS) [18]. A variety of cytosolic adaptor molecules (MyD88, mal, Trif and Tram) stimulated by TLR that activates protein kinases (IRAK1, IRAK4, and IKK) resulted in signal amplification for proinflammatory transcription factors, which induce inflammatory response. TLR4 signaling also caused maturation of alveolar macrophage via activation of NF-κB [84]. In the present study, HDM-induced mice showed up-regulated expression of TLR4 and this results corroborates the findings of the previous researcher [84]. However, administration of AEC did not produce any significant down-regulation in TLR-4 mRNA expression. The similar findings were reported by previous investigators were P fimbriated *E. coli* administration did not produce any alteration in TLR-4 mice [85].
Thus, findings of present investigation demonstrated that oral administration of attenuated *E. coli* inhibited HDM-induced AHR via modulation of pro-inflammatory cytokines (TNF-α, IL-4, IL-5 and IL-8), IgE and IgG responses to improved the Th1/Th2 imbalance and attenuated lung injury.

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