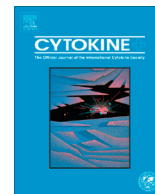




Contents lists available at ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Lipopolysaccharide (LPS) exposure differently affects allergic asthma exacerbations and its amelioration by intranasal curcumin in mice

Asha Kumari^a, D. Dash^b, Rashmi Singh^{a,*}^a Department of Zoology, MMV, Banaras Hindu University, Varanasi 221005, India^b Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221005, India

ARTICLE INFO

Article history:

Received 30 March 2015

Received in revised form 4 July 2015

Accepted 27 July 2015

Available online xxx

Keywords:

Asthma exacerbations

Neutrophils

Lipopolysaccharide

ABSTRACT

Aim: Lipopolysaccharide (LPS) is ubiquitous in the environment and can therefore, exacerbate allergic responses. Studies have suggested immunoregulatory effects of LPS according to route, dose and stage of exposure. Present study has examined whether dose and stage of LPS exposure (during sensitization and challenge with OVA) exacerbates airway inflammations, antigen specific-IgE level, histamine release, Th1/Th2 cytokine response. Further, anti-asthmatic potential of curcumin, through intranasal route has been evaluated for the first time in LPS induced airway inflammation in an ovalbumin (OVA)-challenged mouse asthma model.

Methods: Balb/c mice were first sensitized with OVA on 1st and 8th day and exposed to two LPS doses (0.1/1.0 µg) separately on 2nd day and then further exposed to LPS with OVA-aerosol (from 9 to 14 day). Further, lower LPS dose (0.1 µg) was chosen for OVA exposed mouse model of asthma exacerbation study. Intranasal curcumin was administered from 9th to 14th day before every LPS exposure.

Results: Exposure to LPS (0.1 µg) exacerbates airway inflammations in terms of IgE level, Th2-cytokine response (IL-4 and IL-5), histamine release, EPO and MPO activities and oxidative stress. Intranasal curcumin has effectively ameliorated airway exacerbations whereas dexamethasone, a known glucocorticosteroid, was not promising as compared to intranasal curcumin.

Conclusion: Schedule and dose of LPS exposure determines asthma exacerbations and intranasal curcumin could be better immunomodulatory agent in LPS exposed asthma exacerbations.

© 2015 Published by Elsevier Ltd.

1. Introduction

Asthma is a common respiratory disorder characterized by airway inflammation, bronchoconstriction and airway hyperresponsiveness. Immune response in asthma is mainly driven by T-helper 2 (Th2) cells, mainly IL-4, IL-5 and IL-13 resulting in eosinophil recruitment, mast cell influx, mucous secretion and hyperresponsiveness [1]. Some well known environmental triggers of asthma are smoke, particulate matters, pollutants and bacterial or viral infections [2]. LPS a major component of the outer membrane of gram-negative bacteria and its exposure is a major risk factor for asthma as studies have shown strong relationship between levels of household LPS and asthma exacerbations [3]. Gram negative bacterial infections like *Chlamydia*, *Mycoplasma*, and *Haemophilus influenzae* are most frequently associated with asthmatic exacerbations. There is apparent controversy about the

role of LPS in asthma and allergic inflammation as evidenced by epidemiological and experimental studies, which show that LPS exhibits both protective as well as exacerbating role on asthma [4–6].

LPS binds to Toll like receptor (TLR-4) initiating MyD88 dependent or MyD88 independent signalling which lead to the secretion of pro inflammatory cytokines like, IL-1, IL-6, TNF-α and IFN-γ [7]. Adaptive transfer of bone-marrow-derived mast cells (BMMCs) from wild-type, but not from TLR4-deficient mice, restored eosinophils recruitment in TLR4-deficient mice [8]. Low dose of LPS regulates asthmatic response via TLR-4 signalling pathway in alveolar macrophages with marked infiltration of eosinophils and neutrophils to the lungs with increased Th-2 cytokines production [9]. Mast cells are major regulatory cells in asthma as they release potent physiological and immunological modulators such as eicosanoids, proteases and histamine [10]. Further, they can also respond to LPS directly by producing IL-1, IL-6, IL-13, and TNF-α without degranulation [11,12].

Recent studies have suggested importance of neutrophils in asthma as these are the first cells to be recruited and have been

* Corresponding author.

E-mail addresses: rashmirs98@rediffmail.com, rashmisinghmmv@gmail.com (R. Singh).

linked to severe, chronic and sudden attacks of asthma [13]. Activated neutrophils can release metalloproteinase (MMP-9), elastase and myeloperoxidase (MPO) contributing to the asthma worsening [14]. Reactive oxygen species (ROS) released from activated neutrophils has long been known to induce epithelial cell inflammation by directly causing tissue injury. Reactive oxygen species derived from EPO and MPO can interact with nitrite (NO_2^-) and H_2O_2 leading to the formation of reactive nitrogen species (RNS) whose presence in airways has been correlated with asthma severity [15–18]. Many studies have reported detrimental effects of oxidative damage and low neutrophil clearance from lungs in acute respiratory distress syndrome (ARDS) [19,20].

LPS is one of the most powerful pro-inflammatory factor and can induce acute pulmonary inflammation even lung injury after inhalations or systemic administration. The hygiene hypothesis states that low incidences of childhood infections in industrialized countries modulate Th1 response, thus increasing the tendency to allergic diseases in old age [21]. Recently, new phenotype of asthma has been reported among steroid resistant forms which account for 5–10% of all asthmatics. These stable forms are mainly non-eosinophilic in nature dominated by neutrophils and macrophages with distinct lower airway inflammation [22,23]. Though corticosteroids like dexamethasone are presently used as anti-asthmatic drug but several studies have reported that presence of microbes in airways can inhibit corticosteroid responses, potentially influencing the efficacy of corticosteroid treatment in asthma [24].

Curcumin (diferuloylmethane), a phytochemical present in turmeric as been shown to down regulate numerous cytokines (IL-4, IL-5 and IL-13) and expression of various cell surface adhesion molecules [25,26]. Curcumin has also been investigated and shown to protect endotoxin (LPS) induced acute and chronic lung injury [27]. Studies suggest that curcumin is protective against endotoxin (LPS) shock by improving survival and reducing the severity of shock symptoms such as lethargy, diarrhoea, and watery eyes following LPS challenge. We have reported earlier that intranasal curcumin could be protective in acute and chronic asthma; hence it may be effective in ameliorating LPS exacerbated allergic asthma. Intranasal route of delivery is particularly attractive as it can deliver a drug directly to the airways, allowing efficient onset of action without degradation. Epithelial dysfunction is a fundamental pathology leading to asthma and administration of a drug directly to this site would be highly advantageous [28]. Furthermore, intranasal drug administration offers ease of use and promotes patient adherence to treatment, thereby offering greater feasibility than systemic delivery.

2. Methods

2.1. Animal and reagents

Balb/c mice (6–8 weeks old, 20–24 g) were purchased from Central Animal Facility of Central Drug Research Institute, Lucknow, India and maintained at standard temperature (25 °C) and light: dark cycle. They were given standard diet and water *ad libitum*. All the experimental procedures were approved by the Animal Ethical Committee, Banaras Hindu University, Varanasi,

India. LPS (*Escherichia coli* serotype O111:B4), Ovalbumin (OVA, grade V), Curcumin (diferuloylmethane), hexadecyltrimethylammonium bromide (HTAB) and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St Louis, MO). The Toxin sensor™ chromogenic LAL endotoxin assay and Toxin Eraser™ endotoxin removal kit were purchased from (GenScript, Piscataway, NJ, USA). O-phenyladenine (OPD) O-phthalaldehyde (OPT) was purchased from Ameresco (Solon, USA) and spectrochem, India respectively. HRP labelled anti-mouse IgE secondary was purchased from Southern Biotech (Birmingham, USA). IL-5, IL-4, IFN- γ and TNF- α ELISA kits were purchased from Biolegend (San Diego, CA).

2.2. Detection of LPS contamination in OVA solution and purification

LPS contamination in OVA solution was measured quantitatively using the Toxin Sensor chromogenic LAL endotoxin assay kit with a sensitivity range of 0.005–1 EU/ml. Different concentrations of OVA solution (ranging from 0.5 mg/ml to 10 mg/ml) were used to evaluate LPS content. First, 100 μl of LAL was added to 100 μl of each sample followed by 40 min of incubation at 37 °C. Following incubation, 100 μl of chromogenic substrate was added to each sample followed by 6 min of incubation at 37 °C. After incubation, 500 μl of stop solution, 500 μl of colour stabilizer 2 and 500 μl of colour stabilizer 3 were added to each sample. Absorbance was measured at 550 nm. LPS contamination in 1% OVA solution was 0.2 ng/ml which was purified by using Toxin Eraser™ endotoxin removal kit according to manufacture instructions up to 0.043 ng/ml.

2.3. Determination of Effective LPS dose in mice

To study effective dose of LPS exposure and asthma exacerbations, mice were randomly divided into four groups ($n = 5$). Control, OVA, OVA + LPS (0.1), OVA + LPS (1) (Table 1). Animals were sensitized with 100 μg OVA and 1 mg aluminium hydroxide i.p (Imject alum; Thermo Scientific) on day 1 and 8 in 0.25 ml phosphate buffer saline (PBS), further mice were exposed to 1% aerosolized LPS free OVA for 7 consecutive days from day 9 to 15 in a close plexiglass chamber. Control group was sensitized and challenged with PBS alone (Fig. 1).

2.4. LPS exposure

Mice were exposed to LPS (0.1 and 1.0 μg) on day 2 of sensitization an hour before antigen administration and on each day of OVA-aerosol challenge in 100 μl sterile saline. Control mice were given LPS free saline (Table 1).

2.5. LPS induced asthma exacerbations and Intranasal curcumin treatment

LPS (0.1 μg) was more effective in inducing asthma exacerbations, hence it was chosen to study immunomodulatory effects of curcumin on LPS exacerbated mouse model of asthma. Curcumin was administered 2 h before every OVA challenge.

Table 1
Experimental groups for the determination of effective LPS dose.

S. no	Groups	Sensitization (i.p)	Challenge (Aerosol)	LPS exposure (i.p)	Treatment (i.n)
Group I	Control	PBS	PBS	–	–
Group II	OVA	OVA	OVA	–	–
Group III	OVA + LPS (0.1)	OVA	OVA	0.1 μg	–
Group IV	OVA + LPS (1)	OVA	OVA	1 μg	–

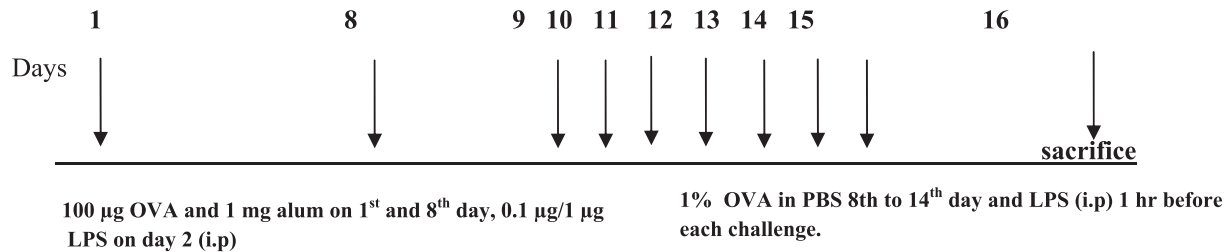


Fig. 1. Sensitization and challenge schedule: LPS exposed asthma exacerbation in mouse model. LPS exacerbated groups were given LPS (0.1/1 µg) on 2nd day and on each day before OVA-aerosol challenge from 9th to 14th day.

Table 2

LPS induced asthma exacerbation and treatment groups.

S. no	Groups	Sensitization (i.p)	Challenge (Aerosol)	LPS exposure (i.p)	Treatment (i.n)
Group I	Control	PBS	PBS	–	–
Group II	OVA	OVA	OVA	–	–
Group III	OVA + L	OVA	OVA	0.1 µg	–
Group IV	OVA + L + DM	OVA	OVA	0.1 µg	–
Group V	OVA + L + CU5 mg	OVA	OVA	0.1 µg	5 mg/kg (i.n)
Group VI	OVA + L + CU10 mg	OVA	OVA	0.1 µg	10 mg/kg (i.n)
Group VII	OVA + L + DEXA	OVA	OVA	0.1 µg	Dexamethasone 1 mg/kg (i.p)

Briefly, curcumin was dissolved in dimethylsulphoxide (DMSO) and administrated (2.5 µl) to each nostril and mice were kept in supine position for few minutes as per dose regime (5 mg/kg and 10 mg/kg respectively, (Table 2).

2.6. Lungs, BAL fluid, serum collection and BAL cell counts

Serum was collected and stored at –20 °C for further analysis. Lungs were removed and stored at –80 °C. Right lung lobe was fixed for 24 h in 10% neutral buffered formalin (NBF) for histopathological analysis. Bronchoalveolar lavage fluid (BALF) was collected by trachea cannulation and flushing lungs with 1 ml of autoclaved PBS three times. BALF was centrifuged and supernatant was stored at –80 °C. Total cell counts in BALF pellet were performed by using haemocytometer and cytospin preparations for differential count were stained by Giemsa for 10 min. Randomly five fields were selected at ×40 and cells were identified on the basis of morphological characteristics.

2.7. Eosinophil peroxidase (EPO) assay

EPO activity was measured in both BALF and lungs [29]. Briefly, lung was homogenized in 5% PBS (w/v) and centrifuged at 6000 rpm at 4 °C for 15 min. The cell pellet was resuspended in PBS containing 0.5% HTAC and homogenized again followed by three freeze thaw cycles. The sample were mixed with reaction mixture containing 1.5 mM OPD and 6.6 mM hydrogen peroxide H₂O₂ in 75 mM Tris–HCl (pH–8.0) followed by 30 min incubation at 20 °C in dark. The reaction was stopped by using 4 M H₂SO₄ and results were shown in absorbance units.

2.8. Myeloperoxidase (MPO) activity

MPO activity was measured in lungs to quantify pulmonary neutrophil infiltration [30]. Briefly, lung homogenate was prepared in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% CTAB (cetyltrimethylammonium bromide) and subjected to three freeze cycles at –80 °C. The reaction mixture containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.002% hydrogen peroxide (H₂O₂) was mixed with homogenate in 300 µl volume.

MPO activity in lungs was calculated by measuring change in absorbance at 460 nm over 20 min.

2.9. Serum IgE level

Ova specific-IgE was measured in serum as described earlier [31]. Microtitre plates were coated with 20 µg OVA in 100 µl coating buffer (sodium carbonate and sodium bicarbonate, pH 9.6) at 4 °C overnight. Plate was washed 5 times on day 2 with PBST (0.05% Tween-20) and blocked with 1% BSA at 37 °C for 2 h, followed by plate washing with PBST. Serum (1:9) was incubated for 2 h at 37 °C, followed by three subsequent washing with PBST. HRP labelled anti-mouse IgE (1:1000) was incubated for 2 h at 37 °C. OVA specific serum IgE was detected using TMB and reaction was stopped by using 100 µl of 2.4 M sulphuric acid. Absorbance was measured at 450 nm.

2.10. Histamine estimation in serum

Histamine release was detected in serum using flat bottom black well plate in dark as reported earlier [31]. In brief, 180 µl of diluted serum in 0.1 M HCl was mixed with 36 µl 1 M NaOH and 9 µl 1% w/v OPT dissolved in acetone-free methanol. The reaction was stopped after 20 min of incubation using 18 µl 3 M HCl. Fluorescence intensities were measured using 360 nm excitation and 450 nm emission filters.

2.11. Cytokine analysis

Cytokines IFN-γ, IL-4, IL-5 and TNF-α levels in BAL fluid were analysed by ELISA according to the manufacturer's recommendations. The optical density was measured at 450 nm in microplate reader (EL×800, BioteK). The concentration of cytokines were determined and expressed in pg/ml.

2.12. Lung histology

Paraffin embedded lung sections (5 µm) were stained with haematoxylin–eosin (H&E). The degree of pathological changes (stereology) in lung architecture, bronchoconstriction and

recruitment of inflammatory cells in alveolar space were observed under light microscope ($\times 40$).

2.13. Nitrite level estimation

Nitrite level in serum was detected as a measure of nitric oxide (NO release) [32]. Serum was diluted (1:3) in PBS and mixed with 100 μ l VCl_3 (0.8 g/ml in 1 N HCl). Griess reagent (100 μ l) was added which was prepared by mixing 1:1 solution of 1% sulphanilic acid in 5% orthophosphoric acid and 0.1% N-(1-naphthyl) ethylenediamine. The reaction mixture was incubated for 30 min at 37 °C and absorbance was measured at 540 nm in microplate reader. Nitrite concentration was calculated against standard curves and expressed as (μ M/ml) of serum.

2.14. Catalase activity

Catalase activity was measured in lung homogenate according to previously described method [30]. Briefly, Lung homogenate was diluted in phosphate buffer (pH = 7.4) containing H_2O_2 as substrate. Catalase activity of each sample was measured for 3 min at 240 nm and expressed in katal per second per mg of protein.

2.15. SOD level

Superoxide dismutase (SOD) was measured in lung tissue as previously described [30]. In brief, Lung homogenate (50 μ l) was mixed with reaction mixture containing 75 μ l of 20 mM α -methionine, 40 μ l Triton x-100, 75 μ l of 100 mM hydroxylamine hydrochloride and 100 μ l 50 μ M EDTA in phosphate buffer (pH

7.4). Followed by incubation for 5 min at 37 °C, 80 μ l of 50 μ M riboflavin was added and the reaction mixture was exposed to white light for 10 min. Griess reagent (1 ml) containing 1:1 solution of 0.1% N-(1-naphthyl) ethylenediamine and 1.0% sulphanilic acid in 5% orthophosphoric acid was added to the reaction mixture and absorbance of the mixture was taken at 543 nm. SOD activity was expressed in per mg of protein.

2.16. Lipid peroxidation

LPS induced lipid peroxidation was estimated by measuring malondialdehyde (MDA) level in the form of thiobarbituric acid active substances (TBARS) [30]. In brief, 50 μ l of 10% lung homogenate was mixed with 50 μ l of 8.1% SDS, 375 μ l of 20% acetic acid, 375 μ l of 8.1% thiobarbituric acid and 150 μ l distilled water. The reaction was kept in boiling water bath for 1 h and cooled in tap water. The reaction was shaken vigorously after adding 250 μ l distilled water followed by 1.25 ml 1:1 solution of n-butanol and pyridine. The reaction mixture was centrifuged at 2000 rpm for 10 min and upper organic layer was taken in separate tube. Absorbance of upper layer was taken at 532 nm and MDA concentration was expressed in nmoles/mg of protein.

2.17. Statistical analysis

Data were expressed as mean $\pm$ SEM. Student t-test and ANOVA followed by Tukey's test was used to identify differences between groups. The experiment was repeated three times and statistical significance was considered at $P < 0.05$ by using SPSS 16.

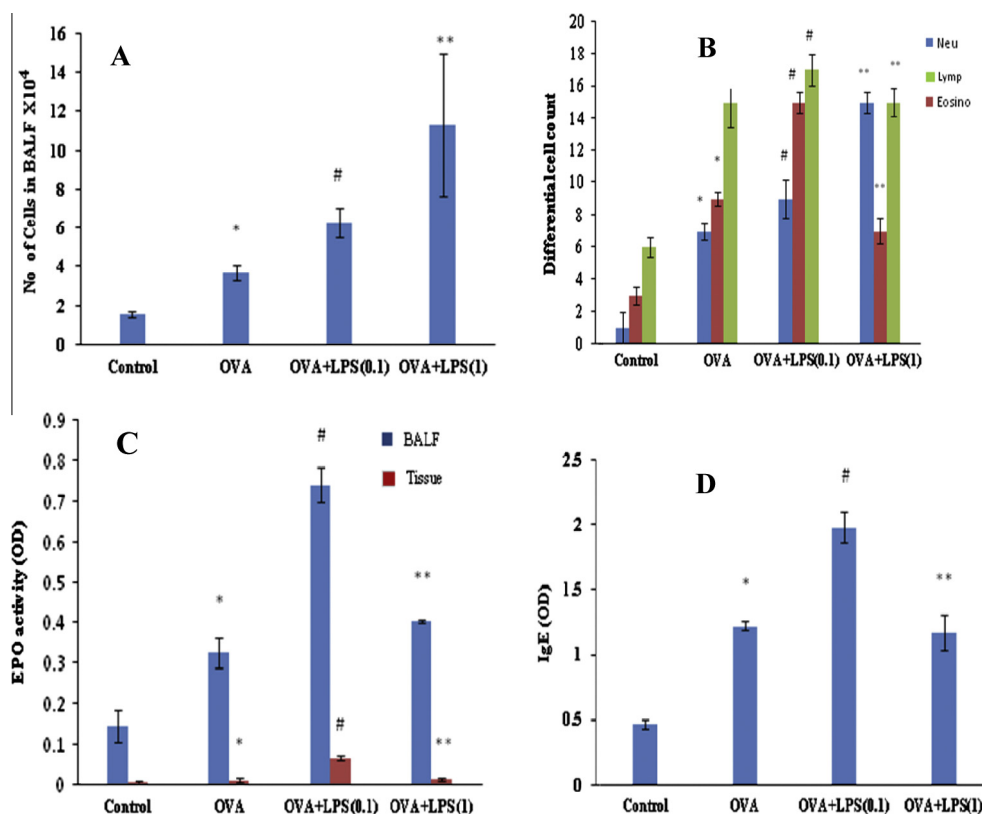


Fig. 2. Effect of lower and higher LPS doses on asthma exacerbations. Total and differential cell count in BALF collected after 24 h of last OVA challenge (A and B). Cell counts were increased significantly after LPS exposure. Eosinophils and neutrophils were significantly higher after LPS exposure (0.1 μ g). However, only neutrophil numbers were significantly enhanced after 1 μ g of LPS exposure. IgE level and EPO activity was significantly higher with 0.1 μ g LPS in OVA exposed group as compared to 1 μ g of LPS exposed group (C and D). * OVA vs. control; # OVA vs. OVA + LPS (0.1) group, ** OVA vs. OVA + LPS (1) $p < 0.05$ ($n = 5$).

3. Results

3.1. LPS exposure to OVA group differently modulates airway inflammation

Higher and lower LPS doses (0.1 and 1 μ g) were used to assess effect of LPS exposure on allergic asthma. Mice exposed to LPS (0.1 and 1 μ g) have significantly enhanced lung inflammation as compared to OVA alone (asthmatic group). After LPS exposure (OVA alone), there was significant infiltration of eosinophils and neutrophils to the lungs ($p < 0.05$). LPS exposure (0.1 μ g) has significantly enhanced eosinophilic inflammation, higher EPO activity and IgE level, whereas higher LPS dose (1 μ g) had resulted in neutrophil dominated inflammation with smaller number of eosinophils which can be correlated with lower EPO activity and IgE level (Fig. 2). However, constricted bronchioles, inflammation and mucous secretion in both LPS exposed asthmatic groups as compared to OVA group could be seen after histological analysis (Fig. 3). Thus, lower LPS dose has markedly enhanced allergic asthma characterized by large numbers of eosinophil and neutrophil recruitments, increased IgE level resulting in LPS exposed mice model of exacerbated asthma.

3.2. Intranasal curcumin modulates asthmatic inflammation by lowering eosinophils and neutrophils recruitment to the lungs

We hypothesized that curcumin could protect against LPS induced asthma exacerbations. Curcumin (5 mg/kg and 10 mg/kg, i.n) had significantly suppressed LPS exposed lung inflammation. After intranasal curcumin treatment, inflammatory cell count in the BALF was significantly reduced as compared to LPS exposed OVA group (43% and 59% respectively; $p < 0.05$) (Fig. 3A and B). Eosinophilic inflammation was significantly ameliorated (34%) after higher dose of curcumin treatment (10 mg/kg, i.n) which was assessed by enhanced EPO activity (Fig. 4A). Suppression in MPO activity (30%) can be correlated with reduced neutrophil recruitments after higher dose of intranasal curcumin treatment (10 mg/kg; $p < 0.05$) (Fig. 4A and B).

3.3. Intranasal curcumin ameliorates serum IgE and histamine levels

Lower LPS dose (0.1 μ g) has enhanced IgE and histamine levels as compared to OVA exposed group (28% and 98% respectively; $p < 0.05$). Curcumin treatment has significantly suppressed serum

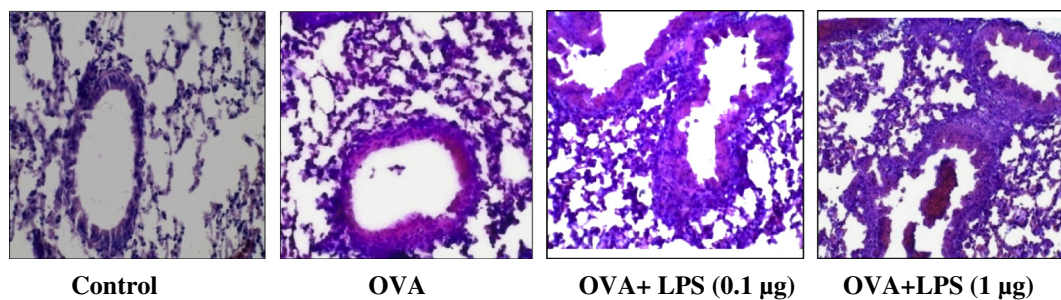


Fig. 3. Histological analysis (H&E) of lungs 24 h after last OVA challenge. LPS exposed groups (0.1 and 1 μ g) are showing increased infiltration of inflammatory cells in alveolar spaces, bronchoconstriction and mucus secretion in the airway lumen. * OVA vs. control; # OVA vs. OVA + LPS (0.1) group, ** OVA vs. OVA + LPS (1) $p < 0.05$ ($n = 5$).

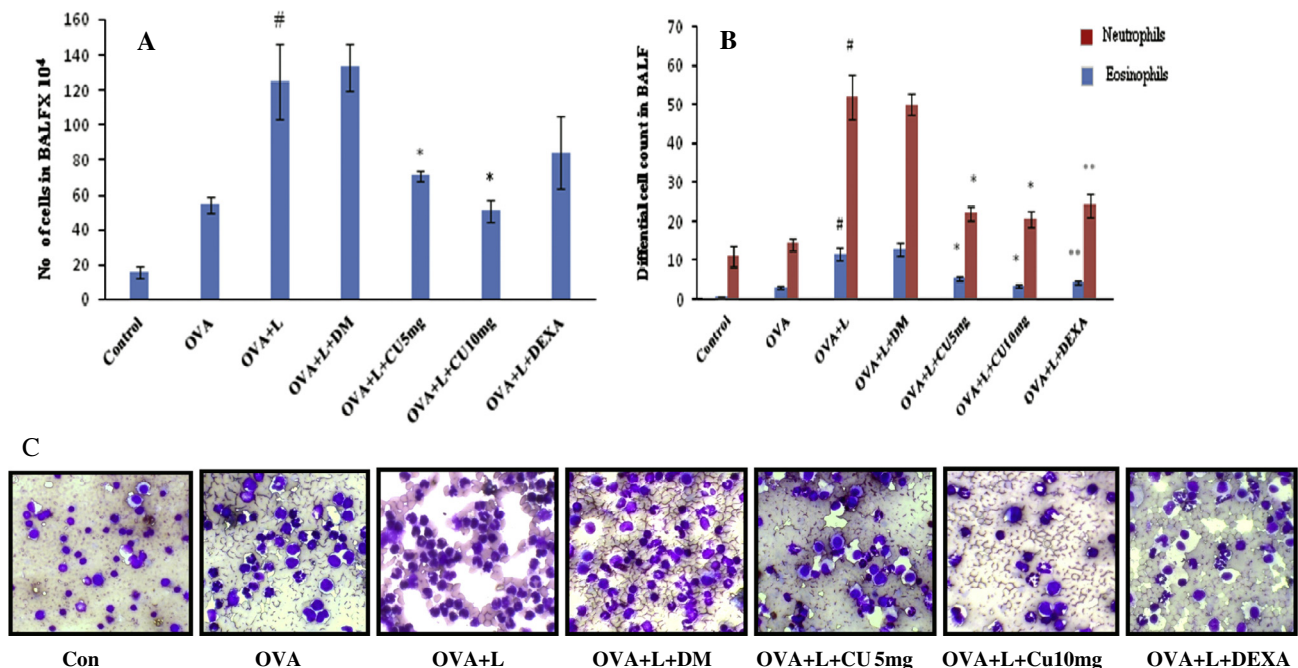


Fig. 4. Effect of curcumin on total and differential cell count in BAL fluid collected after 24 h of last OVA challenge. (A) Total cell count and (B) differential cell count. Eosinophils and Neutrophils were increased significantly in LPS exposed group as compared to OVA group. Curcumin treated groups have ameliorated eosinophil and neutrophil counts as compared to the LPS exposed OVA group and Dexamethasone treated groups. (C) Cytopsin preparations of BALF cells after Geimsa staining. The values are $\pm$ SEM ($n = 5$). # OVA + L vs. OVA group, * OVA + L vs. curcumin treated groups (5 mg/kg, 10 mg/kg). ** OVA + L vs. OVA + L + DEXA $p < 0.05$ ($n = 5$).

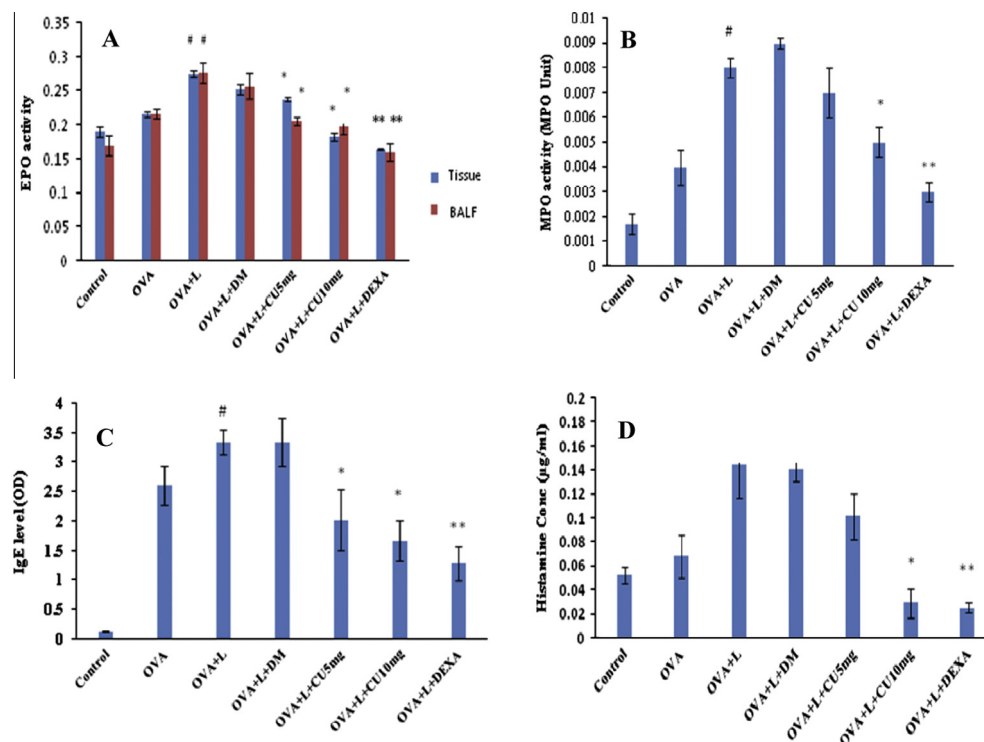


Fig. 5. Effect of intranasal curcumin on EPO and MPO activities (A and B); there was no significant effect on LPS exposure as EPO activities in both BALF and lungs were not much affected whereas MPO activity in lungs were significantly inhibited by higher curcumin dose (10 mg/kg); (C and D) Curcumin treatment had significantly attenuated the serum IgE level and histamine release after LPS exposure. The values are $\pm$ SEM ($n = 5$). # OVA + L vs. OVA group, * OVA + L vs. curcumin treated groups (5 mg/kg, 10 mg/kg). ** OVA + L vs. OVA + L + DEXA $p < 0.05$ ($n = 5$).

levels of both mediators (37% and 50% respectively; $p < 0.05$), as compared to OVA groups (Fig. 4C and D).

3.4. Effect of intranasal curcumin on cytokine release (Th1/Th2) after LPS exposure

Significant increase in TNF- α level after LPS exposure was noted (34% and $p < 0.05$) as compared to OVA alone. Curcumin (10 mg/kg, i.n) has significantly lowered TNF- α level as compared to LPS exposed OVA group (91%; $p < 0.05$). Other cytokine levels (IL-4 and IL-5) which were significantly increased after LPS exposure were significantly suppressed (24% and 46%; $p < 0.05$) after curcumin (10 mg/kg) treatment. IFN- γ level was also found higher after LPS exposure; however, it was not significant, but significantly suppressed by intranasal curcumin (10 mg/kg) (Fig. 5).

3.5. Histopathological changes after LPS exposure and effect of intranasal curcumin

Lungs sections were subjected to H&E staining where OVA groups have shown bronchoconstriction, tissue injury and inflammatory cell infiltration in the alveolar spaces whereas LPS exposed group has shown remarkable bronchoconstriction and inflammation near bronchioles and alveolar spaces. Intranasal curcumin had significantly lowered inflammation and injury as compared to LPS exposed groups (Fig. 6).

3.6. Effect of curcumin on LPS induced oxidative damage

Oxidative damage caused by LPS was assessed by measuring nitrite, antioxidant enzymes and MDA levels. LPS exposed asthmatic groups had higher nitrite level (68%) as compared to OVA alone. Curcumin (10 mg/kg, i.n) had markedly suppressed serum

nitrite level (59%) as compared to LPS exposed group ($p < 0.05$) (Fig. 7A). Significant decrease in major antioxidant enzyme levels (SOD and catalase; 59%) was noted but not significant as compared to OVA group (Fig. 7B and C). Curcumin pretreatment had significantly restored enzymes levels (SOD, 79% and catalase 54%, respectively). Moreover, MDA level was remarkably enhanced in OVA and LPS exposed groups, which was lowered (32%; $p < 0.05$) after curcumin (10 mg/kg, i.n) pre treatment (Fig. 7D).

4. Discussion

Asthma is common respiratory disorder, causing multiple morbidities worldwide especially in the developed countries [3,33]. In our earlier studies, we have established intranasal route of curcumin administration in mice model of acute and chronic asthma, where it has effectively suppressed airway inflammations [34]. Since LPS infections exacerbate allergic asthma inflammations, therefore, in present study, impact of LPS exposure in allergic asthma was studied by using two different doses and exposure schedules where lower dose has effectively enhanced eosinophilic inflammation (Fig. 2A and B) [32,34]. Neutrophil and macrophage numbers were also higher in LPS exposed groups as compared to OVA indicating prominent role of neutrophils in severe asthma. EPO activity in BALF and lung tissue was enhanced after LPS exposure (Fig. 2C and D) which could be correlated with airway inflammations observed in lung sections after H&E staining (Fig. 3). Our results are consistent with earlier studies where release of sputum and eosinophil activation was reported in children with acute asthma [35].

MPO activity in lungs as a marker of neutrophil recruitment has been associated with sudden onset of fatal asthma and airway narrowing where mast cells play a crucial role via TLR-4 activation in LPS exacerbated asthmatic models [36,37]. Marked asthmatic

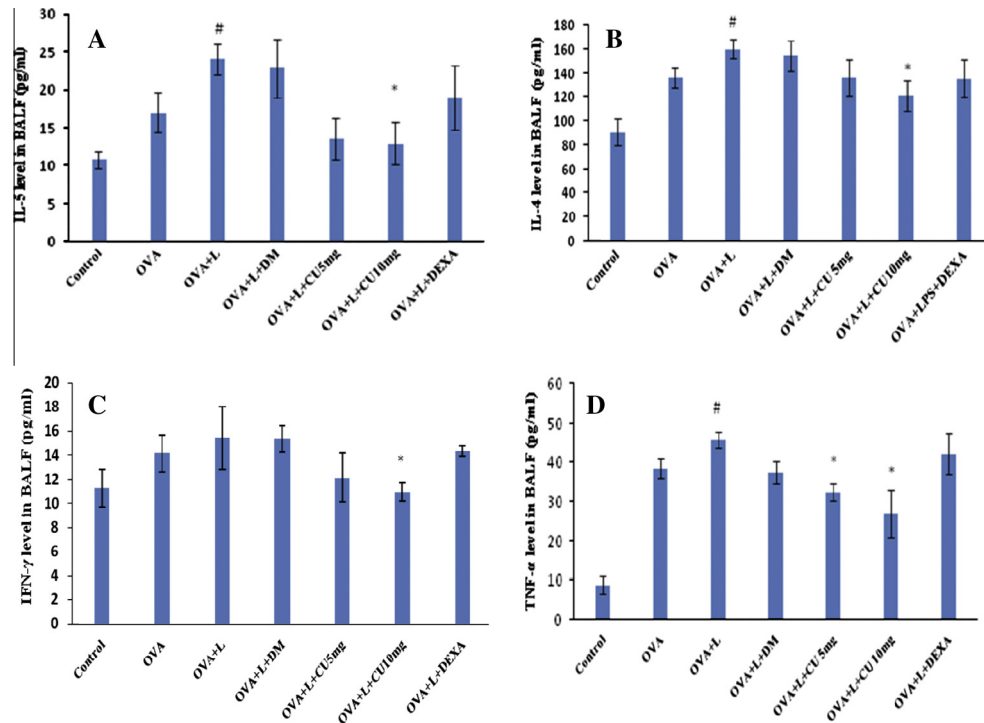


Fig. 6. Effect of intranasal curcumin on cytokine responses after LPS exposure (A–D). Cytokine levels were significantly increased after LPS exposure as compared to OVA group. Intranasal curcumin treatment has lowered the levels of these cytokines, however, significant inhibition was found only at higher curcumin dose (10 mg/kg). [#] OVA + L vs. OVA group, ^{*} OVA + L vs. curcumin treated groups (5 mg/kg, 10 mg/kg). $p < 0.05$ ($n = 5$).

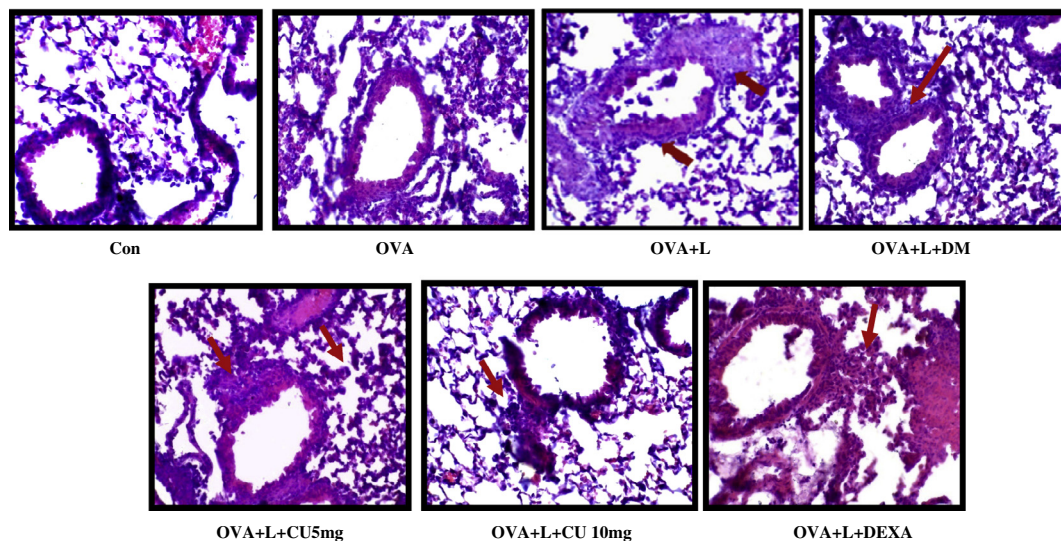


Fig. 7. Effect of intranasal curcumin on lung histology. OVA group is showing inflammation as compared to control group whereas accumulation of inflammatory cells near bronchioles, alveolar spaces and mucus in the airway lumen can be seen after LPS exposure (OVA + L). Curcumin treatment at higher dose (10 mg/kg, i.n.) ameliorates inflammation than dexamethasone (1 mg/kg, i.p.).

exacerbations were noted where OVA specific IgE level and histamine release in serum was found much higher after LPS exposure in asthmatic groups (Fig. 5A–D).

Being an anti-inflammatory molecule, curcumin has been reported to reduce acute lung injury (ALI) phenotypes, which include the reduction of neutrophil recruitment and activation, lung edema, inflammatory cytokines response, most likely via a reduction of the NF- κ B pathway in several ALI models. These models include bleomycin-induced lung injury [38], acute inflammation by *Klebsiella pneumonia* introduction [39], oleic acid-induced ALI [40], and LPS induced acute lung injury [41]. These findings

suggest that curcumin could be an interesting alternative for the LPS exposed asthma exacerbations as well.

We have reported earlier that intranasal curcumin has effectively regulated airway inflammations where BALF of OVA sensitized and challenged mice showed decreased inflammatory cell recruitments, IgE level and Th2-cytokine response [34]. In present study, cytospin preparations of BALF has shown ameliorated inflammatory cells recruitments in LPS exposed asthma exacerbations after intranasal curcumin administration (Fig. 4). Further, eosinophil and neutrophil recruitments, their activation in terms of EPO and MPO, histamine release and IgE levels were

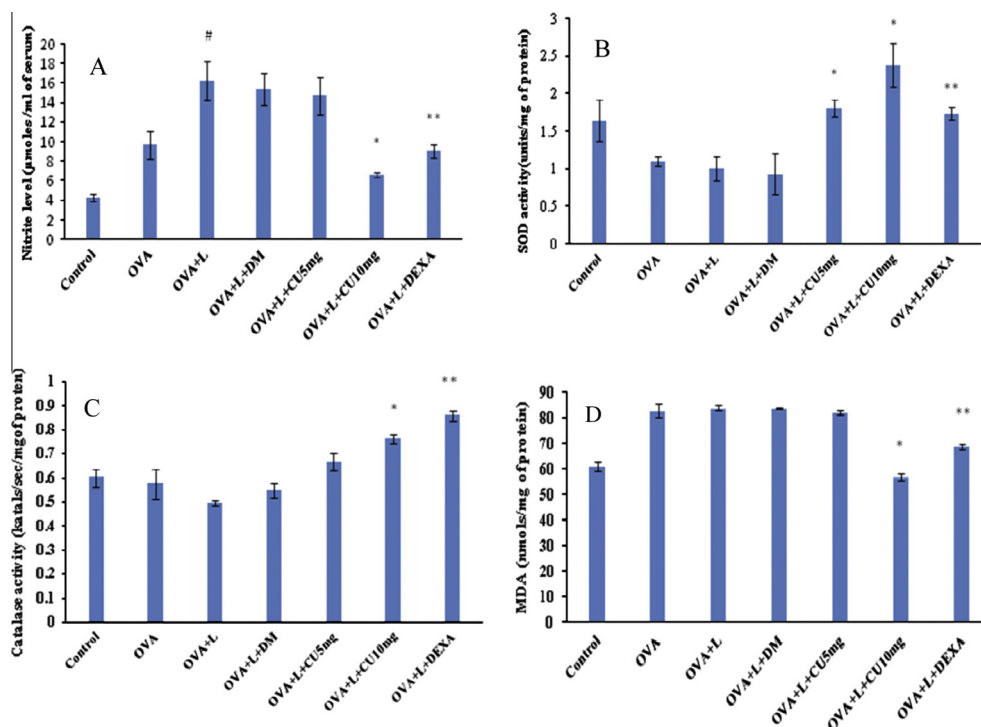


Fig. 8. Effect of intranasal curcumin on Nitrite, SOD, Catalase and MDA levels in lungs (A–D). Intranasal curcumin treatment has lowered nitrite level as compared to LPS treated groups. LPS exposure has not significantly affected catalase and SOD levels as compared to OVA group, however, intranasal curcumin treatment 1 h before LPS exposure has significantly enhanced Catalase and SOD activity. Lipid peroxidation was not significantly affected after LPS exposure. However, intranasal curcumin treatment has significantly lowered MDA level. OVA group [#] OVA + L vs. OVA group, ^{*} OVA + L vs. curcumin treated groups (10 mg/kg) ^{**} OVA + L vs. OVA + L + DEXA $p < 0.05$ ($n = 5$).

dose-dependently ameliorated after intranasal curcumin treatment (Fig. 5A–D).

Earlier studies have reported that asthma is driven by Th2 specific cytokines where IL-4 and IL-5 levels in BALF were upregulated after LPS exposure [42–44]. TNF- α polymorphism has been attributed to asthma susceptibility showing correlation between serum IgE and TNF- α level in patients with atopic asthma [45,46]. We also found higher TNF- α level after low dose of LPS exposure which is consistent with the earlier findings where low LPS dose regulates asthmatic response via TLR-4 signalling pathway in alveolar macrophages with marked infiltration of eosinophils and neutrophils to the lungs with increased Th-2 cytokines production [9]. Level of TNF- α in BALF was also found higher after LPS exposure as compared to the OVA groups (34%) where higher dose of intranasal curcumin (10 mg/kg) had significantly inhibited TNF- α level (91%). But Th1 cytokine, IFN- γ level was higher after LPS exposure (Fig. 6). IFN- γ level in asthma has been described to be protective; however, some epidemiological studies have reported elevated level of IFN- γ in airway tissue of severe asthma patients [47].

Some studies have also reported interaction between IFN- γ and LPS where activated pulmonary macrophages are steroid resistant suggesting a cross talk between IFN- γ and TLR-4 dependent pathways that regulate inflammatory networks in macrophages leading to resistance to glucocorticoids [48,49]. LPS is well known inducer of iNOS, resulting in the production of large amount of NO which impairs lung function by causing lung injury and vascular hyper reactivity. It is well documented that curcumin scavenges superoxide anions, peroxynitrite radicals and quenches singlet oxygen [50]. Our results are consistent with the previous findings as curcumin pre-treatment in ALI model has significantly inhibited NO production (59%; Fig. 8). Significant increase in the SOD and catalase activities were observed after curcumin pre-treatment (79% and 54% respectively, as levels of anti-oxidants were suppressed

in LPS exposed OVA groups (59%; Fig. 8). As reported earlier that presence of microbes in airway can inhibit corticosteroid responses, potentially influencing the efficacy of corticosteroid treatment in asthma [24]. Our results also indicate that Th2 cytokine response and TNF- α level was not much affected after dexamethasone treatment (Fig. 8). Therefore, intranasal curcumin might be better alternative for asthmatics after bacterial infections.

5. Conclusions

In summary, our results suggest that innate immunity plays an important role in modulating allergic asthma. We report here that LPS exposure at lower dose (0.1 μ g) during antigen sensitization and challenge exacerbates allergic asthma where Th2 cytokine, IL-5 is responsible for eosinophil and neutrophil recruitments to the lungs and TNF- α release. So, apart from effectively attenuating allergic airway inflammations, intranasal curcumin do have potential to ameliorate LPS exposed allergic asthma exacerbations. As reported, standard drug, dexamethasone being used in asthma medication might become insensitive in presence of LPS. This first report further supports therapeutic potential of intranasal curcumin in the treatment of severe LPS exposed asthma exacerbations and towards the development of curcumin-aerosol in near future.

Conflict of interest

Authors have no conflict of interest.

Acknowledgment

This work was supported by Grant from Department of Science and Technology – Science and Engineering Research Board, India (DST-SERB), Project No (P-07/526, BHU).

References

- [1] L. Cohn, J.A. Elias, G.L. Chupp, Asthma: mechanisms of disease persistence and progression, *Ann. Rev. Immunol.* 22 (2004) 789–815.
- [2] V. Doreswamy, D.B. Peden, Modulation of asthma by endotoxin, *Clin. Exp. Allergy* 41 (2011) 9–19.
- [3] P.S. Thorne, K. Kulhankova, M.I. Yin, Endotoxin exposure is a risk factor for asthma: the national survey of endotoxin in United States housing, *Am. J. Respir. Crit. Care Med.* 172 (2005) 1371–1377.
- [4] C.E. Reed, D.K. Milton, Endotoxin stimulated innate immunity: a contributing factor for asthma, *J. Allergy Clin. Immunol.* 108 (2001) 157–166.
- [5] C. Delayre Orthez, J. Becker, F. de Blay, Exposure to endotoxins during sensitization prevents further endotoxin-induced exacerbation of airway inflammation in a mouse model of allergic asthma, *Int. Arch. Allergy Immunol.* 138 (2005) 298–304.
- [6] A.H. Liu, Endotoxin exposure in allergy and asthma: reconciling a paradox, *J. Allergy Clin. Immunol.* 109 (2002) 379–392.
- [7] Y.C. Lu, W.C. Yeh, P.S. Ohashi, LPS/TLR4 signal transduction pathway, *Cytokine* 42 (2008) 145–151.
- [8] Y.I. Nigo, M. Yamashita, K. Hirahara, Regulation of allergic airway inflammation through Toll-like receptor 4-mediated modification of mast cell function, *Proc. Natl. Acad. Sci.* 103 (2006) 2286–2291.
- [9] L. Dong, H. Li, S. Wang, Different doses of Lipopolysaccharides regulate the lung inflammation of asthmatic mice via TLR4 pathway in alveolar macrophages, *J. Asthma* 46 (2009) 229–233.
- [10] D.D. Metcalfe, D. Baram, Y.A. Mekori, Mast. Cells Phys. Rev. 77 (1997) 1033–1079.
- [11] R. Malaviya, T. Ikeda, E. Ross, S.N. Abraham, Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF- α , *Nature* 381 (1996) 77–80.
- [12] C. Yang, X. Mo, Lv. Lipopolysaccharide enhances Fc ϵ RI mediated mast cell degranulation by increasing Ca $^{2+}$ entry through store-operated Ca $^{2+}$ channels: implications for lipopolysaccharide exacerbating allergic asthma, *Exp. Physiol.* 97 (2012) 1315–1327.
- [13] J. Monteseirin, Neutrophils and asthma, *J. Investig. Allergol. Clin. Immunol.* 19 (2009) 340–354.
- [14] V.M. Keatings, P.J. Barnes, Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects, *Am. J. Respir. Crit. Care Med.* 155 (1997) 449–453.
- [15] G. Caramori, A. Papi, Oxidants and asthma, *Thorax* 59 (2004) 170–173.
- [16] C.W. Chow, A.M.T. Herrera, T. Suzuki, G.P. Downey, Oxidative stress and acute lung injury, *Am. J. Respir. Cell Mol. Biol.* 29 (2003) 427–431.
- [17] F.J.A. Chabot, J.M. Mitchell, T. Gutteridge, W. Evans, Reactive oxygen species in acute lung injury, *Eur. Respir. J.* 11 (1998) 745–757.
- [18] A. Boueiz, P.M. Hassoun, Regulation of endothelial barrier function by reactive oxygen and nitrogen species, *Microvasc. Res.* 77 (2009) 26–34.
- [19] L. Fialkow, F.L. Fochesatto, M.C. Bozzetti, Neutrophil apoptosis: a marker of disease severity in sepsis and sepsis-induced acute respiratory distress syndrome, *Crit. Care* 10 (2006) 155.
- [20] E.D. Kebir, J.G. Filep, Targeting neutrophil apoptosis for enhancing the resolution of inflammation, *Cell* 2 (2013) 330–348.
- [21] W.O. Cookson, M.F. Moffatt, Asthma – an epidemic in the absence of infection?, *Science* 275 (1997) 41–42.
- [22] K. Ito, C. Herbert, J.S. Siegle, Steroid-resistant neutrophilic inflammation in a mouse model of an acute exacerbation of asthma, *Am. J. Resp. Cell Mol. Biol.* 39 (2008) 543–550.
- [23] P. Haldar, I.D. Pavord, Noneosinophilic asthma: a distinct clinical and pathologic phenotype, *J. Allergy Clin. Immunol.* 119 (2007) 1043–1052.
- [24] Y.J. Huang, H.A. Boushey, The bronchial microbiome and asthma phenotypes, *Am. J. Respir. Crit. Care Med.* 188 (2013) 1178–1180.
- [25] L. Camacho Barquero, I. Villegas, J.M. Sánchez Calvo, Curcumin, a Curcuma longa constituent, acts on MAPK p38 pathway modulating COX-2 and iNOS expression in chronic experimental colitis, *Int. Immunopharmacol.* 7 (2007) 333–342.
- [26] A. Geol, C.R. Boland, D.P. Chauhan, Specific inhibitor of cyclooxygenase (COX-2) expression by dietary curcumin in HT-29 Human colon cancer cell, *Cancer Lett.* 172 (2001) 111–118.
- [27] B. Madan, B. Ghosh, Differuloylmethane inhibits neutrophil infiltration and improves survival of mice in high dose endotoxin shock, *Shock* 19 (2003) 91–96.
- [28] S.G. Royce, X. Li, S. Tortorella, Mechanistic insights into the contribution of epithelial damage to airway remodeling: novel therapeutic targets for asthma, *Am. J. Respir. Cell Mol. Biol.* 50 (2014) 180–192.
- [29] D. Negrao Correa, M.R. Silveira, C.M. Borges, Changes in pulmonary function and parasite burden in rats infected with *Strongyloides venezuelensis* concomitant with induction of allergic airway inflammation, *Infect. Immun.* 71 (2003) 2607–2614.
- [30] A. Kumari, N. Tyagi, D. Dash, R. Singh, Intranasal curcumin ameliorates lipopolysaccharide-induced acute lung injury in mice, *Inflammation* 38 (2014). 1103–1102.
- [31] P.S. Chauhan, Subhashini, D. Dash, R. Singh, Intranasal curcumin attenuates airway remodeling in murine model of chronic asthma, *Int. Immunopharmacol.* 21 (2014) 63–75.
- [32] N.A. Guzel, S. Hazar, D. Erbas, Effects of different resistance exercise protocols on nitric oxide, lipid peroxidation and creatine kinase activity in sedentary males, *J. Sports Sci. Med.* 6 (2007) 417.
- [33] B. Schaub, R. Lauener, E. von Mutius, The many faces of the hygiene hypothesis, *J. Allergy Clin. Immunol.* 117 (2006) 969–977.
- [34] Subhashini, P.S. Chauhan, S. Kumari, et al., Intranasal curcumin and its evaluation in murine model of asthma, *Int. Immunopharmacol.* 17 (2013) 733–743.
- [35] M.Z. Norzila, K. Fakes, R.L. Henry, Interleukin-8 secretion and neutrophil recruitment accompanies induced sputum eosinophil activation in children with acute asthma, *Am. J. Respir. Crit. Care Med.* 161 (2000) 769–774.
- [36] S. Sur, T.B. Crotty, G.M. Kephart, Sudden-onset fatal asthma: a distinct entity with few eosinophils and relatively more neutrophils in the airway submucosa?, *Am. Rev. Respir. Dis.* 148 (1993) 713–719.
- [37] Y. Masakatsu, N. Toshinori, Progress in allergy signal research on mast cells: regulation of allergic airway inflammation through toll-like receptor 4-mediated modification of mast cell function, *J. Pharm. Sci.* 106 (2008) 332–335.
- [38] M.R. Smith, S.R. Gangireddy, V.R. Narala, et al., Curcumin inhibits fibrosis-related effects in IPF fibroblasts and in mice following bleomycin-induced lung injury, *Am. J. Physiol. Lung Cell Mol. Physiol.* 298 (2010) L616–L625.
- [39] S. Bansal, S. Chhibber, Curcumin alone and in combination with augmentin protects against pulmonary inflammation and acute lung injury generated during *Klebsiella pneumoniae* B5055-induced lung infection in BALB/c mice, *J. Med. Microbiol.* 59 (2010) 429–437.
- [40] R.F. Zhu, M. Zhou, J.L. He, F.Y. Ding, S.Q. Yu, G.L. Xu, Protective effect of curcumin on oleic-acid-induced acute lung injury in rats, *Zhongguo Zhong Yao Za Zhi* 33 (2008) 2141–2145.
- [41] Q. Lian, X. Li, Y. Shang, S. Yao, L. Ma, S. Jin, Protective effect of curcumin on endotoxin-induced acute lung injury in rats, *J. Huazhong Uni. Sci. Tech.* 6 (2006) 678–759.
- [42] J.W. Steinke, L. Borish, Th2 cytokines and asthma-Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists, *Respir. Res.* 2 (2001) 66–70.
- [43] S. Greenfeder, S.P. Umland, F.M. Cuss, R.W. Chapman, R.W. Egan, Th2 cytokines and asthma – the role of interleukin-5 in allergic eosinophilic disease, *Respir. Res.* 2 (2001) 71–79.
- [44] S. Sharma, A. Sharma, S. Kumar, Association of TNF haplotypes with asthma, serum IgE levels, and correlation with serum TNF- α level, *Am. J. Respir. Cell Mol. Biol.* 35 (2006) 488–495.
- [45] C. Russo, R. Polosa, TNF- α as a promising therapeutic target in chronic asthma: a lesson from rheumatoid arthritis, *Clin. Sci.* 109 (2005) 135–142.
- [46] J. Shannon, P. Ernst, Yamauchi, Differences in airway cytokine profile in severe asthma compared to moderate asthma, *Chest* J. 133 (2008) 420–426.
- [47] N. Hayashi, T. Yoshimoto, K. Izuhara, et al., T helper 1 cells stimulated with ovalbumin and IL-18 induce airway hyper responsiveness and lung fibrosis by IFN- γ and IL-13 production, *Proc. Natl. Acad. Sci.* 104 (2007) 14765–14770.
- [48] M. Yang, R.K. Kumar, P.S. Foster, Pathogenesis of steroid-resistant airway hyper responsiveness: interaction between IFN- γ and TLR4/MyD88 pathways, *J. Immunol.* 182 (2009) 5107–5115.
- [49] F. Chabot, J.A. Mitchell, J.M. Gutteridge, T.W. Evans, Reactive oxygen species in acute lung injury, *Eur. Respir. J.* 11 (1998) 745–757.
- [50] G. Kaur, N. Tirkey, S. Bharran, V. Chanana, P. Rishi, K. Chopra, Inhibition of oxidative stress and cytokine activity by curcumin in amelioration of endotoxin-induced experimental hepatotoxicity in rodents, *Clin. Exp. Immunol.* 145 (2006) 313–321.