Heliyon 5 (2019) e02864

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.heliyon.com

Heliyon

Research article

An experimental model of asthma in rats using ovalbumin and lipopolysaccharide allergens

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ARTICLE INFO

Keywords:
Immunology
Inflammation
Immune response
Pathophysiology
Allergology
Lipopolysaccharide
Ovalbumin
Eosinophilia
Asthma
Neutrophilia

ABSTRACT

Asthma is chronic and multi-factorial inflammatory disease hence single allergen induced asthma in an animal is not identical to clinical asthma. Therefore, we developed a novel experimental model of asthma in rats using ovalbumin (OVA) and lipopolysaccharide (LPS) allergens. Rats were divided into four groups; normal (NC), OVA, LPS, and OVA-LPS treated. Rats were sensitized with OVA (100 μg/kg, adsorbed in 100 mg/mL aluminum hydroxide, i.p.), LPS (10 μg/kg, i.p.) and both (OVA-LPS) on 7th, 14th, 21st days and was followed by challenge with OVA (1%w/v), LPS (1%w/v), OVA (0.5%w/v) and LPS (0.5%w/v) for 30 min thrice/week for three weeks in the OVA, LPS and OVA-LPS groups, respectively. On 41 day, lung function parameters (respiration rate, tidal volume, and airflow rate), total and differential leukocytes count in the blood as well as BALf and inflammatory cytokines (IL-4, IL-5, and IL-13) in serum were measured. Histology of lungs was performed.

The results suggested that the tidal volume and airflow rate were significantly decreased while respiration rate, total and differential leukocytes count in blood as well as BALf and serum cytokines level were significantly increased in the OVA-LPS as compared to NC, OVA, and LPS.

In conclusion, the combination of OVA and LPS induced phenotypes of severe asthma with eosinophilic, neutrophilic and lymphocytic inflammation.

1. Introduction

Asthma is a chronic multi-factorial inflammatory disease involving numerous inflammatory cells including eosinophils, basophils, neutrophils, monocytes, macrophages and activated mast cells. Various inflammatory mediators (Interleukins, Cysteinyl leukotrienes) released from inflammatory cells and produce the phenotypes of asthma [1]. The underlying pathogenesis of asthma suggested, a role of various allergens in the activation of type 1 helper T cell (Th1) and type 2 helper T cell (Th2) mediated immune responses and thereby resulting in an increased proliferation and differentiation of neutrophils and eosinophils, respectively [2, 3]. Further, Th2 mediated cytokines (IL-4, IL-5, IL-9, and IL-13) is anticipated in orchestrating, prolonging and augmenting the inflammatory response in asthma [4, 5]. Currently, multiple animal models of experimental asthma are available for the preclinical screening of anti-asthmatic drugs. Among them, the ovalbumin induced experimental asthma is widely used to screen the antiasthmatic activity of a drug molecule. The study reported immunization by ovalbumin (OVA) as a standard approach to induce eosinophilic asthma through Th2 mediated inflammatory response [6]. Ovalbumin (OVA) induced eosinophilic asthma showed the characteristic features such as airway inflammation (with significant eosinophils infiltrates), airway hyperresponsiveness (AHR) and airway remodeling (with increased mucus secretion of epithelial goblet cells) [7]. However, asthma being multifaceted disorder merely single allergen is not adequate to indicate all the characteristics of clinical asthma. Recently numerous studies have reported the major proportion of asthma is associated with neutrophilia [8]. The study has demonstrated that severe asthma involved the airway neutrophilia, which govern by Th1 mediated neutrophilic inflammatory responses. LPS is a well reported toll like receptor-4 (TLR4) agonist, activates the TLR4 intracellular signaling, and produce the Th1 mediated neutrophil activation [9, 10]. The reported studies demonstrated that combining LPS with commercial ovalbumin induce pulmonary neutrophilia against a background of Th2-driven eosinophilia in the mouse [11], and guinea-pig [12] during allergen challenging period. Another study has reported that the contamination of ovalbumin with LPS has shown an elevation of neutrophils count in the rats [13]. However, these studies did not demonstrate the effect of LPS on induction of neutrophilic and eosinophilic asthma during the sensitization and challenge period. Therefore, the objective of the present study was to evaluate the

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https://doi.org/10.1016/j.heliyon.2019.e02864
Received 6 March 2019; Received in revised form 14 May 2019; Accepted 12 November 2019
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combined effect of LPS and Ovalbumin during sensitization and challenge period on the induction of eosinophilic and neutrophilic asthma in rats.

2. Materials and methodology

2.1. Animals

Six to seven week old, male Wistar albino rats (200–250 g) were procured (Zydus research center (ZRC), Ahmedabad, India) for the experiment. The protocol (LMCP/COLOGY/15/05) of the experiment was approved by the institutional animal ethical committee (IAEC), and all the experiments were conducted as per the guidance of the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA), Animal Welfare Division, New Delhi, India. The experimental protocol has followed the European Community guidelines [14]. All rats were housed with free access to a standard pellet diet and water ad libitum. They were kept at a constant temperature of 22 ± 1 °C, the relative humidity of 55 ± 5%, and 12/12 h light/dark cycle.

2.2. Chemicals

Ovalbumin and Lipopolysaccharide (E. coli origin, serotype 0111:B4) was purchased from Sigma Chemicals, St Louis, USA. Aluminum hydroxide gel (S.D. Fine-Chemical Pvt. Ltd, India), Heparin (Troikaa Pharma Ltd., India), Ketamine (Themis Medicare Ltd, India).

2.3. Experimental design

Rats were divided into four groups (n = 6) receiving the following treatments:

Group-1 (NC): Rats were sensitized and challenged with vehicle
Group-2 (OC): Rats were sensitized and challenged with OVA
Group-3 (LC): Rats were sensitized and challenged with LPS
Group-4 (OLC): Rats were sensitized and challenged with OVA-LPS

2.4. Study protocol

Rats were sensitized with OVA (100 μg/kg, adsorbed in 100 mg/mL aluminum hydroxide, i.p), LPS (10 μg/kg, i.p.), OVA and LPS (100 μg/kg

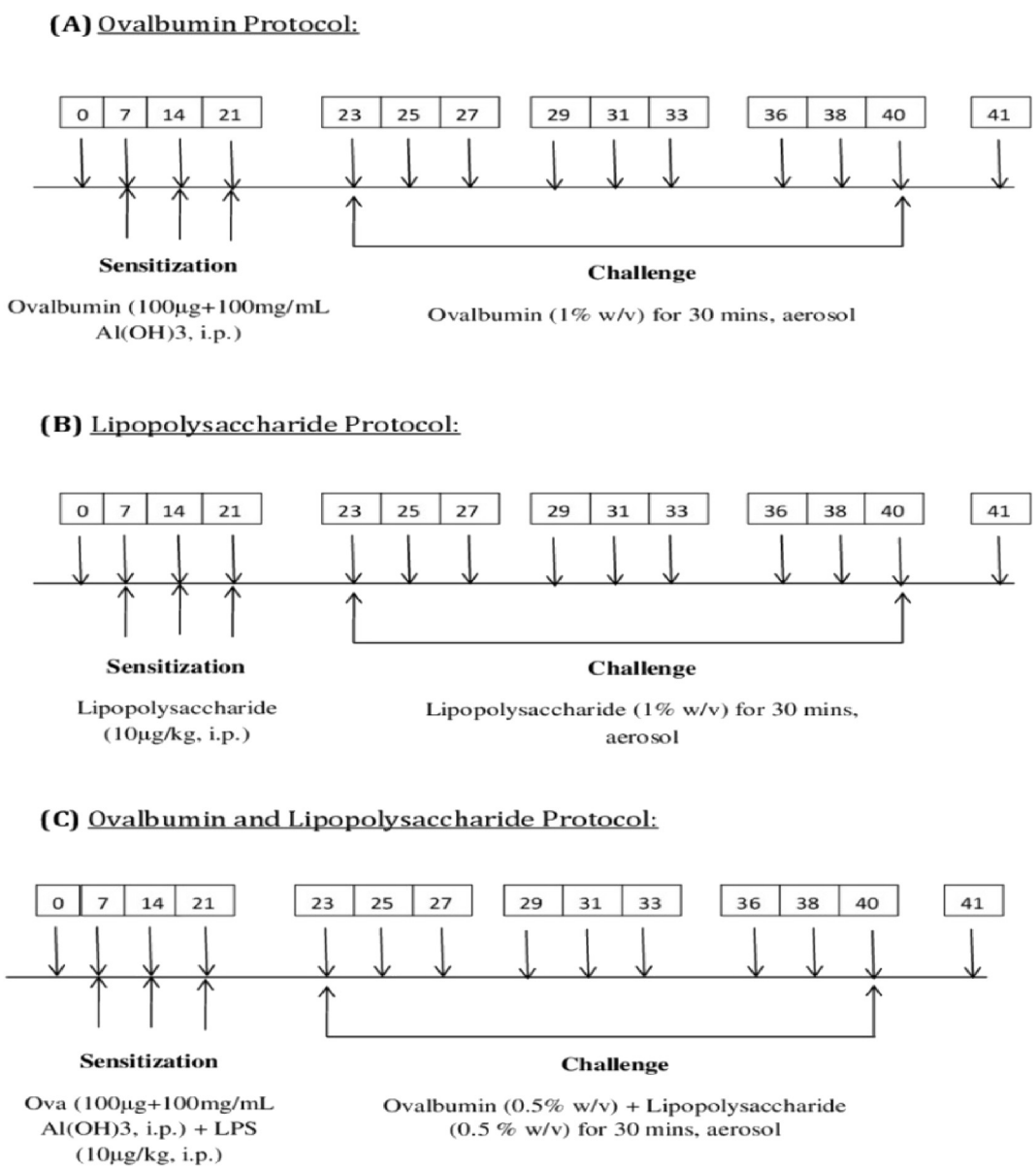

Fig. 1. Schedule for sensitization and challenging protocol for the asthma induction in rats.

and 10 μg/kg, i.p.) on 7th, 14th, 21st days and challenged with OVA (1 % w/v, adsorbed in 100 mg/mL aluminum hydroxide), LPS (1 % w/v in saline), OVA and LPS (both 0.5 % w/v, adsorbed in 100 mg/mL aluminum hydroxide and saline) for 30 min thrice/week for three weeks in the OVA, LPS and OVA-LPS groups, respectively. In the OVA-LPS group, the 10 min time interval was kept between the administration of OVA and LPS during sensitization. For the challenge, rats were placed into a plastic chamber (diameter 70 cm, height 40 cm) attached to a nebulizer (CX4-Omron Healthcare, Kyoto, Japan). The nebulizer rate for aerosol delivery was 0.22 mL/min. In the Normal control (NC) group rats were sensitized and challenged with intraperitoneal injection and aerosolized saline respectively (Fig. 1).

At the end of three weeks, lung function parameters (respiration rate, tidal volume, and airflow rate), total leukocytes count and differential leukocytes (Neutrophils, eosinophils, lymphocytes, and monocytes) count in the blood as well as BALf and inflammatory cytokines (IL-4, IL-5 and IL-13) in serum were measured. Histology of lungs was also performed.

2.5. Determination of lung function parameters

Lung function parameters like respiration rate (breaths/minute), tidal volume (ml), and airflow rate (ml/minute) were measured in conscious rats using Respiromax, Columbus Instrument [15]. Rats were acclimatized, 2 min/day for 15 days before the measurement of lung function parameters.

2.6. Blood sampling and serum isolation

Blood was collected from retro-orbital plexus of rats under light diethyl ether anesthesia in a non-heparinized tube. They were kept at room temperature for 30 min, followed by centrifugation at 1000 g for 15 min, and serum was isolated by aspiration. The separated serum was stored at -80℃ for the later quantitative determination of cytokines [16].

2.7. Determination of total and differential cell counts

Total and differential leukocytes (Neutrophils, Eosinophils, Lymphocytes, and Monocytes) were measured in the blood as well as BALf using a fully automated cell counter (Diatek, West Bengal, India).

2.8. Bronchoalveolar lavage fluid (BALf) collection

Rats were anesthetized by subcutaneous administration of ketamine (24 mg/kg) and then euthanized. The trachea was exposed, followed by the insertion of a polypropylene cannula into the trachea. 0.9% (w/v) phosphate-buffered saline (10 mL) was introduced subsequently into the lungs using a 10 mL syringe at 37 °C and recovered 5 min later with gently squeezing the rat chest. The recovered lavage fluid (5 ml) was centrifuged at 5000 rpm for 10 min at 4 °C. The resultant supernatant was discarded, and cells deposited at the bottom of the centrifuge tube were recovered. These cells in the pellet were resuspended in 0.5 ml saline and total, and differential leukocytes counts were then performed in an automated cell differential count [17].

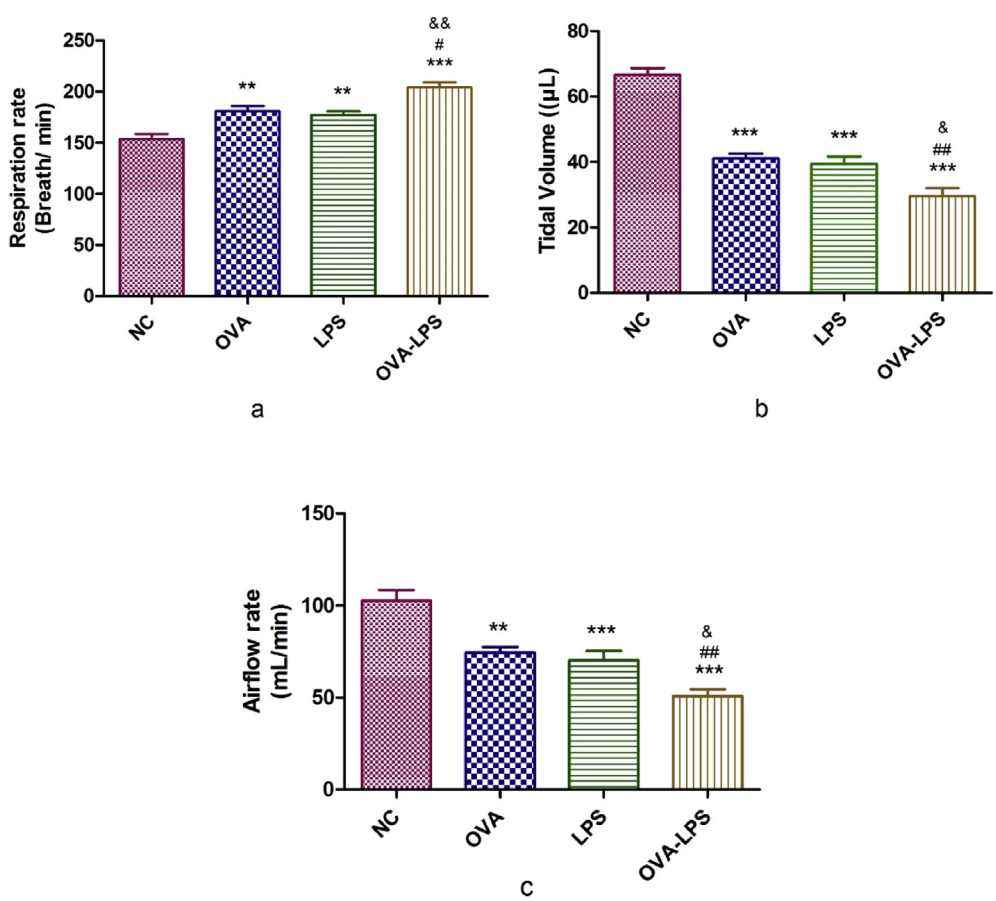

Fig. 2. Effects of allergens sensitization and challenge on (a) respiration rate, (b) tidal volume and (c) airflow rate in normal and asthmatic rats. Normal Control (NC), Ovalbumin Control (OC), Lipopolysaccharide Control (LC), Ova-LPS Control (OLC). $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. NC. $^{\#}p < 0.05$, and $^{\#\#}p < 0.01$ vs. OVA. $^{\&}p < 0.05$ and $^{\&\&}p < 0.01$ vs. LPS.

2.9. Determination of inflammatory cytokines

The inflammatory cytokines (IL-4, IL-5, and IL-13) were measured by enzyme-linked immunosorbent assay (ELISA) in the serum using spectrophotometry based kits (IL-4, RayBio) (IL-5 and IL-13, Bioassay Technology Laboratory, Korain Biotech Co. LTD). All the plates were analyzed on an automated plate reader (Lab System Multiscan 51118220, Thermo Bioanalysis, Helsinki, Finland).

2.10. Histological analysis

Dissected lungs tissues were washed with normal saline and immersed in 10% (v/v) formaldehyde solution and embedded in paraffin. Lung specimens were sectioned and stained with hematoxylin and eosin (H & E) dye. Images of selected sections were captured at 10X magnifications using a zoom digital camera (MLX, Magnus, China).

2.11. Statistical analysis

All the data obtained from the experiments were expressed as mean ± Standard Error of Mean (SEM). Statistical analysis was performed by one-way analysis of variance (ANOVA) for assessing differences amongst multiple groups, followed by Tukey's test using Graphpad Prism 5.0 software (Graphpad, San Diego, CA). $P < 0.05$ was considered statistically significant.

3. Results

The lung function parameters; respiration rate was significantly ($P < 0.01$, $P < 0.01$, $P < 0.001$) increased while tidal volume ($P < 0.001$, $P <$

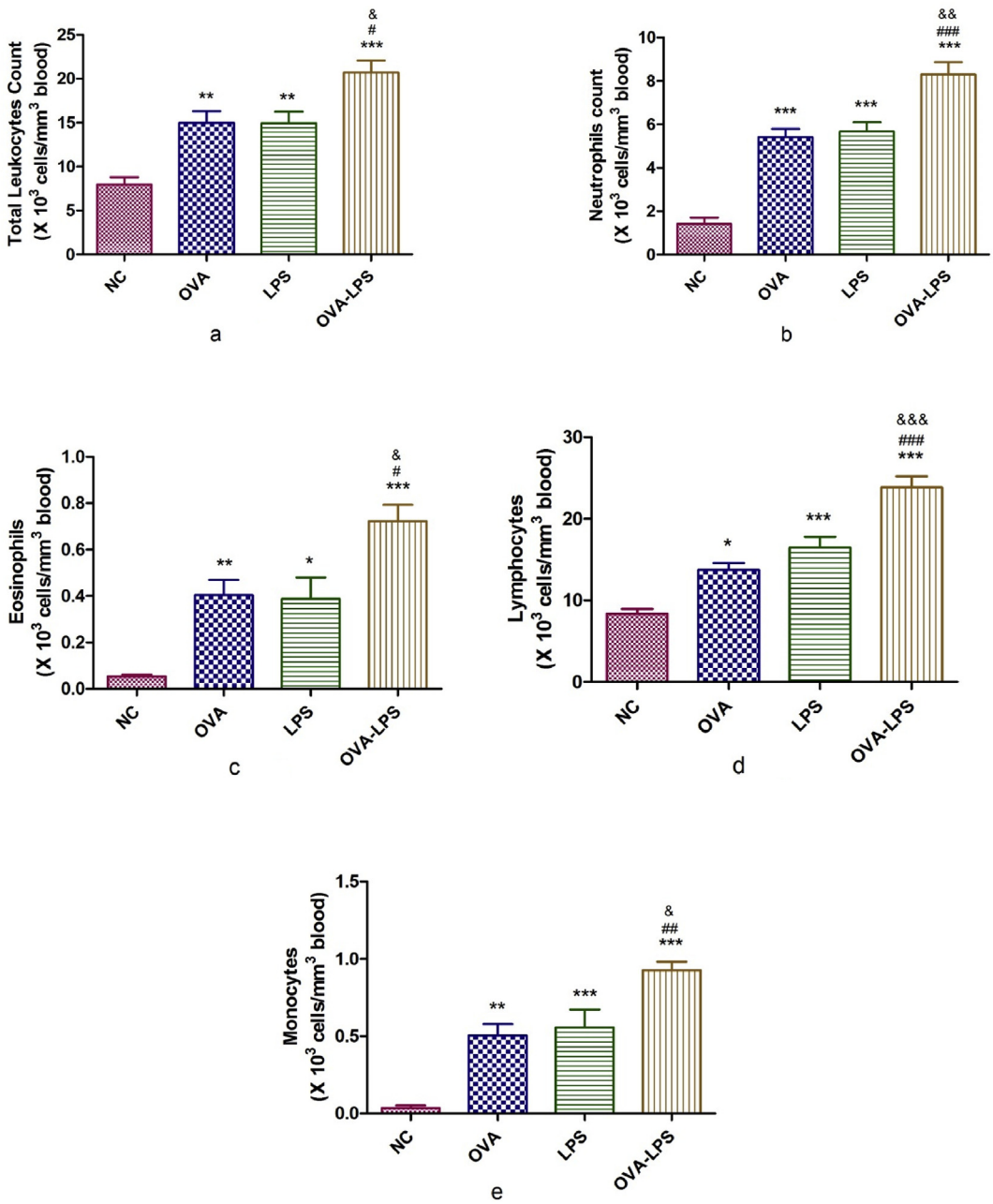

Fig. 3. Effects after 24 h of last allergen challenge on blood (a) total leukocytes count, (b) neutrophils, (c) eosinophils, (d) lymphocytes and (e) monocytes in normal and asthmatic rats. Normal Control (NC), Ovalbumin Control (OC), Lipopolysaccharide Control (LC), Ova-LPS Control (OLC). *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$vs. NC. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. OVA. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ and $^{\&\&\&}p < 0.001$ vs. LPS.

0.001, P < 0.001) and airflow rate (P < 0.01, P < 0.001, P < 0.001) were significantly reduced in the OVA, LPS and OVA-LPS groups as compared to NC. The respiration rate was significantly (P < 0.05, P < 0.01) increased while tidal volume (P < 0.01, P < 0.05) and airflow rate (P < 0.01, P < 0.05) were significantly reduced in the OVA-LPS group as compared to OVA and LPS groups (Fig. 2a–c).

A total leukocytes counts in the blood were significantly (P < 0.01, P < 0.01, P < 0.001) elevated in the OVA, LPS and OVA-LPS groups as compared to NC. A total leukocytes counts in the blood were significantly (P < 0.05, P < 0.05) elevated in the OVA-LPS group as compared to OVA and LPS groups. Compared to the normal control group, a differential leukocytes counts; neutrophils (P < 0.001, P < 0.001, P < 0.001), eosinophils (P < 0.01, P < 0.05, P < 0.001), lymphocytes (P < 0.05, P < 0.001, P < 0.001) and monocytes (P < 0.01, P < 0.001, P < 0.001) were significantly increased in the blood of OVA, LPS and OVA-LPS groups. The differential leukocytes counts; neutrophils (P < 0.001, P < 0.01), eosinophils (P < 0.05, P < 0.05), lymphocytes (P < 0.001, P < 0.001) and monocytes (P < 0.01, P < 0.05) in the blood were significantly increased in the OVA-LPS group as compared to OVA and LPS groups (Fig. 3a–e).

Compared to the Normal Control group, total leukocytes counts in the BALf were significantly (P < 0.001, P < 0.01, P < 0.001) elevated in the OVA, LPS and OVA-LPS groups. However, total leukocytes counts in the BALf of OVA-LPS group was significantly elevated as compared to OVA and LPS groups (P < 0.001, P < 0.001, respectively). In differential leukocytes counts; neutrophils (P < 0.01, P < 0.001, P < 0.001), eosinophils (P < 0.001, P < 0.001, P < 0.001), lymphocytes (P < 0.001, P < 0.001, P < 0.001) and monocytes (P < 0.05, P < 0.01, P < 0.001) in the BALf were significantly increased in the OVA, LPS and OVA-LPS groups

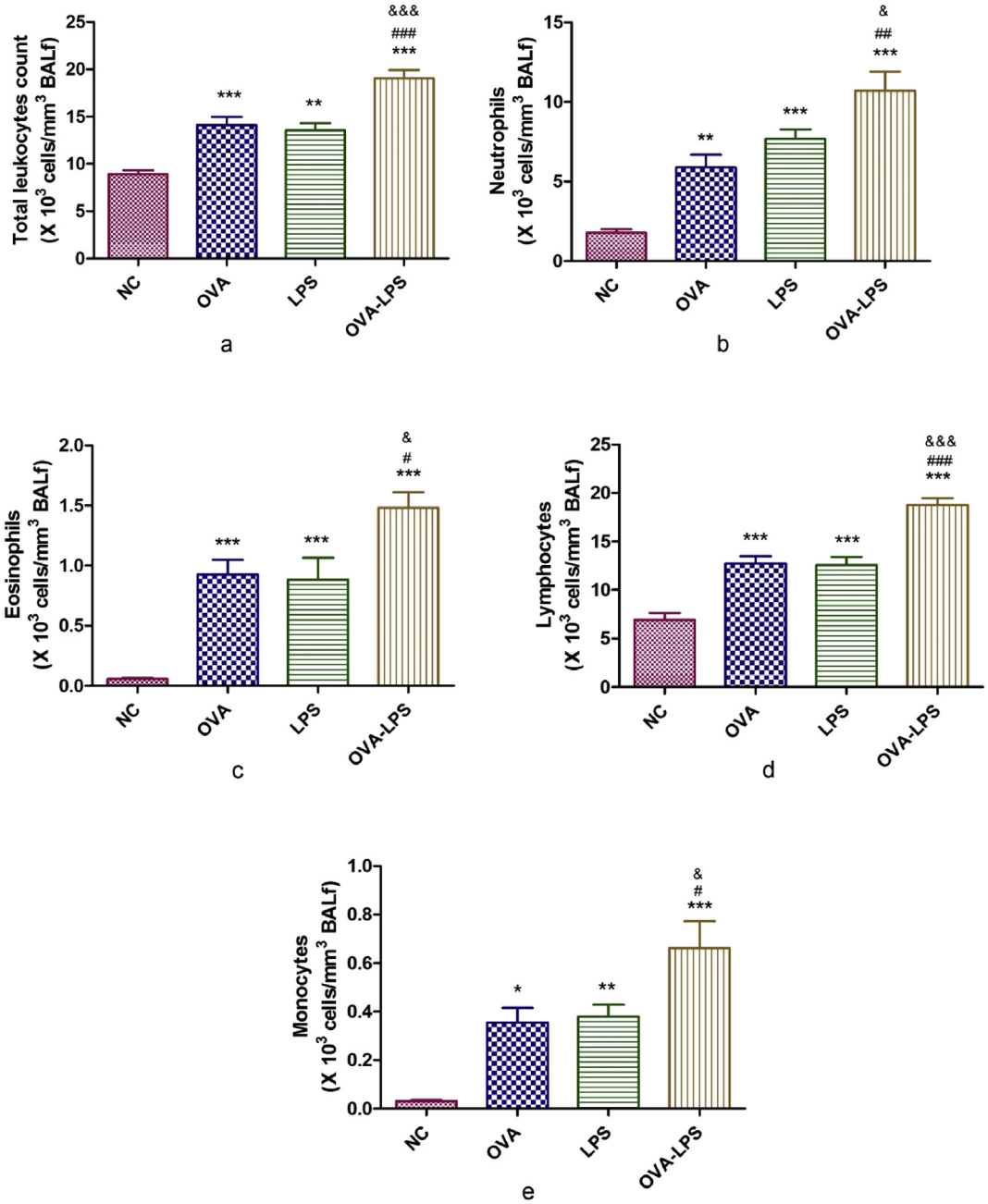

Fig. 4. Effects after 24 h of last allergen challenge on bronchoalveolar lavage (BALf) (a) total leukocytes count, (b) neutrophils, (c) eosinophils, (d) lymphocytes and (e) monocytes in normal and asthmatic rats. Normal Control (NC), Ovalbumin Control (OC), Lipopolysaccharide Control (LC), Ova-LPS Control (OLC). *p < 0.05, **p < 0.01 and ***p < 0.001vs. NC. #p < 0.05, ##p < 0.01 and ###p < 0.001 vs. OVA. &p < 0.05 and &&&p < 0.001 vs. LPS.

as compared to NC. The differential leukocytes counts; neutrophils ($P < 0.01$, $P < 0.05$), eosinophils ($P < 0.05$, $P < 0.05$), lymphocytes ($P < 0.001$, $P < 0.001$) and monocytes ($P < 0.05$, $P < 0.05$) in the BALf were significantly increased in the OVA-LPS group as compared to OVA and LPS groups (Fig. 4a–e).

Compared to the normal control group, the inflammatory cytokines; IL-4 ($P < 0.001$, $P < 0.001$, $P < 0.001$), IL-5 ($P < 0.01$, $P < 0.01$, $P < 0.001$) and IL-13 ($P < 0.001$, $P < 0.001$, $P < 0.001$) were significantly increased in the OVA, LPS and OVA-LPS groups. The inflammatory cytokines; IL-4 ($P < 0.001$, $P < 0.001$), IL-5 ($P < 0.001$, $P < 0.001$) and IL-13 ($P < 0.05$, $P < 0.05$) were significantly elevated in the OVA-LPS group as compared to OVA and LPS groups (Fig. 5a–c).

Histological section of OVA, LPS and OVA-LPS treated lungs showed prominent infiltration of inflammatory cells predominantly eosinophils, neutrophils in peribronchial region. Proliferation of airway epithelium and smooth muscle cell hyperplasia resulted in the thickening of bronchiolar wall. However, these pathological conditions were found comparatively higher in OVA-LPS treated asthmatic rats (Fig. 6a–d).

4. Discussion

The current study showed the phenotypes of OVA, LPS, and OVA-LPS induced experimental asthma. Ovalbumin is a well-known allergen for generating Th2 and eosinophils cells mediated inflammatory responses [18, 19]. Activated Th2 cells produce IL-4, IL-13, and IL-5, which are responsible for IgE production by B cells, eosinophil activation and recruitment, and mucus production. In contrast, Th1 cells differentiate from naıve $CD4^+$ cells in response to microbial activation of antigen-presenting cells under the influence of IL-12. Differentiated Th1 cells secrete interferon-γ, which is vital in the intracellular destruction of phagocytosed microbes. Furthermore, interferon-gamma produced by Th1 cells and IL-4 produced by Th2 counter-regulate each other. The previous reported studies indicated that inflammatory responses to OVA immunization are governed by eosinophil activation and mast cell degranulation [19]. LPS is a selective TLR4 agonist and generating their inflammatory responses through Th1 and neutrophils cells [20, 21]. The reported study demonstrated that inflammatory responses of LPS are governed by neutrophil activation and its cascades [22]. In the current study, the modulation of lung function parameters (tidal volume, flow rate, and respiratory rate) in the OVA-LPS group indicated the more severe bronchoconstriction as compared to OVA and LPS groups. The significant increase in eosinophils, neutrophils and lymphocyte counts in the blood as well as BALf of OVA-LPS as compared to OVA and LPS groups indicated the induction of severe eosinophilia, neutrophilia and lymphocytic inflammation in the OVA-LPS group. Consistent with the above finding, OVA-LPS treated rats showed a significant elevation in the inflammatory cytokines (IL-4, IL-5, and IL-13) as compared to OVA and LPS groups.

The previous reports indicated that ovalbumin combined with a lower dose of LPS induced the Th2 associated responses, which characterized by an increase of neutrophils, eosinophils, and cytokines levels (IL-4, IL-5, IL-13, and IFN-γ) in the BALf. The higher dose of LPS with OVA induced the Th1 associated response, characterized by the increase in neutrophil and IFN-γ level in the BALf of mice [23, 24].

Histological examination of the lung showed prominent infiltration of leukocytes, increased proliferation of epithelial, and smooth muscle hyperplasia in the OVA-LPS group as compared to OVA and LPS groups, which suggests that OVA-LPS induced the severe histological changes as

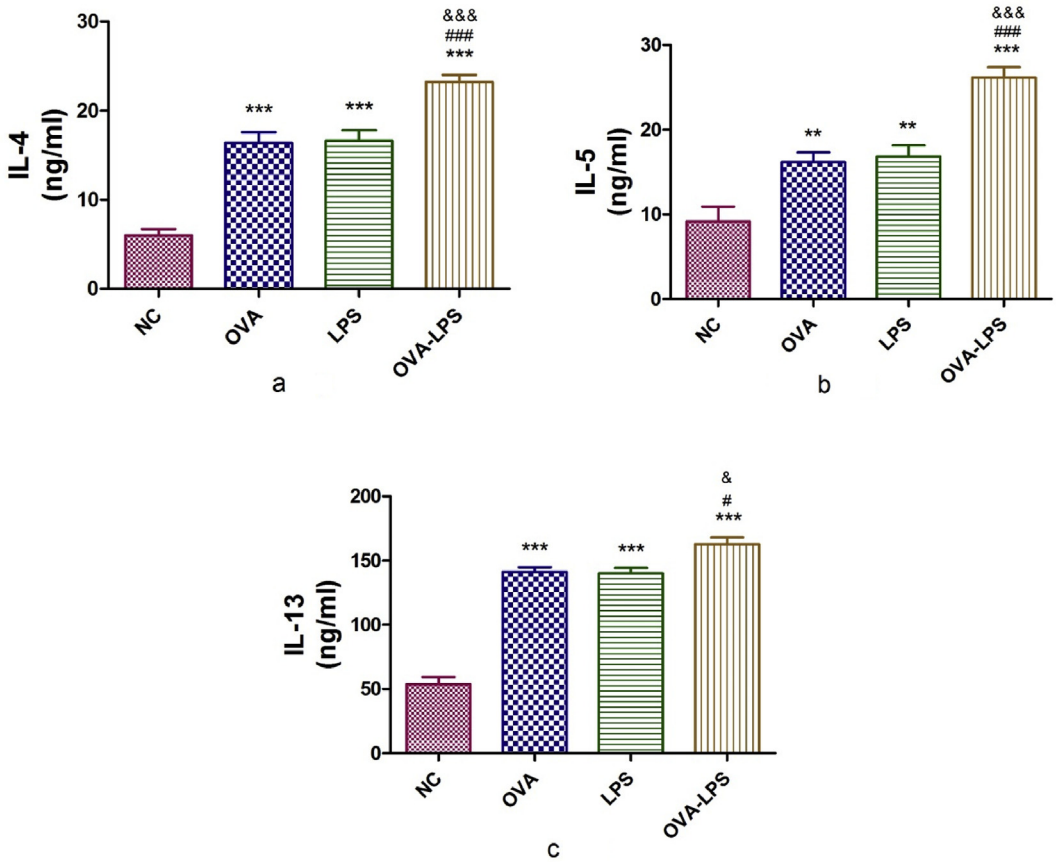

Fig. 5. Effects after 24 h of last allergen challenge on serum (a) IL-4, (b) IL-5 and (c) IL-13 in normal and asthmatic rats. Normal Control (NC), Ovalbumin Control (OC), Lipopolysaccharide Control (LC), Ova-LPS Control (OLC). ***$p < 0.001$ and **$p < 0.01$ vs. NC. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. OVA. $^{\&}p < 0.05$ and $^{\&\&\&}p < 0.001$ vs. LPS.

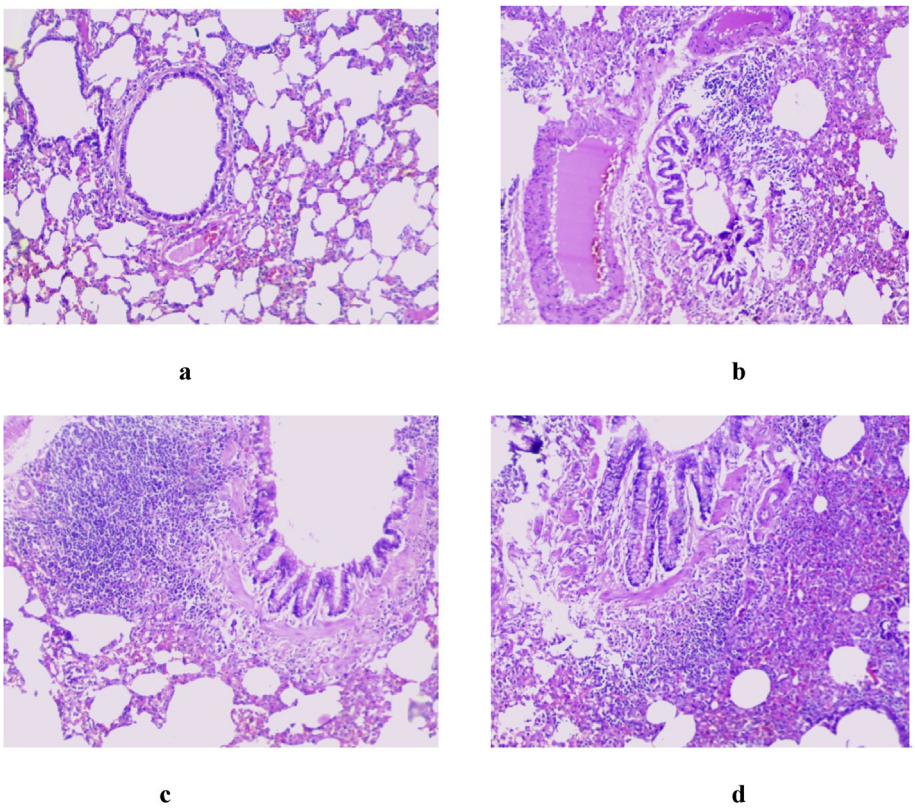

Fig. 6. Microscopic structure of H&E stained lung (transverse section) in normal and asthmatic rats. (100X original magnification) (a) Normal Control (NC), (b) Ovalbumin Control (OC), (c) Lipopolysaccharide Control (LC), (d) Ova-LPS Control (OLC).

compared to an individual. This might be due to the aggregative effect of individual allergen on the eosinophils and neutrophils. Thus, sensitization and challenged rats with OVA and LPS showed the phenotypes of severe bronchoconstriction and eosinophilic, neutrophilic and lymphocytic inflammation as compared to a single allergen.

5. Conclusion

The combination of OVA ad LPS induced the phenotypes of severe asthma with eosinophilic, neutrophilic and lymphocytic inflammation.

Declarations

Author contribution statement

Vandana R. Thakur, Vikas Khuman, Jayesh V. Beladiya, Kiranj K. Chaudagar, Anita A. Mehta: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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