

Protease activated receptor-2 mediated upregulation of IL-17 receptor signaling on airway epithelial cells is responsible for neutrophilic infiltration during acute exposure of house dust mite allergens in mice

Ahmed Nadeem^{a,*}, Naif O. Al-Harbi^a, Sheikh F. Ahmad^a, Khalid E. Ibrahim^b, Moureq R. Alotaibi^a, Nahid Siddiqui^c, Shakir D. Alsharari^a, Sabry M. Attia^a, Mohammad M. Al-Harbi^a

^a Department of Pharmacology & Toxicology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia

^b Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia

^c Amity Institute of Biotechnology, Amity University, Noida, India

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ABSTRACT

Asthma, a chronic inflammatory disease affecting the airways is primarily caused due to immune system dysfunction. Different inhaled allergens such as house dust mites (HDM), fungi, cockroach allergens are the main contributors to allergic asthma. Protease activated receptor-2 (PAR-2) signaling plays an important role in allergic asthma through modulation of immune mediators in airway epithelial cells (AECs). Interleukin-17A (IL-17A) signals via subunits of IL-17 receptor (IL-17R), i.e. interleukin-17 receptor A (IL-17RA) and interleukin-17 receptor C (IL-17RC), and plays a necessary role in neutrophilic infiltration in response to infectious/allergenic stimuli, however it is not known if PAR-2 activation affects IL-17A/IL-17R signaling during acute exposure to house dust mite (HDM) allergens. Therefore, our study exposed mice to HDM allergens for five days and evaluated its effect on IL-17A/IL-17R signaling, chemokine/cytokines and neutrophilic inflammation in mice. Our study shows that HDM allergens upregulate IL-17A levels in the lung and IL-17RA/IL-17RC expression in AECs. PAR-2 activation by trypsin also upregulates neutrophilic influx and IL-17A/IL-17R signaling in the lung. Upregulated IL-17A/IL-17R signaling was associated with increased BAL neutrophils, pulmonary MPO activity and proinflammatory chemokines and cytokines (IL-23, IL-6, and MCP-1 in AECs/lung) in HDM exposed mice. Further, HDM-induced IL-17A, IL-17R and chemokines/cytokines were attenuated by PAR-2 antagonist, ENMD-1068. Furthermore, HDM-primed mice treated with IL-17A had greater neutrophilic inflammation and higher levels of inflammatory cytokines/chemokines than PBS-exposed mice treated with IL-17A. This proposes that acute exposure to HDM allergens activate AECs at a very early stage where PAR-2/IL-17R signaling serves a crucial role in neutrophilic inflammation.

1. Introduction

Asthma is a debilitating and chronic inflammatory airways disease that arises due to genetic and environmental factors. It affects nearly 300 million people worldwide. The characteristics of this disorder are varying degrees of bronchoconstriction, and remodeling of the airways resulting mainly from immune system dysfunction [1,2]. Different immune/non-immune cells including eosinophils, macrophages, neutrophils, dendritic cells and airway epithelial cells (AECs) play a vital role in the pathogenesis of allergic asthma. Inhaled allergens, e.g house dust mites (HDM), fungi, cockroach allergens are the main contributors to allergic asthma. Amongst all the allergens, HDM is most commonly associated with the development of this disease [3,4].

The inflammation that is observed in asthma is very complex and the epithelium has come up as a major player in regulating, managing and localizing the inflammation in the airways. It is also crucial in repair and remodeling [1,5]. Activation of AECs by protease/non-protease components found in HDM determines the immune response towards the development of allergic airway disease such as asthma. Release of inflammatory cytokines and other mediators from AECs usually leads to infiltration of different innate/adaptive immune cells into the lung, which contribute a great deal towards airway inflammation [1,4,6].

Several earlier reports have demonstrated that IL-17A plays a key role in neutrophilic airway inflammation [7–9]. Asthmatics who have elevated numbers of neutrophils in the airways are known to have poor

* Corresponding author. Department of Pharmacology & Toxicology, College of Pharmacy, King Saud University, PO Box 2455, Riyadh, 11451, Saudi Arabia.
E-mail address: anadeem@ksu.edu.sa (A. Nadeem).

lung function, worse asthma control and increase in acute exacerbations. This is reportedly due to elevated levels of IL-17A in the lung [10–12]. IL-17A derived from pulmonary adaptive and innate immune cells regulate neutrophilic inflammation. Moreover, increased IL-17A and neutrophilic inflammation is often associated with corticosteroid resistance [13–16]. Upon binding with its receptor, i.e. IL-17 receptor (IL-17R), IL-17A causes elevation of pro-inflammatory mediators such as chemokines, cytokines, and oxidants in airway epithelial cells (AECs). These mediators aggravate pulmonary inflammation through recruitment of neutrophils and other immune cells [17–19]. However, it is not studied whether acute exposure to HDM allergens modulates IL-17A/IL-17R signaling in AECs.

AECs have a fundamental function in early recognition of pathogenic stimuli via innate immune receptors such as toll like receptors and protease activated receptor-2 (PAR-2). HDM allergens are known to affect airway inflammation through activation of PAR-2 expressed on AECs. Some earlier studies have reported HDM-induced allergic asthma to be PAR-2 dependent while others have shown PAR-2 independent components as well [20–22]. These HDM-induced asthma models in mice have been able to impart valuable information through the employment of chronic/sub-chronic dosing regimen [20,23,24]. In this study, we further aim to analyze the role of PAR-2 activation on IL-17R signaling and its outcome on the pro-inflammatory mediators in AECs/lung using acute HDM exposure.

Our study shows that acute exposure to HDM allergens via intranasal route causes upregulation of IL-17R in AECs and pulmonary IL-17A levels. This is due to activation of PAR-2 as PAR-2 antagonist, ENMD-1068 leads to downregulation of HDM-induced upregulation of IL-17R and subsequent neutrophilic inflammation. This suggests that acute exposure to HDM allergens primes the lung for IL-17A response from an early stage.

2. Materials and methods

2.1. Animals

Male Balb/c mice (20–25 g), free of specific pathogens, used in these experiments were maintained under environmentally controlled conditions in the animal facility of our college (College of pharmacy, KSU). The protocol for the usage of animals was reviewed and approved by The Animal Care and Research Committee of College of Pharmacy, King Saud University.

2.2. Acute exposure to HDM allergens, IL-17A and drug treatment

Mice were administered with 50 µg house dust mite extract (HDM; *Dermatophagoides pteronyssinus*, Greer Laboratories, Lenoir, NC, USA), intranasally (i.n) under light anesthesia for 5 consecutive days. Control animals received PBS in the same volume. To study the role of PAR-2 on HDM-induced changes in lung/AECs, mice were administered PAR-2 antagonist, ENMD-1068 (Enzo Life Sciences, USA) at 5 mg/kg, i.n. 30 min before administration of HDM/PBS daily for five days as reported previously [25]. To further evaluate the role of IL-17A in neutrophilic inflammation in this HDM model, anti-IL-17A antibody/isotype control antibody (Biolegend, USA) at 100 µg/mouse, i.p. was given 30 min before HDM administration on days 1, 3 and 5 as stated earlier [26,27]. There were following groups for this study. Control group (Vehicle): mice were administered PBS (i.n) only; Control group treated with PAR-2 antagonist, ENMD-1068 (ENMD): mice were administered PBS (i.n) and ENMD-1068, i.n as described above; HDM administered group (HDM): mice were administered HDM (i.n) only; HDM and ENMD-1068 administered group (ENMD + HDM): mice were administered ENMD-1068 (i.n) and HDM (i.n.) as described above. Anti-IL17A antibody and HDM administered group (HDM^{Anti-IL-17A}): mice were administered anti-IL17A antibody (i.p) and HDM (i.n.) as described above. Isotype control antibody and HDM administered group (HDM^{Isotype}): mice were

administered isotype control antibody (i.p) and HDM (i.n.) as described above. Mice were sacrificed 12 h after HDM administration for various analyses.

To study the role of IL-17R signaling in HDM-primed mice (HDM was administered from day 1–5 as described above, followed by resolution of airway inflammation for two days). Mice were then challenged with IL-17A, 1 µg i.n. once only on day 7. There were following groups for this study. Control group (PBS): mice were administered PBS (i.n) only; Control group administered IL-17A (IL-17A): mice were administered IL-17A (i.n) as described above; HDM-primed group administered PBS (HDM-Primed): mice were administered HDM (i.n) for 5 consecutive days and then administered PBS (i.n.) on day 7; HDM-primed group administered IL-17A (HDM-Primed^{IL-17A}): mice were administered HDM (i.n) 5 consecutive days and then administered IL-17A (i.n.) on day 7 described above; Control group treated with ENMD-1068 and IL-17A (ENMD + IL-17A): mice were administered ENMD-1068 (i.n.) for 5 consecutive days and then administered IL-17A (i.n.) on day 7 as described above; HDM-primed group administered IL-17A and ENMD-1068 (ENMD + HDM-Primed^{IL-17A}): mice were administered ENMD-1068 (i.n.) and HDM (i.n) for 5 consecutive days and then administered IL-17A (i.n.) on day 7 as described above. Mice were sacrificed 12 h after IL-17A administration for various analyses.

To evaluate the role of specific PAR-2 activation on neutrophilic inflammation and IL-17A/IL-17R signaling, we used PAR-2 agonist, trypsin at 0.2 µg/mouse, i.n. for five consecutive days as stated earlier [25]. Another group was administered ENMD-1068 (Enzo Life Sciences, USA) at 5 mg/kg, i.n. 30 min before administration of trypsin as state above. There were following groups for this study. Vehicle group (Vehicle): mice were administered PBS (i.n) only; Trypsin administered group (Trypsin): mice were administered trypsin (i.n) as described above; ENMD-1068 and trypsin administered group (ENMD + Trypsin): mice were administered ENMD-1068 and trypsin (i.n) for 5 consecutive days as stated above. Mice were sacrificed 12 h after final trypsin administration for various analyses.

2.3. Bronchoalveolar lavage (BAL) and lung histology

The BAL procedure was performed as described previously [25,28,29]. Briefly, trachea was cannulated to obtain alveolar cells in PBS and differential counting was carried out on Diff-Quik stained slides. Data were expressed as number of cells/ml. Lungs were formalin-fixed and sectioned at a thickness of 5 µm followed by staining with haematoxylin and eosin (H&E) for assessment of airway inflammation. Sectioned lung slides were examined by bright-field microscopy.

2.4. Real-time PCR

AECs were harvested by scraping the lumen of the trachea and utilized for extraction of total RNA as elucidated before [25,28,29]. mRNA levels were measured by real-time PCR analysis on ABI PRISM 7500 sequence detection system (Applied Biosystems) as stated earlier [25,26] using Taqman inventoried assays-on-demand probes for IL-23p19, IL-6, MCP-1, IL-17RA, IL-17RC, and GAPDH (Applied Biosystems, USA). The fold difference in expression of different genes was quantified using the relative gene expression (i.e., $\Delta\Delta CT$) method as described earlier [30].

2.5. Measurement of cytokines/chemokines protein levels by ELISA

Protein levels of IL-17A/IL-6/MCP-1/IL-23 in the lung were measured using ELISA kits from Biolegend (USA) and R&D Systems (USA) as per manufacturer's protocol.

2.6. Flow cytometry

For flow experiments, whole trachea along with primary bronchi

was digested with mixture of collagenase/DNAse I in RPMI 1640 (Invitrogen, USA) to get single cell suspension as described previously [25,28,29]. Single cell suspensions were labeled with FITC/APC/APC-Cy7-conjugated anti-CD326 (Ep-CAM) or anti-CD217 (IL-17RA) antibody (BioLegend, USA; R&D Systems, USA) for surface labeling followed by FITC/APC/PE-conjugated anti-IL-6/MCP-1/IL-23 monoclonal antibody (R&D Systems, USA; BioLegend, USA). The immunostained cells were acquired on a flow cytometer (Beckman Coulter, USA) and analyzed for the expression of protein of interest based on different markers using Cytomics FC 500 software as reported previously [25,28,29].

2.7. Measurement of myeloperoxidase activity (MPO)

Lung MPO activity was quantified as a measure of neutrophilic inflammation by recording the absorbance at 450 nm according to our past study [28,29].

2.8. Chemicals

Unless stated otherwise, all chemicals were of the highest grade available ($\geq 95\%$ purity) and were obtained from Sigma-Aldrich Chemicals Company (USA).

2.9. Statistical analysis

The data were expressed as mean $\pm$ SEM. Different groups were compared using ANOVA (analysis of variance) followed by Tukey's multiple comparison tests. A 'P' value of less than 0.05 was considered significant for all statistical tests. All the statistical analyses were performed using Graph Pad Prism statistical package.

3. Results

3.1. Increased neutrophilic inflammation after acute exposure to HDM allergens

HDM allergens have been shown to cause neutrophilic inflammation in long term models, here we wanted to explore whether acute exposure without the involvement of adaptive immune system results in neutrophilic inflammation. Our data show that there is increase in neutrophilic inflammation as depicted by increased neutrophil numbers in BAL and pulmonary MPO activity (Fig. 1A–C). Histopathological analysis depicted mild airway inflammation after acute exposure of HDM (Fig. 1D). HDM-induced neutrophilic airway inflammation was greatly attenuated by PAR-2 antagonist, ENMD-1068 (Fig. 1A–D). These data suggest that even acute exposure to HDM allergens leads to influx of neutrophils in the lung which is dependent on PAR-2 signaling.

3.2. Increased IL-17A/IL-17R signaling after acute exposure to HDM allergens

Our next objective was to delineate the pathway responsible for increased neutrophilic inflammation during acute HDM exposure. It is well established that IL-17R signaling plays a predominant important role in neutrophilic inflammation, there we assessed IL-17RA and IL-17RC expression in AECs. Our data show that acute exposure HDM allergens causes upregulation of both IL-17RA and IL-17RC in the AECs (Fig. 2A–D). Further, IL-17A mRNA and protein levels (Fig. 2E–F) are increased in the lung after acute exposure to HDM allergens. Furthermore, HDM-induced upregulation of IL-17A/IL-17R signaling was greatly downregulated by ENMD-1068 (Fig. 2A–F). These data suggest that acute exposure to HDM allergens leads to upregulation of IL-17A/IL-17R signaling in the lung which is dependent on PAR-2 signaling.

3.3. Increased neutrophilic inflammation and IL-17A/IL-17R signaling after specific PAR-2 activation, and attenuation of HDM-induced neutrophilic inflammation after neutralization of IL-17A

Further to confirm if HDM-induced changes on neutrophilic inflammation and IL-17A/IL-17R signaling were due to PAR2 activation, we used trypsin as a specific PAR-2 agonist. Our data show that trypsin administration causes neutrophilic inflammation (Fig. 3A–B). Further, trypsin administration causes upregulation of IL-17A/IL-17R signaling as depicted by increase in IL-17RA expression in AECs (Fig. 3C) and IL-17A protein levels in the lung (Fig. 3D). Furthermore, antagonism of PAR-2 by ENMD-1068 led to blockade of trypsin-mediated effects on neutrophilic inflammation and IL-17A/IL-17R signaling (Fig. 3A–D). To assess whether neutrophilic influx was IL-17A dependent in this model, IL-17A neutralization was carried out during HDM exposure. HDM-induced neutrophilic infiltration and MPO activity in the lung was greatly diminished by treatment with anti-IL17A antibody (Fig. 3E–F). These data clearly demonstrate that PAR-2 activation is involved in neutrophilic influx as well as upregulation of IL-17A/IL-17R expression in the lung. Further, it elucidates that HDM-induced neutrophilic inflammation is dependent on IL-17A/IL-17R signaling.

3.4. Increased chemokines/cytokines in the AECs/lung after acute exposure to HDM allergens

IL-17A/IL-17R signaling is often associated with increased release of neutrophilic chemoattractants from AECs, so we explored the expression of different cytokines/chemokines responsible for this phenomenon. Our data show increased protein expression of IL-23 (Fig. 4), IL-6 (Fig. 5), and MCP-1 (Fig. 6) in AECs/lung after acute exposure to HDM allergens. Further, HDM exposure led to upregulation of mRNA levels of IL-23, IL-6, and MCP-1 in the AECs (Figs. 4–6). Furthermore, HDM-induced upregulation in cytokines/chemokines was attenuated by ENMD-1068 (Figs. 4–6). These data emphasize that acute exposure to HDM allergens leads to upregulation of chemokines/cytokines in AECs/lung which is attenuated by PAR-2 antagonism.

3.5. Increased neutrophilic inflammation in HDM-primed mice and attenuation of neutrophilic inflammation after antagonism of PAR-2 in HDM-primed mice in response to IL-17A

We showed that IL-17R expression is upregulated after HDM exposure, therefore our next objective was to test if IL-17A administration would lead to enhanced neutrophilic inflammation in these mice as compared to PBS-exposed mice. Firstly, expression of IL-17RA and IL-17RC on day 7 (after resolution of airway inflammation) was still elevated in HDM-primed mice as compared to PBS-exposed mice, however other parameters like neutrophil numbers, MPO activity and IL-17A levels were similar in HDM-primed mice and PBS-exposed mice (data not shown). This shows that HDM-primed mice had resolution of airway inflammation on day 7. Based on this observation, we administered IL-17A to both HDM-primed and PBS-exposed mice to find out differences in IL-17R signaling. Our data show HDM-primed mice treated with IL-17A had much greater neutrophilic inflammation (neutrophilic numbers and MPO activity) as compared to PBS-exposed mice treated with IL-17A (Fig. 7A–B). Further, HDM-primed mice treated with PAR-2 antagonist, ENMD-1068 and IL-17A had diminished neutrophilic inflammation as compared to HDM-primed mice treated with IL-17A (Fig. 7C–D). These data emphasize the point that HDM-primed mice readily respond to IL-17A and have enhanced neutrophilic inflammation due to upregulated IL-17R expression in which PAR-2 plays a considerable role.

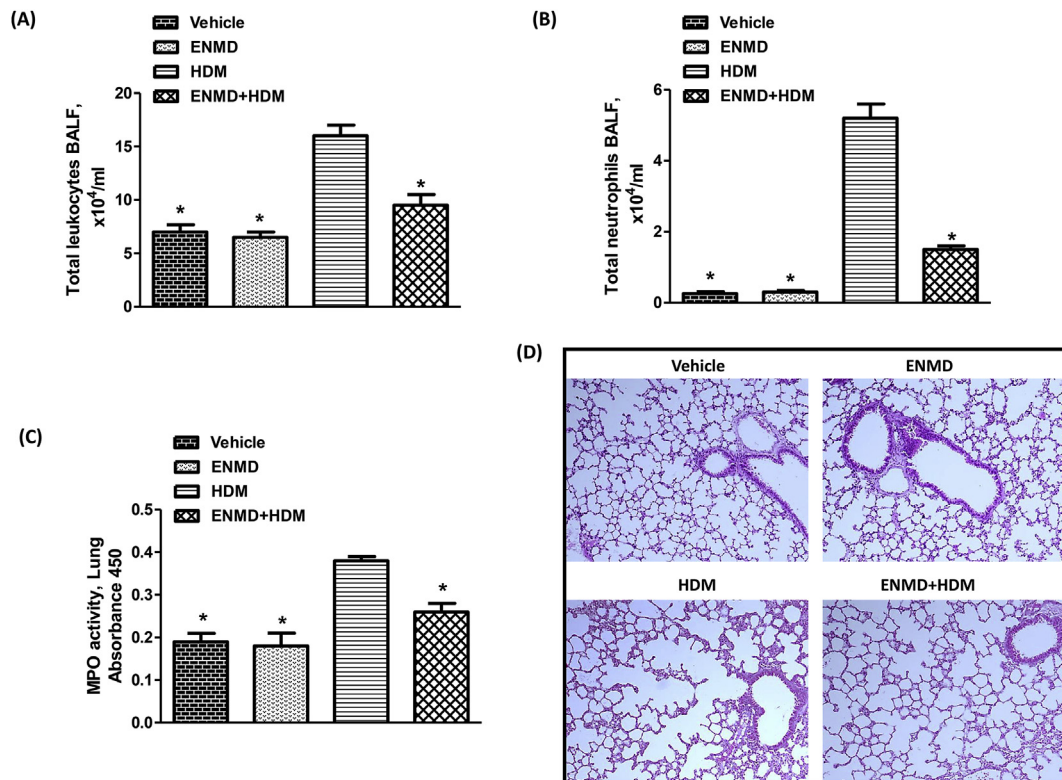


Fig. 1. Effect of PAR-2 antagonist, ENMD-1068 and acute exposure of HDM allergens on airway inflammation in mice. A) Total leukocyte count, B) Total neutrophil count, C) MPO activity, and D) Photomicrographs of formalin-fixed lung slides by H&E staining. Values are shown as mean $\pm$ SE, $n = 6-8/\text{group}$. * $P < 0.05$, vs. HDM group.

3.6. Increased chemokines/cytokines in the AECs/lung in HDM-primed mice in response to IL-17A

Next, we explored if there were differences in expression of

chemokines/cytokines of HDM-primed and PBS-exposed mice after administration of IL-17A. Our data show HDM-primed mice treated with IL-17A had much higher expression of IL-6, and MCP-1 in AECs/lung as compared to PBS-exposed mice treated with IL-17A (Fig. 8A–F).

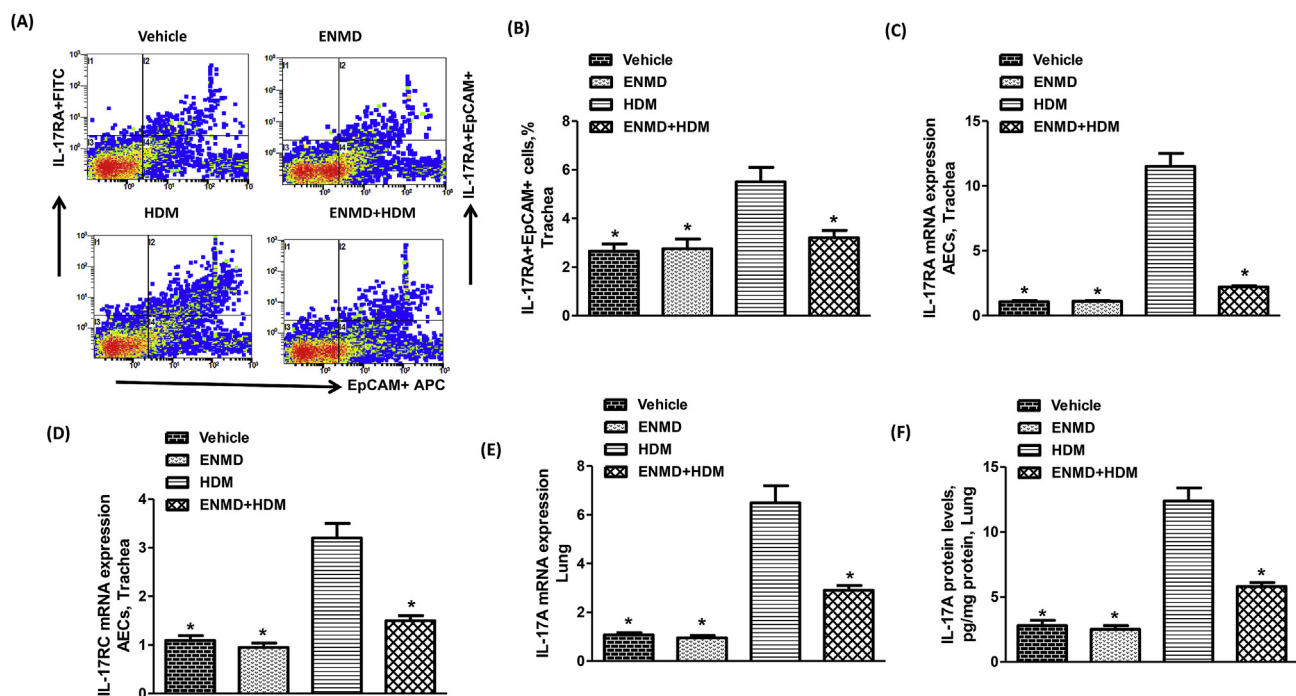


Fig. 2. Effect of PAR-2 antagonist, ENMD-1068 and acute exposure of HDM allergens on IL-17R and IL-17A expression in mice. A) Flow plot depicting immunostaining for IL-17RA+ and EpCAM+ cells, B) % IL-17RA+ EpCAM+ cells, C) IL-17RA mRNA in AECs, D) IL-17RC mRNA in AECs, E) IL-17A mRNA expression, and F) IL-17A protein levels. Values are shown as mean $\pm$ SE, $n = 6-8/\text{group}$. * $P < 0.05$, vs. HDM group.

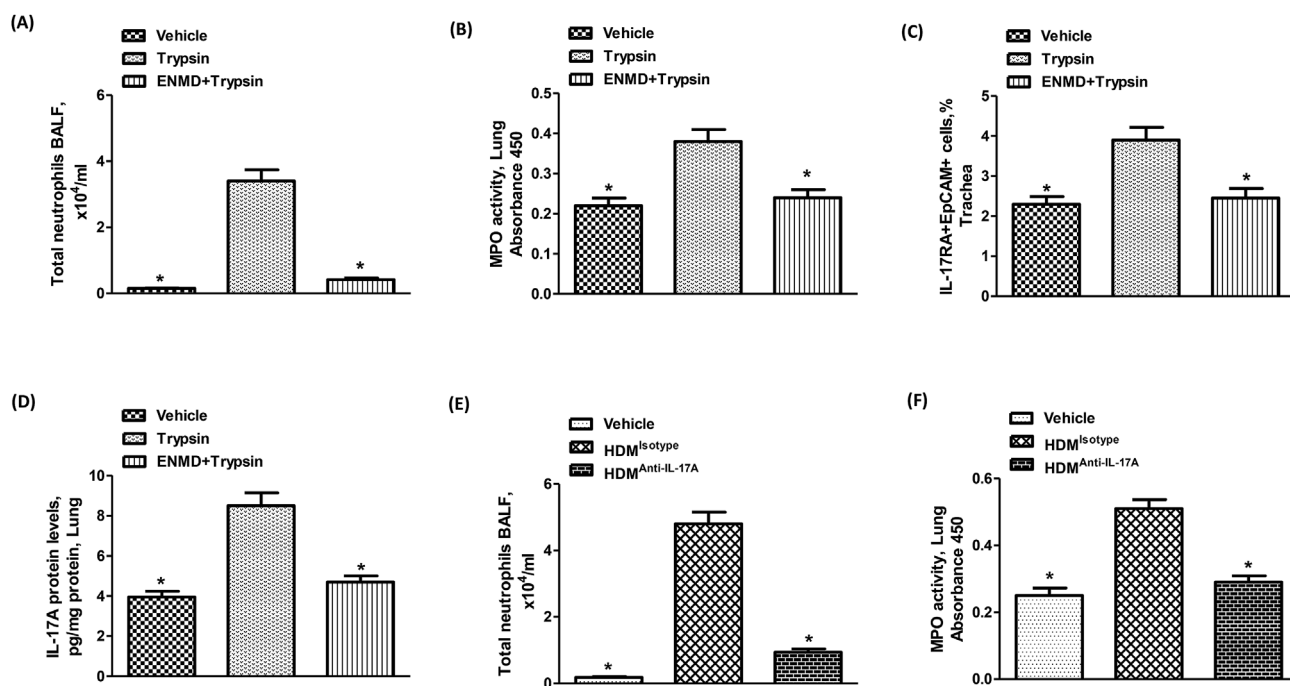


Fig. 3. Effect of PAR-2 antagonist, ENMD-1068 on PAR-2 agonist-induced neutrophilic inflammation/IL-17R signaling, and IL-17A neutralization on HDM-induced neutrophilic inflammation in mice. A) Total neutrophil count, B) MPO activity, C) % IL-17RA + EpCAM + cells, D) IL-17A protein levels, E) Total neutrophil count, and F) MPO activity. Values are shown as mean $\pm$ SE, $n = 6/\text{group}$. * $P < 0.05$, vs. Trypsin/HDM group.

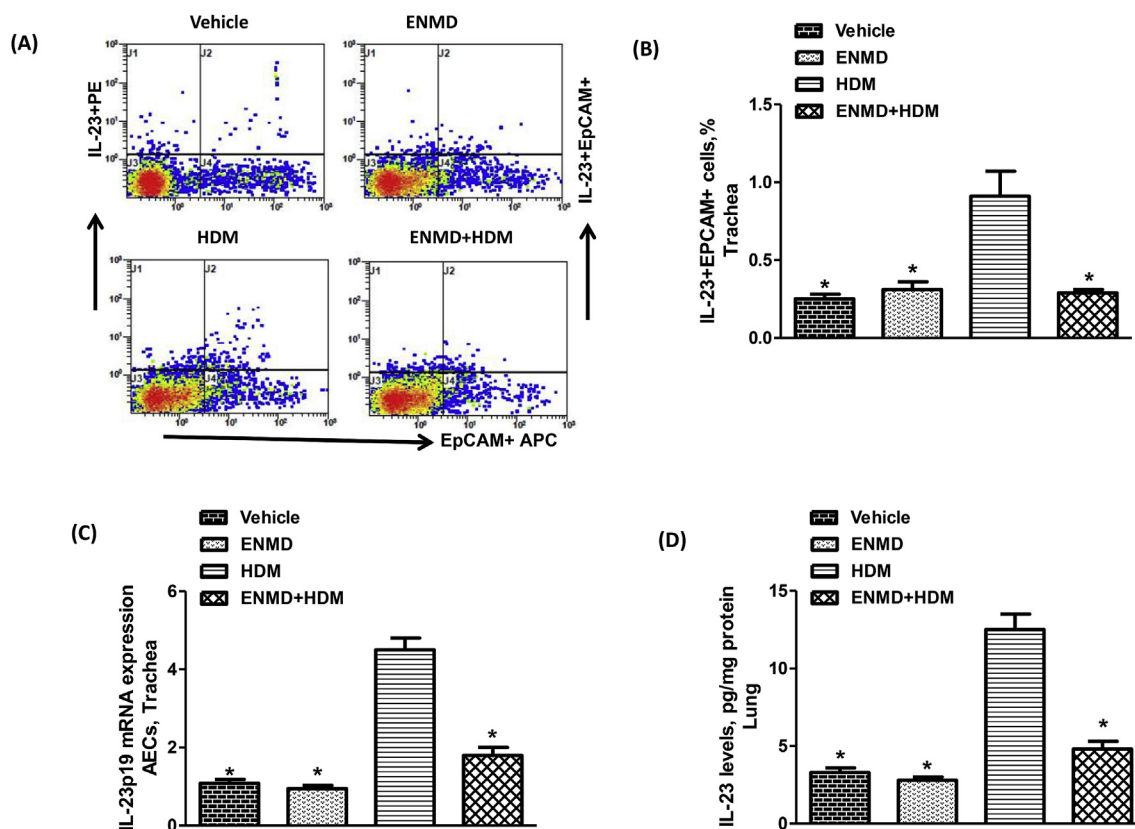


Fig. 4. Effect of PAR-2 antagonist, ENMD-1068 and acute exposure of HDM allergens on IL-23 expression in mice. A) Flow plot depicting immunostaining for IL-23 + and EpCAM + cells, B) % IL-23 + EpCAM + cells, C) IL-23 mRNA in AECs, and D) IL-23 protein levels in lung. Values are shown as mean $\pm$ SE, $n = 6-8/\text{group}$. * $P < 0.05$, vs. HDM group.

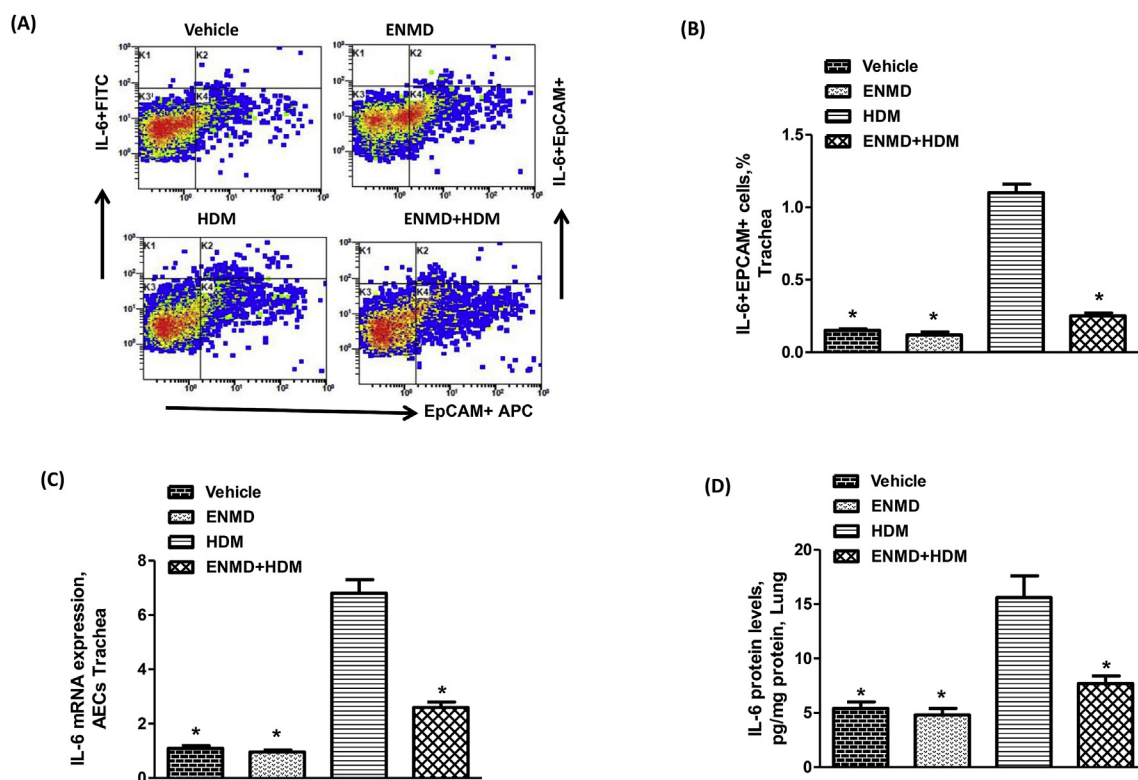


Fig. 5. Effect of PAR-2 antagonist, ENMD-1068 and acute exposure of HDM allergens on IL-6 expression in mice. A) Flow plot depicting immunostaining for IL-6 + and EpCAM + cells, B) % IL-6 + EpCAM + cells, C) IL-6 mRNA in AECs, D) IL-6 protein levels in lung. Values are shown as mean $\pm$ SE, $n = 6-8$ /group. * $P < 0.05$, vs. HDM group.

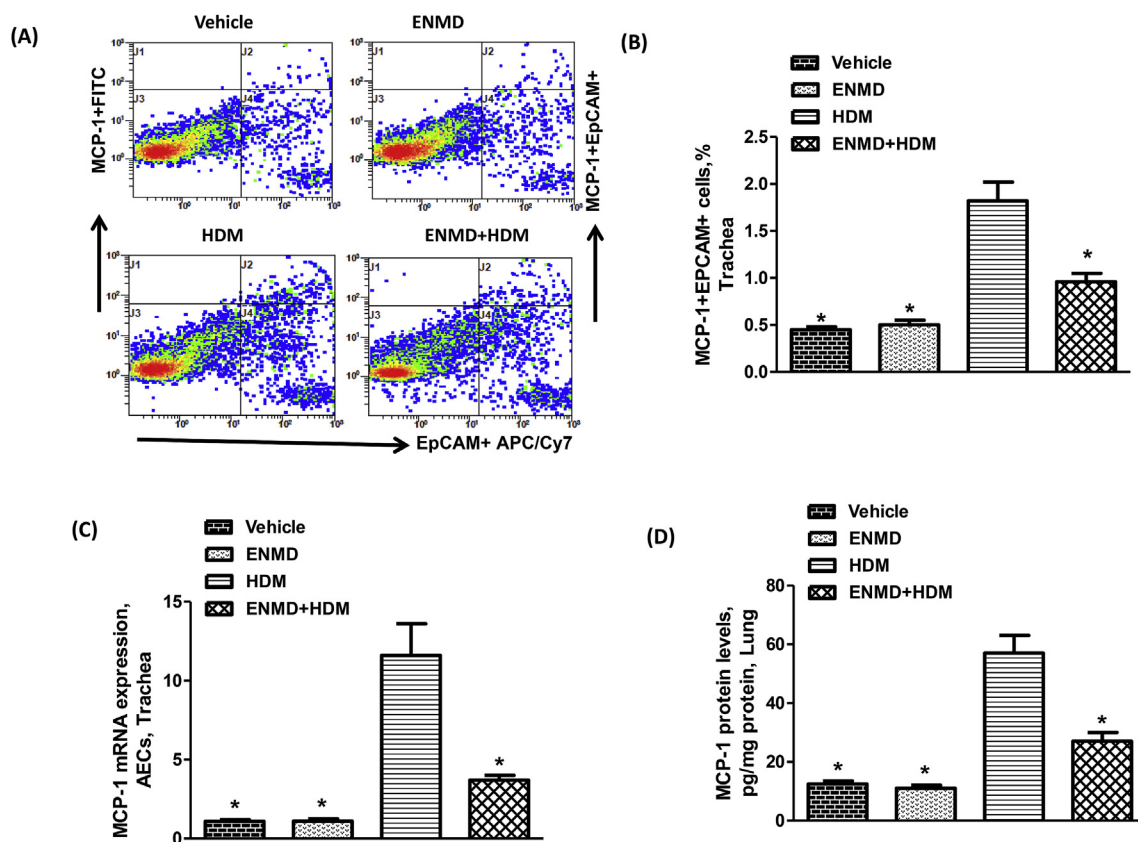


Fig. 6. Effect of PAR-2 antagonist, ENMD-1068 and acute exposure of HDM allergens on MCP-1 expression in mice. A) Flow plot depicting immunostaining for MCP-1 + and EpCAM + cells, B) % MCP-1 + EpCAM + cells, C) MCP-1 mRNA in AECs, and D) MCP-1 protein levels in lung. Values are shown as mean $\pm$ SE, $n = 6-8$ /group. * $P < 0.05$, vs. HDM group.

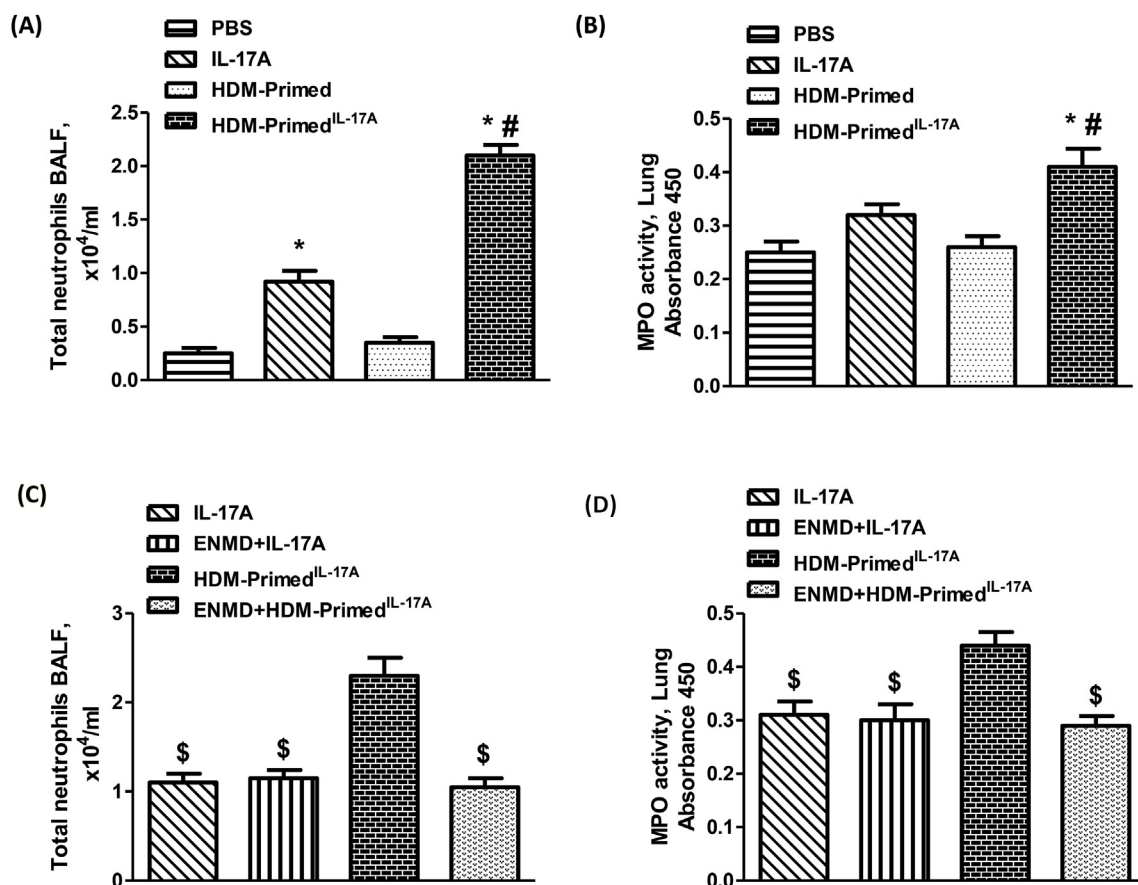


Fig. 7. Effect of IL-17A and anti-IL-17A antibody on airway inflammation in HDM-primed and PBS-exposed mice. A) Total neutrophil count, B) MPO activity, C) Total neutrophil count, and D) MPO activity. Values are shown as mean $\pm$ SE, $n = 6-8/\text{group}$. * $P < 0.05$, vs. PBS/HDM-Primed group; # $P < 0.05$, vs. IL-17A group; \$ $P < 0.05$, vs. HDM-Primed^{IL-17A} group.

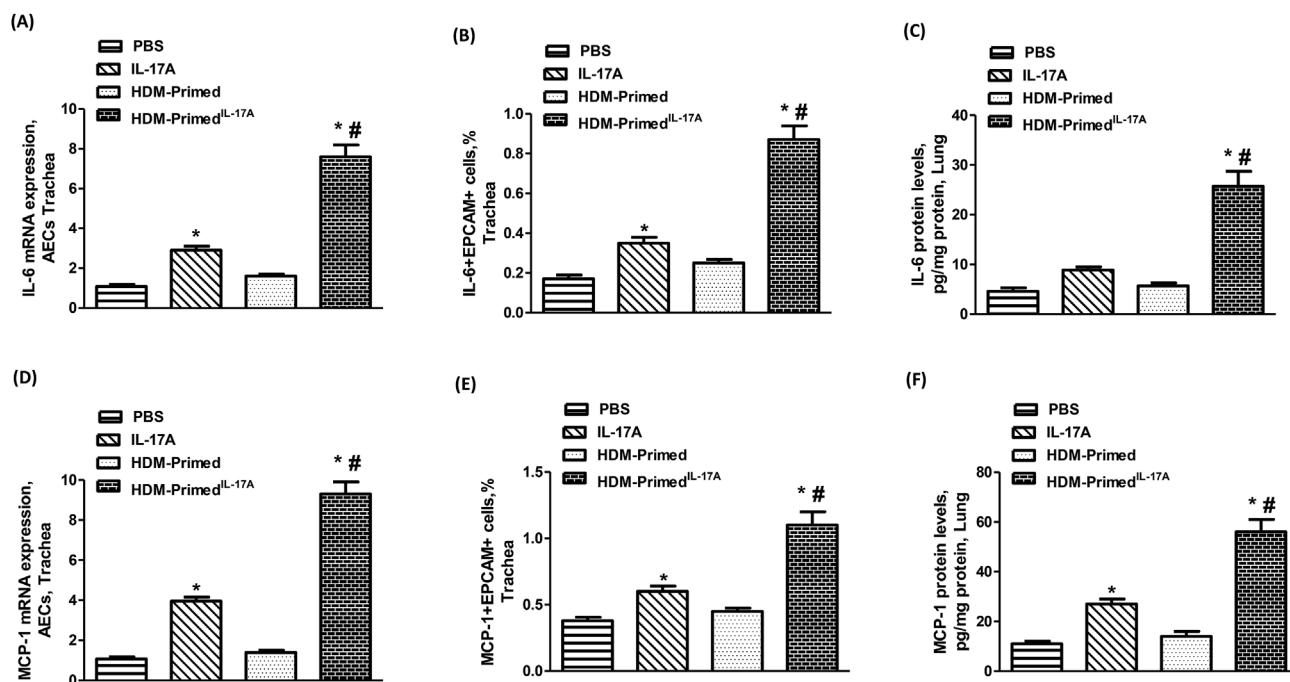


Fig. 8. Effect of IL-17A on inflammatory cytokines in HDM-primed mice and PBS-exposed mice. A) IL-6 mRNA in AECs, B) % IL-6 + EpCAM + cells, C) IL-6 protein levels in lung, D) MCP-1 mRNA in AECs, E) % MCP-1 + EpCAM + cells, and F) MCP-1 protein levels in lung. Values are shown as mean $\pm$ SE, $n = 6-8/\text{group}$. * $P < 0.05$, vs. PBS/HDM-Primed group; # $P < 0.05$, vs. IL-17A group.

IL-23 expression in AECs/lung was not affected by IL-17A administration in HDM-primed and PBS-exposed mice (data not shown). These data put forward the notion that upregulated IL-17R signaling in HDM-primed mice is coupled with elevated expression of inflammatory cytokines/chemokines in AECs. In a succinct form, our study proposes that HDM allergens activate AECs at a very early stage where PAR-2/IL-17R signaling serves an important role in neutrophilic inflammation.

4. Discussion

Asthma is a chronic lung disease where eosinophilic/neutrophilic infiltration and increased Th2/Th17 cells play a vital role. It is now well documented that AECs have a major role in the development of airway inflammation, hyperresponsiveness and mucus secretion, thus contributing to the overall pathogenesis of allergic asthma. Aerial allergens like smoke, particles, viruses, and HDM stimulate AECs to release various key inflammatory mediators which sensitize the individual towards allergic disposition [1,10,31]. Our study reports for the first time that PAR-2 activation by HDM allergens upregulates expression of IL-17R on AECs which primes these cells for increased sensitivity towards IL-17A signaling from an early stage in which adaptive immunity is unlikely to play any significant role.

The interactions that exist among the allergenic stimuli, innate immune response and airway inflammation are still not clearly defined. It has been shown in earlier studies that HDM allergens possess protease activity which can affect AECs function in different ways. HDM allergens have cysteine proteases, trypsin, chymotrypsin, and serine proteases (Fassio et al., 2018; [4]). These proteases by activating PAR-2 are known to increase permeability of AECs by disturbing different junctional proteins with concurrent release of a variety of immune modulators such as cytokines, chemokines and oxidants [6,21,32]. This is thought to provide an easy access of inhaled allergens to dendritic cells underneath the AECs, thereby thus shaping the primary immune response [5,31].

Current models of HDM-induced asthma have been quite useful in understanding the multifaceted immune response that is stimulated as a result of short/long-term allergen exposure. However, these chronic/subchronic models do not furnish information on the early innate events taking place at the allergen-AEC interface which are very critical in deciding the final adaptive immune response [23,33–35]. Therefore, this study probed the effect of PAR-2 activation on IL-17R signaling in AECs/lung after acute HDM exposure in mice. This would give us a better knowledge about the role of HDM allergens in the commencement of innate immune response during acute exposure and the role of PAR-2 in it.

The airway epithelium is proposed to have a predominant role in the orchestration of lung inflammation and immune responses after an allergen exposure. AECs act as the first defense barrier against a wide variety of inhaled allergens/pollutants/toxic substances thereby governing innate/adaptive immune responses to these challenges. They have the potential to identify foreign substances by means of innate receptors such as PAR-2 that are expressed on AECs. Recent reports have shown us that AEC dysfunction is a major contributing factor in the pathogenesis of a variety of inflammatory lung diseases [1,32,36]. Our study further adds to this information by providing evidence that acute HDM exposure leads to dysregulated IL-17R signaling via PAR-2 activation in AECs. Further, it shows that activation of PAR-2 by HDM allergens leads to accumulation of neutrophils early in the immune response. This was verified by the utilization of PAR-2 agonist, trypsin which showed effects similar to HDM allergens on neutrophilic infiltration and IL-17A/IL-17R expression. This suggests that AECs have the potential to amplify PAR-2 induced airway inflammation through IL-17R signaling. No previous study explored the relationship between PAR-2 activation and IL-17R in the lung to the best of our knowledge.

The Th2/Th17 immune responses to inhaled HDM using chronic/subchronic models have been extensively characterized in several

previous studies [9,23,34,35,37], however, very few studies have probed the effect of PAR-2 antagonism on the IL-17A/IL-17R and neutrophilic inflammatory response during acute exposure to HDM allergens. HDM-induced effects on neutrophilic influx and IL-17A/IL-17R were greatly attenuated by ENMD-1068. This was substantiated by the treatment of PAR-2 antagonist, ENMD-1068 before specific activation of PAR-2 which led to blockade of neutrophilic accumulation and IL-17A/IL-17R expression. Further, neutralization of IL-17A during HDM exposure also caused blockade of airway neutrophil influx, thus suggesting involvement of IL-17A/IL-17R signaling in accumulation of airway neutrophils during initial stages of allergen exposure.

Different innate immune cells are incriminated in the pathogenesis of allergic asthma. Each one of them serves a distinct function in regulation of airway inflammation. Our study shows that acute HDM exposure causes elevation in neutrophil numbers in BAL which is dependent on PAR-2/IL-17R signaling. Neutrophils are one of the most critical effectors of airway inflammation. They are easily recruited to the site of inflammation by chemoattractants released by AECs [11,38]. IL-17A/IL-17R signaling is well known to recruit neutrophils to the lung which may be either involved in airway inflammation or clearance of infections depending on the context (Chen et al., 2016; [8,19]). This is owing to the release of different inflammatory cytokines/chemokines such as IL-6, GM-CSF, MCP-1 and TNF- α from AECs because of IL-17A/IL-17R signaling [1,19,38,39].

With regards to allergic airway inflammation induced by HDM allergens in long term chronic models, IL-17A has been reported to cause influx of neutrophils in the lung. However, there is scarcity of data related to effect of acute HDM exposure on IL-17A/IL-17R signaling in AECs of mice. IL-17A acts as a determining factor for severe asthma as it aggravates the neutrophil inflammatory response. This is also confirmed by reports that show increased severity of asthma when there are increased IL-17A levels and neutrophil numbers in the airways. This is also reportedly involved in resistance to traditional corticosteroid therapy as neutrophilic inflammation remains uncontrolled in such a scenario [26–29]; [12,14–16,37]. Our study further proposes that neutrophilic inflammation starts developing at a very early stage in response to acute HDM exposure which could be a considerable factor in corticosteroid resistance upon chronic exposure. However, this needs to be probed in a future study.

IL-6, IL-1 β and TGF- β are required for differentiation of Th17 cells, however they may not be pathogenic in nature. Th17 cells polarized in the presence of IL-23, IL-6, and IL-1 β but without TGF- β are reported to have higher pathogenic potential due to induction of both transcription factors, i.e. RORC/T-bet. This leads to IL-17A/IFN- γ dual positive Th17 cells *in vivo* which correlate with disease severity in a mouse model of multiple sclerosis [40]. A recent study has also confirmed this notion in which it was documented that T cells from patients with steroid resistant asthma generated higher IL-17A and IFN- γ as compared to patients with steroid sensitive asthma [41]. Our data suggest that IL-6/IL-23 released by HDM exposure may lead to generation of pathogenic Th17 cells upon chronic exposure. It has been documented previously that A/J mice have pulmonary milieu (high IL-6/IL-23 from DCs) that promotes pathogenic Th17 cell polarization whereas C3H/HeJ mice are deficient in this environment. This is proposed to be the main reason behind the development of airway hyperresponsiveness in the former strain [42]. Based on these findings, Th17 cells in the lung may have distinct inflammatory profiles in response to different allergens. It may be an interesting future study to compare *in vivo* production of IL-23, IL-6, IL-1 β and TGF- β in the lung along with airway inflammation profile using well known allergens such as HDM, cockroach extract, *Aspergillus fumigatus*, and ragweed. This may give us an idea on the pathogenicity of pulmonary Th17 cells under different allergenic conditions.

Elucidation of the pathways in response to allergenic stimuli could provide a deeper understanding to tackle allergic airway disease in a better way. Our study is an effort in that direction as it attempts to

furnish an insight into the interactions between AECs and HDM allergens, and the role played by PAR-2/IL-17R signaling in it. In conclusion, our study provides evidence that HDM allergens activate the AECs for an early neutrophilic response which is dependent on PAR-2 and IL-17A/IL-17R signaling. This may later develop into a full-blown inflammatory cascade when adaptive immunity comes into play due to reexposure of HDM allergens.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cbi.2019.03.001>.

Transparency document

Transparency document related to this article can be found online at <https://doi.org/10.1016/j.cbi.2019.03.001>.

Disclosure

All authors declare no conflicts of interest.

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