

Double-stranded RNA evokes exacerbation in a mouse model of corticosteroid refractory asthma

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Abstract

RNA viruses are a major cause of respiratory infections and are known to exacerbate asthma and other respiratory diseases. Our aim was to test the ability of poly(I:C) (polyinosinic:polycytidylic acid), a viral surrogate, to elicit exacerbation in a model of severe asthma driven by HDM (house dust mite) in FCA (Freund's complete adjuvant). Poly(I:C) was administered intranasally around the HDM challenge in FCA–HDM-sensitized animals. Changes in AHR (airway hyperresponsiveness), BALF (bronchoalveolar lavage fluid) inflammatory infiltrate, HDM-specific immunoglobulins and cytokine/chemokine release were evaluated at different points after the challenge. The effect of oral dexamethasone was also assessed. Exacerbation was achieved when poly(I:C) was administered 24 h before the HDM challenge and was characterized by enhanced AHR and an increase in the numbers of neutrophils, macrophages and lymphocytes in the BALF. Th1, Th2 and Th17 cytokines were also elevated at different time points after the challenge. Peribronchial and alveolar inflammation in lung tissue were also augmented. AHR and inflammatory infiltration showed reduced sensitivity to dexamethasone treatment. We have set up a model that mimics key aspects of viral exacerbation in a corticosteroid-refractory asthmatic phenotype which could be used to evaluate new therapies for this condition.

Key words: airway hyperresponsiveness; Freund's complete adjuvant, house dust mite, neutrophilic asthma, poly(I:C), steroid resistance.

INTRODUCTION

Asthma is a chronic and heterogeneous respiratory condition characterized by airflow obstruction, airway inflammation and AHR (airway hyperresponsiveness) with symptoms of wheeze, cough, chest tightness and shortness of breath [1]. Although eosinophil accumulation and Th2 cell activation is fundamental to mild–moderate asthma, patients with severe asthma manifest a more diverse inflammatory pattern with several prominent subphenotypes [2,3]. Severe corticosteroid-refractory asthma is poorly understood and difficult to treat, and these patients pose a major healthcare problem as they have greater morbidity and mortality accounting for most asthma-related healthcare expenditure [2,4].

The U-BIOPRED (Unbiased BIOmarkers in PREdiction of respiratory disease outcomes) project aims to reduce bottlenecks

in the development of new therapies for severe asthma by identifying different phenotypes and biomarkers and developing better models of severe asthma [2]. A model of allergic inflammation in mice driven by HDM (house dust mite) in FCA (Freund's complete adjuvant) has been developed as part of U-BIOPRED [5]. This reflects many characteristics of human severe asthma, showing a reduced sensitivity to corticosteroids, AHR and a mixed T-helper phenotype with an important neutrophilic component [5].

Acute exacerbations are significant events in severe asthma and have enormous implications for patients, their caregivers and healthcare providers [6]. Asthma exacerbations are characterized by progressive shortness of breath, chest tightness, cough and wheezing, or a combination of these, and increased airflow obstruction that is manifested by reductions in lung function. Exacerbations accelerate disease progression, impair quality of life,

Abbreviations: AHR, airway hyperresponsiveness; AUC, area under the curve; BALF, bronchoalveolar lavage fluid; FCA, Freund's complete adjuvant; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; HDM, house dust mite; IFN- γ , interferon γ ; KC, keratinocyte chemoattractant; IL, interleukin; i.n., intranasally; MCh, methacholine; MCP-1, monocyte chemoattractant protein 1; MIP, macrophage inflammatory protein; OVA, ovalbumin; Penh, enhanced pause; p.o., per os; poly(I:C), polyinosinic:polycytidylic acid; RANTES, regulated on activation normal T-cell expressed and secreted; TNF- α , tumour necrosis factor α ; U-BIOPRED, Unbiased BIOmarkers in PREdiction of respiratory disease outcomes.

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cause significant morbidity and are a major cause of mortality. Preventing exacerbations is a major therapeutic goal not achieved with currently available treatments [6].

Viral infections are major triggers for asthma exacerbations in both adults and children [7]. The need for understanding the mechanisms underlying asthma exacerbations and for testing new therapies has driven the development of animal models that mimic asthma exacerbations [8].

Most viruses, including those that cause respiratory infections (rhinovirus, influenza, respiratory syncytial virus), produce dsRNA at some point during their replication [9]. Consequently, the TLR3 (Toll-like receptor 3) agonist poly(I:C) (polyinosinic:polycytidylic acid), a synthetic analogue of dsRNA, is used as a surrogate to mimic viral infections [10].

The objective of the present study was to use poly(I:C) to elicit an exacerbated response in a model that resembles a severe asthma phenotype and profile the changes associated with this exacerbation.

MATERIALS AND METHODS

Animals

Male BALB/c mice (20–25 g) were purchased from Charles River Laboratories and housed at 20–24°C, humidity 40–70% with cycles of room air (10 per h) under a 12-h light/12-h dark cycle. Animals were allowed free access to standard laboratory food (Harlan Teklad 2014) and water. Care and use of animals was undertaken in compliance with the European Community Directive 86/609/CEE for the use of laboratory animals. Experimental procedures were approved by the Almirall Ethics Committee.

HDM extract

Purified HDM extract from *Dermatophagoides pteronyssinus* (*Der p1*, 32.8 µg/mg of total protein; GREER Laboratories) was used in these experiments. HDM doses used refer to the total protein in the extract.

Poly(I:C) preparation

High-molecular-mass Poly(I:C) was prepared according to the manufacturer's instructions (CAYLA-InVivoGen).

Drug preparation

For oral administration, vehicle was 0.5% methyl cellulose and 0.2% Tween 80 in water; dose 2 ml/kg.

Poly(I:C) dose–response and time course in naïve animals

Naïve male BALB/c mice (5–6 weeks of age) were administered with 3, 10, 30 and 100 µg of poly(I:C) i.n. (intranasally) per animal in 50 µl of saline or saline alone and AHR was measured 24 h after challenge. At 48 h after challenge, BALF (bronchoalveolar lavage fluid) was collected to measure inflammatory cells. This was used to choose a submaximal dose to perform a time-course study. BALF was collected at 2, 6, 24, 48, 72, 96 and 168 h after poly(I:C) challenge.

Effect of poly(I:C) on the FCA–HDM model

Male BALB/c mice were sensitized subcutaneously on day 0 with HDM (100 µg) in FCA (Sigma–Aldrich) as described previously [5]. On day 14, mice were exposed to saline or HDM (25 µg) under isoflurane anaesthesia via intranasal instillation. In order to decide the best time to administer the viral surrogate, poly(I:C) (30 µg) or saline was administered under isoflurane anaesthesia via intranasal instillation 24 h before (–24 h), at the same time (0 h) or after (+6 h, +24 h) HDM challenge. For further experiments, the time of 24 h before HDM challenge was selected.

At 24 h after the HDM challenge, non-invasive whole-body plethysmography (Buxco) was used to assess Penh (enhanced pause) AUC (area under the curve) to aerosolized MCh (methacholine) (0–16 mg/ml, Sigma) as a measurement of AHR. At 48 h after challenge, the trachea was cannulated and the lungs were lavaged three times with 0.4 ml of PBS containing 5% FBS (Sigma). Differential counts were performed using a Sysmex XT-2000i Automated Haematology Analyser. BALF samples were centrifuged at 300 g for 5 min and the supernatant was stored at –80°C for cytokine/chemokine analysis. In some experiments, blood from intracardiac puncture was collected in heparinized tubes, centrifuged at 3000 g for 10 min at 4°C and the plasma was stored at –80°C in order to determine changes in HDM-specific immunoglobulin levels in plasma. Lung tissue was collected for histological analysis.

Time course after poly(I:C)/HDM challenge

To explore the changes in cytokine/chemokine expression and HDM-specific immunoglobulins associated with poly(I:C) administration, a time-course study after poly(I:C)/HDM challenge was carried out. BALB/c mice were sensitized and challenged as described in Figure 2(A). Samples were collected 4, 24 and 48 h after HDM challenge. BALF was collected to measure cytokine/chemokine levels. Blood was also collected by intracardiac puncture in order to determine changes in HDM-specific immunoglobulin levels in plasma.

Effect of dexamethasone in poly(I:C)-associated exacerbation

Sensitization and poly(I:C)/HDM challenge was carried out as described above. Dexamethasone (1 mg/kg) or vehicle was administered orally 1 h before HDM challenge. AHR was assessed 24 h post-challenge, and BALF and lung tissue were collected 48 h after challenge.

Histological analysis

Following BALF collection, the left lobe of the lung was inflated with 0.6 ml of 10% neutral-buffered formalin and immersed further in fixative until processing. Two cross-sections containing the first-generation bronchus were paraffin-embedded, and 3 µm sections were cut before staining with haematoxylin/eosin for the evaluation of inflammation as well as bronchial and bronchiolar mucosal thickness. Additional sections were stained with AB (Alcian Blue)/PAS (periodic acid–Schiff) to demonstrate presence of goblet cells. The severity of pulmonary inflammation was assessed using a semi-quantitative score on a scale from 0 to 4,

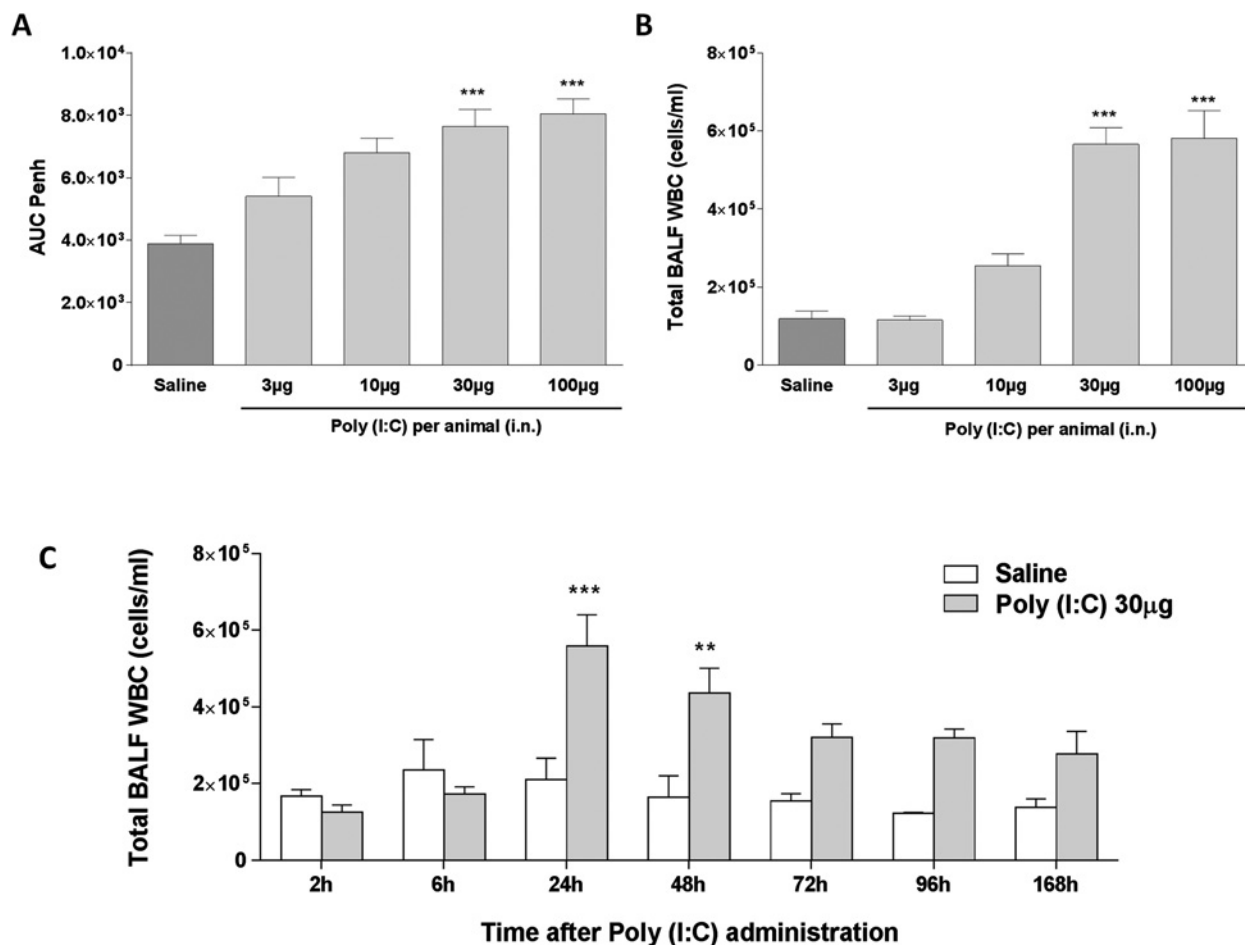


Figure 1 Determining poly(I:C) dose and time of administration in naïve animals

Saline or poly(I:C) (3–100 μg per animal) was administered to naïve mice. AHR (Penh AUC) to MCh challenge (A) and inflammatory infiltration (B) were evaluated. A time course of inflammatory infiltration after administering a submaximal dose of poly(I:C) or saline was performed (C). Results ($n = 8–16$) are presented as means ± S.E.M. and were analysed by one-way ANOVA (A and B) or an unpaired Student's *t* test (C). ** $P < 0.01$; *** $P < 0.001$ compared with saline.

where 0 is not inflamed and 4 is markedly and diffusely inflamed tissue. Peribronchovascular interstitium as well as parenchymal involvement were evaluated. Bronchial and bronchiolar epithelial thickness was analysed using a semi-quantitative score from 0 to 3, where 0 is normal epithelial height and 3 is marked increase in height. Proportion of goblet cells was evaluated using a four-point scoring system, where 0 indicates absence of goblet cells and 4 indicates 75–100% replacement of bronchial mucosa by goblet cells.

Cytokine/chemokine BALF analysis

The concentration of BALF cytokines was determined using Luminex® technology (Bioplex 200 system, Bio-Rad Laboratories) according to the manufacturer's instructions. A 23-plex panel including IL (interleukin)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, eotaxin, G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte/macrophage colony-stimulating factor), IFN-γ (interferon γ), KC (keratinocyte chemoattractant), MCP-1 (monocyte chemoattractant protein 1), MIP (macrophage inflammatory

protein)-1α, MIP-1β, RANTES (regulated on activation normal T-cell expressed and secreted) and TNF-α (tumour necrosis factor α) was measured in the samples. IL-33 was also measured in these samples using a Quantikine® ELISA kit from R&D Systems. The cytokines explored were selected on the basis of being elevated after HDM challenge in previous studies carried out within the U-BIOPRED consortium or due to their particular relevance in asthma exacerbation.

HDM-specific immunoglobulin levels in plasma

HDM-specific immunoglobulin levels were determined as described previously [11]. Immunoglobulin biotinylated antibodies were supplied by BD Pharmingen.

Data analysis

Results are expressed as means ± S.E.M. for n observations. An unpaired Student's *t* test was used when comparing two groups and a one-way ANOVA when comparing multiple groups with the appropriate post-hoc test analysis. A Mann–Whitney for comparing two groups and a Kruskal–Wallis test for multiple groups with

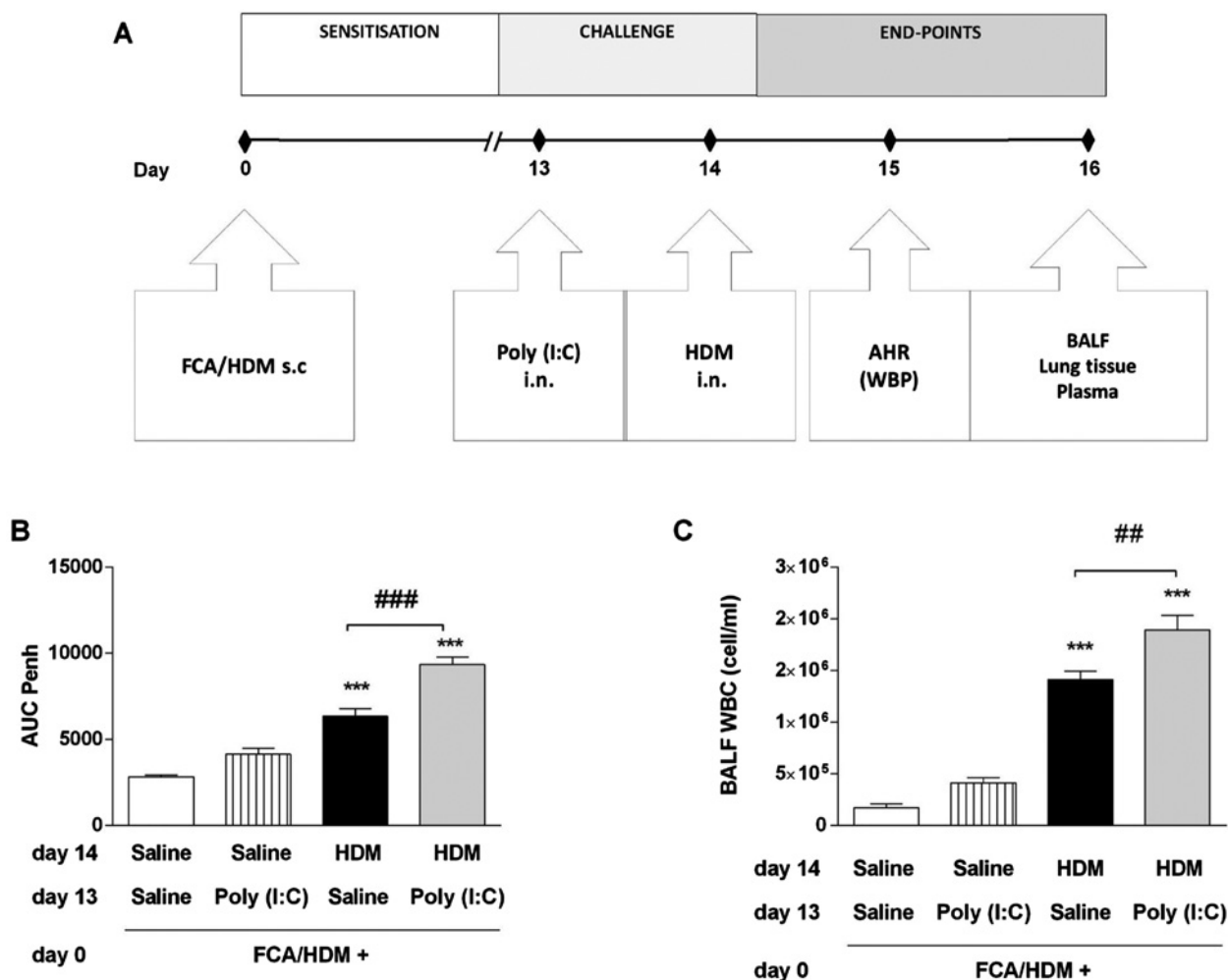


Figure 2 Effect of poly(I:C) administered 24 h before HDM challenge on AHR and BALF total cells

(A) BALB/c mice were sensitized subcutaneously with HDM in FCA. At day 13, they received poly(I:C) or saline i.n. After 24 h they received either HDM extract or saline. (B) AHR (Penh AUC) to MCh was measured 24 h after HDM/saline challenge by whole-body plethysmography. (C) Total inflammatory cells ('WBC') in the lavage fluid were evaluated 48 h after HDM or saline challenge. A group of naïve animals was also included as a control for inflammatory infiltration (results not shown). Results ($n = 16$; naïve $n = 6$) are expressed as means $\pm$ S.E.M. and were analysed by a non-parametric Mann-Whitney test or Kruskal-Wallis test followed by a Dunn's post-hoc test. *** $P < 0.001$ compared with the saline/saline group; ## $P < 0.01$, ### $P < 0.001$ compared with the saline/HDM group.

the appropriate post-test analysis were used for non-normally distributed data. All treatments were compared with relevant vehicle control groups; differences were significant when $P < 0.05$. These analyses were performed using GraphPad Prism 6.0.

RESULTS

Poly(I:C) dose-response and time course in naïve animals

Poly(I:C) in naïve animals showed a dose-response effect on AHR (Figure 1A) and on BALF inflammatory cells (Figure 1B).

We chose a submaximal dose (30 μ g) to perform the subsequent time-course study. In this study, poly(I:C) challenge caused a significant inflammatory infiltration with a maximum at 24 h (Figure 1C). This time was taken as a reference when choos-

ing the time of superimposing the viral mimetic to the HDM model.

Poly(I:C) effect on the FCA-HDM model

Poly(I:C) caused the most significant and reproducible exacerbation when given 24 h before HDM, and this was selected for later studies (Figure 2A). Poly(I:C) administered at this time caused a significant increase in the AHR (AUC Penh; Figure 2B) and in BALF inflammatory cells (Figure 2C; see also Figures 8A and 8B). This exacerbation was associated with increased BALF neutrophils, macrophages and lymphocytes (Figures 3A, 3C and 3D respectively; see also Figures 8C, 8E and 8F respectively). The increase in macrophages after poly(I:C) challenge achieved statistical significance consistently across experiments, whereas the increase in neutrophils and lymphocytes did not always reach significance. Eosinophilic infiltration was not significantly

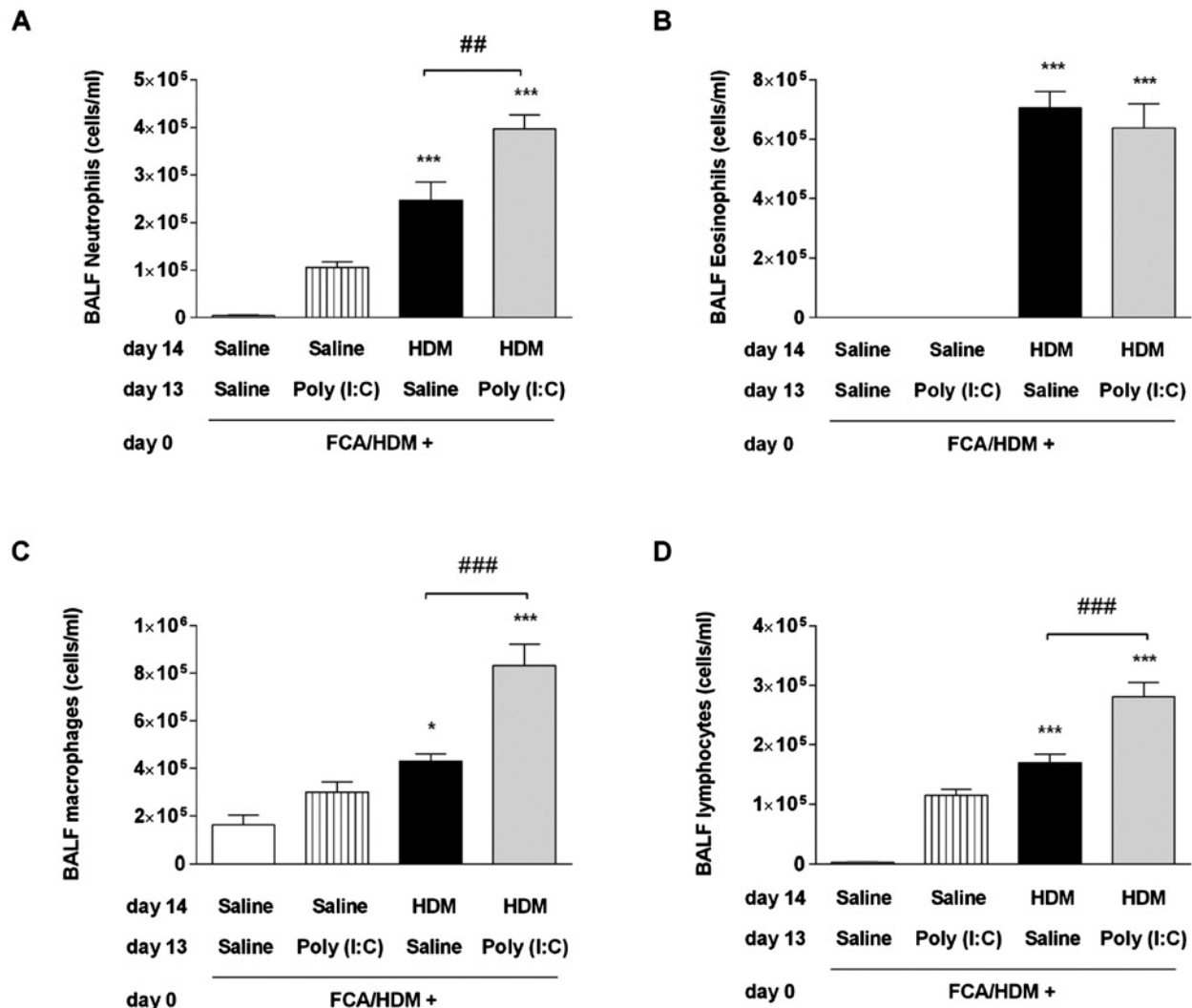


Figure 3 Effect of poly(I:C) administered 24 h before HDM challenge on BALF inflammatory infiltrate (differential counts) BALB/c mice were sensitized subcutaneously with HDM in FCA. At day 13 they received poly(I:C) or saline i.n. After 24 h, they received either HDM extract or saline. Inflammatory cells in the lavage fluid were evaluated 48 h after HDM or saline challenge. Effects on neutrophils (A), eosinophils (B), macrophages (C) and lymphocytes (D) are shown. A group of naïve animals was also included as a control for inflammatory infiltration (results not shown). Results ($n = 16$; naïve $n = 6$) are expressed as means $\pm$ S.E.M. and were analysed by a non-parametric Mann–Whitney test or Kruskal–Wallis test followed by a Dunn's post-hoc test. * $P < 0.05$, *** $P < 0.001$ compared with the saline/saline group; ## $P < 0.01$, ### $P < 0.001$ compared with the saline/HDM group.

affected (Figure 3B; see also Figure 8D). Other times tested also showed significant exacerbation in AHR (0 h), BALF neutrophils (0 and +6 h), macrophages (0 h) and lymphocytes (0 h) (see Supplementary Figure S1).

Histological changes associated with poly(I:C) challenge

The lungs from naïve mice (results not shown) and saline/saline mice (Figures 4 and 5A) were histologically unaltered. In the poly(I:C)/saline group, mild peribronchovascular and alveolar inflammation was present (Figures 4A, 4B and 5B). In the poly(I:C)/HDM group, pulmonary inflammation was significantly increased compared with saline/HDM, especially in the alveolar compartment (Figures 4A, 4B, 5C and 5D). The bron-

chial and bronchiolar epithelial thickness was increased in the saline/HDM group compared with saline/saline mice, consistent with epithelial hypertrophy. There was a trend towards further epithelial thickening in the poly(I:C)/HDM group (Figure 4C). In both the saline/HDM and poly(I:C)/HDM groups, there was a moderate to marked increase in goblet cells (Figure 4D).

Time course after poly(I:C)/HDM challenge: evaluation of cytokine, chemokine expression and HDM-specific immunoglobulins

HDM-specific immunoglobulins time course in plasma
FCA–HDM-sensitized groups were all associated with significant increases in HDM-specific IgE, IgG1, IgG2a and IgG2b in the HDM-challenged group as reported previously [5]. Poly(I:C)

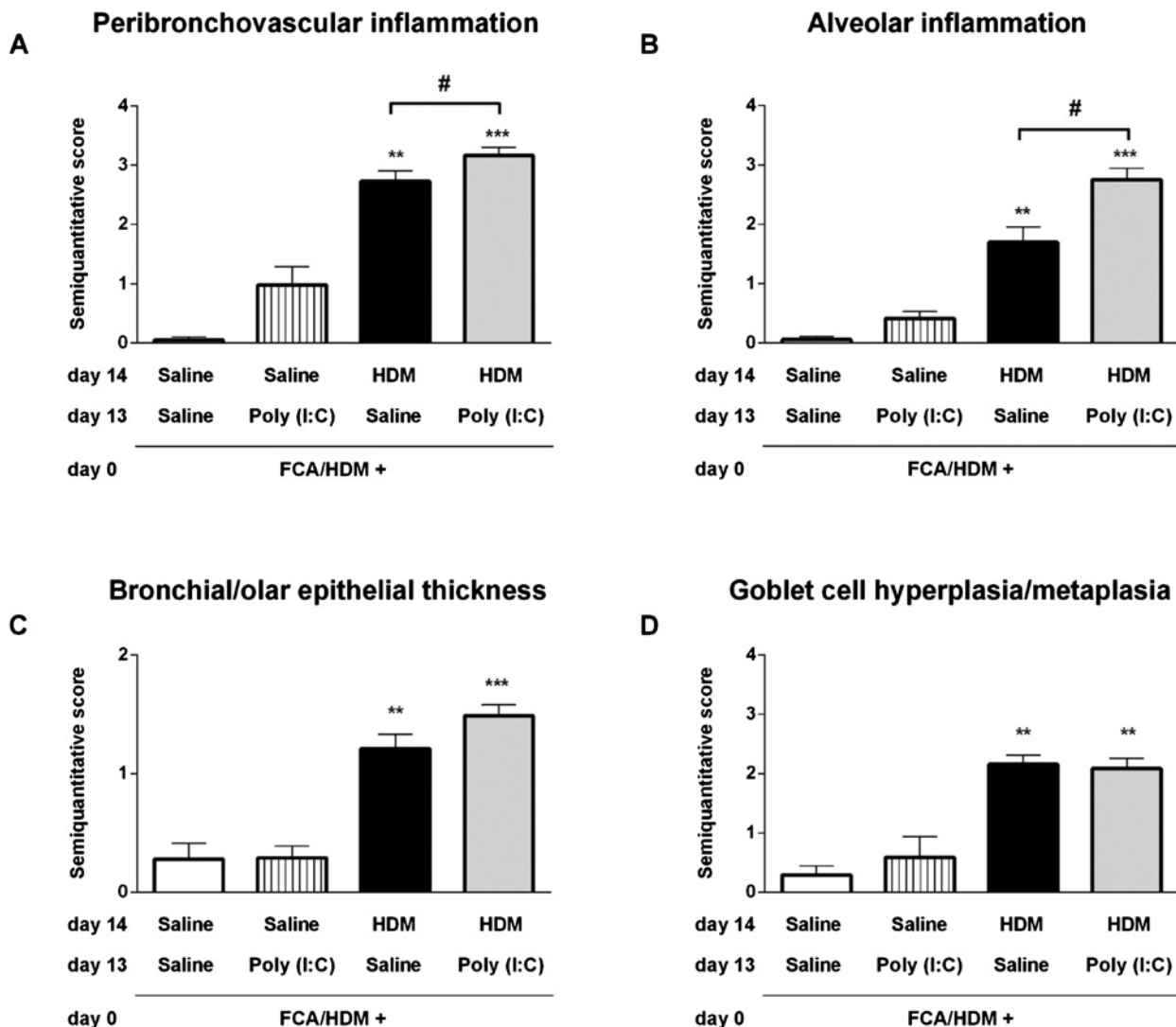


Figure 4 Effect of poly(I:C) administered 24 h before HDM challenge on lung histology

BALB/c mice were sensitized subcutaneously with HDM in FCA. At day 13 they received poly(I:C) or saline i.n. After 24 h, they received either HDM extract or saline i.n. Pulmonary lesions were evaluated histologically using semi-quantitative scores 48 h after HDM/saline challenge. Effects on peribronchovascular inflammation (A), alveolar inflammation (B), bronchial/bronchiolar epithelial thickness (C) and goblet cell metaplasia/hyperplasia (D) are shown. Results ($n = 8-16$) are expressed as means $\pm$ S.E.M. and were analysed by a non-parametric Mann-Whitney test or Kruskal-Wallis test followed by a Dunn's post-hoc test. # $P < 0.05$ compared with the saline/HDM group; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the saline/saline group.

administration did not further increase HDM-specific IgE, IgG1 and IgG2b at any of the times tested (results not shown). However, the levels of IgG2b showed a significant reduction at 24 h (optical densities of 387.1 for saline/HDM compared with 183.45 for poly(I:C)/HDM; $P < 0.05$) and 48 h (optical densities of 615.25 for saline/HDM compared with 294.1 for poly(I:C)/HDM; $P < 0.01$) after challenge.

Cytokine/chemokine time course in BALF

The saline/HDM group showed significant increases in IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN- γ , KC, MCP-1,

MIP-1 α , MIP-1 β , RANTES and TNF- α (Figure 6 and Table 1). At 4 h after HDM challenge, the poly(I:C)/HDM group showed a significant increase in all cytokines except IL-3, IL-5, IL-13, KC, MIP-1 α , MIP-1 β and TNF- α (Figure 6 and Table 1). At 24 h after HDM challenge, only IL-1 α , IL-1 β , IL-2, IL-5, IL-12(p40), IL-12(p70), G-CSF, MCP-1, RANTES, IFN- γ and TNF- α were significantly increased in the poly(I:C)/HDM group. Finally, at 48 h after HDM challenge, only RANTES and IL-12(p40) seemed to remain exacerbated (Figure 6 and Table 1). IL-33 has been postulated to have a key role in viral exacerbations [12]. Levels of IL-33 BALF were very low at every time point and no changes could be detected in these samples (results not shown). This has

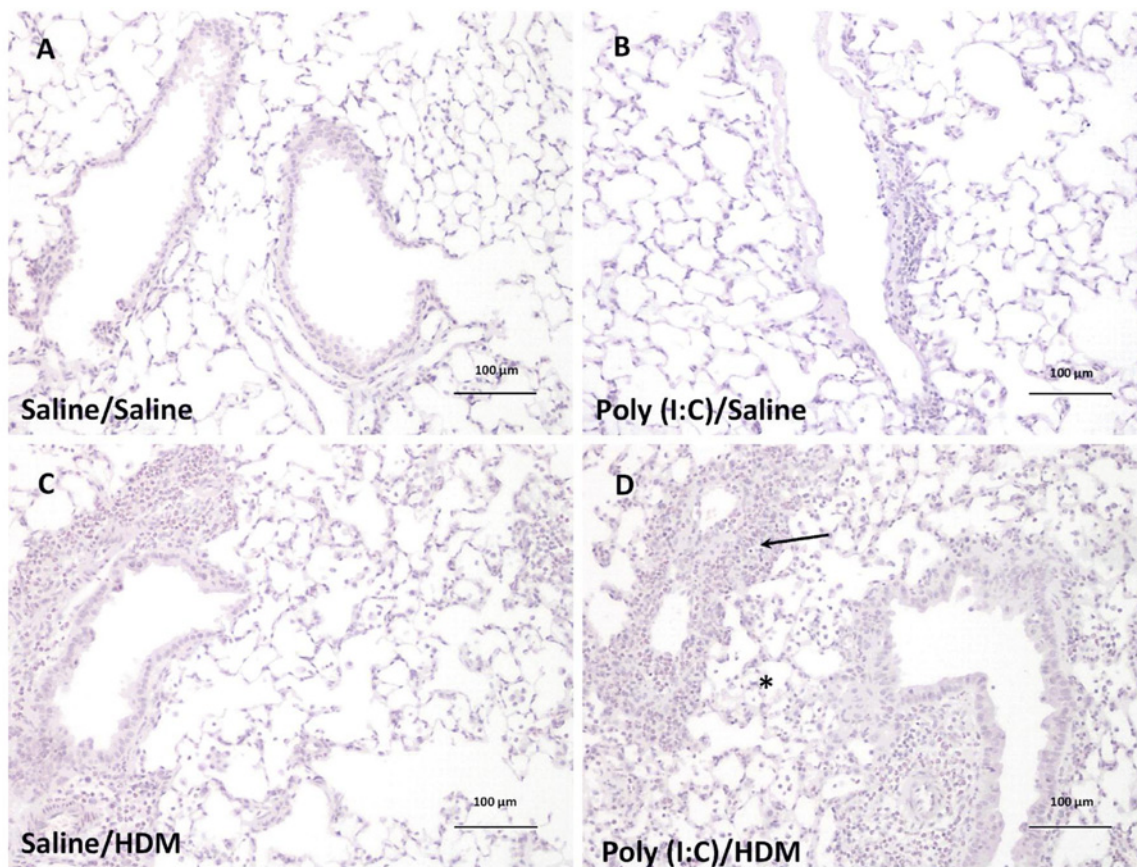


Figure 5 Exacerbation of inflammation in the lung tissue when administering poly(I:C) 24 h before HDM challenge BALB/c mice were sensitized subcutaneously with HDM in FCA. At day 13 they received poly(I:C) or saline i.n. After 24 h, they received either HDM extract or saline i.n. Inflammation within the peribronchovascular (arrow) and alveolar (asterisk) compartments were evaluated semi-quantitatively 48 h after HDM/saline challenge. Representative microphotographs of inflammation in saline/saline (A), poly(I:C)/saline (B), saline/HDM (C) and poly(I:C)/HDM (D) groups are shown. Note the exacerbated inflammation in the latter group, especially at the alveolar level. Haematoxylin and eosin stain was used. Scale bar, 100 μ m.

been shown previously by other groups [13]. However, IL-33 was elevated in the lungs of saline/HDM animals. Moreover, IL-33 was also significantly exacerbated in the poly(I:C)/HDM group (Figure 7).

Effect of dexamethasone in poly(I:C)-associated exacerbation

Dexamethasone had no significant effect on either the AHR associated with the saline/HDM group or on the exacerbated AHR due to poly(I:C) exposure (Figure 8A). Dexamethasone caused a small, but non-significant, decrease in BALF inflammatory cells in both the saline/HDM and the poly(I:C)/HDM groups (Figure 8B). This dose and dosing regimen caused 80–90% inhibition of the inflammatory response induced in the acute OVA (ovalbumin) mouse model of allergic inflammation [OVA/dexamethasone 1 mg/kg p.o. (per os) 4854.2 compared with OVA/vehicle 126741 eosinophils/ml in BALF 48 h after last OVA challenge; $P < 0.001$]. Dexamethasone significantly inhibited HDM-induced eosinophilia (Figure 8D), whereas it failed to reduce neutrophils, macrophages and lymphocytes (Figures 8C,

8E and 8F). Histologically, dexamethasone did not show any relevant inhibitory effects on any of the evaluated lesions in either the saline/HDM or the poly(I:C)/HDM groups (results not shown).

DISCUSSION

We have profiled a new murine model of allergic inflammation exacerbated with poly(I:C) that reflects several features of viral exacerbations associated with severe asthma. The unique mixed T-cell profile caused by the use of FCA in the sensitization of our model results in an exacerbated profile unlike any other reported asthma exacerbation models. Our model shows an exacerbation of the response to the bronchoconstrictor MCh, increased inflammatory infiltrate in BALF, enhanced alveolar inflammation and a range of elevated pro-inflammatory cytokines and chemokines which have been associated with human asthma exacerbation, including IL-33. Furthermore, this model also shows a reduced sensitivity to steroids, both in AHR and inflammation,

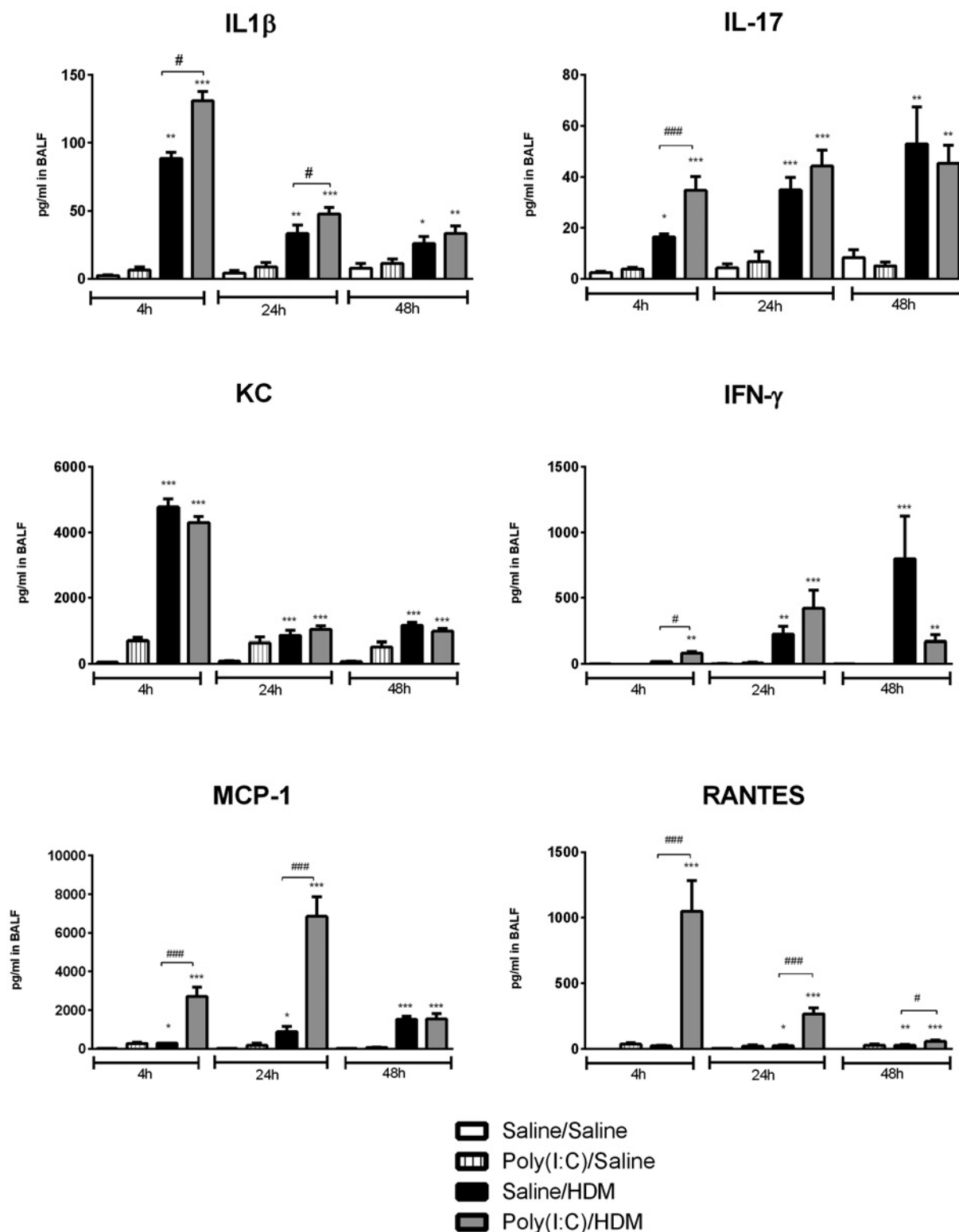


Figure 6 Effect of poly(I:C) administered 24 h before HDM challenge on BALF IL-1 β , IL-17, KC, INF- γ , MCP-1 and RANTES. BALB/c mice were sensitized subcutaneously with HDM in FCA. At day 13 they received poly(I:C) or saline i.n. After 24 h, they received either HDM extract or saline i.n. Cytokine/chemokine levels in the BALF were evaluated 4, 24 and 48 h after HDM challenge. Results ($n = 4-8$ saline/saline; $8-16$ other groups) are expressed as means $\pm$ S.E.M. and were analysed by Kruskal–Wallis test followed by a Dunn's post-hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the saline/saline group) or Mann–Whitney test (# $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with the saline/HDM group). For a complete cytokine/chemokine profile, see Table 1.

Table 1 Effect of poly(I:C) administered 24 h before HDM challenge on BALF cytokines/chemokines

BALB/c mice were sensitized subcutaneously with HDM in FCA. At day 13, they received poly(I:C) or saline i.n. After 24 h, they received either HDM extract or saline i.n. Cytokine/chemokine levels in the BALF were evaluated 4, 24 and 48 h after HDM challenge. Results ($n = 4-8$ for saline/saline; $n = 8-16$ for other groups) are expressed as means $\pm$ S.E.M. and analysed by Kruskal–Wallis test followed by a Dunn's post-hoc test (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the saline/saline group) or Mann–Whitney test (# $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with the saline/HDM group). Results that are significant compared with both groups are presented in italics.

Time after HDM challenge (h)	Saline/Saline (pg/ml in BALF)	Poly(I:C)/Saline (pg/ml in BALF)	Saline/HDM (pg/ml in BALF)	Poly(I:C)/HDM (pg/ml in BALF)
IL-1 α				
4	0.4 $\pm$ 0.2	1.6 $\pm$ 0.5	11.7 $\pm$ 0.7**	33.3 $\pm$ 5***###
24	0.4 $\pm$ 0.1	1.5 $\pm$ 1.2	10.7 $\pm$ 2.4**	12.9 $\pm$ 1.6**#
48	0.5 $\pm$ 0.1	0.03 $\pm$ 0.1	3.2 $\pm$ 0.5*	2.3 $\pm$ 0.4*
IL-1 β				
4	2.4 $\pm$ 0.4	6.4 $\pm$ 2.4	88.5 $\pm$ 4.4**	130.9 $\pm$ 7***#
24	4.3 $\pm$ 1.7	8.7 $\pm$ 3	33.3 $\pm$ 6**	47.7 $\pm$ 5**#
48	7.9 $\pm$ 3.5	11.4 $\pm$ 3	26.0 $\pm$ 5*	33.4 $\pm$ 6*
IL-2				
4	2.3 $\pm$ 0.5	3.5 $\pm$ 0.1	18.6 $\pm$ 5**	68.8 $\pm$ 14***###
24	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1	6.9 $\pm$ 1.4	29.3 $\pm$ 6**###
48	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1	3.5 $\pm$ 1	3.8 $\pm$ 1.5
IL-3				
4	1.0 $\pm$ 0.3	1.2 $\pm$ 0.4	15.1 $\pm$ 1**	22.2 $\pm$ 5***
24	3.1 $\pm$ 1.6	2.4 $\pm$ 1.7	8.2 $\pm$ 2	9.6 $\pm$ 2*
48	7.5 $\pm$ 3.5	1.7 $\pm$ 1.4	8.7 $\pm$ 4	11.9 $\pm$ 5.6
IL-4				
4	3.9 $\pm$ 1.2	3.4 $\pm$ 1.1	172.6 $\pm$ 26*	617.2 $\pm$ 70***###
24	9.5 $\pm$ 4	832.3 $\pm$ 827	4821.8 $\pm$ 641***	4445.8 $\pm$ 384***
48	18.6 $\pm$ 7	12.5 $\pm$ 6	559.3 $\pm$ 69***	241.5 $\pm$ 29*
IL-5				
4	2.1 $\pm$ 0.5	5.3 $\pm$ 0.8	40.1 $\pm$ 6***	41.6 $\pm$ 6***
24	5.1 $\pm$ 2.6	35.7 $\pm$ 33.5	208.6 $\pm$ 24**	403.9 $\pm$ 37***#
48	10.5 $\pm$ 4.4	5.9 $\pm$ 2	241.5 $\pm$ 21**	306.7 $\pm$ 37.5***
IL-6				
4	0.8 $\pm$ 0.1	25.5 $\pm$ 8.6	137.5 $\pm$ 18**	1080.8 $\pm$ 107***###
24	1.0 $\pm$ 0.4	66 $\pm$ 62.6	399.9 $\pm$ 57***	572 $\pm$ 32***
48	2.1 $\pm$ 0.9	2.8 $\pm$ 0.7	117.9 $\pm$ 24***	208.1 $\pm$ 58***
IL-10				
4	1.9 $\pm$ 0.3	1.4 $\pm$ 0.3	28.8 $\pm$ 1.4**	32.9 $\pm$ 1.8***
24	1.6 $\pm$ 0.4	13.6 $\pm$ 12.6	65.7 $\pm$ 5**	62.3 $\pm$ 4***
48	2.9 $\pm$ 1	1.3 $\pm$ 0.3	19.7 $\pm$ 1.4**	12.6 $\pm$ 2**
IL-12 (p40)				
4	20.0 $\pm$ 3	66.6 $\pm$ 9.4	59.9 $\pm$ 4.5*	564.3 $\pm$ 104***###
24	18.9 $\pm$ 2.7	210.9 $\pm$ 75	171.2 $\pm$ 36*	542.5 $\pm$ 68***###
48	20.9 $\pm$ 2	1010.2 $\pm$ 360	372.7 $\pm$ 91*	1276.9 $\pm$ 259***###
IL-12 (p70)				
4	2.8 $\pm$ 0.8	0.3 $\pm$ 0.1	106.8 $\pm$ 7*	160.5 $\pm$ 7***###
24	2.5 $\pm$ 0.9	56.3 $\pm$ 56	329.4 $\pm$ 31***	281.7 $\pm$ 21**
48	2.8 $\pm$ 0.9	0.1 $\pm$ 0.1	41.5 $\pm$ 9***	18.3 $\pm$ 5*
IL-13				
4	23.1 $\pm$ 9	5.9 $\pm$ 2.7	290.2 $\pm$ 12***	298.5 $\pm$ 11***
24	22.8 $\pm$ 9	53.7 $\pm$ 53	392.3 $\pm$ 39**	565.5 $\pm$ 59***
48	22.8 $\pm$ 9	0.7 $\pm$ 0.1	392.9 $\pm$ 57***	448.5 $\pm$ 76***

Table 1 Continued

Time after HDM challenge (h)	Saline/Saline (pg/ml in BALF)	Poly(I:C)/Saline (pg/ml in BALF)	Saline/HDM (pg/ml in BALF)	Poly(I:C)/HDM (pg/ml in BALF)
IL-17A				
4	2.4 ± 0.5	3.8 ± 0.7	16.5 ± 1*	34.7 ± 5***###
24	4.3 ± 1.5	6.9 ± 4	34.9 ± 5***	44.2 ± 6***
48	8.4 ± 3	5.1 ± 1.5	53 ± 14.4**	45.3 ± 7**
Eotaxin				
4	90.8 ± 9.4	63.1 ± 0.1	535.5 ± 69.2*	838.2 ± 67.4***##
24	87 ± 9.7	78.3 ± 15	574.9 ± 66.4**	495.6 ± 48.7**
48	63.1 ± 0.1	63.1 ± 0.1	63.1 ± 0.1	63.1 ± 0.1
G-CSF				
4	6.3 ± 0.7	45.6 ± 7	733.8 ± 69.7***	1394.8 ± 180***##
24	7.7 ± 1	30.7 ± 9	201.6 ± 72***	388.2 ± 95***#
48	7.3 ± 0.5	12.9 ± 1.8	74.5 ± 16***	166.9 ± 50.7***
GM-CSF				
4	13.4 ± 3.7	4.8 ± 2.2	136.8 ± 7**	165.7 ± 4***#
24	14.6 ± 2.9	17.9 ± 15.3	105.7 ± 7***	101.4 ± 5**
48	2.6 ± 0.1	2.6 ± 0.1	15.8 ± 6.4	14.5 ± 9.2
IFN- γ				
4	2.0 ± 0.6	0.2 ± 0.01	17.7 ± 1.9	81.0 ± 15**#
24	2.6 ± 0.6	7.7 ± 7.5	221.7 ± 61.6**	419.8 ± 141***
48	2.0 ± 0.6	0.2 ± 0.01	798.6 ± 324***	167.7 ± 52**
KC				
4	53.3 ± 5.6	697.4 ± 103.6	4776 ± 248.7***	4298 ± 179***
24	83.9 ± 9	639.7 ± 169	856.2 ± 154***	1042.5 ± 97***
48	72.2 ± 7	514.2 ± 148	1165 ± 98***	980.8 ± 86.6***
MCP-1				
4	16.8 ± 1.6	272.6 ± 53.7	286 ± 14*	2710.4 ± 470***###
24	16.8 ± 1.6	176.6 ± 114	879.1 ± 272*	6855.7 ± 996***###
48	16.8 ± 1.6	71.2 ± 25.5	1511 ± 180***	1547 ± 265.6***
MIP-1 α				
4	1.2 ± 0.4	7.7 ± 2.4	5354 ± 607***##	3189 ± 388**
24	2.6 ± 0.9	19.4 ± 16.2	127.2 ± 22.4**	164.2 ± 17.6***
48	4.4 ± 2	2.3 ± 1.1	44.7 ± 6.5***	35 ± 5**
MIP-1 β				
4	1.5 ± 0.3	5.7 ± 1	729.7 ± 116.7***##	361.8 ± 41.95***
24	2.2 ± 0.5	8.4 ± 4.7	56.2 ± 14**	81 ± 11***##
48	3.3 ± 1	3.1 ± 0.5	25 ± 3***	22.2 ± 2.7**
RANTES				
4	0.5 ± 0.1	35.8 ± 9	22.5 ± 3.4	1049.1 ± 234.5***###
24	2.2 ± 0.7	20.3 ± 10.5	24.7 ± 6.4*	267.5 ± 47***###
48	0.7 ± 0.2	26.2 ± 10	27.4 ± 7**	55.5 ± 13***(#)
TNF- α				
4	1.3 ± 0.3	3.8 ± 0.6	3716 ± 1020***	2190.5 ± 587***
24	2.1 ± 1.2	4.8 ± 3.6	101.9 ± 56***	117.6 ± 90***
48	5.4 ± 2.6	2.0 ± 1.2	9.3 ± 3.4	11.6 ± 4.5

in agreement with what happens in a range of human severe asthma phenotypes.

Historically, asthma has been considered as an allergic, eosinophilic, Th2-mediated and corticosteroid-responsive disease [1]. However, it is now evident that asthma is not a single disease,

but a heterogeneous condition consisting of clinically recognizable phenotypes [3]. In adults, severe asthma is often associated with increased Th1 and/or Th17, and neutrophilic inflammatory responses, and is refractory to steroid treatment [14]. Research into severe asthma has also led to identifying pathophysiological

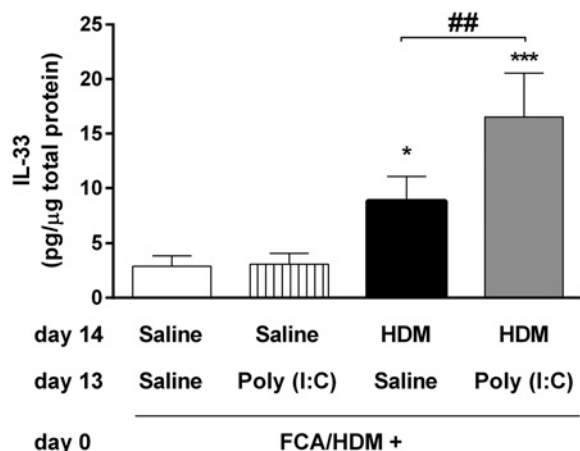


Figure 7 Effect of poly(I:C) administered 24 h before HDM challenge on tissue IL-33 levels

BALB/c mice were sensitized subcutaneously with HDM in FCA. At day 13 they received poly(I:C) or saline i.n. After 24 h, they received either HDM extract or saline i.n. IL-33 levels in the lung tissue were evaluated 48 h after HDM/saline challenge. Results ($n = 8$) are expressed as means $\pm$ S.E.M. and were analysed by a non-parametric Mann-Whitney test or Kruskal-Wallis test followed by a Dunn's post-hoc test. * $P < 0.05$, *** $P < 0.001$ compared with the saline/saline group; ## $P < 0.01$ compared with the saline/HDM group.

characteristics (i.e. early-onset compared with late-onset; atopic compared with non-atopic; Th2 high compared with Th2 low) that might become part of specific phenotypes and define the way that treatment is delivered [15]. In contrast with this clinical heterogeneity, the majority of the animal models available are intrinsically eosinophilic, Th2-driven and highly sensitive to steroid treatment [16,17]. Therefore these models do not mimic the more severe mainly neutrophilic phenotype.

The effort to define asthma phenotypes for a more personalized therapy [18] should be complemented by finding animal models of asthma that better represent these phenotypes. The current data suggest that the FCA-HDM model provides a good match to a severe asthma phenotype associated with mixed T-cell involvement and neutrophilia, and may be valuable in elucidating the mechanisms of pathogenesis and identifying new therapeutic targets.

In adults with asthma, upper respiratory infections are associated with severe decreases in peak expiratory flow, enhanced airway reactivity and the magnitude of bronchoconstriction in response to constrictor agonists [19,20]. In our model, poly(I:C) alone was able to elicit a small non-significant increase in AHR in unchallenged animals similar to that seen with influenza A infection [21]. Moreover, poly(I:C) was also able to enhance HDM-induced AHR. These data are consistent with recent data, using force oscillations, where poly(I:C) exacerbated OVA-induced AHR [22]. The up-regulation of IL-17A and IL-33 associated with our model could co-operate to exacerbate AHR by enhancing neutrophilic inflammation [23]. Alternatively, the observed up-regulation of IFN- γ has also been involved in the development and enhancement of AHR in asthma and asthma exacerbation [24], and may drive the relative corticosteroid insensitivity of AHR in our model [25].

Several studies have identified neutrophils as a key inflammatory cell in virus-induced asthma exacerbations [26,27]. Neutrophil elastase can stimulate mucus production, contributing to the pathogenesis of asthma exacerbations. Poly(I:C) in our model significantly increased BALF neutrophilia. Several cytokines that have been related with neutrophilic inflammation, such as IL-1 or IL-6 are exacerbated in our model and could therefore have a role in this neutrophilia. Interestingly, KC, the mouse equivalent of IL-8, does not seem to be involved in the exacerbation associated with our model unlike in other reported models of asthma exacerbation using poly(I:C), where this particular cytokine seemed to play a key role [28,29].

Although increases in BALF eosinophilia in asthma exacerbation models after poly(I:C) [30] or live virus challenge, particularly rhinovirus [31,32], have been reported, in our model eosinophilia remained unaltered. However, our data are consistent with data published by several other authors in exacerbation models after poly(I:C) challenge [28,29,33]. These discrepancies could be due to signalling differences between live virus and poly(I:C) [34], the inflammatory background of the model or the timing of the infection or poly(I:C) challenge.

On the other hand, poly(I:C) also caused a significant increase in the number of lymphocytes and, more remarkably, macrophages. This increase in lymphocytes has been reported previously in models of asthma exacerbation using both poly(I:C) or live virus [29–31]. Interestingly, neither poly(I:C) nor HDM alone increased macrophage numbers which would suggest a key role for macrophages in exacerbations. Increased macrophage numbers have been occasionally reported in models of asthma exacerbation [33,35,36]. It has been shown that after rhinovirus infection, alternatively activated (M2) airway macrophages can release type 2 cytokines, particularly IL-13, contributing to a Th2-exacerbated phenotype [37,38]. However, in our model, IL-13 is not increased and does not seem to play a role in the exacerbation. Interestingly, the same authors showed that, when using IL-4R (IL-4 receptor)-knockout mice, there was a polarization towards conventionally activated (M1) macrophages contributing to the potentiation of the Th1–Th17 profile and enhancing neutrophilia. Owing to the particular mixed phenotype of the FCA-HDM model, macrophages could be contributing to the exacerbation in a similar way.

Unlike most models of asthma which have a highly Th2-biased profile [16,17], our model shows a mixed Th1 (TNF- α , IFN- γ , IL-2), Th2 (IL-4, IL-5, IL-6) and Th17 (IL-17) cytokine profile. This particular profile, which reflects the neutrophilic severe asthma phenotype [3], is maintained after poly(I:C) and probably more accurately reflects what happens in human severe asthma. Moreover, inflammatory mediators, such as MCP-1, IFN- γ and RANTES, which are increased during asthma exacerbations, are also up-regulated in our model [39,40].

IL-33, which has been recently proposed as a key player in viral exacerbations [12], was also up-regulated in our model. Viral infection could amplify Th2 responses through the production of IL-33 by alveolar macrophages during asthma exacerbations [12] and anti-IL-33 suppresses the inflammatory response in different asthma models of asthma and asthma exacerbation [13,41,42]. IL-33 has been shown to promote neutrophilic inflammation,

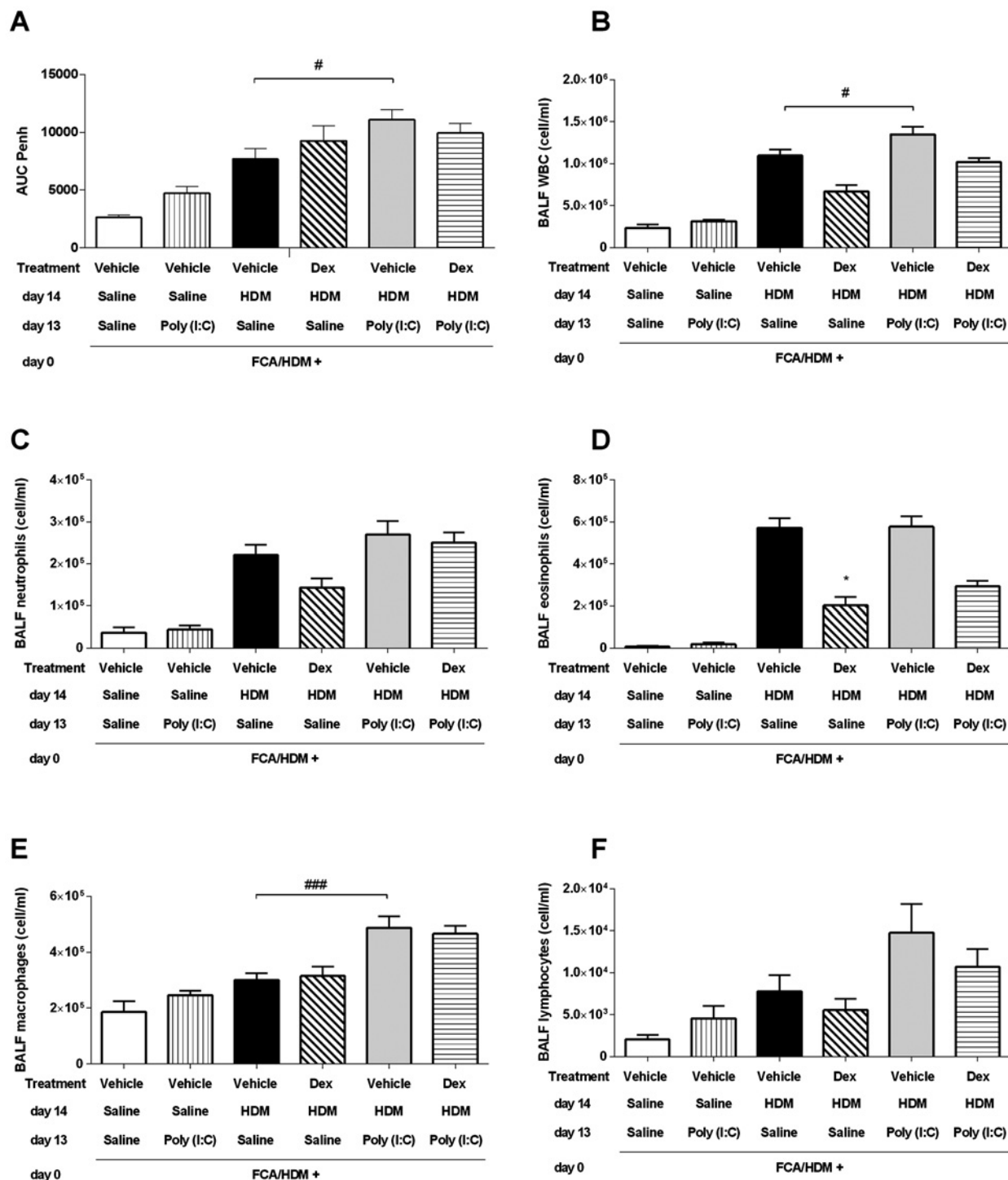


Figure 8 Effect of dexamethasone on poly(I:C) exacerbation of the FCA/HDM model (AHR and BALF total cells)

BALB/c mice were sensitized subcutaneously with HDM in FCA. At day 13 they received poly(I:C) or saline i.n. After 24 h, they received an intranasal challenge of HDM extract or saline. At 1 h before challenge, animals received a single dose of dexamethasone (1 mg/kg p.o.). AHR to MCh was measured at day 15 by whole-body plethysmography (A) and total inflammatory cells in the lavage fluid were evaluated 48 h after HDM/saline challenge (B). Effects on neutrophils (C), eosinophils (D), macrophages (E) and lymphocytes (F) are shown. A group of naïve animals was also included as a control for inflammatory infiltration (results not shown). Results ($n = 14-16$; naïve $n = 8$) are expressed as means $\pm$ S.E.M. and were analysed by a non-parametric Mann-Whitney test or Kruskal-Wallis test followed by a Dunn's post-hoc test. * $P < 0.05$ compared with the saline/HDM+vehicle group, # $P < 0.05$, ### $P < 0.001$ compared with the saline/HDM+vehicle group.

therefore it may be also contributing to the neutrophilia observed in our model [43].

In our model, dexamethasone failed to reduce AHR or inflammatory infiltration (neutrophils and macrophages) associated with the FCA–HDM model. This reduced steroid sensitivity of the AHR and inflammatory infiltration was maintained after exacerbation with poly(I:C) and, in the case of the neutrophilia, it seemed to be enhanced. IL-17, which is increased in our model and further augmented after exacerbation, has been involved in neutrophilic severe asthma [44]. In particular, the IL-23–Th17 axis has been linked with steroid-resistant airway inflammation and AHR [45], two important characteristics of this asthma phenotype [44]. It is therefore possible that a potentiation of the IL-23–Th17 pathway in our model after poly(I:C) challenge could be accountable for the increased steroid resistance of the exacerbated group.

In summary, we have described a mouse model that mimics some crucial aspects of severe neutrophilic asthma viral exacerbation. Samples from this model will be used in the U-BIOPRED effort to develop a ‘pawprint’ that, by comparing with the ‘handprint’ obtained in the clinical data, will help to advance our knowledge in severe asthma. The model displays a range of features that are only partially achieved by other existing models and is easily replicated without the need of special containment facilities. This model could be used to investigate new mechanisms of action underlying viral exacerbation in persistent asthma and for the assessment and evaluation of novel therapies for such conditions.

CLINICAL PERSPECTIVES

- Acute exacerbations are significant events in severe corticosteroid-refractory asthma and have enormous implications for patients, posing a major healthcare problem.
- We have established a translational mouse model that mimics some crucial aspects of severe neutrophilic asthma viral exacerbation, such as increased airway hyperresponsiveness, enhanced inflammation and elevated pro-inflammatory cytokines and chemokines associated with human asthma exacerbation.
- This model could be used to investigate new mechanisms of action underlying viral exacerbation in severe neutrophilic asthma and for the assessment and evaluation of novel therapies for such conditions. Samples/data from this model will be also used by the U-BIOPRED (Unbiased BIOMarkers in PREdiction of respiratory disease outcomes) consortium to compare with clinical samples/data with the hope of finding translational biomarkers of severe asthma that will help to advance our knowledge in the disease.

AUTHOR CONTRIBUTION

Jorge De Alba, Raquel Ota, Elena Calama and Montserrat Miralpeix conceived and designed the experiments. Raquel Ota and Elena Calama carried out the experiments/assays described. Jorge De Alba, Raquel Ota and Elena Calama carried out the interpretation

and analysis of the data. Neus Prats and Anna Domenech performed the histopathological analysis. Jorge De Alba wrote the main body of the paper with contributions from all other authors. Montserrat Miralpeix critically reviewed the paper before submission and provided substantial intellectual contribution. Neil Gozzard provided intellectual contribution and transfer of key knowledge from UCB.

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