LPS induces steroid-resistant exacerbations in a mouse model of allergic airway disease collectively through IL-13 and pulmonary macrophage activation

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Running Title: LPS-induced exacerbations require IL-13 and lung macrophages

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ABSTRACT

Background: Acute exacerbations of asthma represent a major burden of disease and are often caused by respiratory infections. Viral infections are recognised as significant triggers of exacerbations, however less is understood about the how microbial bioproducts such as the endotoxin (lipopolysaccharide (LPS)) trigger episodes. Indeed, increased levels of LPS have been linked to asthma onset, severity and steroid-resistance

Objective: The goal of this study was to identify mechanisms underlying bacterial-induced exacerbations by employing LPS as a surrogate for infection.

Methods: we developed a mouse model of LPS induced exacerbation on the background of pre-existing Type-2 allergic airways disease (AAD).

Results: LPS induced exacerbation was characterised by steroid resistant airways hyper-responsiveness (AHR) and an exaggerated inflammatory response distinguished by increased numbers of infiltrating neutrophils/macrophages and elevated production of lung inflammatory cytokines, including TNFα, IFNγ, IL-27 and MCP-1. Expression of the Type-2 associated inflammatory factors such as IL-5 and IL-13 were elevated in AAD but not altered by LPS exposure. Furthermore, AHR and airways inflammation were no longer suppressed by corticosteroid (dexamethasone) treatment after LPS exposure. Depletion of pulmonary macrophages by administration of 2-chloroadenosine into the lungs suppressed AHR and reduced IL-13, TNFα and IFNγ expression. Blocking IL-13 function, through either IL-13-deficiency or administration of specific blocking antibodies, also suppressed AHR and airway inflammation.

Conclusions & Clinical Relevance: We present evidence that IL-13 and innate immune pathways (in particular pulmonary macrophages) contribute to LPS-induced exacerbation of pre-existing AAD and provide insight into the complex molecular processes potentially underlying microbial-induced exacerbations.

INTRODUCTION

Asthma is a complex and heterogeneous chronic airway disease, which is characterised by airways inflammation, mucus hyperplasia and airway hyper-responsiveness (AHR)[1]. Acute asthma exacerbations are a significant clinical issue as they worsen disease symptoms, often fail to respond to corticosteroid treatment and contribute significantly to healthcare costs[2]. Respiratory infections are associated with the induction, progression and exacerbation of asthma and have been associated with up to 80% of acute asthma exacerbations in children and 50% in adults[3]. Bacterial infections including *Haemophilus influenzae*, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* are associated with exacerbations[4].

Further, exposure to the bacterial component lipopolysaccharide (LPS; endotoxin) has independently been linked to asthma onset, severity and steroid-resistance[5,6]. Microbiome studies have also highlighted potential pathogenic roles for microbiota and microbial products on disease pathogenesis, including asthma onset, severity and disease phenotype[7,8]. However, the mechanisms linking endotoxin exposure to asthma exacerbations remain poorly understood.

A number of lines of evidence link lung bacterial exposure with asthma outcomes. Individuals with asthma have altered bacterial profiles in their lungs as determined by 16S rRNA sequencing, compared to healthy controls[9]. Differences in bacterial colonisation of the lungs are associated with altered bronchial hyperreactivity[10] and clinical phenotypes of asthma[11]. Pathogenic bacterial infection is associated with more severe airway obstruction and neutrophil recruitment to the airways, which fails to respond to corticosteroid therapy[12]. A number of epidemiological studies have also linked high exposure levels to the bacterial cell-wall component LPS with severity of asthma[5,6]. Low-dose LPS inhalation in healthy controls can induce sputum neutrophilia[13], but limited data is available on the effect of LPS challenge in individuals with pre-existing asthma.

A number of mouse models have been developed to assess the effects of bacterial infection or LPS exposure on the development of allergic airways disease (AAD)[4]. AAD models of bacterial infection have highlighted a range of mechanisms contributing to pathogenesis, including induction of neutrophilic inflammation, potentiation of T_H2 responses and induction of T_H1 and/or T_H17 responses[14] in infected airways. Most studies assessing the pathogenic role of LPS have focussed on the effects of pre-exposure on subsequent allergen induced induction of AAD[15] or co-exposure during allergen sensitisation and/or aerochallenge[16-18]. LPS co-exposure has been demonstrated to modulate the local immune response, through activation of T_H1 and/or T_H2

responses and can induce steroid-resistant inflammation. However, there is only limited data on the effects of LPS exposure in the lung after allergen challenge and establishment of Type-2 AAD, which more appropriately models disease exacerbations in individuals with existing asthma.

Asthma exacerbations often occur on the background of an underlying Type-2 inflammatory response. In the current study, we have generated a model of LPS-induced exacerbation on the background of pre-existing AAD. This builds on our previous work demonstrating that prolonged AHR persists for at least 1 week after allergen challenge in models of ovalbumin (OVA) induced AAD[19]. Importantly, this prolonged AHR is associated with AHR originating specifically from the airways (like asthma) and activation of pulmonary macrophages[19], which we hypothesised may be modulated by allergen-associated LPS exposure. We now demonstrate that LPS administration to mice with pre-existing type-2 AAD led to an exacerbation characterised by increased AHR and dampened responsiveness to dexamethasone (DEX) treatment. Exacerbation was accompanied by significant infiltration of neutrophils (24hrs) and macrophages into the lung, increased inflammatory cytokine production and persistence of Type-2-inflammation (eosinophils, IL-5, IL-13 and eotaxin). Macrophage depletion or neutralisation of IL-13 (or genetic IL-13-deficiency) after allergen challenge suppressed AHR and the severity of LPS induced inflammatory response and background Type-2 inflammation. These investigations provide a model of bacterial-induced exacerbations of pre-existing Type-2 AAD and demonstrate that both activated pulmonary macrophages and IL-13 are required for LPS-induced exacerbation. Thus, Type-2 inflammatory factors and macrophages may cooperate to regulate endotoxin-induced exacerbations of asthma. Characterising the interplay between underlying chronic Type-2 responses in asthma and activation of innate immune responses by infection may improve our understanding of the mechanisms of exacerbations and identify new approaches to treatment.

METHODS

Mice

Adult male wild type mice (6-8 weeks old) and IL-13 gene-deficient mice (IL-13^{-/-} mice) on a BALB/c background were obtained from the University of Newcastle Animal Services Unit and experiments performed in the Hunter Medical Research Institute (HMRI) animal facility, under specific pathogen-free conditions. IL-13^{-/-} mice have been backcrossed onto a BALB/c background for greater than 10 generations. All protocols were approved by the local Animal Care and Ethics Committee (The University of Newcastle; Approval Number A-2013-317).

Experimental procedures

As previously described[20], mice were systemically sensitized by intra-peritoneal (i.p. day 0) injection of chicken egg ovalbumin (OVA, 50 μg; Sigma) or saline control Alhydrogel (1 mg; Sigma) in 200μl sterile saline. Mice were then aerosol-challenged with OVA (10 mg/ml in 0.9% saline) for 30 min/day on days 13-16. Where indicated, mice were instilled intratracheally (i.t.) with LPS (50 ng; Sigma-Aldrich) under Aflaxan anaesthetic (1:4; Jurox) in PBS, on days 19 and 21. Endpoints were assessed on day 24.

To assess responsiveness to corticosteroid treatment, mice were treated with DEX (1mg/kg i.p.; Sigma-Aldrich) on days 19 and 21. To deplete macrophages, mice were treated with 2-Chloroadenosine (2-CA, 50μl of 1mM, i.t.; Sigma-Aldrich) or PBS control on days 22 and 23, as previously described[19]. To neutralize IL-13, mice were injected with anti-IL-13 antibody (150μg, i.p, clone eBio1316H; eBioscience) or isotype control (rat IgG1$_K$, clone eBRG1, eBioscience) on days 19 and 21.

Lung function

Airway resistance (Raw) in response to methacholine (MCh, Sigma-Aldrich) was measured using a Flexivent apparatus (FX1 system; Scireq)[20]. Briefly, mice were anesthetized by injection of xylazine (2 mg/ml, i.p.; Troy Laboratories), ketamine (40 mg/ml; Parnell) and PBS (5:4:1). A cannula was inserted into the trachea and mice were ventilated with a tidal volume of 8 ml/kg at a frequency of 450 breaths/minute. Mice were challenged with saline aerosol followed by increasing concentrations of Methacholine (0.1, 0.3, 1, 3, 10 and 30 mg/ml). Measurements were excluded if the coefficient of determination was lower than 95%. Airway resistance was recorded and presented as percentage increase over baseline.

Bronchoalveolar lavage fluid (BALF) differential cell counts

BALF was collected immediately following lung function measurements, as previously described[21]. The left lobe of the lung was tied off and the right lung was flushed twice with 700µl Hanks Buffered Saline Solution (HBSS; Invitrogen). BALF samples were centrifuged to pellet cells and supernatant stored at -80˚C for protein analysis. Red blood cells were lysed in hypotonic red cell lysis buffer and remaining cells cytospun onto glass slides. Differential leukocyte counts were determined based on morphological criteria by light microscopy (×100) on May-Grunwald and Giemsa-stained slides, counting of 300 cells/slide/sample.

Macrophage isolation and treatment

Mouse pulmonary macrophages, which include alveolar macrophages, interstitial macrophages and monocytes, were isolated from mouse lungs as previously described[22]. Briefly, lung tissues were minced and forced through a 70µm cell strainer. Macrophages were purified by density gradient centrifugation (Histopaque-1083; Sigma-Aldrich) and seeded onto tissue culture-treated 6 well plates at 37ºC at a concentration of 1×10^6 cells/ml in DMEM containing 20% fetal calf serum (FCS). After 3 h, >95% of adherent cells were macrophages. Where indicated, isolated primary macrophages were collected into Trizol and stored at -80⁰C for RNA analysis.

In specified experiments, purified macrophages were isolated from naïve mice and cultured *in vitro*. Isolated macrophages were cultured in fresh complete Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% FCS, 2 mM L-glutamine, 0.1 mM sodium pyruvate, 20 mM HEPES and 2-mercaptoethanol (β-ME). Where indicated, macrophages were incubated with recombinant murine IL-13 (100 ng/ml; Lonza) for 16 hours, then exposed to LPS (100 ng/ml; Sigma) and incubated for a further 12 hours. Cells were collected into Trizol and stored at -80⁰C for RNA analysis and supernatant were collected and stored at -80⁰C for protein analysis.

RNA isolation, reverse-transcription (RT)-PCR and quantitative PCR (qPCR) assessment

As previously described[23], lung tissue was immediately placed in RNAlater (Ambion, Austin, TX) and stored at -80˚C for further analysis. RNA was isolated using Trizol reagent, following manufacturer instructions (Invitrogen Life Technologies), followed by phenol-chloroform separation and isopropanol precipitation. RNA samples were quantified on a NanoDrop 1000 spectrophotometer (ND-1000; Thermo Scientific). cDNA was synthesised using random hexamer primers and MMLV reverse transcriptase (Invitrogen) on a T100 thermal cycler (Bio-Rad). Relative qRT-PCR quantification was performed on a Viia7 real-time PCR machine (Life Technologies), using SYBR reagents with following primer sequences: 5'-AGGCCAGACTTTGTTGGATTTGAA-3' (forward) and 5'-CAACTTGCGC

TCATCTTAGGCTTT-3' (reverse) for HPRT; 5'-CTGTTGCTGCTACCCTTGCTT-3' (forward) and 5'-CACTCCTGGCAATCGAGATTC-3' (reverse) for IL-27 (p28); 5'-GTCTA CTGAACTTCGGGGTGATCG-3' (forward) and 5'-AGCCTTGTCCCTTGAAGAGAACC-3' (reverse) for TNFα; 5'-TCTTGAAAGACAATCAGGCCATCA-3' (forward) and 5'-GAAT CAGCAGCGACTCCTTTTCC-3' (reverse) for IFNγ; 5'-AGAGCCAGACGGGAGGAAG-3' (forward) and 5'-CCAGCCTACTCATTGGGATC-3' (reverse) for MCP-1; 5'-AGCTGAGC AACATCACACAAGACC-3' (forward) and 5'-TGGGCTACTTCGATTTTGGTATCG-3' (reverse) for IL-13; 5'-CGGCCGGAGAAAGTTGGTCCC-3' (forward) and 5'-GCACACCCGCCTGGTATGTCC-3' (reverse) for Muc5ac. Thermal cycling conditions consisted of an initial denaturing step (95°C: 3 min) followed by 40 cycles of denaturing (95°C, 5 s) and annealing (60°C, 30 s). All mRNA levels were normalized to hypoxanthine-guanine phosphoribosyl transferase (HPRT).

Flow cytometry analysis

Single-cell suspensions from lungs were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) followed by Brefeldin A (5 μg/ml) for a total of 5 hours of incubation. Cells were then washed in PBS and resuspended in ZombieYellow Fixable Viability Kit (BioLegend) for exclusion of dead cells. Fc receptor block (CD16/32) was added followed by anti-mouse CD3, CD4, CD45, CD90.2, ICOS and ST2 antibodies (BD Pharmingen, BioLegend) and washed with FACS buffer (PBS + 1% FCS + 2 mM EDTA) twice. Cells were then incubated on ice in 4% paraformaldehyde, permeabilized using BD Perm/Wash buffer (BD Pharmingen) and stained for intracellular marker (anti - IL-13). Analysis was performed using FACS Fortessa (BD Pharmingen). IL-13-producing ILC2 cells are defined as $CD45^+Lineage^-ICOS^+CD90.2^+ST2^+IL-13^+$.

ELISA

Lung tissue was disrupted in radioimmunoprecipitation assay buffer (RIPA; Sigma-Aldrich) with protease/phosphatase inhibitor cocktail (Cell Signaling Technology), on a Tissuelyser LT tissue disruptor (Qiagen) at 50 Hz for 5 minutes and stored at -80°C. Levels of TNFα, IFNγ, IL-5, IL-13, IL-27 (p28), eotaxin-1 and MCP-1 were assessed using ELISA kits (DuoSet, R&D Systems) according to manufacturer's instructions. Total protein concentrations were determined using a BCA Protein Assay kit (Thermo Scientific). Individual cytokine levels were normalised to total protein.

Lung histology

Lung sections presenting central and peripheral airways were fixed in 10% formalin buffer for 24h, then transferred to 70% ethanol. The fixed tissue was embedded in paraffin and cut into 8μm sections. After heat-affixing the section to the glass slide, the slides were immersed in xylene to remove the paraffin from the section and stained with AB-PAS (Alcian Blue/Periodic Acid-Schiff) for recognition of mucus secreting cells (MSC) or Carbol's Chromotrope-hematoxylin to detect eosinophils. Peribronchial eosinophil numbers were determined by counting the number of eosinophils surrounding the airways in 20 high power fields (HPF×1000) using a green lens filter (Olympus, Japan). To count mucus-secreting cells, we identified intact airways and quantified cells at 100x magnification under oil immersion. A gradicule was used along the edge of the airway. Pink-staining MSCs were counted in 10 gradicule areas and data were then averaged to provide results in cells/100 μm.

Statistical analysis

Statistical analysis was performed using Prism version 6.0 (GraphPad Software, USA). Two-way ANOVA was used to identify differences between two or more experimental groups, and Student's unpaired t-tests was used where comparison were made between two treatment groups. All results are presented as means ± SEM and *P* values < 0.05 were considered statistically significant.

RESULTS

LPS administration exacerbates AHR, increases lung macrophage numbers and attenuates steroid-responsiveness.

To establish a mouse model of LPS-induced exacerbation of AHR and airway inflammation, BALB/c WT mice were sensitized with OVA (or PBS control) in Alum and exposed to aerosolised OVA to induce AAD. Three days after the final OVA aerosol challenge, some groups were treated with LPS (OVA/LPS) or LPS + DEX (OVA/LPS/DEX) on days 19 and 21 and lung function and inflammatory responses were assessed on day 24 (Fig. 1).

OVA-sensitized/challenged (OVA) mice exhibited significantly increased AHR in response to increasing doses of methacholine, compared with control PBS-sensitized/OVA-challenged (PBS) mice (Fig. 1A), as previously described[19]. This response is reduced in comparison to day 17, where allergic airway inflammation (type 2 cytokine levels, eosinophil and Th2 cell numbers) is most prominent after allergen challenge (data not shown). LPS administration exacerbated AHR in OVA-treated mice (OVA/LPS), as compared to that in OVA-treated mice (OVA) (Fig. 1A). We previously demonstrated that DEX treatment of OVA-treated mice in this model effectively suppressed AAD and decreased AHR to baseline (PBS) levels on day 17 and at this time point[20]. Conversely, following LPS administration, DEX treatment (OVA/LPS/DEX) failed to suppress AHR (Fig. 1A). These findings indicate that LPS exposure exacerbates AHR in mice with pre-existing Type-2 AAD, which is resistant to systemic corticosteroid treatment.

Differential BALF immune cell numbers were quantified to characterize the extent and type of inflammatory infiltration (Fig. 1B). OVA-treated mice had increased neutrophil, macrophage, eosinophil and lymphocyte numbers in BALF, compared to the PBS control group (Fig. 1B). LPS exposure induced a transient increase in neutrophils at day 22 (data not shown) and increased macrophage numbers at day 24 (Fig. 1B), compared to OVA-treated mice. Eosinophil numbers were decreased following LPS exposure (Fig. 1B). We previously demonstrated that DEX treatment of OVA-treated mice decreased total inflammatory cell numbers in BALF, by reducing eosinophil and lymphocyte numbers at the same timepoint[20]. DEX treatment following LPS exposure further reduced eosinophil numbers but had no effect on macrophage numbers (Fig. 1B), which remained elevated compared to OVA-treated mice. These findings indicate that LPS exacerbation of pre-existing AAD is accompanied by a corticosteroid-resistant increase in macrophage numbers in BALF.

LPS administration to OVA-treated mice induces steroid-resistant inflammatory cytokine production but has no effect on T_H2-associated cytokines.

We next assessed the effect of LPS exposure of OVA-treated mice on cytokine production within the lung. As previously described[24], OVA-treated mice had increased protein levels of IL-5, IL-13, eotaxin-1, IL-27(p28), IFNγ and MCP-1 in the lung (Fig. 1D). DEX treatment significantly decreased IL-5 and IL-13 production in OVA-treated mice. Following LPS administration, levels of IL-5 and IL-13 remained elevated, but were not changed compared with the OVA group (Fig. 1D). Of note, increased levels of IL-13 were not altered by DEX treatment in OVA/LPS/DEX-treated mice. The same was observed for muc5ac expression in lung (Fig. 1C), in the OVA/LPS/DEX treated mice.

We also quantified the production of inflammatory cytokines, which have previously been implicated in asthma exacerbations. TNFα levels were unchanged in OVA-treated mice, compared to PBS controls (Fig. 1D). We previously demonstrated that DEX treatment has no effect on the levels of eotaxin, IL-27p28, TNFα and IFNγ in OVA-treated mice[20-25]. Eotaxin-1, IL-27 (p28), TNFα, IFNγ and MCP-1 protein levels were all increased in OVA/LPS-treated mice compared to those in OVA-treated mice (Fig. 1D). DEX treatment had no effect on the protein levels of IL-27 (p28), TNFα or IFNγ induced by LPS administration (Fig. 1D). Thus, LPS exposure increased the production of multiple inflammatory cytokines, without altering levels of IL-5 and IL-13, and this increase in cytokine (IL-27 (p28), TNFα and IFNγ) production was steroid-resistant.

Pulmonary macrophages from OVA/LPS-treated mice upregulate expression of inflammatory cytokines.

Based on the proposed role of pulmonary macrophages in steroid-resistant asthma exacerbations[26] and the increase in BALF macrophage numbers following LPS exposure, we also assessed expression of innate cytokines elevated in the lung of purified primary lung macrophages isolated at day 24 (as it is not experimentally practical to measure protein levels in these cells). TNFα expression was only significantly increased in macrophages isolated from OVA-treated mice (Fig. 2). By contrast, in macrophages isolated from OVA/LPS-treated mice, the levels of IL-27 (p28), TNFα, IFNγ and MCP-1 expression were all increased (Fig. 2), compared to the OVA-treated group. DEX treatment partially reduced expression of IL-27 (p28), TNFα and MCP-1 and completely abolished expression of IFNγ, compared with macrophages from OVA/LPS-treated mice (Fig. 2). These data provide evidence that pulmonary macrophages upregulate expression of

inflammatory cytokines following OVA/LPS stimulation and expression is resistant to steroid treatment.

Macrophage depletion suppresses AHR and reduces pro-inflammatory cytokine levels during LPS-induced exacerbation of AAD.

To determine whether macrophages have a functional role in disease exacerbation in our model, we depleted macrophages using the established method of 2-CA administration [a purine analogue that specifically depletes pulmonary macrophages[19,27]]. 2-CA administration reduced OVA/LPS-induced AHR, compared with vehicle-treated controls, to the same level observed in OVA-treated mice (Fig. 3A). 2-CA administration decreased macrophage and lymphocyte numbers in the OVA/LPS group (Fig. 3B). BALF neutrophil and eosinophil numbers were not affected by 2-CA administration (Fig. 3B). 2-CA administration decreased the expression of muc5ac in OVA and OVA/LPS treated mice (Fig. 3C). Furthermore, 2-CA significantly decreased the levels of IL-13, IL-27 (p28), TNFα and IFNγ in OVA/LPS group, compared to those in OVA treated group, as assessed by ELISA (Fig. 3D). In contrast, 2-CA administration had no effect on IL-5, eotaxin-1 or MCP-1 levels (Fig. 3D). Together, these findings indicate that macrophages are required for LPS-induced exacerbation of AHR and that macrophage ablation results in a reduction in the levels of a subset of cytokines (IL-13, IL-27 (p28), TNFα and IFNγ) in the lung.

IL-13 blockade suppresses AHR, inflammatory cell recruitment and pro-inflammatory cytokine levels during LPS-induced exacerbation.

IL-13 plays a key role in contributing to induction of AHR and goblet cell hyperplasia[28]. While IL-13 expression was not increased following LPS exposure, we did note that IL-13 levels were significantly elevated in all groups (as compared to that in PBS group) and that steroid treatments did not suppress IL-13 levels after LPS exposure (by contrast to OVA treated group) (Fig. 1). We next assessed the potential role of IL-13 crosstalk with LPS activation of the innate macrophage driven immune response by employing both IL-13^{-/-} mice and treatment with neutralising anti-IL-13 antibodies just prior to induction of the exacerbation by LPS.

Induction of AHR was suppressed in IL-13^{-/-} mice, compared to that in WT controls, following either OVA sensitisation/challenge alone or subsequent LPS exposure (Fig. 4A). In differential counts, all inflammatory cell types were reduced in IL-13^{-/-} mice following OVA- and OVA/LPS- treatments, compared to the respective WT responses to these treatments (Fig. 4B). The expression of muc5ac was not altered in OVA or OVA/LPS groups in IL-13^{-/-} mice when compared to PBS treated controls (Fig. 4C). Protein levels of IL-13 were not detected after OVA

or OVA/LPS exposure in IL-13$^{-/-}$ mice (Fig. 4D). Further, eotaxin-1, IL-27 (p28), IFNγ and MCP-1 levels were lower in IL-13$^{-/-}$ mice following OVA/LPS treatment, compared to the respective WT responses to these treatments (Fig. 4D). IL-5 and TNFα levels were not increased in IL-13$^{-/-}$ mice (Fig. 4D). These findings provide evidence that IL-13 production is required for OVA and subsequently LPS-induced exacerbation of AHR, and severity of inflammation and cytokine production. However, these experiments do not differentiate between the role of IL-13 during the initial induction of AAD by allergen exposure and the LPS-induced exacerbation phase.

To assess the functional role of IL-13 during LPS induced exacerbation, we administered neutralising antibody against IL-13 after AAD was established (Fig. 5). IL-13 neutralization abolished OVA/LPS-induced AHR, compared with isotype control (Fig. 5A). Further, anti-IL-13 treatment reduced neutrophil and eosinophil numbers and to a lesser extent macrophage and lymphocyte numbers (Fig. 5B). The expression of muc5ac was significantly reduced in the OVA/LPS group that was treated with anti-IL-13 when compared to OVA/LPS group (Fig.5C). Blocking IL-13 decreased IL-5, IL-13 and MCP-1 production in lung tissue (Fig. 5D). In contrast, TNFα, IL-27 (p28) and eotaxin-1 levels were unaffected by anti-IL-13 administration (Fig. 5D). These results indicate that IL-13 is required during the LPS induced exacerbation phase, to maintain the Type-2 response and that this cytokine plays a cooperative role with macrophages (Fig. 3) in the induction of AHR and airway inflammation.

IL-13 and LPS stimulation of isolated naive alveolar macrophages modulates inflammatory cytokine production.

Our intervention studies indicate roles for both pulmonary macrophages and IL-13 in LPS-induced exacerbations. Next, we assessed the direct response of naïve pulmonary macrophages to LPS and/or IL-13 stimulation *in vitro*. LPS stimulation alone induced marked expression of IL-27 (p28), TNFα, IFNγ and MCP-1 in primary macrophages (Fig. 6). IL-13 stimulation alone had limited effect on cytokine expression when compared to PBS. Interestingly, IL-13/LPS co-stimulation further increased MCP-1 expression, compared to LPS or IL-13 stimulation alone (Fig. 6), but had no further effect on IL-27, TNFα or IFNγ expression. These findings provide evidence that IL-13 and LPS modulate inflammatory cytokine expression by pulmonary macrophages and indicate additive effects of IL-13/LPS co-stimulation on chemokine expression.

Steroid insensitivity in Group 2 ILC cells in the lungs following LPS exposure.

To further understand the pathways involved in DEX resistance to LPS induced exacerbation (OVA/LPS group) we stain lung cells with antibody markers for ILC2s and enumerated their

numbers by flow cytometry (Fig. 7A). We observed a significantly increased amount of ILC2s in the OVA treated group but numbers were not increased by LPS exposure (OVA/LPS group), and numbers were not suppressed by DEX treatment (Fig. 7A). The protein levels of IL-25, IL-33 and TSLP were also increased by OVA treatment group by not further by LPS exposure and levels were not reduced by DEX treatment (Fig. 7B).

Effect of DEX, 2-CA and anti-IL-13 treatments on histopathological changes in lung tissues during OVA/LPS-induced allergic asthma.

To characterize the changes in lung histopathology caused by OVA and OVA/LPS exposure, and the effects of different treatments (DEX, 2-CA and anti-13), we stained the lung samples with AB-PAS and CR (Fig 8A). Mucus producing cells and eosinophils numbers were then quantified (Fig. 8B). Goblet cell hyperplasia, mucus hypersecretion and increased numbers of eosinophils were observed after OVA treatment but not further increased by LPS exposure (OVA/LPS). DEX treatment of the OVA/LPS group significantly reduced eosinophil numbers but not the levels of PAS positive cells. By contrast the OVA/LPS group treated with 2-CA or anti-IL-13 had diminished levels of PAS-positive cells. Evidence of inflammatory cell infiltration was observed in CR stained samples. OVA, OVA/LPS and DEX treated groups showed an extensive cell infiltration around the airways and blood vessels when compared to control group. Mice treated with 2-CA and anti-IL-13 after OVA or OVA/LPS exposure had significantly reduced inflammatory infiltrates.

DISCUSSION

Respiratory infections are clinically associated with asthma exacerbations, which negatively contribute to disease burden, healthcare utilisation and associated healthcare costs for asthma[3]. Further, epidemiological studies have linked endotoxin (LPS) exposure to worsened disease outcomes and exacerbations[5,6]. There is also growing interest in the effects of the microbiota and microbial products on disease pathogenesis in asthma[7,8]. We provide evidence that LPS exposure exacerbates AHR in the context of pre-established AAD, which models the effect of microbial exposure in patients with pre-existing allergic asthma. LPS-induced exacerbations were steroid-resistant, which is an important clinical feature of severe asthma and disease exacerbations[29]. In functional studies, we provide evidence that pulmonary macrophages and IL-13 are required for disease exacerbation. These data highlight that integration of IL-13 and innate immune responses predispose to steroid-resistant inflammatory pathways and AHR associated with LPS induced exacerbation.

The effects of LPS or bacterial exposure on AAD have previously been assessed in a range of protocols[4, 30, 31]. Repeated administration of high-dose LPS alone (4 consecutive days; 100 μg) in the absence of pre-existing AAD induced AHR[32]. This may be linked to neutrophil recruitment and/or macrophage activation. However, at the dose used in the current study (0.05 μg per exposure to naïve WT mice), LPS alone transiently increases neutrophil numbers (not shown) but does not induce AHR[21]. Early-life LPS exposure has been shown to protect from development of AAD, by dampening T_H2 responses[15]. However, LPS exposure during allergen sensitisation or challenge was shown to exacerbate disease, through increased T_H1 activation and/or increased mast cell activation and T_H2 immune responses[16,17]. Similarly, *M. pneumoniae* bacterial lung infection prior to allergen sensitisation protected from AAD induction, while infection after sensitisation promoted Type-2 inflammation and severity of inflammation[33]. In contrast, *Chlamydia muridarum* lung infection before induction of AAD resulted in an allergen driven mixed T_H1/T_H2 response and increased IL-13 production[34] or increased neutrophil accumulation and T_H1/T_H17-associated gene expression[35]. *H. influenzae* infection prior to allergen sensitisation led to chronic bacterial infection and persistent steroid-resistant, neutrophilic airways disease[14]. Furthermore, *H. influenzae* infection worsened disease through increased T_H17 responses in an exacerbation model[36]. LPS co-exposure during allergen challenge has also been shown to dampen steroid-responsiveness[18]. We now provide evidence that LPS exposure after establishment of AAD

activates innate proinflammatory pathways and activates macrophage to induce AHR in concert with IL-13 regulated Type-2 responses.

In our model, LPS-induced exacerbation was associated with increased expression of a number of inflammatory cytokines that have been implicated in asthma severity and/or exacerbations. Notably, IL-27, TNFα, IFNγ and IL-13 were increased in lung tissue, and expression persisted despite DEX treatment (Fig. 1C). IL-27 is a heterodimeric cytokine consisting of two subunits (EBI3 and p28), which promotes T_H1 polarization and IFNγ production[37]. IFNγ is associated with Type-1 anti-viral responses and bacterial infections, and has been suggested as a potential target in chronic asthma[38]. IFNγ expression is increased in individuals with severe asthma, compared to moderate disease[39]. Increased IL-27 and IFNγ production may also reflect a shift towards T_H1 polarisation, from a T_H2 allergic background. We previously demonstrated that administration of LPS and recombinant IFNγ into the lungs is sufficient to induce steroid-resistant AHR in a short-term model[21]. Further, administration of recombinant IL-27 and IFNγ to mice also induced steroid-resistant AHR, through activation of pulmonary macrophages[40]. IFNγ and IL-27 expression was also found to be increased in sputum samples from patients with neutrophilic asthma, a patient subset that are typically less responsive to corticosteroid treatment[40]. TNFα promotes AHR induction and neutrophil recruitment (as shown in animal and clinical studies) in mouse models and control human subjects[41-43]. TNFα is significantly increased in samples from individuals with severe asthma[44,45], individuals with neutrophilic asthma[20] and following exacerbation, where levels were associated with exacerbation severity[46]. Clinical trials targeting TNFα in asthma have yielded some positive results, with reduced exacerbation rates[47,48], improved quality of life, lung function[44] and asthma control[49]. However, trial outcomes have been variable and there are concerns about increased risk of infections following treatment. MCP-1 is produced by activated macrophages and promotes macrophage migration[50]. We have detected heightened expression of MCP-1 in purified pulmonary macrophages from OVA/LPS treated mice and LPS/IL-13 stimulated naive macrophages (Fig 2 & 6). MCP-1 expression is increased by virus infection in individuals with asthma[51] and in sputum from individuals with neutrophilic asthma[20]. Interestingly, MCP-1 may not only regulate macrophage activation but also attract either Th2 cells or ILC2s into inflamed tissues, as these cells express receptor specific to this chemokine[52]. Of note, the latter two cells are the predominant cellular source of IL-13[53]. We identified increased expression of each of these molecules in our LPS-induced exacerbation model. However, the specific role each of these cytokines play in asthma exacerbation remains unclear.

A growing body of literature has highlighted functional roles for macrophages in steroid-resistant asthma[16,54]. In line with a role for pulmonary macrophages in our model, we observed increased production of IL-27, IFNγ, TNFα and MCP-1 in primary pulmonary macrophages isolated from OVA/LPS-exposed mice (Fig. 2). By contrast to the Type 2 factors (IL-5, eosinophil and eotaxin, Fig. 1), DEX treatment did not affect the levels of IL-13, IL-27, IFNγ or TNFα production in mice treated with LPS (LPS/DEX group, Fig. 1). In intervention studies, macrophage depletion markedly reduced LPS induced AHR, which was associated with reduced IL-13, TNFα and IFNγ production in lung tissue. We have previously demonstrated that macrophage depletion also reduces AHR induced by OVA treatment alone at the examined time point, with no effect on the numbers of eosinophils or mucus-secreting cells[19]. Further, we have observed similar role for macrophages in regulating AHR induced by exposure of the lung to both IFNγ/LPS- or RSV- induced exacerbation of pre-existing AAD[19,20]. In the current study we demonstrate that macrophages play a critical role in LPS-induced exacerbations of AHR and inflammation in concert with IL-13 and possibly downstream of these factors through processes involving TNFα and/or IFNγ.

Key roles for Type-2 inflammation (including eosinophils, IL-5, IL-13 and eotaxin) are well-recognised in the pathogenesis of AAD[55]. However, the role of Type-2 inflammation in exacerbations is less clear. LPS co-exposure during allergen challenge has been shown to increase[17] or decrease[18] Type-2 cytokine levels. IL-13 in particular plays a key role in allergic asthma pathogenesis by inducing AHR and promotes eosinophil recruitment and goblet cell hyperplasia[28]. IL-13 administration to the lung alone is sufficient to induce AHR[56] and viral delivery of IL-13 to the lung induces mucus hypersecretion, neutrophilia and AHR, which is resistant to DEX treatment[57]. Further, IL-13 over-expressing transgenic mice develop spontaneous AHR and mixed granulocytic lung inflammation[58]. In our model, LPS exposure did not alter levels of IL-5 or IL-13. However, treatment with DEX (LPS/DEX group) reduced IL-5 (and eotaxin) but not IL-13 levels and the reduction in IL-5 and eotaxin correlated with decreased numbers of eosinophils in BALF. Notably, IL-13 levels remain elevated after corticosteroid treatment in individuals with steroid-insensitive asthma[59]. By contrast, DEX treatment reduced Type-2 cytokine expression, including IL-13 production, in the absence of LPS exposure[20], and as demonstrated in our Fig. 1. Thus, persistent and steroid-resistant IL-13 expression following LPS exposure may contribute to the pathogenesis. It should be noted that steroid resistance in asthma is heterogenous[60]. For instance, the expression of defective glucocorticoid receptors in non-

hematopoietic lung cells is closely associated with steroid resistance in a mouse model of allergic asthma[61]. Persistent eosinophilic inflammation is also considered as one hallmark of a subgroup of severe asthma[62].

IL-13 production was required for LPS-induced exacerbations in our model, as IL-13^{-/-} mice failed to develop AHR and airway inflammation was significantly attenuated. These findings were consistent with previous observations that IL-13-deficient mice fail to develop AAD, including in the context of *Chlamydia*-induced persistent AHR[63]. To confirm a role for IL-13 in the exacerbation phase, we administered IL-13 neutralising antibodies after the establishment of AAD (on days 19 and 21). Neutralization of IL-13 inhibited AHR, reduced infiltrating inflammatory cell numbers (neutrophils, eosinophils and lymphocytes) and decreased IL-5, IFNγ, MCP-1 and IL-13 protein levels. Numerous studies have demonstrated that genetic or pharmacologic blockade of IL-13 protects from induction of characteristic features of AAD, including AHR, inflammation and airway remodelling[28]. In clinical trials, treatment with lebrikizumab (anti-IL-13) significantly reduced asthma exacerbations[64]. However, targeting IL-13 has also had only limited effects on exacerbations[65]. Further stratification of patients and identification of the exacerbation trigger (e.g. viral and/or bacterial) may demonstrate greater therapeutic benefit. Our findings provide evidence that blocking IL-13 and/or macrophage function can also inhibit LPS-induced steroid-resistant exacerbations of AAD, suggesting a potential role for this cytokine in bacterial regulated components of exacerbations.

Understanding how microbial bioproducts such as LPS/endotoxin contribute to the progression and exacerbations of asthma will provide insights in new approaches to treat disease. Here we demonstrate that LPS induces an exacerbation of AAD characterised by steroid resistant AHR and increased numbers of macrophages in the lung activation of innate inflammatory pathways and elevated levels of IL-13. Depletion of macrophages suppressed AHR and reduced IL-13, IL-27, TNFα and IFNγ levels in the lung. Further, IL-13 was required for LPS-induced exacerbations and blocking IL-13 function reduced IL-5, eotaxin, MCP-1 and IFNγ production. Our findings provide support for a mechanism whereby LPS exposure in the context of Type-2 AAD exacerbates disease through parallel activation of innate inflammatory pathways in macrophages and the induction of a steroid-resistant IL-13 pathway. These findings provide further insight into the mechanisms underlying LPS and bacterial-induced exacerbations, which are relevant to existing therapies (e.g. anti-IL-13) and identify further potential targets for future intervention.

FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. LPS administration induces clinical features of asthma exacerbation characterised by steroid-resistant AHR and an exaggerated inflammation response in mice with pre-existing AAD. BALB/c mice were sensitized with OVA or PBS plus alum on day 0 and exposed to aerosol OVA on days 13-16. LPS or PBS was administrated i.t. on days 19 and 21 and endpoint assessed on day 24. DEX or PBS was administered i.p., three hours before LPS administration. (A) Airway resistance to increased doses of methacholine (mg/ml), presented as percentage change over baseline (saline). (B) Differential BALF cell counts of neutrophils, macrophages, eosinophils and lymphocytes presented as 1×10^4/ml of BALF. (C) Mucus levels were assessed by qPCR, normalised to HPRT. (D) Cytokine levels in lung tissue were assessed by ELISA, normalised to total lung protein (pg/ml). Presented as mean±SEM (n=8-10 mice per group); *p<0.05, **p<0.01, ***p<0.001, #p<0.0001. # symbol on top of the bar column shows significant difference (p<0.0001) compared to all groups.

FIGURE 2. LPS administration to OVA-sensitised and challenged mice increased innate inflammatory cytokine expression in primary lung macrophages. Primary pulmonary macrophages were isolated from treated mice on day 24. Cytokine levels were assessed by qPCR, normalised to HPRT. Presented as mean±SEM (n=8-10 mice per group); *p<0.05, **p<0.01, ***p<0.001, #p<0.0001.

FIGURE 3. 2-CA–mediated macrophage depletion suppresses AHR and inflammatory cytokine levels in OVA/LPS-treated mice. Mice were treated i.t. with 2-CA or PBS (vehicle control) after LPS challenge in OVA-treated mice on days 22/23 and endpoints assessed on day 24. (A) Airway resistance to increased doses of methacholine (mg/ml), presented as percentage change over baseline (saline). (B) Differential BALF cell counts of neutrophils, macrophages, eosinophils and lymphocytes presented as 1×10^4/ml of BALF. (C) Mucus levels were assessed by qPCR, normalised to HPRT. (D) Cytokine levels in lung tissue assessed by ELISA, normalised to total lung protein, showed as pg/ml. Presented as mean±SEM (n=8-10 mice per group); *p<0.05, **p<0.01, ***p<0.001, # p<0.0001. # symbol on top of the bar column shows significant difference (p<0.0001) compared to all groups.

FIGURE 4. IL-13-deficiency suppresses AHR, inflammatory cell infiltrates and cytokine levels in OVA/LPS-treated mice. (A) Airway resistance presented as percentage change over baseline (saline) on day 24 in wild-type (WT) and IL-13$^{-/-}$ mice. (B) Differential BALF cell counts of neutrophils, macrophages, eosinophils and lymphocytes presented as 1×10^4/ml of BALF. (C)

Mucus levels were assessed by qPCR, normalised to HPRT. (D) Cytokine levels in lung tissue assessed by ELISA, normalised to total lung protein (pg/ml). Presented as mean±SEM (n=5-6 mice per group); *p<0.05, **p<0.01, ***p<0.001, # p<0.0001. # symbol on top of the bar column shows significant difference (p<0.0001) compared to all groups.

FIGURE 5. IL-13 neutralization suppresses AHR and inflammatory cell numbers in OVA/LPS-treated mice. Anti-IL-13 antibody or isotype control was administered i.p. to OVA/LPS-treated mice, 4 h before LPS administration on day 19 and 21 and endpoints assessed on day 24. (A) Airway resistance to increased doses of methacholine (mg/ml), presented as percentage change over baseline (saline). (B) Differential BALF cell counts of neutrophils, macrophages, eosinophils and lymphocytes presented as $1x10^4$/ml of BALF. (C) Mucus levels were assessed by qPCR, normalised to HPRT. (D) Cytokine levels in lung tissue assessed by ELISA, normalised to total lung protein (pg/ml). Presented as mean±SEM (n=8-10 mice per group); *p<0.05, **p<0.01, # p<0.0001. # symbol on top of the bar column shows significant difference (p<0.0001) compared to all groups.

FIGURE 6. Cytokine expression is induced in naïve pulmonary macrophages following LPS and/or IL-13 exposure *in vitro*. Primary lung macrophages were isolated from naïve mice and stimulated with IL-13 (100 ng/ml) for 16 hours, before addition of LPS (100 ng/ml) for 12 hours. Cytokine or chemokine levels (as indicated) were assessed by qPCR, normalised to HPRT. Presented as mean±SEM (n=6 samples per group); ***p<0.001, # p<0.0001.

FIGURE 7. Steroid insensitivity in Group 2 ILC cells in the lungs following LPS exposure. Mice were treated i.p. with DEX after LPS challenge in OVA-treated mice on days 22/23 and endpoints assessed on day 24. (A) Levels of IL-13-producing ILC2 cells (CD45^{+}Lineage^{-} ICOS^{+}CD90.2^{+}ST2^{+}IL-13^{+}) in lung tissue were assessed by flow cytometry analysis. (B) Type 2 cytokine levels in lung tissue were assessed by ELISA, normalised to total lung protein. Presented as mean±SEM (n=6 mice per group); *p<0.05, **p<0.01, ***p<0.001, # p<0.0001.

FIGURE 8. Effect of DEX, 2-CA and anti-IL-13 treatments on histopathological changes in lung tissues during OVA/LPS-induced allergic asthma.

Lungs samples were collected on day 24, fixed in 10% formalin, paraffinized and cut into slices. The slices were stained with Alcian Blue/Periodic Acid-Schiff (AB-PAS) to examine mucus production or Carbol's Chromotrope-hematoxylin (CR) to examine cell infiltration and hypertrophy of structural cells. (A) shows histopatology of the lung samples from different groups and treatments, all the histological images were randomly selected using 20X magnification lens.

(B) Positive mucosal goblet cells and eosinophils around the bronchial airway were counted as described in the Methods section. Presented as mean±SEM (n=8-10 mice per group); *p<0.05, #p<0.0001.
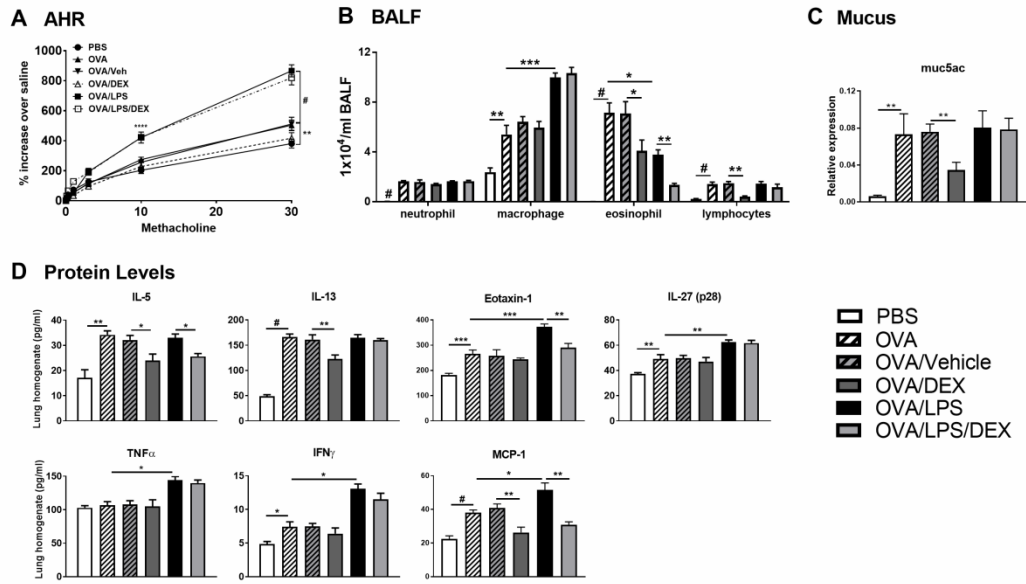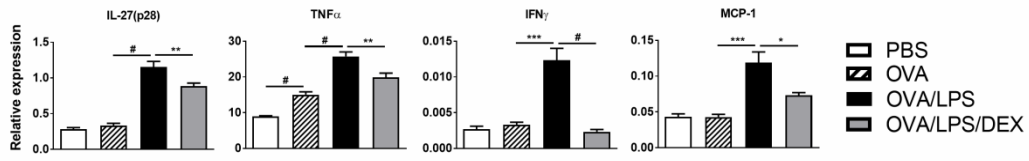
Figure 1.

cea_13505_f1.tif

Figure 2.

cea_13505_f2.tif

A AHR

B BALF

C Mucus

D Protein levels
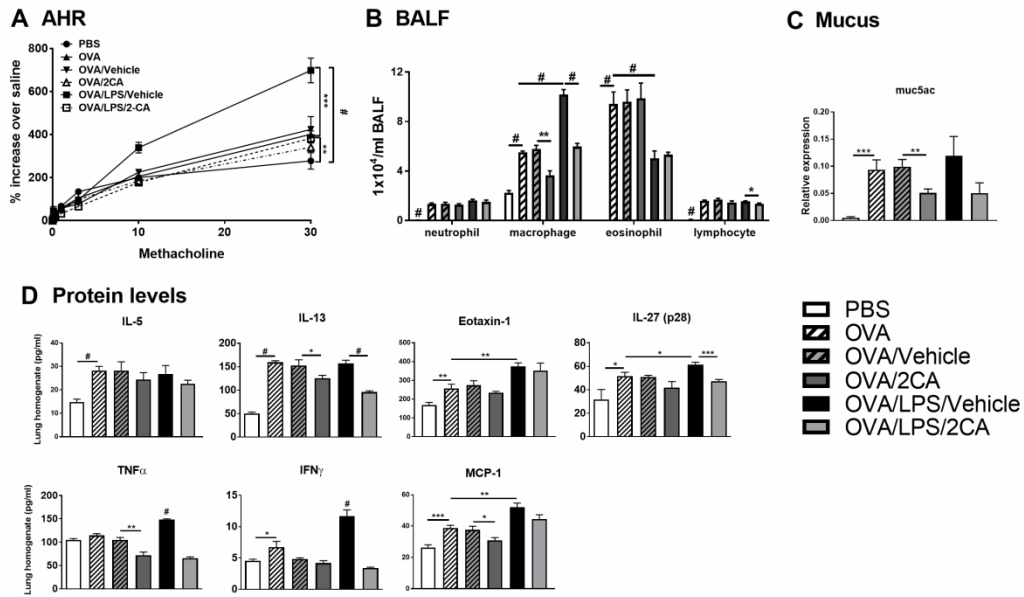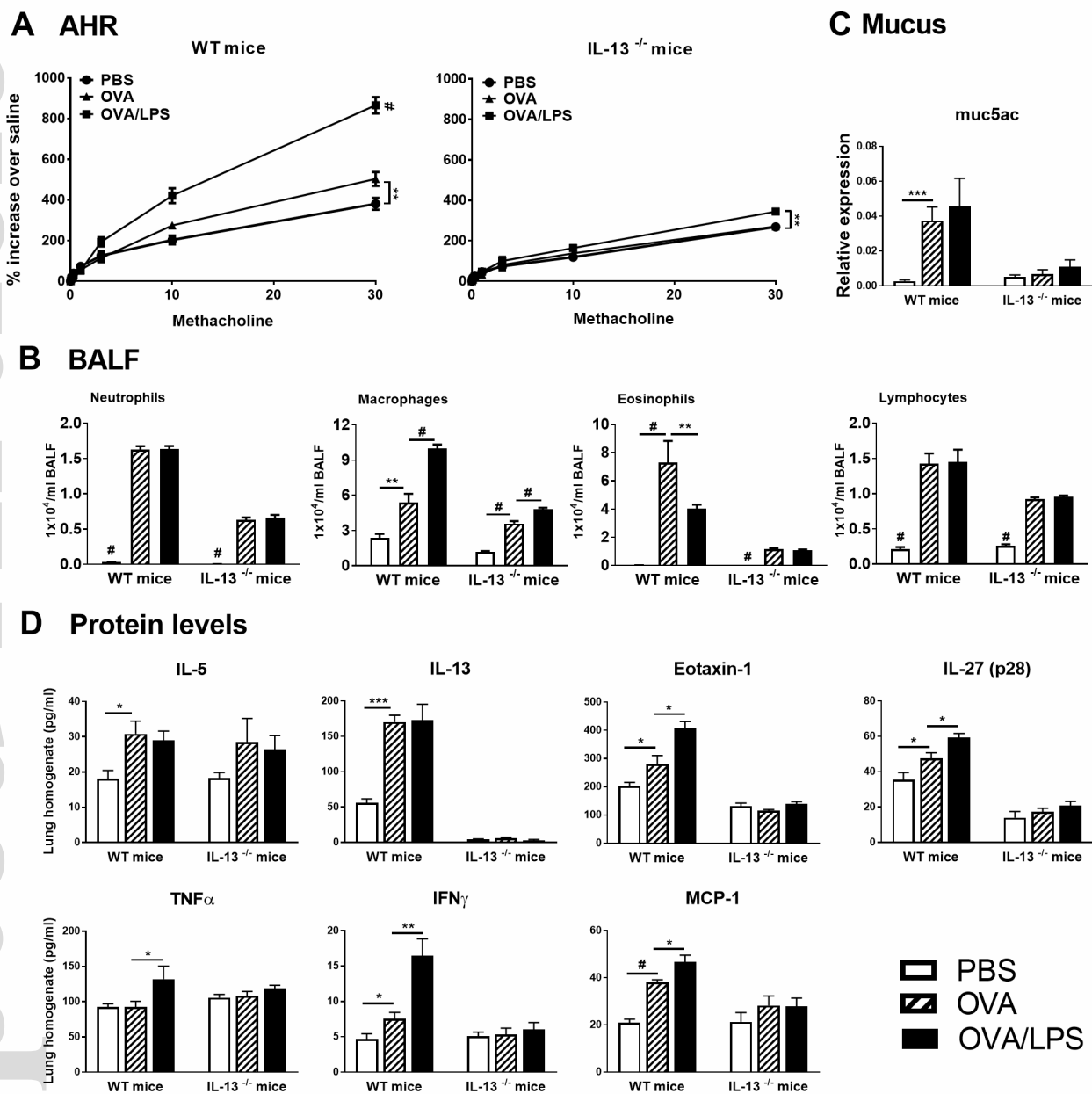
Figure 3.

cea_13505_f3.tif

Figure 4.

cea_13505_f4.tif
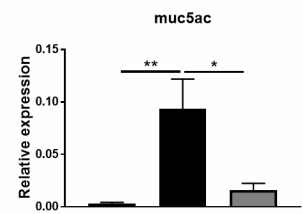
A AHR

B BALF

C Mucus

D Protein level

Legend:
- ☐ PBS
- ■ OVA/LPS + Isotype
- ▨ OVA/LPS + anti-IL-13
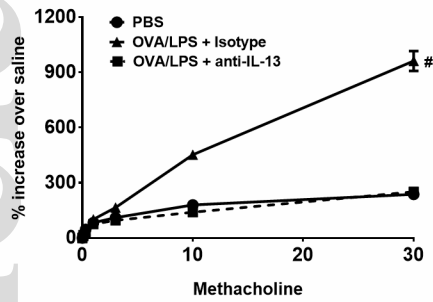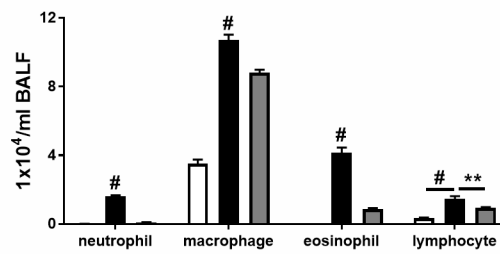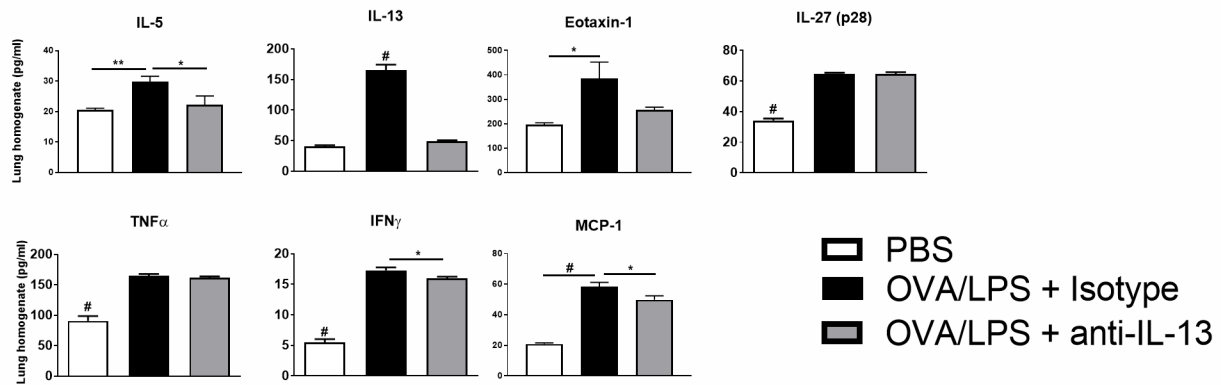
Figure 5.

cea_13505_f5.tif

Figure 6.

cea_13505_f6.tif

A Flow cytometry   **B Protein levels**

ILC2

Figure 7.

cea_13505_f7.tif

A

| | PBS | OVA | OVA/LPS | OVA/LPS/DEX | OVA/LPS/2CA | OVA/LPS + anti-IL-13 |

AB-PAS

CR

B

MSCs/100μm
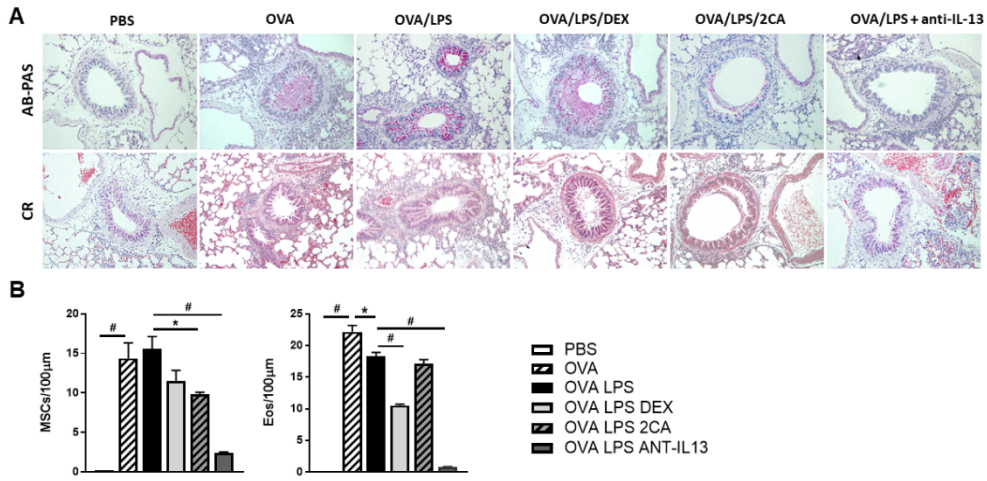
Eos/100μm

- PBS
- OVA
- OVA LPS
- OVA LPS DEX
- OVA LPS 2CA
- OVA LPS ANT-IL13

Figure 8.

cea_13505_f8.tif