Mucosal Immunology 18 (2025) 656–667

Contents lists available at ScienceDirect

Mucosal Immunology

journal homepage: www.elsevier.com/mi

Female sex hormones and the oral contraceptive pill modulate asthma severity through GLUT-1

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ARTICLE INFO

Keywords:
Asthma
Estrogen
Progesterone
oral contraceptive pill (OCP)
GLUT-1

ABSTRACT

Females are disproportionately affected by asthma. An increased understanding of how female sex hormones influence key pathophysiological processes that underpin asthma may identify new, more effective asthma therapies, particularly for females with severe, poorly controlled asthma.

We assessed the effects of oral ethinylestradiol/levonorgestrel (representing OCP use) and depot-medroxyprogesterone acetate (DMPA) and estradiol injections on key features of experimental asthma, and determined their effects on glucose transporter-1 (GLUT-1). The effects of OCP use on clinical asthma outcomes, and the relationships between estrogen receptors and type 2 (T2), non-T2, and GLUT-1 responses, in clinical asthma were also determined.

OCP and DMPA reduce T2 responses, disease features, and lung expression of GLUT-1, whereas estradiol increases lung expression of GLUT-1, and results in severe, corticosteroid-insensitive, neutrophil-enriched disease, in experimental asthma. OCP use is associated with reduced T2 cytokine and GLUT-1 responses in clinical asthma. GLUT-1 expression is increased in sputum of severe asthmatics, and positively correlates with estrogen receptor expression and both T2 and non-T2 inflammatory responses. Significantly, OCP or GLUT-1 inhibition protects against obesity-associated or estradiol-induced, severe, experimental asthma, respectively.

Together, these data show how female sex hormones and the OCP likely modulate asthma severity by modifying GLUT-1 responses in the airways.

Abbreviations: 2DG, 2-deoxy-d-glucose; AHR, Airway hyperresponsiveness; BALF, Bronchoalveolar lavage fluid; BMI, Body mass index; CC, Control chow; DEX, Dexamethasone; DMPA, Depot-medroxyprogesterone acetate; Est, 17β-estradiol; ESR, Estrogen receptor; FEV, Forced expiratory volume; FVC, Forced vital capacity; GLUT-1, Glucose transporter-1; HFD, High-fat diet; IL, Interleukin; Mch, Methacholine; NLRP, NLR family, pyrin domain containing; OCP, Oral contraceptive pill; Ova, Ovalbumin; Sal, Saline; T2, Type 2.

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https://doi.org/10.1016/j.mucimm.2025.02.006
Received 27 September 2024; Accepted 20 February 2025
Available online 26 February 2025
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Introduction

In adulthood, there is an increased prevalence of asthma in females compared to males (60 % *vs* 40 % of non-severe asthma)[1] indicating that asthma disproportionately affects females. Furthermore, up to 82 % of patients with severe asthma are female[2]. Significantly, the mechanisms that underpin increased disease burden in females are largely unknown but increasing evidence suggests that this outcome could be influenced by female sex hormones.

Hormonal fluctuations occurring throughout a female's lifespan are associated with variable asthma severity and control[3]. The female sex hormones, estrogen and progesterone, have been shown to play important roles in regulating airway inflammation[4], airways hyper-responsiveness (AHR)[5], and mucus production, in asthma[6–9]. However, the findings of these studies are often contradictory, describing both pro- and anti-inflammatory functions for female sex hormones [5,10]. For many females with asthma, symptoms worsen during the late-luteal and early follicular phases of the menstrual cycle[11–16]. This phenomenon is termed perimenstrual asthma and affects up to 40 % of pre-menopausal females with asthma[15,17], and represents a unique, highly symptomatic, disease phenotype associated with increased bronchial reactivity[12] and reduced lung function[15].

Interestingly, data suggests that the use of hormonal contraceptives, that control fluctuations of female sex hormones during the menstrual cycle in pre-menopausal females, modify risk of asthma exacerbation and asthma severity[18]. Importantly, we have previously shown that females with asthma using the OCP have significantly lower sputum neutrophil percentages, reduced inhaled corticosteroid (ICS) use, and better lung function and asthma control compared to those not using the OCP[19]. The mechanisms underpinning these sex-hormone mediated effects on disease outcomes remains unknown.

Increasing evidence suggests that sex hormones modify immune cell metabolism. Specifically, increased glucose uptake and metabolism have been shown to play important roles in driving inflammation[20–24]. In immune cells, GLUT-1 is the dominant glucose uptake transporter and increased GLUT-1 responses have been implicated in the pathogenesis and severity of asthma[25,26], however, our understanding of the interactions between female sex hormones and GLUT-1 mediated responses in asthma is unknown.

In summary, the OCP and female sex hormones play an important role in modulating the severity of asthma, however, the nature of the interplay remains poorly understood. Furthermore, whether this interplay is governed through GLUT-1 responses is unknown. A greater understanding of how female sex hormones and the OCP modulate disease may provide novel therapeutic targets and approaches for asthma, particularly for severe asthma. In this study, we investigated how female sex hormones and the OCP affect asthma using a complementary combination of murine models of experimental asthma and clinical data and samples.

Methods

Study approvals and clinical data

All experiments were conducted with approval of the Human/Animal Ethics Committees of the University of Newcastle, Hunter New England and Metro South Local Health Districts Ethics Committees, Australia, and the local ethics committees of the Unbiased Biomarkers in Prediction of respiratory disease outcomes (U-BIOPRED) clinical centres. Full details on clinical datasets are provided in supplementary material.

Experimental asthma models in non-obese and obese mice and treatments with female sex hormones, the oral contraceptive pill (OCP) or metabolic modulators BAY876 or 2-deoxy-D-glucose (2DG); airway inflammation; lung function; gene expression; airway mucus secreting cell (MSC); histopathological; single cell metabolic analyses in murine lung tissue.

Female BALB/c mice, fed a high fat diet (HFD) or control chow (CC), sensitized and challenged with ovalbumin (Ova), as previously described[27–29], were treated subcutaneously with 17β-estradiol (β-Estradiol 3-benzoate) or progesterone (depot-medroxyprogesterone acetate; DMPA) in corn oil, or sham-treated (Fig. A.1**A**). From days 21–35, some groups of mice were treated daily by oral gavage with a combination of ethinylestradiol and levonorgestrel in PBS to mimic the OCP (Fig. A.1**B**). To assess the role of glucose metabolism, some groups of mice were treated with the GLUT-1 inhibitor, BAY876, or the competitive glycolysis inhibitor, 2-deoxy-d-glucose (2DG) (Fig. A.1**D**). To assess the response to ICS, some groups of mice were treated with dexamethasone (DEX). Lung function, inflammation, airway MSC numbers and histopathology, and T2, non-T2 and GLUT-1 gene expression was assessed after the final Ova challenge in treated, and sham-treated, groups, as previously described[28–32] and outlined in the Online Repository. In a separate set of analyses, mice were treated subcutaneously with 17β-estradiol and metabolism was assessed in single cell suspensions of whole lung tissue using a puromycin uptake-based flow cytometry assay[33] as outlined in the Online Repository.

Statistics

Comparisons between two groups were performed using unpaired Student's *t*-tests, or a non-parametric equivalent, where appropriate. Comparisons between multiple groups were performed using ordinary one-way analysis of variance (ANOVA) and an appropriate post-hoc test, or a non-parametric equivalent. AHR data were analysed using two-way ANOVA with Tukey's post-hoc test. Correlation analyses were performed using Spearman's rank correlation. Analyses were performed using GraphPad Prism Software (version 10.1.1).

Results

OCP use is associated with decreased T2 cytokine responses and key features of disease in clinical and experimental asthma

Spirometry data analysis was retrospectively performed on pre-menopausal, female asthmatics using, and not using, the OCP (Table 1), in a separate cohort than previously reported[19]. Microarray data from a subset of female asthmatics, using, and not using, the OCP, was used to measure gene expression in sputum (Table A.2). We show that female asthmatics using the OCP have higher pre-bronchodilator FEV1% compared to those not using the OCP (Fig. 1**A**), which is consistent with previous findings showing the OCP protects against asthma disease features[18,19,34]. Importantly, we now show that OCP use

Table 1
Subject demographics (Australian Cohort).

Clinical Characteristic	No OCP use (n = 24)	OCP use (n = 18)	p-value
Sex (female/male), % female	24/0, 100 %	18/0, 100 %	n/a
Age (y), mean (range)	34.7 (20.6, 47.9)	32.8 (18.5, 46.2)	0.615
Weight (kg), mean (range)	86.7 (53.5, 172.7)	85.7 (49.9, 132.3)	0.554
BMI, mean (range)	31.8 (22.4, 61.0)	31.1 (17.3, 52.9)	0.711
FEV_1% predicted, mean (range)	84.5 (40.6, 108.0)	99.2 (78.0, 124.0)	**0.0049****
FVC% predicted, mean (range)	93.7 (72.1, 124.0)	103.1 (88.0, 125.0)	**0.0258***
FEV_1/FVC %, mean (range)	74.7 (47.4, 94.0)	80.8 (64.2, 98.4)	0.0503

OCP, oral contraceptive pill; BMI, body mass index; FEV1, forced expiratory volume in one second; FVC, forced vital capacity.

corresponds with reduced IL-5 and IL-13 gene expression in sputum compared with pre-menopausal, female asthmatics not using the OCP (Fig. 1**B,C**).

Similar to our clinical findings, we show that daily oral treatment of mice with a combination of ethinylestradiol and levenorgesterel (Fig. A.1), which mimics the most commonly used OCP combination in Australia, reduces AHR in experimental asthma (Fig. 1**D,E**). Significantly, OCP treatment reduces IL-13 and IL-5 gene expression in the lungs in experimental asthma (Fig. 1**F,G**). Importantly, our findings suggest that T2 responses and asthma disease features in females are amenable to modulation by female sex hormones.

Estradiol promotes, whereas progesterone suppresses, key features of experimental asthma

We next sought to determine how different female sex hormones affect disease by examining the effects of treatment with estradiol and progesterone (DMPA) on airway inflammation and AHR in the presence, or absence, of experimental asthma. We show that estradiol treatment increases the magnitude of AHR (Fig. 2**A,B and A.4A,B**) in both the absence (Est/Sal) and presence (Est/Ova) of experimental asthma, compared to sham-treated controls (Sham/Sal and Sham/Ova, respectively). Whilst estradiol treatment has no effect on total leukocyte numbers (Fig. 2**C**), it decreases eosinophil and lymphocyte numbers (Fig. 2**D, G**), and increases neutrophil numbers(Fig. 2**E**) without altering macrophages (Fig. 2**F**) in bronchoalveolar lavage fluid (BALF) during experimental asthma (Sham/Ova *vs* Est/Ova). Estradiol treatment increases IL-13, Muc5ac and NLRP3 gene expression in the lungs (Fig. 2**H-K**) in the absence of experimental asthma (Sham/Sal *vs* Est/Sal), but has no additional effect on their expression in experimental asthma (Sham/Ova *vs* Est/Ova). Indeed, estradiol treatment decreased Muc5ac gene expression in experimental asthma. In contrast to estradiol treatment, DMPA treatment significantly reduces AHR (Fig. 2**A,B and A.4A,B**) and leukocyte, eosinophil, macrophage and lymphocyte numbers (Fig. 2**C,D, F,G**) in BALF in experimental asthma (Sham/Ova *vs* DMPA/Ova). Importantly, DMPA treatment decreases IL-5, Muc5ac, NLRP3 and Caspase-1 (Fig. 2**H, J-L**) gene expression during experimental asthma (Sham/Ova *vs* DMPA/Ova) and reduces Caspase-1 and IL-1β gene expression (Fig. 2**L,M**) in the absence of experimental asthma (Sham/Sal *vs* DMPA/Sal). This reduction in DMPA-induced Caspase-1 and IL-1β gene expression corresponds with a decrease in macrophages in the airways in the absence of experimental asthma (Fig. 2**F**). Together, our data demonstrate that estradiol increases, whereas progesterone decreases, the magnitude of key features of disease in experimental asthma.

Estradiol increases, whilst DMPA and the OCP decrease, GLUT-1 expression in experimental and clinical asthma

Considering the growing evidence of the relationship between female sex hormones and glucose metabolism, we next sought to examine the relationship between hormone treatments and GLUT-1 expression in the lungs of mice treated with estradiol, DMPA, or experimental OCP, in the absence, or presence, of experimental asthma, and in sputum from females with asthma using, or not using, the OCP.

We show that estradiol increases, whilst DMPA decreases, lung gene expression of GLUT-1 in experimental asthma (Sham/Ova *vs* Est/Ova and DMPA/Ova, respectively; Fig. 3**A**). Importantly, we also show that OCP treatment decreases GLUT-1 gene expression in the sputum of females with asthma (Fig. 3**B**). These data show that treatment with different female sex hormones exert robust and contrasting effects on lung GLUT-1 responses, and highlight the potential importance of GLUT-

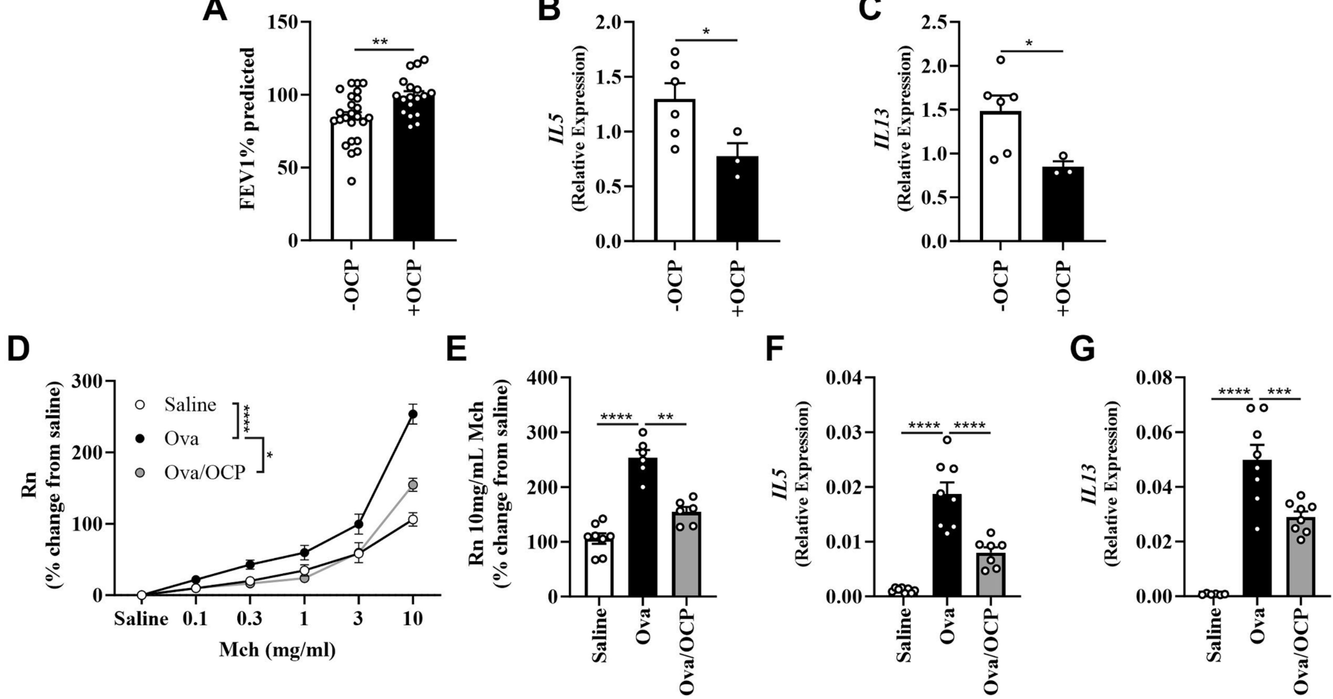

Fig. 1. The oral contraceptive pill (OCP) is associated with better outcomes in asthmatic females and experimental asthma. Spirometry and sputum microarray analyses were performed on pre-menopausal, female asthmatics using and not-using the OCP. Females on the OCP have a higher pre-bronchodilator FEV1% (forced expiratory volume over one second, % predicted) (**A**), (n = 18–24 Table 1) and lower expression of IL-13 (**B**) and IL-5 (**C**) (n = 3–6 Table A.2). Female BALB/c mice with ovalbumin (Ova)-induced experimental asthma were gavaged daily with a combination of ethinylestradiol and levonorgestrel, mimicking the oral contraceptive pill. OCP treatment protects against airways resistance in response to increasing doses of methacholine (Mch; **D**), and at the maximal dose of 10 mg/ml (**E**). Similar to clinical findings, OCP treatment reduces the expression of IL-5 (**F**) and IL-13 (**G**) in the lungs in experimental asthma, n = 6–8 *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

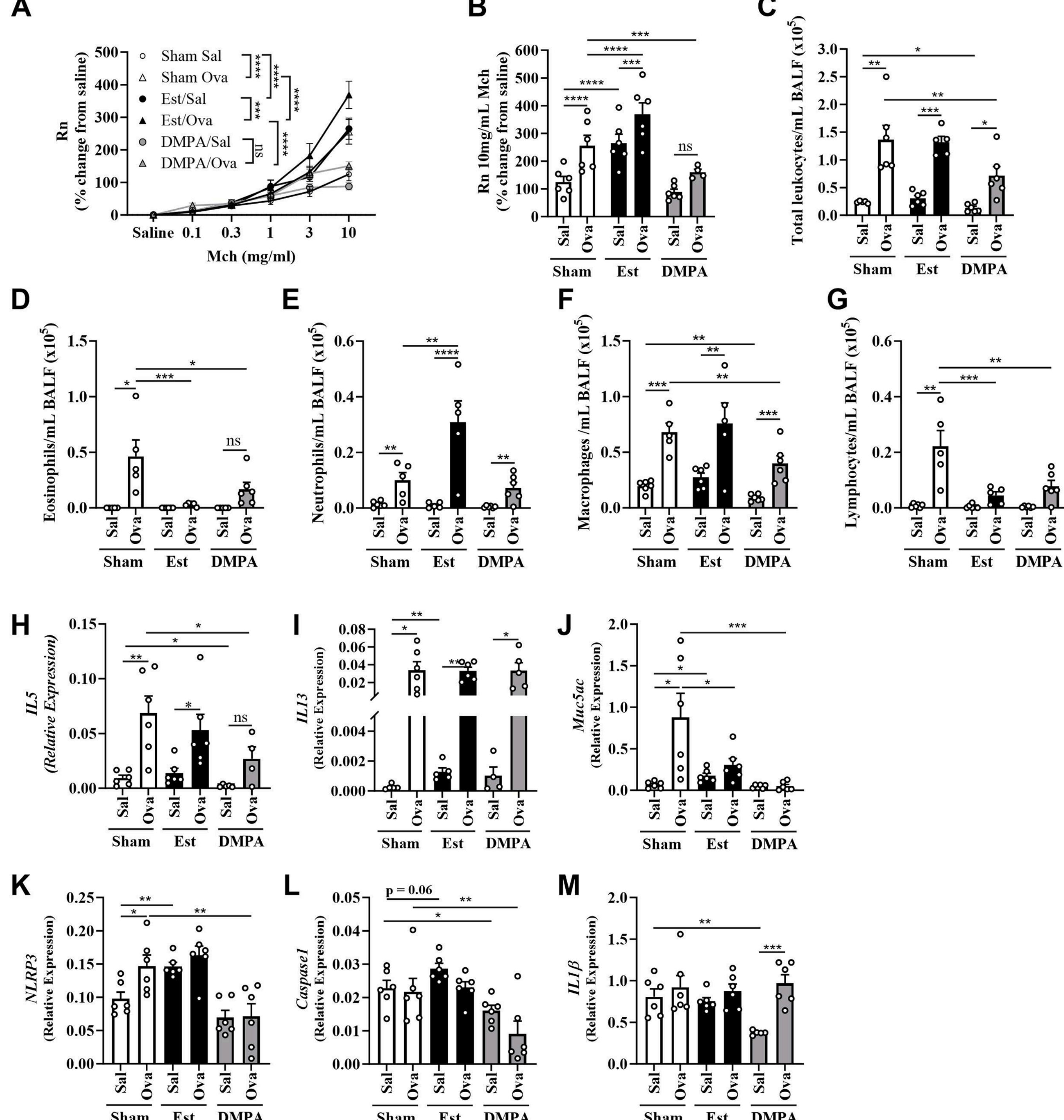

Fig. 2. Estradiol promotes, whereas progesterone suppresses, key features of experimental asthma. Female BALB/c mice with ovalbumin (Ova)-induced experimental asthma were treated subcutaneously with 17β-estradiol (Est, estradiol); medroxyprogesterone acetate (DMPA); or sham-injected with corn oil (**Fig. A.1**). The effects of estradiol and DMPA on airways hyper-responsiveness (AHR) in terms of central airways resistance (Rn) in response to increasing doses of methacholine (Mch; **A**), and at the maximal dose of Mch (10 mg/ml) (**B**) in Ova-indued asthma was determined. Effects of treatments on total leukocyte (**C**), eosinophil (**D**), neutrophil (**E**), macrophage (**F**) and lymphocyte (**G**) numbers in the bronchoalveolar lavage fluid (BALF), as well as IL-5 (**H**), IL-13 (**I**), Muc5ac (**J**), NLRP3 (**K**), Caspase-1 (**L**) and IL-1β (**M**) expression in the lung, was also determined. n = 5–15, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

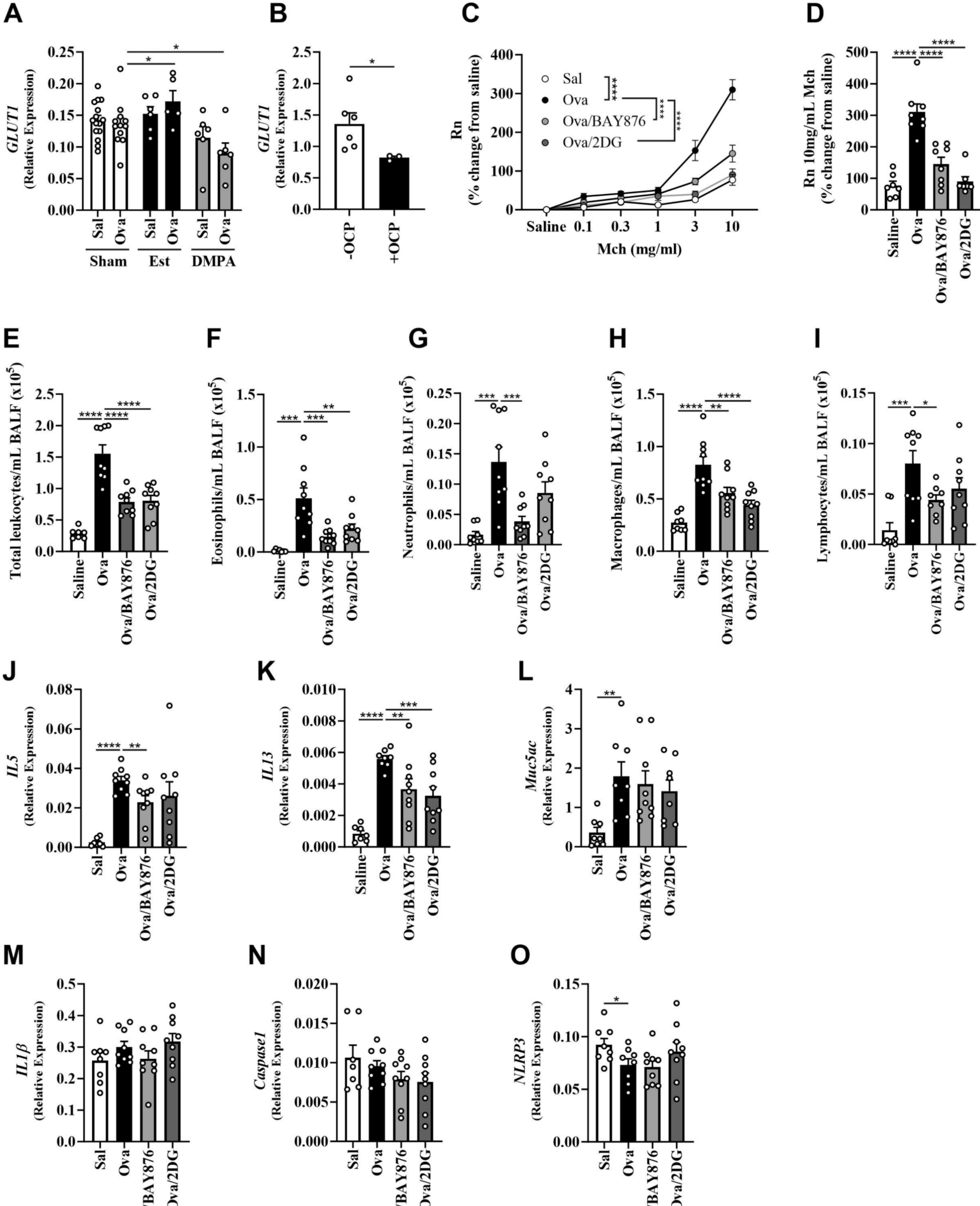

Fig. 3. Targeting glucose metabolism protects against key features of experimental asthma. Female BALB/c mice with ovalbumin (Ova)-induced experimental asthma were treated subcutaneously with 17β-estradiol (Est; estradiol); medroxyprogesterone acetate (DMPA); or sham-injected with corn oil (**Fig. A.1**) and the mRNA expression of glucose transporter-1 (GLUT-1) by qPCR on day 35 (**A**). Sputum microarray analyses were performed on pre-menopausal, female asthmatics using and not-using the OCP and the expression of GLUT-1 determined (**B**). Wild-type, female, BALB/c mice with ovalbumin (Ova)-induced experimental asthma were treated intranasally with glucose transporter-1 (GLUT-1) inhibitor, BAY876, or 2-deoxyglucose (2DG), or sham treated. AHR in terms of airways resistance in response to increasing doses of methacholine (Mch; **C**), and at the maximal dose of 10 mg/ml (**D**) was determined on day 35 of the model (**Fig. A.1**). Total leukocytes (**E**), eosinophils (**F**), neutrophils (**G**), macrophages (**H**) and lymphocytes (**I**) were also determined on day 35. IL-5 (**J**), IL-13 (**K**), Muc5ac (**L**), IL-1β (**M**), Caspase-1 (**N**) and NLRP3 (**O**) mRNA expression in the lung was determined by qPCR. n = 6–9, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Rn, Airway resistance.

1, and its downstream metabolic activities, in driving key features of asthma in females.

Inhibition of GLUT-1 reduces AHR and T2 cytokine responses in experimental asthma

Our data show that GLUT-1 is altered in response to OCP and/or female sex hormones in clinical and experimental asthma. We next investigated the roles of GLUT-1 on AHR and T2 cytokine responses in experimental asthma. We show that, intranasal administration of the potent and selective GLUT-1 inhibitor, BAY876 (Fig. A.1), reduces AHR (Fig. 3**C,D and A.4C,D**) and total leukocyte (Fig. 3**E**), eosinophil, neutrophil, macrophage, and lymphocyte numbers (Fig. 3**F-I**) in BALF during experimental asthma. Importantly, BAY876 treatment reduces IL-5 and IL-13 gene expression in experimental asthma (Fig. 3**J,K**). To investigate a potential role for GLUT-1-mediated glucose metabolism in driving these responses, we also assessed the effects of intranasal administration of the competitive glycolysis inhibitor, 2-deoxy-D-glucose (2DG). Similar to treatment with BAY876, 2DG treatment reduces AHR (Fig. 3**C,D and A.4C,D**) and total leukocyte (Fig. 3**E**), eosinophil(Fig. 3**F**) and macrophage numbers (Fig. 3**H**), and IL-13 (Fig. 3**K**) gene expression, in the airways in experimental asthma. Administration of BAY876 or 2DG had no effects on Muc5aC, IL-1β, Caspase-1, or NLRP3 gene expression (Fig. 3**L-O**).

OCP suppresses AHR and GLUT-1-expressing cell populations in the airways and lungs in both non-obese and obese mice with experimental asthma

We previously showed that OCP use is associated with decreased sputum neutrophils and ICS use in a cohort that contains both obese and non-obese females[19]. However, this study was not specifically designed

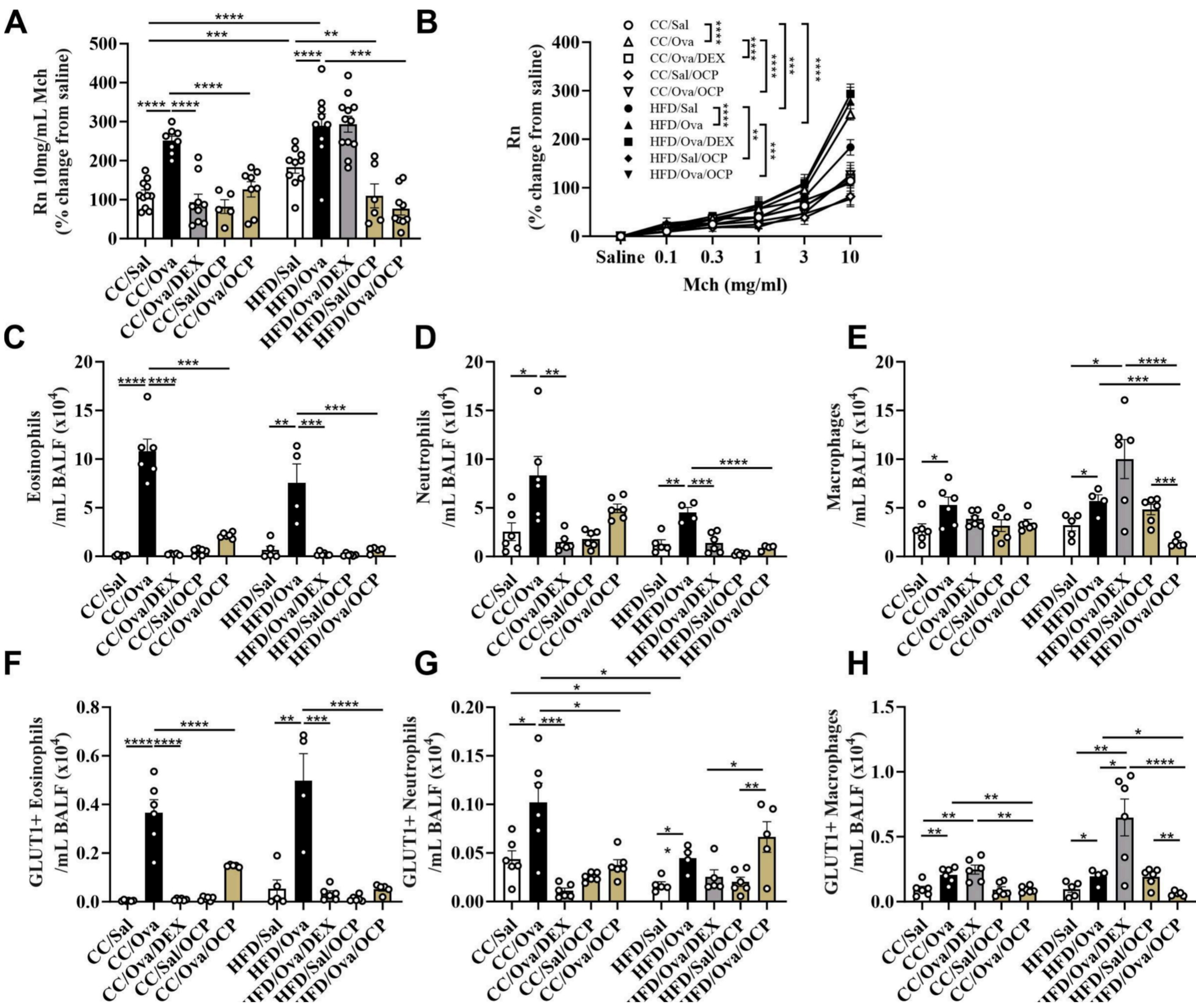

Fig. 4. The oral contraceptive pill (OCP) protects against steroid insensitive airways hyper-responsiveness in obese experimental asthma and GLUT-1 expressing cells in the airways in non-obese and obese experimental asthma. Female BALB/c mice with non-obese (CC, control chow) and obese (HFD, high fat diet) ovalbumin (Ova)-induced experimental asthma were gavaged daily with a combination of ethinylestradiol and levonorgestrel, mimicking the oral contraceptive pill (OCP) and treated with dexamethasone (DEX) on days 32–34 of the model (**Fig. A.1**). OCP treatment protects against central airways resistance (Rn, % change from saline) in response to increasing doses of methacholine (Mch; **A**), and at the maximal dose of 10 mg/ml (**B**). The number of eosinophils (**C**), neutrophils (**D**), macrophages (**E**) and their GLUT-1 expressing populations (**F-H**) in the airways of these mice were determined by multi-colour flow cytometry. n = 4–12 *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Data from CC fed controls in **A-B** are shared in Fig. 1**D,E**.

to examine the effects of the OCP use on specific disease parameters in obese compared to non-obese females. We and others have established that neutrophilic inflammation and ICS use are higher, and asthma severity and disease control are worse, in obese compared to non-obese females with asthma[27,35–38]. In this study, to assess how the OCP affects GLUT-1 responses and disease in non-obese and obese asthma, and to identify the cellular source/s of GLUT-1, we next examined GLUT-1-expressing cells in the lungs in non-obese versus obese mice, with and without experimental asthma, with and without OCP treatment..

We show that OCP treatment suppresses AHR in both non-severe, corticosteroid-sensitive experimental asthma (Fig. 1**D,E**) and in our previously published model[29] of obesity-associated, severe, corticosteroid-insensitive experimental asthma (Fig. 4**A,B and A.4E,F**). We also show that the numbers of GLUT-1 protein-expressing eosinophils, neutrophils, total macrophages, interstitial macrophages, and monocytes are increased in BALF (Fig. 4**F-H**) and/or lungs (Fig. A.5 **I-K, M**), in non-severe, corticosteroid-sensitive, experimental asthma (CC/Sal *vs* CC/Ova), and in obesity-associated severe, corticosteroid-insensitive asthma (HFD/Sal *vs* HFD/Ova). Importantly, we show that OCP treatment suppresses Ova-induced increases in the numbers of GLUT-1 protein-expressing cells in BALF (Fig. 4**F-H**) as effectively as ICS in non-severe, corticosteroid-sensitive experimental asthma (CC/Ova *vs* CC/Ova/OCP *vs* CC/Ova/DEX). We also show that obesity increases GLUT-1 protein-expressing neutrophils, monocytes and $CD3^+$ T-cells, whilst decreases GLUT-1 protein-expressing alveolar macrophages in lung tissue (Fig. A.5**J,M,N**) compared to non-obese controls (CC/Sal *vs* HFD/Sal). Furthermore, whilst obesity decreases GLUT-1 protein-expressing neutrophils in BALF (Fig. 4**G**) in both the presence (CC/Ova *vs* HFD/Ova) and/or absence (CC/Sal *vs* HFD/Sal) of experimental asthma, compared to non-obese controls, obesity increases GLUT-1 protein-expressing cells in lung tissue (Fig. A.5**J**). Importantly, we also show that corticosteroid treatment increases the number of GLUT-1 protein-expressing macrophages in BALF (HFD/Ova *vs* HFD/Ova/DEX; Fig. 4**H**) in obesity-associated, severe, corticosteroid-insensitive experimental asthma and that this effect is suppressed by OCP treatment (HFD/Ova *vs* HFD/Ova/OCP).

GLUT-1 and estrogen receptor expression are increased in severe asthma, and are associated with one another, with both associated with decreased lung function and increased T2 and non-T2 inflammatory responses in asthma

We next assessed the relationships between GLUT-1 and estrogen receptor (ESR) gene expression, and asthma severity and lung function, and T2 and non-T2 responses, in sputum from subjects (males and females) in a different clinical cohort (U-BIOPRED; Table 2). We show that GLUT-1 gene expression is increased in the sputum of severe asthmatics compared to non-severe asthmatics and healthy subjects (Fig. 5**A**). We also show that GLUT-1 expression negatively correlates with FEV1% predicted, FVC% predicted, and FEV1/FVC ratio in asthmatics (Fig. 5**B-D**). We then show that GLUT-1 expression positively correlates with the gene expression of T2 (IL-5 and IL-13) and non-T2 (NLRP3 and IL17A) immune factors in sputum from asthmatics (Fig. 5**E-H**). Elevated eosinophilic and neutrophilic inflammation are also associated with increased GLUT-1 gene expression in asthmatics (Fig. A.6**A,B)**. Importantly, we show that sputum GLUT-1 gene expression positively correlates with ESR2 gene expression (Fig. 5**I)**. ESR2 gene expression, like with GLUT-1 gene expression, is increased in the sputum of severe asthmatics compared to non-severe asthmatics and healthy subjects (Fig. 5**J**), negatively correlates with FEV1% predicted, FVC% predicted, and FEV1/FVC ratio (Fig. 5**K-M**), and positively correlates with increased gene expression of T2 (IL-5 and IL-13) and non-T2 (NLRP3 and IL17A) factors (Fig. 5**N-Q**). Similar to GLUT-1, we also show that elevated eosinophilic and neutrophilic inflammation are associated with increased ESR2 gene expression in asthmatics **(**Fig. A.6**C,D)**. Importantly, we also show that ESR2 gene expression is reduced in the sputum of females with asthma who are using the OCP compared to those not using the OCP (Fig. 5**R**). In contrast to ESR2, ESR1 gene expression is unchanged in non-severe and severe asthmatics compared to healthy subjects (Fig. A.7**A**), and is not correlated with changes in FEV1% predicted, FVC% predicted, and FEV1/FVC ratio (Fig. A.7**B-D**). However, ESR1 gene expression has a weak, but significant, positive correlation with GLUT1 gene expression **(**Fig. A.7**E)**, and positively correlates with IL-5, IL-13, and IL-17A, but not NLRP3, gene expression (Fig. A.7**F-I**). Furthermore, when these analyses are stratified for sex, whilst the strength of the correlations are stronger in female subjects (Fig. A.8) they still exist in male subjects (Fig. A.9) indicating that the associations between GLUT-1, ESR and cytokine gene expression in the sputum applies to both males and females.

Table 2
Subject demographics (U-BIOPRED Cohort).

Clinical Characteristic	HC (n = 16)	Non-SA (n = 20)	SA (n = 61)
Sex (female/male), % female	4/16, 20 %	11/20, 55 %	37/61, 61 %
Age (y), mean (range)	38.1 (18.0, 65.0)	42.1 (18.0, 68.0)	53.9 (21.0, 78.0) ****,###
Weight (kg), mean (range)	81.6 (62.2, 107.0)	75.7 (50.0, 120.0)	78.2 (46.5, 127.1)
BMI, mean (range)	25.8 (21.8, 30.8)	25.8 (19.0, 36.6)	27.9 (19.3, 42.5)
FEV_1% predicted, mean (range)	105.9 (83.8, 123.6)	92.5 (63.6, 118.3)**	61.3 (18.4, 120.6) ****,####
FVC% predicted, mean (range)	113.6 (96.1, 131.9)	108.6 (84.3, 143.0)	87.3 (40.2, 130.6) ****,####
FEV_1/FVC, mean (range)	77.9 (69.7, 88.8)	71.6 (59.9, 89.5)**	57.1 (29.8, 86.5) ****,####

HC, healthy control; Non-SA, non severe asthma; SA, severe asthma; BMI, body mass index; FEV1, forced expiratory volume in one second; FVC, forced vital capacity. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$ compared to HC. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ compared to Non-SA.

Targeting GLUT-1 responses in the airways protects against estradiol-induced, severe, neutrophilic experimental asthma

We next sought to determine whether inhibiting GLUT-1 responses with the potent, selective GLUT-1 inhibitor BAY876 reduces features of estradiol-induced, severe experimental asthma. Initially, we reproduced our earlier findings (Fig. 2), showing that estradiol treatment increases the magnitude of AHR (Fig. 6**A,B**) in both the absence (Est/Sal), and presence (Est/Ova), of experimental asthma compared to sham-treated controls (Sham/Sal and Sham/Ova, respectively). Notably, we now show that estradiol treatment results in partial corticosteroid insensitivity, with over 40 % increase in maximal AHR, compared to sham-treated controls (Sham/Ova/DEX *vs* Est/Ova/DEX) (Fig. 6**A,B**). Similar to our previous observations, whilst estrogen treatment has no effect on the total number of inflammatory cells in the airways (Fig. 6**C**), estrogen treatment alters the nature of inflammatory response in experimental asthma by decreasing eosinophilic (Fig. 6**D**) and increasing neutrophilic (Fig. 6**E**) inflammation. in experimental asthma. Importantly, we show that intranasal BAY876 treatment (Ova/Est/BAY876), reduces estradiol-induced increases in AHR (Fig. 6**A,B**), and total leukocyte (Fig. 6**C**) and neutrophil numbers (Fig. 6**D**) in BALF in estradiol-induced, severe experimental asthma.

Airways MSC numbers and histopathological scoring was also conducted in lung tissue sections from mice treated with estradiol or sham-treated, in the presence and absence of experimental asthma, with and without BAY876 treatment (Fig. A.10). We show that estradiol increases the number of MSCs in the airways in the absence of experimental asthma (Fig. A.10). This finding agrees with our qPCR data that shows increased expression of Muc5ac gene expression in this group (Fig. 2**J**). BAY876 treatment had no effect on the number of airways MSCs in

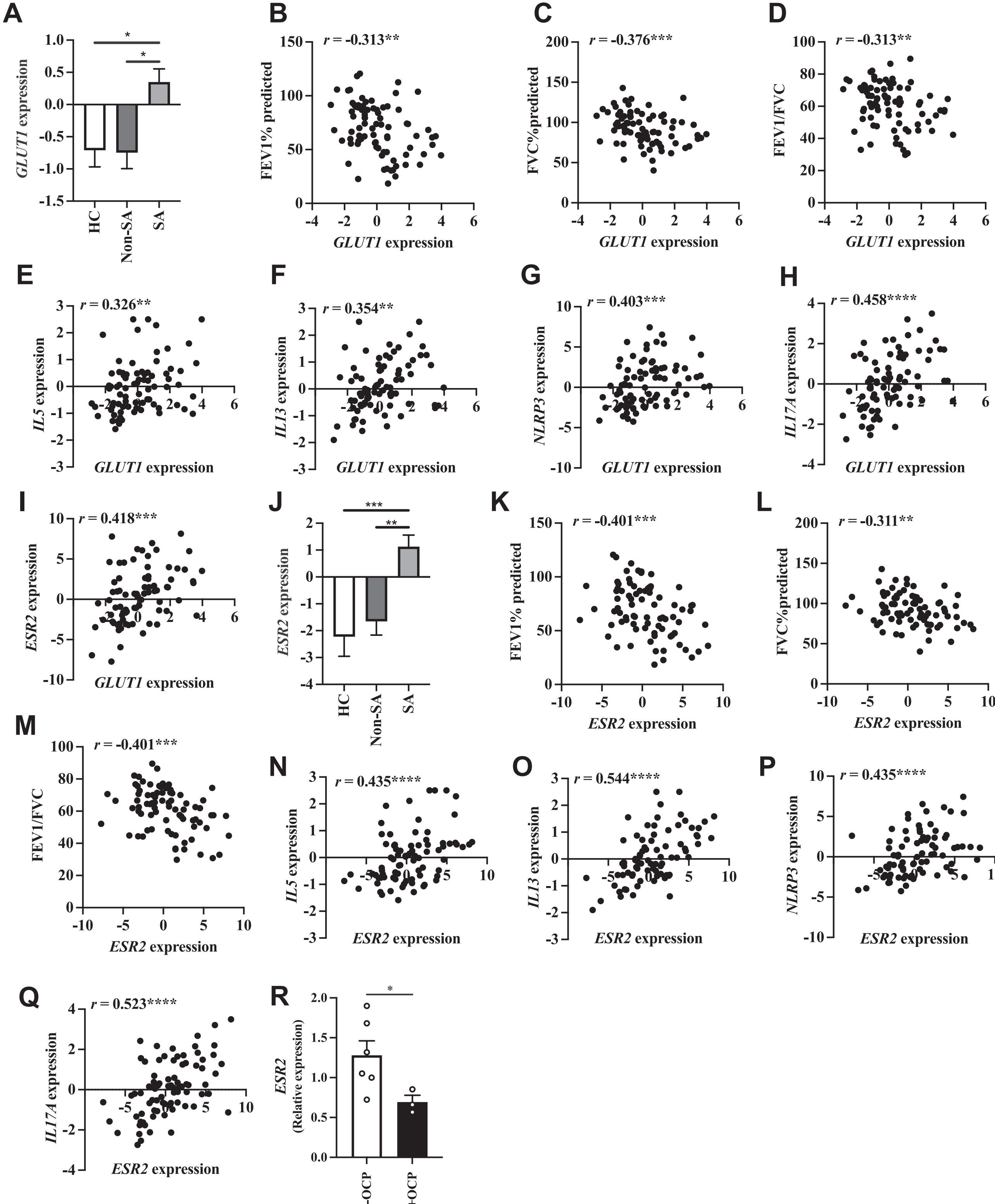

Fig. 5. GLUT-1 and ESR2 expression is higher in severe asthma, and both are positively associated with increased T2 and non-T2 responses in the airways. GLUT-1 expression is increased in sputum RNA in (**A**) severe asthma (SA) compared to non-SA and healthy controls (HC). GLUT-1 expression in sputum RNA in subjects with asthma negatively correlates with FEV1% (**B**), FVC% (**C**), and FEV1/FVC (**D**). GLUT-1 is associated with increased T2 responses; IL-5 (**E**), IL-13 (**F**), non-T2 responses; NLRP3 (**G**), IL-17A (**H**), and estrogen receptor-2 (ESR2) (**I**) responses. ESR2 expression is also increased in sputum RNA in (**J**) SA compared to non-SA and HC. ESR2 expression in sputum RNA in subjects with asthma is negatively associated with FEV1% (**K**), FVC% (**L**), and FEV1/FVC (**M**). ESR2 is associated with increased T2 responses; IL-5 (**N**), IL-13 (**O**), and non-T2 responses NLRP3 (**P**) and IL-17A (**Q**). ESR2 expression is decreased in the sputum of females with asthma who use the oral contraceptive pill (OCP) **(R)**. Associations for each comparison are expressed as Spearman rank correlation coefficient (Spearman rho;r) with P values. n = 3–81, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. ESR2, estrogen receptor 2.

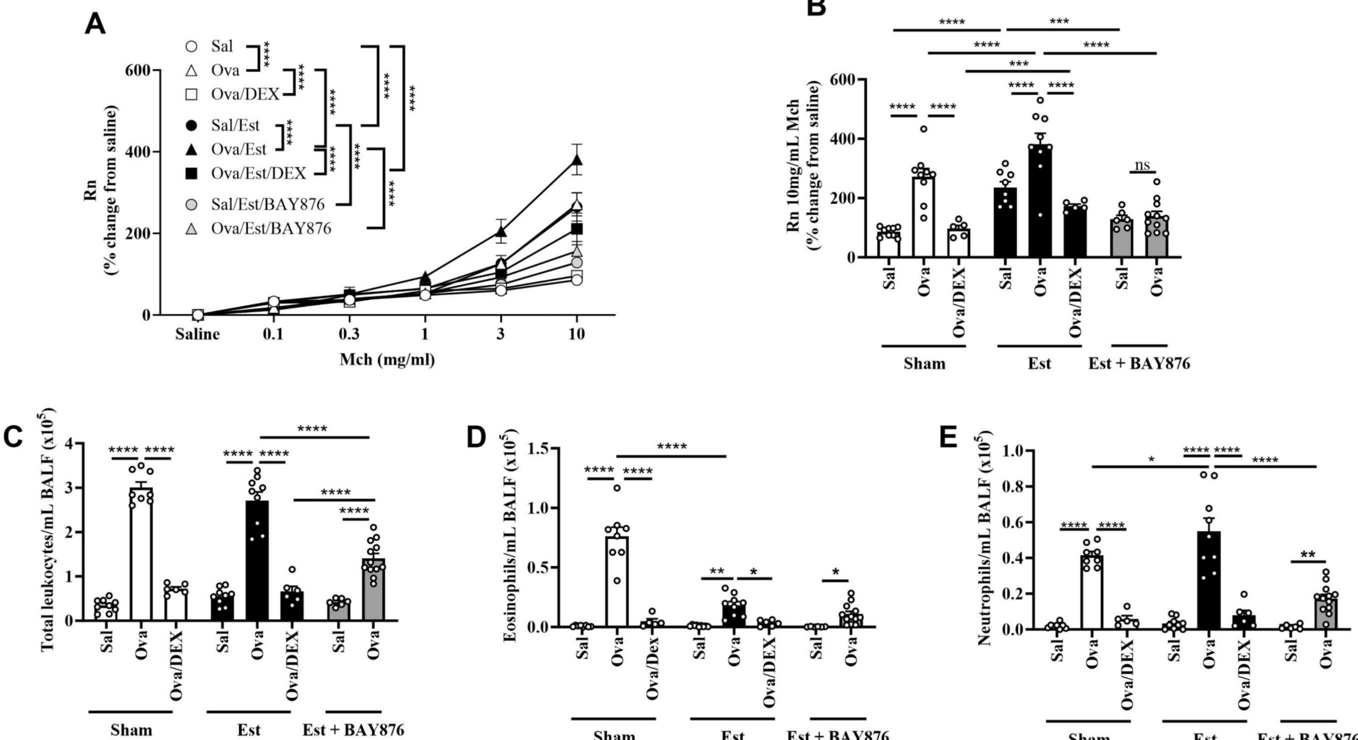

Fig. 6. Targeting glucose metabolism protects against estradiol-induced worsening of experimental asthma. Female, BALB/c mice with ovalbumin (Ova)-induced experimental asthma were treated subcutaneously with 17β-estradiol (Est, estradiol) one week prior to intranasal treatment with dexamethasone (DEX), glucose transporter-1 (GLUT-1) inhibitor, BAY876, or sham treated. AHR in terms of airways resistance in response to increasing doses of methacholine (Mch; **A**), and at the maximal dose of 10 mg/ml (**B**) was determined on day 35 of the model (**Fig. A.1**). Total leukocytes (**C**), eosinophils (**D**), and neutrophils (**E**), were also determined on day 35 of the model. n = 6–12, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Rn, Airway resistance.

estradiol-treated mice in the absence of experimental asthma (Fig. A.10), which suggests that estradiol-induced increases in MSC metaplasia are uncoupled from estradiol-induced increases in GLUT-1, or that BAY876 treatment was administered too late to affect estradiol-induced MSC metaplasia. Given we show that BAY876 reduces estradiol-induced AHR, but not MSC metaplasia, in the absence of experimental asthma, our data suggest that estradiol-induced increases in MSC metaplasia is likely not a major driver of the estradiol-induced AHR we observed in the absence of experimental asthma. We also assessed the effects of estradiol treatment on tissue inflammation, as assessed by histopathology scoring. We show that whilst estradiol slightly increased tissue inflammation in absence of experimental asthma, treatment had no effect on tissue inflammation in the presence of, experimental asthma (Fig. A.10). This suggests that the changes induced by estradiol, and that are reduced by BAY876, are largely limited to the airways (inflammation in BALF and AHR).

In summary, our findings link female sex hormones and GLUT-1 responses in the airways to the pathogenesis and severity of asthma for the first time, highlighting the potential for harnessing sex hormone-mediated effects on GLUT-1 responses for the treatment of disease in both females and males.

Discussion

Using a complementary combination of experimental and clinical data, we demonstrate, for the first time, that the OCP and female sex hormones modify T2 and non-T2 inflammatory processes and disease severity in asthma, and that these effects occur through the modulation of GLUT-1-dependent responses. We, and others, have previously shown that females with asthma who are using an OCP have better asthma control, are taking a lower dose of ICS, and have lower sputum neutrophil percentages[19,39]. However, other studies show that females using an OCP have increased risk of developing asthma, especially when OCP use commences at < 18 years old[18,40,41]. Here, we show that the OCP reduces AHR in experimental asthma and this observation agrees with previous findings reported in the literature showing that pre-menopausal females with asthma, using an OCP, have better lung function compared to asthmatic females who are not using the OCP[39]. Importantly, we now show that OCP use corresponds with lower airway T2 cytokine expression in both experimental and clinical asthma. Additionally, the OCP is as effective as corticosteroids at decreasing key features of non-severe, corticosteroid-sensitive experimental asthma, and potently suppresses features of obesity-associated, severe, corticosteroid-insensitive experimental asthma. Importantly, the OCP dosing/kg in obese animals is lower than in non-obese animals, as occurs in clinical use, highlighting the effectiveness of OCP in protecting against key features of experimental asthma even at lower dosing. Given that we observed similar protective responses with DMPA (synthetic progestogen) and OCP (contains the synthetic progestogen, levonorgestrel along with ethinylestradiol) treatments in experimental asthma, these findings highlight a potential role for progestogens in the disease-suppressing effects of combined oral hormone contraceptives in asthma. Indeed, in older females, menopausal hormone therapy (MHT), consisting of only progestogen reduces the risk of asthma[42]. We also show that DMPA treatment reduces the number of macrophages and IL-1β expression in the absence of experimental asthma. Given that macrophages are potent producers of IL-1β and increased IL-1β increases macrophages, whether these results are due to DMPA-induced reductions in IL-1β or DMPA-induced reductions in macrophage infiltration and activation in the airways requires further investigation that are beyond the scope of the current study. Nevertheless, these data suggest that progestins may dampen inflammatory responses in the lungs by suppressing baseline IL-1β and macrophage responses. Conversely, MHT formulations of either estrogen alone, or a combination of estrogen and a

progestogen, leads to a 63 % greater risk of new asthma development, and increased corticosteroid use and asthma exacerbations in females with pre-existing asthma. Similarly, we show that estradiol worsens features of experimental asthma.

Previous studies have shown roles for female sex hormones[4–9] or GLUT-1[25, 26] driving the pathogenesis of asthma but not how these two key factors interact to influence the severity of disease. This is the first study to show that female sex hormones and the OCP likely mediate their effects on asthma through modification of GLUT-1 responses. We demonstrate that OCP and DMPA treatments decrease, whereas estradiol treatment increases, GLUT-1 expression in the airways in experimental asthma and we also show that female asthmatics taking an OCP have lower sputum GLUT-1 expression. GLUT-1 facilitates the transport of glucose into cells and is ubiquitously expressed in human tissues, including the lungs and immune system[43,44]. Proinflammatory immune responses associated with asthma require immune cells to rapidly adapt their metabolism to meet the energy demand required to switch from a state of naivety to an effector phenotype[24,45]. This metabolic shift is enabled by an upregulation of GLUT-1, which allows increased cellular glucose uptake and fuels downstream glycolysis[46,47]. Furthermore, this glycolytic switch promotes the synthesis of key nucleotide and fatty acid precursors that are essential for fuelling immune cell effector functions, including cytokine production and inflammatory cell proliferation[22,48,49]. To support a role for hormone-mediated effects on GLUT-1 in the pathogenesis of asthma, we show that inhibition of GLUT-1 in the airways reduces T2 cytokine responses, airway inflammation, and AHR in experimental asthma. We also show that inhibiting glycolysis, has similar protective effects to GLUT-1 inhibition, in experimental asthma, which suggests that glycolysis may be playing an important role in GLUT-1-mediated effects. Our findings agree with studies showing inhibition of GLUT-1 reduces airway inflammation and AHR in models of house dust mite/LPS and toluene disocyanate-induced experimental asthma [25,26], highlighting the role of GLUT-1 as a common target in several mechanisms in the development of asthma. Importantly, our interventional studies suggest that estrogen is upstream of/driving GLUT-1-mediated responses as we demonstrate that GLUT-1 inhibition protects against estrogen-associated worsening of experimental disease. To further interrogate the effects of hormones on inflammatory cell metabolism in the lungs, we established a single cell flow cytometric technique that is based on cellular puromycin incorporation, as a surrogate marker of energy metabolism/energy production, to assess metabolic activity in different populations of cells in the lungs[33] (Fig. A.11). Using this technique we show that estradiol treatment results in increased metabolism/energy production in neutrophils and, eosinophils (Fig. A.11) and that this increase in metabolism is largely dependent on glycolysis in neutrophils (as evidenced by inhibition of puromycin incorporation with 2-DG, but not oligomycin), whereas metabolism/energy production is largely dependent on oxidative phosphorylation downstream of glycolysis in the other cells (as evidenced by equal levels of inhibition of puromycin incorporation with 2-DG compared to oligomycin). Further studies are now required to dissect the effects of increased estrogen-mediated GLUT1 expression on the cellular metabolic pathways in different cell populations in the lungs, how these changes affect cell function and the role that these processes play in asthma pathogenesis. Together, our findings suggest that the OCP and female sex hormones may influence key features of asthma through GLUT-1-mediated pathways that are required to activate inflammatory responses.

Our investigations of sputum samples from the U-BIOPRED cohort show that GLUT-1 and ESR2 expression positively correlate with one another and are associated with increased disease severity in both females and males. We also show that increased GLUT-1 and ESR2 expression in asthma correspond with increased percentages of both sputum eosinophils and neutrophils, as well as both T2 (IL-5, IL-13) and non-T2 (NLRP3, IL-17A) responses. Importantly, we also show that the OCP use, which we show protects against key features of asthma, not only reduces GLUT-1, but also ESR2, gene expression in the airways of females with asthma. We, and others, have previously shown that severe forms of asthma are often associated with increased non-T2-mediated, neutrophilic inflammatory responses in the airways and that these responses play important roles in driving severe, corticosteroid-insensitive asthma[28,50–54]. Our latest data suggest that hormone-mediated GLUT-1 responses may play a role in mediating both T2 and non-T2 forms of severe disease. We have also previously shown a role for increased NLRP3 inflammasomes in severe disease[28,29] and our latest findings suggest that estrogen and GLUT-1 responses may play a role in promoting increased NLRP3 responses. In support of this, previous investigations have highlighted the role of estrogen response elements in promoting NLRP3 expression in mouse mast cells[55], and the importance of estrogen signalling through ERα versus ERβ in driving this NLRP3 activation[55,56]. We have previously demonstrated that the NLRP3 inflammasome plays a key role in driving obesity-associated severe, corticosteroid-insensitive asthma[29]. Here, we show that GLUT-1-expressing eosinophils and neutrophils are increased in lungs of both non-obese and obese mice with experimental asthma. Interestingly, we show that corticosteroid treatment increases GLUT-1-expressing macrophages in the airways in obesity-associated severe, corticosteroid-insensitive asthma. These findings highlight a potential role for GLUT-1-expressing macrophages, in obesity-associated corticosteroid-insensitive disease. Importantly, we show that OCP treatment reduces GLUT-1-expressing inflammatory cell numbers in both non-obese and obese mice with experimental asthma. Of note, OCP treatment reduces GLUT-1-expressing macrophages that are increased by corticosteroid treatment in obesity-associated, severe, corticosteroid-insensitive experimental asthma. Together these findings not only demonstrate a clear link between female sex hormone and GLUT-1 responses in mediating different T2 and non-T2 inflammatory responses in asthma, but also may play key roles in driving severe forms of disease. Importantly, our findings demonstrate the therapeutic potential for targeting hormone- and/or GLUT-1-mediated responses for the treatment of different phenotypes of severe asthma.

In conclusion, our clinical and experimental findings show a clear link between the OCP and female sex hormones, and GLUT-1 responses in the pathogenesis and severity of asthma. We also show that several GLUT-1-expressing cell populations are increased in experimental asthma and are suppressed by OCP treatment. Whilst a role for GLUT-1 in asthma and asthma severity has been described, the nature and reason for GLUT-1 being increased has not been well established. Furthermore, whilst a role of sex hormones has been identified in asthma, the mechanistic nature of the link between sex hormone levels and the pathogenesis and severity of disease is not well understood. In this manuscript we show that female sex hormones modify immunopathological and pathophysiological responses in the airways to influence AHR in the absence of asthma, and alter the phenotype, steroid responsiveness and severity of disease in the presence of asthma. We provide strong evidence, for the first time, that the effects of female sex hormones on airways disease and asthma are dependent on their effects on modulating GLUT-1 responses in cells and tissues in the airways. Whilst our data show that female sex hormones and/or the OCP alter the expression of GLUT-1 and/or number of GLUT-1^{+} cells in the lungs, the precise mechanisms that underpin how sex hormones differentially regulate GLUT-1 at the cellular and molecular level, and whether these effects are direct (e.g. directly altering expression in cells) or indirect (e.g. altering the infiltration of GLUT-1^{+} cells), in different populations of cells in the airways, is yet to be determined. Furthermore, whilst our studies have focussed on the role of GLUT-1 based on it being the major glucose transporter in immune cells, whether other GLUT family molecules are also involved requires further studies. Collectively, the findings of this research highlight the urgent need for prospective clinical investigations to identify how the OCP and different female sex hormones affect cellular function in different GLUT-1-expressing cells in non-obese and obese, females and males, with different phenotypes of non-severe and

severe asthma. Such studies will allow for a more comprehensive understanding of how GLUT-1 responses affect disease and will highlight how these factors can be harnessed therapeutically.

Funding

This work was supported by grants from the Priority Research Centre for Healthy Lungs, The University of Newcastle, and the Hunter Medical Research Institute. The U-BIOPRED (Unbiased Biomarkers for the Prediction of Respiratory Diseases Outcomes) cohort was established by financial contribution from the European Union's Seventh Framework Programme and participating EFPIA (European Federation of Pharmaceutical Industries Association) partners under the Innovative Medicines Initiative (IMI)

CRediT authorship contribution statement

Alexandra C. Brown: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. **Olivia R. Carroll:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Jemma R. Mayall:** Writing – review & editing, Validation, Methodology, Investigation. **Nazanin Zounemat-Kermani:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation. **Samantha L.E. Vinzenz:** Methodology, Investigation, Formal analysis. **Henry M. Gomez:** Methodology. **Ed F. Mills:** Investigation. **Richard Y. Kim:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Chantal Donovan:** Validation, Methodology, Investigation, Formal analysis. **Katherine J. Baines:** Validation, Methodology, Investigation, Formal analysis. **Evan J. Williams:** Validation, Methodology, Investigation, Formal analysis. **Bronwyn S. Berthon:** Methodology, Investigation, Formal analysis. **Katie Wynne:** Methodology. **Hayley A. Scott:** Supervision, Methodology, Investigation, Formal analysis. **James W. Pinkerton:** Methodology, Investigation. **Yike Guo:** Methodology, Investigation, Formal analysis. **Philip M. Hansbro:** Methodology, Investigation. **Paul S. Foster:** Methodology, Investigation. **Peter A.B. Wark:** Methodology, Investigation. **Sven-Erik Dahlen:** Methodology, Investigation. **Ian M. Adcock:** Validation, Supervision, Methodology, Investigation. **Lisa G. Wood:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Jay C. Horvat:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mucimm.2025.02.006.

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