The Journal of Allergy and Clinical Immunology

Relationship between type 2 cytokine and inflammasome responses in obesity-associated asthma

--Manuscript Draft--

Abstract:

Background: Obesity is a risk factor for asthma and obese asthmatics are more likely to have severe, steroid-insensitive disease. How obesity affects the pathogenesis and severity of asthma is poorly understood. Roles for increased inflammasome-mediated neutrophilic responses, type-2 immunity and eosinophilic inflammation have been described.

Objective: To investigate how obesity affects the pathogenesis and severity of asthma and identify effective therapies for obesity-associated disease.

Methods: We assessed associations between body mass index and inflammasome

responses with type-2 immune responses in the sputum of 25 subjects with asthma. Functional roles for NLRP3 inflammasome and type-2 cytokine responses in driving key features of disease were examined in experimental high fat diet-induced obesity and asthma.

Results: Body mass index and inflammasome responses positively correlate with increased IL-5 and IL-13 expression, and C-C chemokine receptor type 3 expression in the sputum of subjects with asthma. High fat diet-induced obesity results in steroid-insensitive airway hyper-responsiveness in both the presence and absence of experimental asthma. High fat diet-induced obesity is also associated with increased NLRP3 inflammasome responses and eosinophilic inflammation in airway tissue, but not the lumen in experimental asthma. Inhibition of NLRP3 inflammasome responses reduces steroid-insensitive airway hyper-responsiveness but has no effect on IL-5 or IL-13 responses in experimental asthma. Depletion of IL-5 and IL-13 reduces obesity-induced NLRP3 inflammasome responses and steroid-insensitive airway hyper-responsiveness in experimental asthma.

Conclusion: We show a relationship between type-2 cytokine and NLRP3 inflammasome responses in obesity-associated asthma, highlighting the potential utility of type-2 cytokine-targeted biologics and inflammasome inhibitors.

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Prof. Zuhair Ballas, Editor-in-Chief   21st September 2021
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Dear Professor Ballas,

We thank the Editors and Reviewers for their positive appraisal of our original manuscript JACI-D-21-00373 "Relationship between type 2 cytokine and inflammasome responses in obesity-associated asthma".

As suggested by Reviewer #2 we have changed all the relevant bar graphs to box and whisker plots and revised the associated figure legends to reflect these changes.

We have also revised the following based on the comments from the Editorial office:
  - Added a phone number in the Corresponding Author's contact information.
  - Added a Funding Statement after the Conflict-of-Interest statement.
  - Revised the Key Messages to be 48 words.
  - Unlinked Endnote references.
  - Updated the references to follow the standard JACI format.
  - Moved the Author Contributions section to the end of the manuscript after the Acknowledgements section.

We hope that these changes are appropriate and look forward to having our work published in *Journal of Allergy and Clinical Immunology.*

Kindest Regards,

Jay Horvat

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Relationship between type 2 cytokine and inflammasome responses in obesity-associated asthma

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This article has an online data supplement, which is accessible from this issue's table of content at the Online Repository at www.jacionline.org

50   **Abstract**

51   **Background:** Obesity is a risk factor for asthma and obese asthmatics are more likely to have

52   severe, steroid-insensitive disease. How obesity affects the pathogenesis and severity of asthma

53   is poorly understood. Roles for increased inflammasome-mediated neutrophilic responses,

54   type-2 immunity and eosinophilic inflammation have been described.

55   **Objective:** To investigate how obesity affects the pathogenesis and severity of asthma and

56   identify effective therapies for obesity-associated disease.

57   **Methods:** We assessed associations between body mass index and inflammasome responses

58   with type-2 immune responses in the sputum of 25 subjects with asthma. Functional roles for

59   NLRP3 inflammasome and type-2 cytokine responses in driving key features of disease were

60   examined in experimental high fat diet-induced obesity and asthma.

61   **Results:** Body mass index and inflammasome responses positively correlate with increased IL-

62   5 and IL-13 expression, and C-C chemokine receptor type 3 expression in the sputum of

63   subjects with asthma. High fat diet-induced obesity results in steroid-insensitive airway hyper-

64   responsiveness in both the presence and absence of experimental asthma. High fat diet-induced

65   obesity is also associated with increased NLRP3 inflammasome responses and eosinophilic

66   inflammation in airway tissue, but not the lumen in experimental asthma. Inhibition of NLRP3

67   inflammasome responses reduces steroid-insensitive airway hyper-responsiveness but has no

68   effect on IL-5 or IL-13 responses in experimental asthma. Depletion of IL-5 and IL-13 reduces

69   obesity-induced NLRP3 inflammasome responses and steroid-insensitive airway hyper-

70   responsiveness in experimental asthma.

71   **Conclusion:** We show a relationship between type-2 cytokine and NLRP3 inflammasome

72   responses in obesity-associated asthma, highlighting the potential utility of type-2 cytokine-

73   targeted biologics and inflammasome inhibitors.

74   **Abstract word length:** 250

Key messages:

- How obesity affects the pathogenesis and severity of asthma is poorly understood.

- Using clinical and experimental studies, we highlight a novel link between increased type-2 and NLRP3 inflammasome responses in the airways in obesity-associated severe asthma and the therapeutic potential of targeting type-2 cytokine and/or NLRP3 inflammasome responses.

Capsule summary:

Using a complementary combination of clinical and experimental studies, we show a relationship between type-2 cytokine and NLRP3 inflammasome responses in obesity-associated asthma and highlight the potential utility of type-2 cytokine-targeted biologics and inflammasome inhibitors.

Key Words: Asthma, Obesity, IL-5, IL-13, NLRP3 inflammasomes

Abbreviations:

AHR: Airway hyper-responsiveness

Alum: Aluminium hydroxide

BMI: Body mass index

CC: Control chow

CCR: Chemokine receptor type 3

DEX: Dexamethasone

FEV_1: Forced expiratory volume over one second

HFD: High fat diet

i.n.: Intranasal

100   i.p.: Intraperitoneal

101   Iso: Isotype

102   NOD: Nucleotide-binding oligomerization domain

103   NLR: NOD-like receptor

104   NLRP: NLR family, pyrin domain-containing

105   Ova: Ovalbumin

106   Sal: Saline

107

Introduction

Obesity is highly prevalent, affecting between 25-40% of the populations of the US, UK, and Australia (1). This high prevalence places a major burden on healthcare systems and is associated with many high burden diseases, such as cardiovascular disease and diabetes. Obesity is linked to the pathogenesis and/or increased severity of respiratory diseases, notably asthma. Obesity is associated with airway hyper-responsiveness (AHR) in some studies (2) and increases the risk of developing asthma, and asthma prevalence is higher in obese compared to lean individuals with the disparity greatest in women (3, 4). Increased weight gain and obesity precedes asthma development, particularly females, suggesting that these factors can play a causal role in disease pathogenesis (5-8). Importantly, studies show that obese asthmatics are more likely to have severe, steroid-insensitive disease and large multi-centre clustering analyses in both the US and Europe have identified a unique subtype of severe asthmatics that are obese and predominantly female (9-13). Collectively, these data suggest that obesity has roles in both the pathogenesis and increased severity of asthma, however, how obesity affects disease remains poorly understood. An improved understanding of the complex interactions that occur between obesity and inflammatory processes that underpin asthma is needed to enable the identification of effective therapies, particularly for obesity-associated, severe, steroid-insensitive forms of disease.

We recently showed that body mass index (BMI) correlates with increased expression of components of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family, pyrin domain-containing (NLRP)3 inflammasome (14), which is a multimeric protein complex that plays critical roles in innate immune signalling (15, 16) . Critically, we and others have shown that increased NLRP3 inflammasome responses have important roles in severe, neutrophilic steroid-insensitive asthma (15-18). Thus, increased BMI and NLRP3 inflammasome responses may drive neutrophil-enriched inflammation in the sputum as well as

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steroid-insensitivity in obesity-associated disease (19-21). It has also been shown that obesity is associated with increased type-2 immune responses and eosinophilic inflammation in the airway tissue (22-24). It is likely that these seemingly disparate findings reflect the complex nature of the associations between obesity in adult- *versus* early-onset, and atopic *versus* non-atopic asthma as well as differences in inflammatory responses in the airways tissue compared to sputum (23, 25).

Increasing the understanding of how obesity affects both NLRP3 inflammasome and type 2 responses in the airways in the absence and presence of asthma, and the role these responses play in disease pathogenesis and severity, may identify effective therapeutic strategies for obesity-associated, severe, steroid-insensitive asthma. In this study, we use a combination of clinical analyses and mouse models of obesity and experimental asthma to show a relationship between obesity-induced type-2 cytokine and NLRP3 inflammasome responses in the airways and that these may play a role the pathogenesis and severity of steroid-insensitive disease.

158　**Methods**

159　**Study Approvals**

160　All procedures were performed with approval from the University of Newcastle Human and

161　Animal Ethics committees.

162

163　**Human data: subject characterization and sputum collection and processing**

164　Baseline data was collected from 23 adults with stable asthma, who were participating in

165　dietary intervention trials (*see* **Table E1** in the online supplement) (14, 26, 27). Subjects were

166　recruited from ambulatory care clinics at John Hunter Hospital, Newcastle, Australia. Asthma

167　was defined by physician diagnosis. Stable asthma was defined as no exacerbation, respiratory

168　tract infection, or oral corticosteroid use in the past 4 weeks. Skin prick allergy tests determined

169　atopic status. Subjects fasted overnight, and asthma medications were withheld (short-acting

170　bronchodilators, 6 hours; long-acting bronchodilators and inhaled corticosteroids, 24 hours).

171　Blood was collected, and spirometry and sputum induction were performed during hypertonic

172　saline challenge. Lower respiratory tract sputum portions were selected and dispersed with

173　dithiothreitol (26, 27). Differential sputum cell counts, RNA extraction, reverse transcription

174　and gene expression were performed and analysed as previously described (14). Sputum

175　supernatant IL-1β concentrations were analysed by ELISA DuoSet® (R&D Systems,

176　Minneapolis, Minnesota, USA).

177

178　**Experimental studies; Murine models of high fat diet (HFD)-induced obesity and**

179　**experimental asthma with corticosteroid, NLRP3 inflammasome inhibitor and anti(α)-**

180　**IL-5 (α-IL-5) and α-IL-13 treatment; Assessment of adiposity, AHR, airway lumen and**

181　**tissue inflammatory cell and mucus secreting cell numbers, histopathology and gene**

182　**expression and protein levels in lung tissues.**

Murine models of experimental HFD-induced obesity and ovalbumin (Ova)-induced asthma were superimposed to investigate the impact of obesity on lung disease. Intranasal treatment with dexamethasone (DEX), MCC950, or α-IL-5 and α-IL-13 monoclonal antibodies was used to assess the effects of corticosteroids, NLRP3 inflammasomes and type-2 cytokine responses in obesity-induced disease, respectively. Airway inflammation, AHR, RNA and protein analyses, were determined as previously described and as in the online supplement (17, 28-30).

Statistics

Comparisons between two groups were performed using unpaired Student's t tests or a nonparametric equivalent as appropriate. Comparisons between multiple groups were performed using a one-way analysis of variance and an appropriate post-test or a nonparametric equivalent, as appropriate. Lung function data were assessed using a two-way analysis of variance with an appropriate post-test. Correlation analyses of sputum data were made using Spearman rank correlation. Analyses were performed using GraphPad Prism Software (San Diego, California, USA).

208 **Results**

209 **BMI and NLRP3 inflammasome/IL-1β responses correlate with type-2 immune responses**

210 **in human asthma**

211 To assess the clinical relationships between obesity, NLRP3 inflammasome and type-2

212 responses and extend our previous findings (14, 17), we correlated BMI and NLRP3/IL-1β

213 responses with the numbers of eosinophils and IL-5, IL-13 and C-C chemokine receptor type

214 (CCR)3 gene expression in the sputum of asthma patients (*see* **Table E1** in the online

215 supplement) (14). BMI positively correlates with the absolute numbers of sputum eosinophils

216 (r=0.44; p=0.06), and IL-5 (r=0.50; p=0.02), IL-13 (r=0.45; p=0.04) and CCR3 (r=0.53;

217 p=0.01) mRNA expression (**Figure 1A–D**). Absolute numbers of sputum eosinophils also

218 trended towards a statistically significant positive correlation with NLRP3 mRNA expression

219 (r=0.40; p=0.09) but not with IL-1β mRNA expression (r=0.30; p=0.21) (**Figure E1A and B**).

220 Furthermore, IL-5 mRNA expression positively correlates with NLRP3 (r=0.45; p=0.04) and

221 IL-1β (r=0.42; p=0.05) expression but not sputum IL-1β protein levels (r=0.43; p=0.11)

222 (**Figure E1C**), and IL-13 expression positively correlates with IL-1β protein levels (r=0.57;

223 p=0.04) (**Figure 1E–K**). CCR3 expression positively correlates with both NLRP3 (r=0.48;

224 p=0.02) (**Figure 1G**) and sputum IL-1β (r=0.55; p=0.04) protein levels (**Figure E1D**). These

225 data demonstrate potential clinical relationships between obesity, NLRP3 inflammasome and

226 type-2 responses in the airways of asthmatics and extend our previous findings that show roles

227 for NLRP3 inflammasomes in both severe, steroid-insensitive and obesity-associated asthma.

228

229 **HFD induces obesity**

230 We next established a murine model of HFD-induced obesity to determine functional

231 relationships between obesity, NLRP3 inflammasome and type-2 cytokine responses in

232 obesity-associated disease. BALB/c mice were fed a HFD or control chow (CC) diet for 13

233   weeks (*see* **Figure E2** in the online supplement). Mice fed a HFD have significant increases in

234   total body mass from weeks 3-13 compared to mice fed a CC diet (**Figure 2A and B**). This

235   involved substantial increases in the mass of parametrial (81.14% increase), inguinal (65.23%

236   increase) and retroperitoneal (127.50% increase) fat pads when compared to mice fed a CC

237   diet (**Figure 2C-E**).

238

239   **HFD-induced obesity promotes steroid-insensitive AHR**

240   We next examined the effects of HFD-induced obesity on airway inflammation and AHR in

241   the presence and absence of Ova-induced experimental asthma. Mice were fed a HFD or CC

242   diet and after 9-weeks were systemically sensitized to Ova by intraperitoneal (i.p.) injection of

243   Ova in aluminium hydroxide (alum) (*see* **Figure E2A** in the online supplement). On days

244   (d)12-13, and 33-34, mice were intranasally (i.n.) challenged with Ova to induce and

245   recapitulate experimental asthma (17). Non-allergic controls were sham-sensitized with an i.p.

246   injection of saline (Sal) and alum and were treated i.n. with Ova.

247   HFD-induced obesity had no significant effect on the numbers of total leukocytes,

248   macrophages, neutrophils or eosinophils in bronchoalveolar lavage fluid (BALF) in the

249   absence (CC/Sal *vs* HFD/Sal) or presence (CC/Ova *vs* HFD/Ova) of Ova-induced experimental

250   asthma (**Figure 2F-J**). Inflammatory cell numbers were also sensitive to i.n. treatment (d32–

251   34) with the corticosteroid dexamethasone (DEX) in both lean (CC/Ova *vs* CC/Ova/DEX) and

252   obese (HFD/Ova *vs* HFD/Ova/DEX) mice with Ova-induced experimental asthma.

253   Importantly, HFD-induced obesity induces AHR in the absence of experimental asthma

254   (CC/Sal *vs* HFD/Sal) and, unlike in lean mice (CC/Ova *vs* CC/Ova/DEX), AHR in obese mice

255   is not suppressed by DEX treatment in experimental asthma (HFD/Ova *vs* HFD/Ova/DEX;

256   **Figure 2K and L**). Similar effects of HFD-induced obesity were observed in terms of tissue

257   damping and elastance (**Figure E3**).

258   These data demonstrate that whilst our murine model of HFD-induced obesity does not

259   have a significant effect on the numbers of inflammatory cells in the airway lumen, obesity

260   alone induces AHR in the absence of experimental asthma and steroid-insensitive AHR when

261   superimposed with Ova-induced disease.

262

263   **Obesity increases tissue eosinophil numbers and NLRP3 inflammasome responses in the**

264   **absence and presence of experimental asthma**

265   We next examined inflammatory cell responses in the lung tissues of obese mice to understand

266   how obesity induces the effects observed on AHR (**Figure 2K and L**). HFD-induced obesity

267   increases the number of eosinophils in airway tissue in the absence (CC/Sal *vs* HFD/Sal) and

268   presence (CC/Ova *vs* HFD/Ova) of Ova-induced experimental asthma (**Figure 3A**). Whilst

269   obesity did not significantly increase the magnitude of inflammation in the lung tissues

270   (histopathology score) during experimental asthma (CC/Ova *vs* HFD/Ova), HFD-induced

271   obesity alone (CC/Sal *vs* HFD/Sal; Figure 3B) trended towards increasing inflammatory score

272   (p=0.074). We also show that whilst HFD-induced obesity did not affect the numbers of mucus

273   secreting cells in the airways in the absence or presence of experimental asthma compared to

274   CC diet-fed controls (**Figure 3C**), obese mice with Ova-induced experimental asthma had

275   increased lung *Muc5ac* expression, indicating increased mucus responses (**Figure 3D**).

276   We next investigated how obesity affects NLRP3 inflammasome responses by

277   assessing the levels of IL-1β, NLRP3 and active caspase-1 in lung tissues. HFD-induced

278   obesity increases IL-1β levels and NLRP3 staining in the lungs in the absence of Ova-induced

279   experimental asthma (CC/Sal *vs* HFD/Sal; **Figure 3E and H**). Interestingly, the levels of IL-

280   1β are lower, and NLRP3 staining similar, in HFD-fed obese mice with experimental asthma

281   compared to CC diet-fed controls (CC/Ova *vs* HFD/Ova; **Figure 3E, G and I**). However, most

282   importantly, we show that HFD-induced obesity increases the levels of active caspase-1 in lung

tissues, indicating that obesity increases inflammasome activation in the lungs in the absence

and presence of experimental asthma (**Figure 3J**).

These data show that whilst HFD-induced obesity induces subtly different effects in the

absence and presence of experimental asthma, obesity increases eosinophil numbers in the

airways tissue in association with increased IL-1β, NLRP3 and caspase-1 responses. This

indicates that obesity increases both eosinophilic inflammation and NLRP3 inflammasome

activity in the lung tissues irrespective of asthma status.

NLRP3 inflammasome inhibition suppresses obesity-induced, steroid-insensitive AHR

We next determined whether increased inflammasome responses have roles in obesity-induced,

steroid-insensitive AHR. HFD-fed mice with or without Ova-induced experimental asthma

were treated i.n. (d32-34) with the highly specific NLRP3 inflammasome inhibitor, MCC950

(HFD/Sal/MCC950, HFD/Ova/MCC950), or DEX (HFD/Sal/DEX, HFD/Ova/DEX; *see*

Figure E2B in the online supplement). The effects of treatment on airway inflammation and

AHR were assessed compared to CC diet-fed controls with and without DEX treatment. We

show that treatment with MCC950 reduced total leukocyte, lymphocyte, and neutrophil

numbers in BALF in obese mice with Ova-induced experimental asthma, compared to

untreated controls on a HFD (**Figure 4A-E**). MCC950 treatment had no statistically significant

effect on macrophage or eosinophil numbers in BALF in any of the groups with HFD-induced

obesity although there were trends to a reduction (**Figure 4B and E**). Importantly, we show

that treatment with the NLRP3 inflammasome inhibitor, MCC950, but not the corticosteroid,

DEX, completely suppresses AHR in the absence and presence of Ova-induced experimental

asthma in mice with HFD-induced obesity (**Figure 4F and G**). Similar effects of HFD-induced

obesity were observed in terms of tissue damping and elastance (**Figure E4**) and MCC950

treatment had similar suppressive effects on tissue damping in obese mice in the absence and

11

308 presence of Ova-induced experimental asthma. However, MCC950 treatment only suppressed

309 tissue elastance in obese mice in the absence of Ova-induced experimental asthma.

310

311 **Treatment with α-IL-5 and α-IL-13 suppresses obesity-induced, steroid-insensitive AHR**

312 **and NLRP3 inflammasome responses**

313 Our findings demonstrate that NLRP3 inflammasome responses play an important role in the

314 pathogenesis of steroid-insensitive AHR that is observed in obese mice. Interestingly, we show

315 that treatment with MCC950 has no suppressive effects on lung IL-5 or IL-13 protein levels

316 (**Figure 5A and B**). To determine whether a functional relationship exists between type-2

317 cytokine and NLRP3 inflammasome responses we next assessed the effects of IL-5 and IL-13

318 depletion on inflammation, AHR and NLRP3 inflammasome responses in HFD-induced

319 obesity. HFD-fed obese mice with and without Ova-induced experimental asthma were treated

320 i.n. with a combination of α-IL-5 and α-IL-13 (HFD/Sal/α-IL-5/α-IL-13, HFD/Ova/α-IL-5/α-

321 IL-13), or isotype control (Iso) monoclonal antibodies with or without DEX (HFD/Sal/Iso,

322 HFD/Sal/Iso/DEX,   HFD/Ova/Iso, HFD/Ova/Iso/DEX; *see* **Figure E2C** in the online

323 supplement) and the effects of treatment on airway inflammation and AHR assessed compared

324 to CC diet-fed controls with or without DEX treatment.

325   Treatment with α-IL-5 and α-IL-13 reduces total leukocyte, macrophage, lymphocyte,

326 neutrophil and eosinophil numbers in BALF in obese mice with experimental asthma (**Figure**

327 **5C-G**). Importantly, treatment with α-IL-5 and α-IL-13, but not the corticosteroid, DEX,

328 completely suppresses AHR that is induced in the absence and presence of Ova-induced

329 experimental asthma in mice with HFD-induced obesity (**Figure 5H and I**, and **Figure E5**).

330   To determine the effects of depletion of IL-5 and IL-13 on NLRP3 inflammasome

331 responses in obesity induced disease, we next assessed the effects of α-IL-5/α-IL-13 treatment

332 on NLRP3 levels in lung histological sections from HFD-fed obese mice with or without Ova-

induced experimental asthma. Significantly, α-IL-5/α-IL-13 treatment completely suppresses increased NLRP3-positive staining observed in lung tissues of obese mice with or without Ova-induced experimental asthma (**Figure 6A-G**). Interestingly, treatment with α-IL-5/α-IL-13, but not MCC950, suppresses airways eosinophils in HFD-fed obese mice in the absence or presence of experimental asthma (**Figure E6**). This is the first study to demonstrate functional links between type-2 cytokine and NLRP3 inflammasome responses in airways tissue.

To support our experimental findings, we interrogated data from the U-BIOPRED initiative and also show that IL-13 expression correlates with NLRP3 expression in bronchial and nasal brushings, and bronchial biopsies, in asthmatics (**Figure 6H-J**). Furthermore, IL-5 expression correlates with NLRP3 expression in nasal brushings, but not bronchial brushing or biopsies, in asthmatics.

Discussion

Extensive evidence shows that obesity has an important role in both the pathogenesis and severity of asthma and other respiratory diseases. This is likely due to the complex relationships between age, asthma and atopic status of the individual during which obesity occurs. The prevalence of asthma is increased in obese children and adults (31). Whilst it has been suggested that the increased prevalence of obesity in asthma patients may be due to reduced physical activity and/or other factors associated with asthma, studies also show that obesity often precedes asthma development (5-8). Obese asthmatics are more likely to be non-atopic and have more severe forms of disease suggesting that obesity promotes different phenotypes of asthma (13, 32, 33). Furthermore, bariatric surgery and weight loss improves respiratory symptoms in obese individuals (33-35). These findings suggest that obesity plays important roles in both the development and modification of asthma. In this study, we performed a series of clinical and experimental studies that examined the effects of obesity on immune responses in the lungs in the absence or presence of asthma to identify key drivers that link obesity and disease.

We previously reported associations between BMI and NLRP3 inflammasome responses and neutrophils in the sputum of asthmatics (14). Here, we extend these findings to show that BMI is also associated with increased type-2 cytokine expression, and trends towards correlation with the total numbers of eosinophils in the sputum of asthmatics. We also show a strong association between BMI and increased CCR3 expression. Since CCR3 is highly expressed by eosinophils, and since it has important roles in eosinophil chemoattraction, activation and mediator release, this finding supports a potential link between obesity and increased eosinophil infiltration and priming in the lungs (36, 37). We also show that NLRP3 and/or IL-1β responses are associated with increased type-2 cytokine and CCR3 expression, which highlights a potential link between type-2 immunity and NLRP3 inflammasome

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responses in obesity-associated asthma. Interestingly, we also show that IL-5 and IL-13 expression strongly correlate with NLRP3 expression in nasal brushings in a different cohort of asthma patients, and that IL-13 expression also correlates with NLRP3 expression in bronchial brushings and biopsies in this cohort. These data further support a relationship between type-2 immunity and inflammasome responses in lower and upper airway mucosa in asthma.

We next established a murine model of HFD-induced obesity in BALB/c mice to assess how obesity affects immune responses in the lung. We demonstrate that BALB/c mice fed a HFD display greater weight gain (>16%) associated with increased adiposity (>128% in retroorbital adipose tissue) compared to mice on a CC diet. Significantly, we show that HFD-induced obesity results in the development of AHR in the absence of experimental asthma, which further supports a role for obesity alone in inducing respiratory disease in the absence of allergic asthma (12, 13, 38, 39). We show that obesity-induced AHR is associated with increased NLRP3 inflammasome responses in the lungs of mice and that AHR is suppressed by intranasal administration of the NLRP3 inflammasome-specific inhibitor MCC950. Our findings are supported by a previous study that showed that obese mice develop spontaneous AHR in the absence of experimental asthma, which did not occur in NLRP3-deficient mice (40). Given that obesity is associated with increased inflammasome responses in the lung as well as adipose tissues and systemically, our findings provide new evidence that obesity-induced NLRP3 inflammasome responses in the airways play a critical role in disease pathogenesis (17, 39).

We also show that obesity-induced NLRP3 inflammasome and IL-1β responses are associated with concomitant increases in IL-5 and IL-13 protein levels in the lungs of mice. This supports our observation of a link between inflammasome and type-2 immune responses in the airways in obesity-associated disease in human subjects. Several studies report that the

407   NLRP3 inflammasome plays a critical role in the breaking of tolerance to antigen which is

408   required for the induction of allergic responses in murine models of experimental asthma (41,

409   42). Here, we show that MCC950 treatment has no effect on the levels of type-2 cytokines in

410   the lungs of mice, however, treatment with α-IL-5 and α-IL-13 completely ablates NLRP3-

411   positive staining in lung tissues. Our data suggest that type-2 responses can drive increased

412   inflammasome activation in the lung that promotes AHR. Interestingly, we show that MCC950,

413   which protects against obesity-associated steroid-insensitive AHR but does not suppress T2

414   cytokines, has no effect on obesity-associated increases in tissue eosinophil numbers (Figure

415   E6). Furthermore, treatment with α-IL-5 and α-IL-13, which decreases obesity-associated

416   NLRP3 responses and steroid-insensitive AHR, suppresses tissue eosinophil numbers in the

417   absence or presence of AAD (Figure E6). These data demonstrate that tissue accumulation of

418   eosinophils and/or the release of eosinophil-associated inflammatory mediators, as a feature of

419   increased T2 immune responses, are associated with increased T2 cytokine-induced NLRP3

420   responses, and that increased NLRP3 responses are not the driver of eosinophilic inflammation

421   in the airways tissue of obese mice. Furthermore, our data suggests that suppression of type-2

422   responses &/or inhibition of the NLRP3 inflammasome is sufficient to restore AHR to basal

423   levels, suggesting that AHR is primarily driven by aberrant immune responses rather than

424   altered lung mechanics associated with obesity (43).

425   Significantly, we show that obese mice have increased eosinophil numbers in the

426   airway tissue both in the absence or presence of experimental asthma. This agrees with clinical

427   data showing increased eosinophil numbers in the airway wall of obese individuals (12, 13,

428   33). Increased type-2 cytokine responses and eosinophilic inflammation in the airway tissue of

429   obese mice suggests that obesity may induce increased type-2 cytokine responses in the lung

430   that increases the homing of eosinophils to the airway tissues and that this can occur with or

431   without the presence of asthma. Our findings are supported by a recent study showing that

432 obesity promotes increased type-2 cytokine responses and eosinophilic inflammation in the

433 oesophagus in a murine model of eosinophilic oesophagitis (44). That study showed that

434 obesity alone was associated with increased eosinophilic inflammation in the lung and gut.

435 Thus, obesity may also play a role in the induction of type-2 immunity and eosinophilic

436 inflammatory responses in diseases of other mucosal sites.

437   Importantly, our findings provide insights into how obesity may modify asthma to

438 promote more severe forms of the disease. Severe, steroid-insensitive asthma is a heterogenous

439 disease with many phenotypes now recognized that are underpinned and/or associated with

440 different immunopathological processes. Large cohort clinical studies of adult asthmatics, such

441 as the European U-BIOPRED and the US SARP program, have stratified patients with

442 moderate to severe asthma based on clinico-physiologic parameters and tissue 'omics analyses

443 (12, 13). Both initiatives have identified unique severe asthma cohorts, which are associated

444 with obesity. Typically, these patients develop asthma in adulthood, are more likely to

445 experience more exacerbations and hospitalizations, and be on higher doses of inhaled steroids

446 (12, 13). However, the mechanisms that drive severe forms of obesity-associated asthma are

447 unclear. Previous studies utilized models of HFD-induced obesity to examine the effects on

448 airway disease (39, 45-49). Unfortunately, these studies did not examine the effect of obesity

449 on steroid responses in the airways, which is a critical factor in assessing severe asthma. In this

450 study, we show that obesity drives AHR that is steroid-insensitive in experimental asthma.

451 Interestingly, we also show that obesity does not affect intraluminal airway inflammatory cells

452 in Ova-induced experimental asthma, and that corticosteroid treatment does not affect AHR in

453 obese mice despite suppressing intraluminal inflammatory cell numbers in this T-helper type

454 2 (Th2) cell-mediated model. Together, these findings suggest that obesity likely drives innate

455 responses in the airways that are independent of the classical Th2 pathways that are associated

456 with atopic asthma. Importantly, we also show that increased NLRP3 inflammasome and/or

type-2 cytokine responses, that we and others have shown to be increased in obesity-associated severe disease (14, 23, 50), may be therapeutically targeted in the lung to suppress obesity-induced, steroid-insensitive disease. Whilst we show increased body weight and adiposity in our model of high fat diet-induced obesity, a limitation of this study is that we did not examine hyperglycemia and high cholesterol as other, common manifestations of obesity that are often observed in humans. Given the links between altered metabolism and regulation of immune responses, such indices and their role in driving increased T2 immunity and altered lung physiology would be interesting to follow up in these models in future studies.

In conclusion, we show that obesity increases NLRP3 inflammasome and type-2 cytokine responses in the lung and promotes steroid-insensitive AHR in both the absence and presence of experimental asthma. We also show that therapeutic targeting of either NLRP3 inflammasomes or type-2 cytokines can suppress obesity-induced, steroid-insensitive AHR. Importantly, we show that type-2 cytokine and NLRP3 inflammasome responses correlate with one another in the airways in clinical and experimental asthma, and that suppressing type-2 cytokine responses suppresses NLRP3 inflammasome responses in experimental disease. To our knowledge, these data are the first to provide a potential mechanistic link between increased type-2 and NLRP3 inflammasome responses that have been reported in obese asthmatics in the literature. Importantly, these data highlight the therapeutic potential of targeting type-2 cytokine and/or NLRP3 inflammasome responses in obesity-associated disease.

481 Author contributions: JWP, RYK, PMH & JCH wrote the manuscript and prepared the figures.

482 JWP, RYK, PMH & JCH conceived and designed the studies. JWP, RYK, CD, ACB, BER,

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484 HAS, BSB, KJB, PGG & LGW collected, analysed and validated clinical data and provided

485 intellectual input on obesity-associated asthma. IMA, YG, & NZK provided access to, and

486 analysis of, data collected as part of the U-BIOPRED Study Group. LAO, AABR & MAC

487 synthesised the NLRP3 inhibitor for *in vivo* experimental studies and provided intellectual

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491

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635

Figure 1. Type-2 immune responses correlate with body mass index (BMI) and nucleotide-binding oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3)/IL-1β responses in human asthma. (*A*) Sputum eosinophil absolute number (per mL), and sputum (mRNA) expression of (*B*) IL-5, (*C*) IL-13, and (*D*) C-C motif chemokine receptor 3 (CCR3), correlate with BMI (kg/m^2) in a population of subjects with stable asthma (*n*=23, described previously (14)). Sputum (mRNA) expression of NLRP3 correlated with that of (*E*) IL-5, (*F*) IL-13, and (*G*) CCR3. Sputum (mRNA) expression of IL-1β correlated with that of (*H*) IL-5, (*I*) IL-13, and (*J*) CCR3. Sputum (protein) levels of IL-1β correlated with sputum (mRNA) expression of (*K*) IL-13. Associations for each comparison are expressed as Spearman rank correlation coefficient (Spearman rho; *r*) with *p* values.

Figure 2. High fat diet (HFD) exposure induces obesity that promotes steroid-insensitive airway hyperresponsiveness (AHR). Wild-type female BALB/c mice were fed either a HFD or control chow (CC) diet for 13 weeks and (*A and B*) whole body mass was measured weekly. The mass of major white adipose pads ([*C*] parametrial, [*D*] inguinal, and [*E*] retroperitoneal) was determined at 13 weeks (2 experiments; *n*=24-40). Total leukocytes (*F*), macrophages (*G*), lymphocytes (*H*), neutrophils (*I*), and eosinophils (*J*) were enumerated in bronchoalveolar lavage fluid (BALF) on day 35 of the study protocol (*see* **Figure E2A** in the online supplement) in HFD- and CC-fed groups with ovalbumin (Ova)-induced experimental asthma with or without steroid (dexamethasone [DEX]) treatment compared to non-allergic controls (Sal) (2 experiments; *n*=6-12). AHR in terms of airway resistance in response to increasing doses of methacholine (Mch; *K*), and at the maximal dose of 10mg/mL Mch (*L*) was also determined in all groups on day 35 (≥2 experiments; *n*=10-21). Data in *A* and *K* are presented as means ± SEM. Data in *B-J* and *L* are presented as box (Q2 to Q3 with the median) and whisker (min to max). *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$. Rn = airway resistance.

661

Figure 3. High fat diet (HFD)-induced obesity increases tissue eosinophil numbers and nucleotide-binding oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3) inflammasome responses in the absence and presence of experimental asthma. (*A*) Airway basement membrane-associated eosinophils, (*B*) histopathological scores for gross tissue inflammation, and (*C*) mucus secreting cells (MSCs) in the airways, were enumerated on day 35 of the study protocol (*see* **Figure E2A** in the online supplement) in lung histological sections from HFD- and control chow (CC)-fed groups with ovalbumin (Ova)-induced experimental asthma, with or without steroid (dexamethasone, DEX) treatment compared to non-allergic controls (Sal). Lung (*D*) mRNA expression of *Muc5ac*, and (*E*) protein levels of IL-1β. (*F-I*) Representative photomicrographs of NLRP3 immunofluorescence (Alexa Fluor® 488 with Hoechst 33342 nuclear counterstain) in lung histology sections. (*J*) Lung protein levels of CASP1 (10kDa) normalized to β-actin (ACTB; 42kDa) were determined by quantification of immunoblot by densitometry and are expressed as fold change from CC/SAL from one experiment; (*n*=5-6). Data are presented as box (Q2 to Q3 with the median) and whisker (min to max). *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.

678

Figure 4. Nucleotide-binding oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3) inhibition suppresses obesity-induced, steroid-insensitive airway hyperresponsiveness (AHR). (*A*) Total leukocytes, (*B*) macrophages, (*C*) lymphocytes, (*D*) neutrophils, and (*E*) eosinophils were enumerated in bronchoalveolar lavage fluid (BALF) on day 35 of the study protocol (*see* **Figure E2B** in online supplement) in high fat diet (HFD)- and control chow (CC)-fed groups with ovalbumin (Ova)-induced experimental asthma, with or without steroid (dexamethasone, DEX or MCC950) treatment compared with

686 non-allergic controls (Sal). AHR in terms of airway resistance in response to increasing doses

687 of methacholine (Mch; *F*), and the maximal dose of 10mg/mL Mch (*G*). Data in *A-E* and *G* are

688 presented as box (Q2 to Q3 with the median) and whisker (min to max), and data in *F* is

689 presented as means ± SEM from ≥2 experiments (*n*=6-22). *$P<0.05$; **$P<0.01$; ***$P<0.001$;

690 ****$P<0.0001$. Rn = airway resistance.

691

692 **Figure 5. Treatment with anti(α)-IL-5/α-IL-13 suppresses obesity-induced, steroid-**

693 **insensitive airway hyperresponsiveness (AHR).** Lung (*A*) IL-5 and (*B*) IL-13 protein levels

694 were measured by ELISA on day 35 of the study protocol (*see* **Figure E2B** in the online

695 supplement) in high fat diet (HFD)- and control chow (CC)-fed groups with ovalbumin (Ova)-

696 induced experimental asthma, with or without MCC950 treatment compared to non-allergic

697 control subjects (Sal) (2 experiments; *n*=5-6). (*C*) Total leukocytes, (*D*) macrophages, (*E*)

698 lymphocytes, (*F*) neutrophils, and (*G*) eosinophils were enumerated in bronchoalveolar lavage

699 fluid (BALF) on day 35 of the study protocol (*see* **Figure E2C** in the online supplement) in

700 HFD- and CC-fed groups with Ova-induced experimental asthma, with or without steroid

701 (dexamethasone, DEX) or α-IL-5/α-IL-13 or isotype (Iso) antibody treatment compared to non-

702 allergic controls (Sal). AHR in terms of airway resistance in response to increasing doses of

703 methacholine (Mch; *H*), or at the maximal dose of 10mg/mL Mch (*I*). Data in *A-G* and *I* are

704 presented as box (Q2 to Q3 with the median) and whisker (min to max), and data in *H* is

705 presented as means ± SEM from 2 experiments (*n*=4-8). *$P<0.05$; **$P<0.01$; ***$P<0.001$;

706 ****$P<0.0001$. Rn = airway resistance.

707

708 **Figure 6. Treatment with anti(α)-IL-5/α-IL-13 suppresses nucleotide-binding**

709 **oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3)**

710 **inflammasome responses in obesity-induced experimental asthma, and type-2 cytokine**

711 **responses are associated with NLRP3 responses in the airways in human asthma.** (*A-F*)

712 Representative photomicrographs of NLRP3 immunofluorescence (IF; Alexa Fluor® 488 with

713 Hoechst 33342 nuclear counterstain) in lung histological sections on day 35 of the study

714 protocol (*see* **Figure E2C** in the online supplement) in high fat diet (HFD)- and control chow

715 (CC)-fed groups with ovalbumin (Ova)-induced experimental asthma, with or without α-IL-

716 5/α-IL-13 or isotype (Iso) antibody treatment compared to non-allergic controls (Sal). (*G*)

717 Quantification of NLRP3 IF (Integrated Density) in lung histological sections (representative

718 images in *A-F*; data are presented as box [Q2 to Q3 with the median] and whisker [min to

719 max]). Correlations between NLRP3 and IL-13 and IL-5 mRNA expression in (*H*) bronchial

720 and (*I*) nasal brushings, and (*J*) bronchial biopsies, in a population of subjects with stable

721 asthma (data collected as part of the U-BIOPRED Study).

1   **Relationship between type 2 cytokine and inflammasome responses in obesity-associated**

2   **asthma**

3

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Supplementary Methods

Ethics statement

All studies were performed in strict accordance with the Australian code of practice for the care and use of animals for scientific purposes issued by the National Health and Medical Research Council of Australia. All experiments were approved by the Animal Care and Ethics and Human Ethics Committees of the University of Newcastle, Australia.

Human data: characterization and sputum collection and processing

Baseline data was collected from 25 adults with stable asthma, who were participating in dietary intervention trials (*see* **Table E1** in the online supplement) (1-3). Subjects were recruited from ambulatory care clinics at John Hunter Hospital, Newcastle, Australia. Asthma was defined by clinical history and AHR to hypertonic saline (4.5%), defined as ≥15% decrease in FEV_1 from baseline. Stable asthma was defined as no exacerbation, respiratory tract infection, or oral corticosteroid use in the past 4 weeks. Skin prick allergy tests determined atopic status. Subjects fasted overnight, and asthma medications were withheld (short-acting bronchodilators, 6 hours; long-acting bronchodilators and inhaled corticosteroids, 24 hours).

Blood was collected, and spirometry and sputum induction were performed during hypertonic saline challenge. Lower respiratory tract sputum portions were selected and dispersed with dithiothreitol (1-3). Differential sputum cell counts, RNA extraction, reverse transcription and gene expression were performed and analysed as previously described (3). Sputum supernatant IL-1β concentrations were analysed by ELISA DuoSet® (R&D Systems, Minneapolis, Minnesota, USA).

Subject characteristics	All Subjects n=23	Non-Obese n=13	Obese n=10	P
Age years (range)[†]	50.3 (21-72)	37.6 (21-72)	52.9 (26-71)	0.292
Diagnosis age years (range)[†]	12 (0-58)	6 (0-12)	21.5 (2-58)	**0.011**
Weight kg[*]	85.2 ± 15.8	78.3 ± 14.9	94.3 ± 12.6	**0.013**
BMI kg/m^{2}[*]	29.4 ± 4.5	26.4 ± 2.7	33.3 ± 3.2	**<0.001**
Male n (%)	10 (43.5)	6 (46.2)	4 (40.0)	0.552
Female n (%)	13 (56.5)	7 (53.9)	6 (60.0)	
Atopic n (%)	20 (90.1) n=22	13 (100)	7 (77.9) n=9	0.156
Ex-smokers n (%)	4 (17.4)	2 (15.4)	2 (20.0)	0.596
Smoking history (pack years)[†]	2.1 (0.8, 6.5)	1.7 (0.4, 3.0)	5.6 (1.3, 10)	0.439
GINA patternˆ (1/2/3/4)	9/3/2/9	5/1/1/6	4/2/1/3	0.917
FEV$_1$ % predicted[*]	76.8 ± 22.2	76.1 ± 23.3	77.8 ± 22.0	0.861
FVC % predicted[*]	91.4 ± 16.7	93.0 ± 15.9	89.2 ± 18.3	0.605
FEV$_1$/FVC %[*]	66.8 ± 12.1	65.4 ± 12.9	68.7 ± 11.4	0.531
AHR n (%)	10 (43.5)	6 (46.2)	4 (40.0)	0.552
PD15‡ (mL)[†]	4.2 (0.2, 10.3)	1.0 (0.1, 3.8)	6.9 (1.9, 10.4)	0.394
DRS (%fall FEV$_1$/ mL saline)[†]	0.9 (0.2, 3.8)	0.9 (0.2,11.5)	0.9 (0.3, 1.7)	0.887
ACQ-7[†]	0.7 (0.4, 1.1)	0.9 (0.3, 1.0)	0.7 (0.6, 1.1)	0.576
Induced Sputum				
Total cell count (x10^6/mL)[†]	4.6 (3.4, 5.9)	3.7 (2.3, 5.1)	5.7 (3.9, 7.3)	0.078
Eosinophils %[†]	1.8 (0.8, 5.3)	1.5 (0.8, 6.3)	2.6 (0.8, 5.3)	0.671
Neutrophils %[†]	27.3 (19.0, 64.0)	20.8 (18.8, 74.8)	30.3 (23.3, 64.0)	1.000
Macrophages %[*]	58.0 (31.5, 70.8)	60.3 (21.0, 73.3)	55.8 (31.5, 70.8)	0.944
Asthma Medications				
SABA n (%)	21 (91.3)	11 (84.6)	10 (100)	0.308
Maintenance ICS n (%)	13 (56.5)	5 (38.5)	8 (80.0)	0.057
ICS dose§ (μg/day)[*]	981 ± 505	850 ± 224	1063 ± 623	0.484

BMI, body mass index; GINA, Global Initiative for Asthma; FEV_1, forced expiratory volume in 1 second; FVC, forced vital capacity; DRS, Dose response slope; ACQ, Asthma Control Questionnaire(4); SABA, short acting β^2-agonist; ICS, inhaled corticosteroids; *Data are normally distributed and presented as mean ± SD; †Data are non-parametric and presented as median (IQR); ^1=Intermittent/ 2=Mild/ 3=Moderate/ 4=Severe Persistent; ‡PD15, provocation dose resulting in 15% fall in baseline FEV_1. §Beclomethasone equivalents.

57 **Table E1. Subject Characteristics – patient sputum data**

58

59 **Murine model of high fat diet (HFD)-induced obesity**

60 Five-eight-week-old, specific pathogen-free (SPF), adult, female BALB/c mice were obtained

61 from the central animal house at the University of Newcastle, Newcastle, Australia. Three to

62 four mice were housed in individually ventilated cages under 12-hour light/dark cycling

63 conditions with food and water available *ad libitum.* Mice were either placed on a HFD (60%

64 energy derived from lipids, 15% energy from protein [SF14-154], Specialty Feeds, Glen

65 Forrest, Western Australia, Australia) or control chow (CC) diet (16% energy derived from

66 lipids, 21% energy from protein [SF09-091], Specialty Feeds, Australia) for 13 weeks (**Figure**

67 **E2**). Some groups of mice were weighed weekly throughout the course of the model to confirm

68 a HFD-induced increase in weight gain. Following euthanasia by intraperitoneal (i.p.) injection

69 with sodium pentobarbitone (60mg/kg) in 200μl phosphate buffered saline (PBS), perigonadal,

70 retroperitoneal and inguinal fat pads were collected and weighed to confirm HFD-induced

71 increase in weight and adiposity.

72

73 **Murine model of experimental asthma and intranasal treatment with corticosteroids,**

74 **inflammasome inhibitors or anti(α)-interleukin(IL)-5 and α-IL-13 monoclonal antibodies**

75 Following 9 weeks of HFD or CC diet, mice were randomly selected and administered with an

76 intraperitoneal (i.p.) injection of ovalbumin (Ova) and Rehydragel® (Ova; 50μg, Sigma-

77 Aldrich, Sydney, Australia and Rehydragel®; 1mg, Reheis, Berkeley Heights, New Jersey,

78 USA) in 200μl PBS in order to induce allergic sensitization to Ova. Mice were subsequently

79   challenged i.n. with Ova (10μg, 50μl PBS) under isoflurane anaesthesia (5% isoflurane,

80   2.5L/min O_2) on days 12 and 13 before being subsequently rechallenged i.n. on days 33 and 34

81   (10μg, 50μl PBS) to induce experimental asthma (**Figure E2**). Controls were mice on a HFD

82   or CC diet that were sham-sensitized with an i.p. injection of Rehydragel® (1mg, 200μl PBS).

83   Some groups of mice were also treated i.n. with the corticosteroid dexamethasone (Dex;

84   2mg/kg, 50μl PBS, Sigma-Aldrich, Sydney, Australia), the highly specific, NLRP3

85   inflammasome inhibitor, MCC950 (10mg/kg, 50μl PBS (5)), or a combination of α-IL-5 (Clone

86   TRFK5, 10μg, 50μl PBS, Bioxcell, Lebanon, New Hampshire, USA) & α-IL-13 (Clone 38213,

87   10μg, 50μl PBS, R&D Systems, Minneapolis, Minnesota, USA) on days 32-34 (**Figure E2**).

88   Controls for α-IL-5 or α-IL-13 treatments were administered with isotype control IgG1 (Clone

89   TNP6A7, 10μg, 50μl PBS, BioXcell, USA) and IgG2A (Clone 54447, 10μg, 50μl PBS, R&D

90   Systems, USA) respectively. Lung tissues were collected and all subsequent analyses

91   performed 24 hours after the final Ova challenge (day 35) in all treated and sham-treated groups

92   with and without experimental asthma fed a HFD or CC diet.

93

94   **Inflammatory cell numbers in airway lumen**

95   Bronchoalveolar lavage fluid (BALF) was collected by washing the lungs twice with 1ml of

96   Hanks buffered salt solution (HBSS; Life Technologies, Australia) *via* a cannula inserted into

97   the trachea. The volume of liquid collected was recorded and the BALF was centrifuged

98   (300xg, 10min, 4°C). Cell pellets were resuspended in red blood cell (RBC) lysis buffer (500μl;

99   Tris-buffered NH_4Cl), and centrifuged (300xg, 10min, 4°C) again before being resuspended in

100 HBSS (200μl). Total leukocyte numbers per ml of BALF were determined using trypan blue

101 dye staining of cell pellets enumerated in a haemocytometer (Improved Neubauer) under light

102 microscopy. Cells were cytocentrifuged (15xg, 10min, 25°C) onto a glass slide and stained

103 with May-Grunwald Giemsa. Differential immune cells were counted (≈175) using light

104 microscopy at 40x magnification based on key morphological characteristics (6-10). All

105 samples were coded and differential counts performed in a blinded manner.

106

107 **Airways hyper-responsiveness (AHR)**

108 AHR in terms of central airway resistance (Rn) in response to nebulised methacholine (MCh)

109 was measured using the FlexiVent apparatus (FX1 System; SCIREQ, Montreal, Canada) (6-

110 10). Mice were anaesthetised with a mixture of ketamine (100mg/kg, 200μl PBS, Parnell

111 Laboratories, Alexandria, New South Wales, Australia) and xylazine (10mg/kg, 200μl PBS,

112 Troy Laboratories, Smithfield, New South Wales, Australia). Following tracheostomy,

113 cannulae were inserted into their tracheas and ligated. Rn (tidal volume of 8mL/kg at a

114 respiratory rate of 450 breaths/min) was measured in response to increasing doses of nebulised

115 MCh (up to 10mg/kg, 15μl saline; Sigma-Aldrich, Sydney, Australia) and expressed as a

116 percentage change to saline nebulization (6-10).

117

118 **Lung tissue histopathological, eosinophil and mucus secreting cell analyses**

119 The whole left lungs were collected, formalin-fixed and embedded in paraffin. Lung sections

120 (4-6μm) were stained haematoxylin and eosin (for pathology score), chrome salt fixation (for

121 eosinophils) or periodic acid–Schiff (for mucus-secreting cells). Histopathology was assessed

122 at 40x magnification with set criteria as previously described (10-12). Numbers of eosinophils

123 and PAS positive cells (i.e. mucus secreting cells) were counted per 100μm around the airways

124 at 100x magnification as previously described (10-12).

125

126 **Lung tissue collection and RNA extraction for gene expression analyses**

127 Right lungs were excised and snap frozen before storage at -80˚C. Total RNA was isolated

128 using TRIzol® Reagent (ThermoFisher Scientific, Waltham, Massachusetts, USA) according

129   to the manufacturer's instructions. Whole lung tissues were homogenized on ice in TRIzol®

130   Reagent (~1ml) using a Tissue-Tearor™ (BioSpec Products, Bartlesville, Oklahoma, USA).

131   Phase separation was achieved through addition of chloroform (250µl) and incubation (10min,

132   25˚C) before centrifugation (15mins, 12,000xg, 4˚C). The aqueous phase containing nucleic

133   acids was carefully isolated without disrupting the interphase. Nucleic acids were then

134   precipitated with ice cold molecular grade isopropanol (500µl, 4˚C, Sigma-Aldrich, USA).

135   Samples were incubated (10min, 25˚C) before centrifugation (10min, 12,000xg, 4˚C). The

136   resultant RNA pellet was washed with 70% ethanol (1ml) and centrifuged (2x, 5min, 8,000xg,

137   4˚C), before air drying (30 min, 4˚C). The RNA pellet was then resuspended in nuclease-free

138   water (100µl, Thermo Fisher Scientific, USA). RNA purity and concentration was determined

139   using a Nanodrop® Spectrophotometer ND-1000 (Thermo Fisher Scientific, USA).

140   Phenol/carbohydrate contamination was assessed using the 260/230nm ratio ($\geq$ 2.0). Total

141   RNA concentration was quantified in ng/µl using the 260/280nm ratio ($\geq$ 1.8-2.0). Samples

142   meeting both requirements were stored at -80˚C for future use.

143

144   **Gene expression analyses**

145   RNA samples (8µl, 100ng/µl of RNA) from whole lung tissues were treated with DNase 1 Mix

146   (1µl of amplification grade DNase I and 1µl of 10x DNase Reaction Buffer, 15min, 25˚C,

147   Sigma-Aldrich, USA) to remove any potential DNA. Reactions were inactivated with DNase

148   Stop solution (1µl, 25mM EDTA, Sigma-Aldrich, USA). Reverse transcriptions were

149   performed in a Bio-Rad T100™ Thermal Cycler (Bio-Rad, Gladesville, NSW, Australia).

150   Samples were heated to remove secondary structures (10min, 65˚C) before addition of random

151   primers (2µl, hexamers at 50ng/µL, Bioline, Eveleigh, NSW, Australia) and dNTPs (1µl,

152   2.5mM, Bioline, Australia) and further incubated (5min, 65°C). Samples were then cooled and

153   incubated (10min, 25˚C) to facilitate annealing of primers to RNA. Samples were then

154   supplemented with BioScript™ Master Mix (5x Reaction Buffer, 4µl, 100mM dithiothreitol,

155   1µl, BioScript™ MMLV reverse transcriptase, 1µl, and nuclease-free water, 1µl) and further

156   incubated (10min, 25˚C). Reverse transcription was facilitated by incubation (60min, 42°C)

157   before the enzymatic reactions were terminated by heat-inactivated (15min, 70˚C). The

158   resulting cDNA was resuspended in nuclease-free water to a final volume of 40µl. SYBR-

159   based real-time qPCR was performed with a Mastercycler® ep realplex2 system (Eppendorf

160   South Pacific Pty. Ltd., NSW, Australia) and normalized against the housekeeping gene

161   hypoxanthine-guanine phosphoribosyltransferase (*Hprt*) to calculate relative expression of

162   *Muc5ac* using the formula $2^{-(Ct\ gene\ of\ interest\ -\ Ct\ Hprt)}$. For each reaction, cDNA (2µl) was combined

163   with SYBR Green Supermix (6.25µl; KAPA Biosystems, Inc., Massachusetts, USA), nuclease-

164   free   water   (3.25µl),   housekeeping   gene,   *Hprt*   (forward   5'-

165   AGGCCAGACTTTGTTGGATTTGAA-3';   reverse   5'-

166   CAACTTGCGCTCATCTTAGGCTTT-3'; 0.5µl each, 5µM) and *Muc5ac* (forward 5'-

167   GCAGTTGTGTCACCATCATCTGTG-3';   reverse   5'-

168   GGGGCAGTCTTGACTAACCCTCTT-3'; 0.5µl each, 5µM). Cycling conditions were as

169   follows: 50°C for 2min, 95°C for 2min, two-step cycle of 95°C and an optimal annealing

170   temperature for 40 cycles, with an 8min dissociation curve. Real-time qPCR Ct values were

171   generated using the CalQPlex algorithm (6-10).

172

173   **Lung tissue collection and processing for protein analyses**

174   Whole right lung tissues were placed into 1ml of PBS (for ELISA) or RIPA buffer (for

175   immunblot, Thermo Fisher Scientific, USA) supplemented with protease/phosphatase

176   inhibitors (10ml PBS or RIPA buffer supplemented with 1x tablet of complete protease

177   inhibitor cocktail and 1x tablet of PhoStop, phosphatase inhibitor, Roche, Dee Why, New South

178   Wales, Australia). Whole lungs were homogenized on ice using a Tissue-Tearor (BioSpec

179   Products, USA) and incubated (5min, 4°C). Homogenates were centrifuged (8000xg, 10min,

180   4°C) and supernatants collected for whole lung protein quantitation by BCA assay, ELISA or

181   immunoblot.

182

183   **BCA assay for whole lung protein quantification**

184   Whole lung protein isolates were quantitated using Pierce™ BCA Assay Kit (Thermo Fisher

185   Scientific, USA) as per the manufacturer's instructions. A standard curve using known

186   concentrations of BSA (2mg/ml-0.125mg/ml, Sigma-Aldrich, USA) was prepared to determine

187   the protein concentrations of samples. Lung protein isolates were diluted 1:4 with PBS or RIPA

188   buffer. BSA standards or protein isolates (25μl) were assayed in duplicate in a 96-well plates

189   (Corning Inc., Corning, New York, USA). BCA Reagent was added to each well and incubated

190   in the dark (200μl, 30min, 37°C). Following incubation, absorbance readings were determined

191   using a SpectraMax® M5 plate reader (570nm; Molecular Devices, San Hose, California,

192   USA).

193

194   **Quantification of cytokines in lung tissue homogenates by ELISA**

195   Whole lung IL-1β, IL-5 and IL-13 levels were measured using mouse DuoSet® ELISA

196   Development Systems (R&D Systems, USA) as per the manufacturer's instructions. Flat-

197   bottomed 384-well high-binding ELISA plates (Corning Inc., USA) were coated with capture

198   antibody in PBS (20μl, overnight, 25°C). Plates were washed 3x with PBS-Tween (PBS-T;

199   0.05% Ecoteric T20 in PBS, Ajax Finechem, NSW, Australia in PBS) and blocked in BSA (1%

200   BSA in PBS; 60μl, 120mins, 25°C). Plates were then washed 3x with PBS-T before whole lung

201   protein isolates (20μl) and known concentrations of standard in reagent diluent (20μl) were

202   added with incubation (120min, 25°C). Plates were washed 3x with PBS-T and coated with

203   detection antibody (20μl in reagent diluent, 60min, 25°C) and then washed 3x again with PBS-

204 T. Plates were then incubated with streptavidin-horseradish peroxidise (HRP) conjugate (1:40

205 dilution, 20μl in reagent diluent, 20min, 25°C, R&D Systems, USA) and washed 3x with PBS-

206 T followed by 2x with PBS. Colorimetric reactions were developed by incubating with

207 tetramethylbenzidine (20μl, 1mg/ml, 15mins, 25°C, Sigma-Aldrich, USA) and terminated by

208 the addition of sulphuric acid (50μl, 1M). Optical absorbances were read on a SpectraMax®

209 M5 plate reader (450nm). The concentrations of proteins were calculated by relating optical

210 densities of unknown samples to those of the known concentrations in the standard curve. Lung

211 protein concentrations were then normalized to total lung protein and expressed in pg

212 cytokine/mg lung protein (8).

213

214 **Immunofluorescence staining for NLRP3 in mouse lung tissues**

215 Formalin-fixed, paraffin-embedded lung sections (4-6μm thickness) were deparaffinised in

216 xylene, rehydrated in ethanol/water gradients and washed in PBS (8). Antigen retrieval was

217 performed using citrate-EDTA buffer (10mM citric acid, 2mM EDTA, 0.05% Ecoteric T20

218 [Ajax Finechem, NSW, Australia], pH 6.2). Sections were then washed 3x in PBS-T, dried and

219 blocked in a humidified chamber prior to immunostaining (Blocker™ Casein in PBS, 90min,

220 25°C; Life Technologies, Australia). Primary anti-NLRP3 goat polyclonal antibody was added

221 (ab4207; 90min, 37°C, Abcam, Massachusetts, USA) followed by 3x washes in PBS-T and

222 then the addition of secondary rabbit anti-goat IgG (ab150145; 90min, 25°C, Abcam, USA).

223 Slides were then washed 3x in PBS-T prior to the addition of the nuclear stain (Hoechst33342;

224 5min, 25°C, Life Technologies, Australia). Slides were further washed 3x in PBS-T and

225 mounted with aqueous mounting media (FluorSave™ 5 min, 25°C, EMD Millipore,

226 Massachusetts, USA). Slides were analyzed on an Olympus BX51 Fluorescent Microscope

227 with an Olympus DP73 (17.28 megapixel, 14-bit) digital color camera and a 40x Olympus

228 UPlanFl (numerical aperture 0.75) objective. Images were acquired with Image-Pro® Plus

229 software (Version 7.0.1.658; Media Cybernetics, Inc., MD, USA). Images of individual color

230 channels were then merged using ImageJ (National Institutes of Health, Bethesda, Maryland,

231 USA).

232

233 **Immunoblot analyses of pro- and active-caspase-1 in lung tissue protein**

234 Whole lung protein was diluted 1:4 in 4x sodium dodecyl sulphate (SDS; BDH Laboratory

235 Supplies, Dubai, UAE) sample loading buffer (60mM Tris pH 6.8, 25% glycerol, 2% SDS,

236 375mM dithiothreitol and 0.1% bromophenol blue), boiled (5 min, 95°C) and cooled on ice

237 (4°C). Protein from lung homogenates (30μg) were run through 4% stacking gels (4%

238 Bis/Acrylamide, 375mM Tris [pH 6.8], 0.1% SDS, 0.1% TEMED, 1% ammonium persulphate

239 [APS], Bio-Rad, Gladesville, New South Wales, Australia), and separated using 15% SDS-

240 polyacrylamide resolving gels (15% Bis/Acrylamide, 375mM Tris [pH 8.8], 0.1% SDS, 0.05%

241 tetramethylethylenediamine, 0.05% APS, Bio-Rad, Gladesville, New South Wales, Australia).

242 Precision Plus™ WesternC™ protein standard (10μl, 30% (w/v) glycerol, 2% SDS, 62.5mM

243 Tris, pH 6.8, 50mM DTT, 5mM EDTA, 0.02% NaN$_3$, 0.01% Bromophenol Blue, Bio-Rad,

244 Australia) was used as a molecular weight marker. SDS-PAGE gels were loaded into tanks

245 filled with electrophoresis running buffer (25mM Tris, 191mM glycine, 0.1% SDS) and

246 electrophoresed (3hr, 90V). Proteins were transferred from gels onto Immobilon®-P PVDF

247 nitrocellulose membranes (2hr, 90V; 0.45μm pore size, Millipore, USA) in methanol transfer

248 buffer (25mM Tris, 190mM glycine, 20% methanol, 4°C). Membranes were stained with

249 Ponceau S (0.1% Ponceau S in 5% acetic acid) and then blocked with 5% BSA (5% BSA in

250 TBS-T; 1mM Tris pH 8, 150mM NaCl, 0.05% Ecoteric T-20, overnight, 4°C). Following

251 blocking, membranes were washed 3x with TBS-T (10min) and immunoblotted with the anti-

252 Caspase-1 primary antibody (overnight, 4°C; sc514, Santa-Cruz, Texas, USA). Membranes

253 were washed 3x with TBS-T (10min) and incubated in anti-rabbit secondary polyclonal

254 antibody (#HAF008; 120min, 25°C, R&D Systems, USA). Following a final 3x washes in

255 TBS-T a protein detection assay was performed using an ECL kit as per manufacturer's

256 instructions (SuperSignal West Femto Maximum Sensitivity Subtrate, 2min, 25°C, Thermo

257 Fisher Scientific, USA) and bands were visualized using a ChemiDoc MP System (Bio-Rad,

258 Australia) (7).

259

260 **Statistics**

261 Comparisons between two groups were performed using unpaired Student's t tests or a

262 nonparametric equivalent as appropriate. Comparisons between multiple groups were

263 performed using a one-way analysis of variance and an appropriate post-test or a nonparametric

264 equivalent, as appropriate. Lung function data were assessed using a two-way analysis of

265 variance with an appropriate post-test. Correlation analyses of sputum data were made using

266 Spearman rank correlation. Analyses were performed using GraphPad Prism Software (San

267 Diego, California, USA).

268

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307

308

309 **Figure E1.** Type-2 immune responses correlated with IL-1β and nucleotide-binding

310 oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3) responses

311 in human asthma. In a population of subjects with stable asthma (*n*=23, described previously

312 (14)): Sputum eosinophil absolute number (per mL) correlated with sputum (mRNA)

313 expression of (*A*) NLRP3, and (*B*) IL-1β. Sputum (mRNA) expression of (*C*) IL-5 and (*D*)

314 CCR3 correlated with sputum (protein) levels of IL-1β. Associations for each comparison are

315 expressed as Spearman rank correlation coefficient (Spearman rho; *r*) with *p* values.

316

317 **Figure E2.** Murine models of high-fat diet (HFD)-induced obesity, ovalbumin (Ova)-induced

318 experimental asthma and treatments with dexamethasone (DEX), MCC950 and anti(α)-IL-5

319 and α-IL-13. Mice were fed a HFD or control chow (CC). After 9 weeks (d0) mice were

320 injected (intraperitoneally, i.p.) with ovalbumin (Ova) and Rehydragel® and challenged

321 (intranasally, i.n.) with Ova on days 12 and 13 and 33 and 34 to induce experimental asthma.

322 Some groups of mice were also treated (i.n.) with (*A*) DEX alone, (*B*) the NLRP3

323 inflammasome inhibitor, MCC950 or (*C*) a α-IL-5 and α-IL-13 on days 32-34. Tissues were

324 collected and analyses performed on d35.

325

326 **Figure E3. High fat diet (HFD) exposure induces obesity that promotes steroid-insensitive**

327 **airway hyperresponsiveness (AHR).** Wild-type female BALB/c mice were fed either a HFD

328 or control chow (CC) diet for 13 weeks and AHR assessed on day 35 of the study protocol (*see*

329 **Figure E2A** in the online supplement) in HFD- and CC-fed groups with ovalbumin (Ova)-

330 induced experimental asthma with or without steroid (dexamethasone [DEX]) treatment

331 compared to non-allergic controls (Sal). AHR in terms of tissue (*A* and *B*) damping and (*C* and

332 *D*) elastance in response to increasing doses of methacholine (Mch; *A* and *C*), and at the

333 maximal dose of 10mg/mL Mch (*B* and *D*) was also determined in all groups on day 35 (≥ 2

334 experiments; *n*=10-21). Data in *A* and *C* are presented as means ± SEM. Data in *B* and *D* are

335 presented as box (Q2 to Q3 with the median) and whisker (min to max). *$P<0.05$;

336 ****$P<0.0001$. G = tissue damping, H = tissue elastance.

337

338 **Figure E4. Nucleotide-binding oligomerization domain–like receptor family, pyrin**

339 **domain–containing 3 (NLRP3) inhibition suppresses obesity-induced, steroid-insensitive**

340 **airway hyperresponsiveness (AHR).** AHR was assessed on day 35 of the study protocol (*see*

341 **Figure E2B** in online supplement) in high fat diet (HFD)- and control chow (CC)-fed groups

342 with ovalbumin (Ova)-induced experimental asthma, with or without steroid (dexamethasone

343 [DEX] or MCC950) treatment compared with non-allergic controls (Sal). AHR in terms of

344 tissue (*A* and *B*) damping and (*C* and *D*) elastance in response to increasing doses of

345 methacholine (Mch; *A* and *C*), and at the maximal dose of 10mg/mL Mch (*B* and *D*) was also

346 determined in all groups on day 35 (≥ 2 experiments; *n*=6-22). Data in *A* and *C* are presented

347 as means ± SEM. Data in *B* and *D* are presented as box (Q2 to Q3 with the median) and whisker

348 (min to max). ***$P<0.001$; ****$P<0.0001$. G = tissue damping, H = tissue elastance.

349

350 **Figure E5. Treatment with anti(α)-IL-5/α-IL-13 suppresses obesity-induced, steroid-**

351 **insensitive airway hyperresponsiveness (AHR).** AHR was assessed on day 35 of the study

352   protocol (*see* **Figure E2B** in the online supplement) in high fat diet (HFD)- and control chow

353   (CC)-fed groups with ovalbumin (Ova)-induced experimental asthma, with or without steroid

354   (dexamethasone [DEX]) or α-IL-5/α-IL-13 or isotype (Iso) antibody treatment compared to

355   non-allergic controls (Sal). AHR in terms of tissue (*A* and *B*) damping and (*C* and *D*) elastance

356   in response to increasing doses of methacholine (Mch; *A* and *C*), and at the maximal dose of

357   10mg/mL Mch (*B* and *D*) was also determined in all groups on day 35 (2 experiments; *n*=4-8).

358   Data in *A* and *C* are presented as means ± SEM. Data in *B* and *D* are presented as box (Q2 to

359   Q3 with the median) and whisker (min to max). ****$P<0.0001$. G = tissue damping, H = tissue

360   elastance.

361

362   **Figure E6. Treatment with anti(α)-IL-5/α-IL-13, but not MCC950, suppresses airways**

363   **eosinophils in high fat diet (HFD)-fed obese mice in the absence or presence of**

364   **experimental asthma.** (*A*) Airway basement membrane-associated eosinophils were

365   enumerated on day 35 of the study protocol (*see* **Figure E2** in the online supplement) in lung

366   histological sections from HFD- and control chow (CC)-fed groups with ovalbumin (Ova)-

367   induced experimental asthma, with or without MCC950 or α-IL-5/α-IL-13 or isotype (Iso)

368   antibody treatment compared to non-allergic controls (Sal). Data are presented as box (Q2 to

369   Q3 with the median) and whisker (min to max) from ≥2 experiments (*n*=5-7). *$P<0.05$;
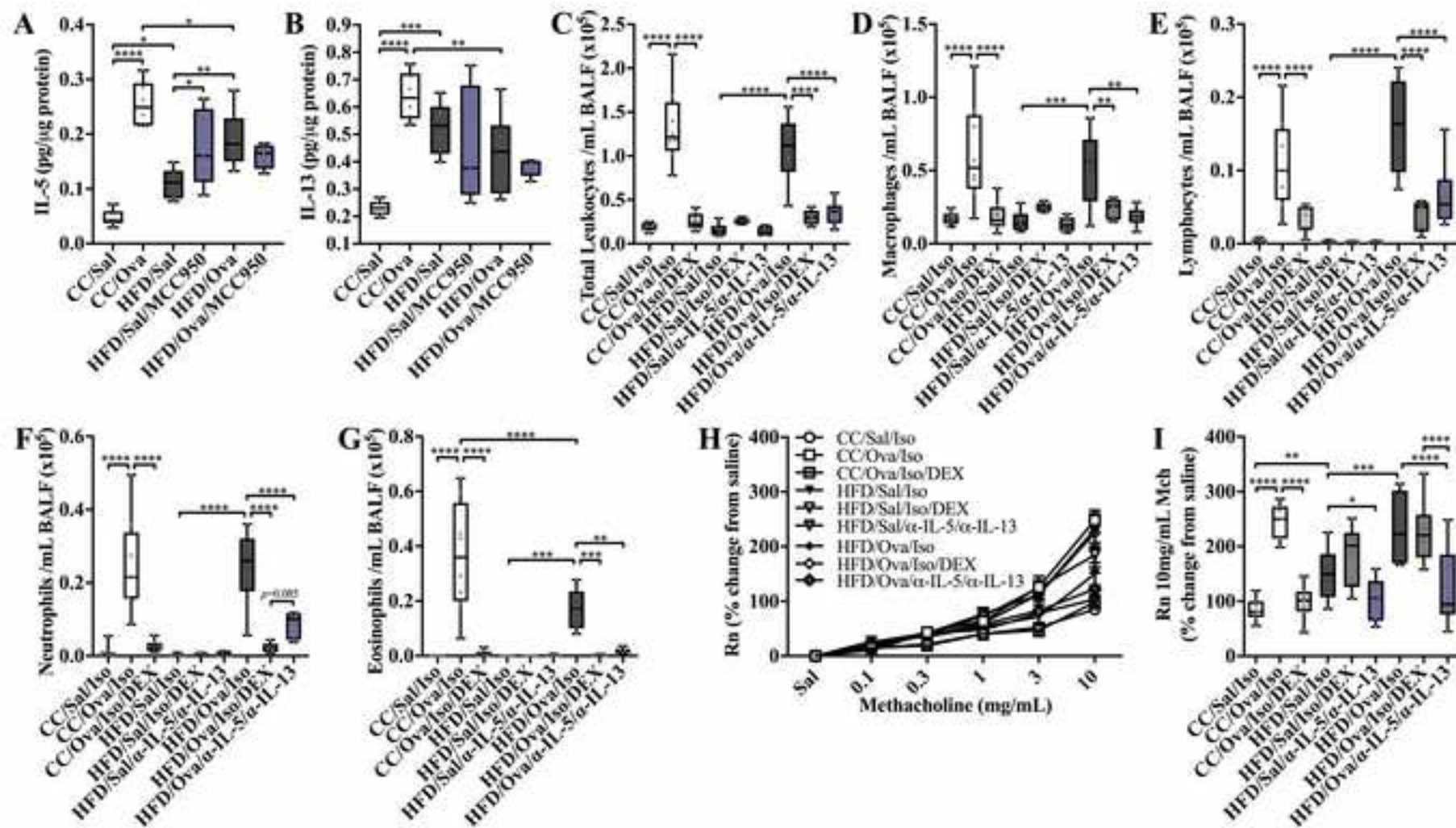
370   **$P<0.01$; ****$P<0.0001$.

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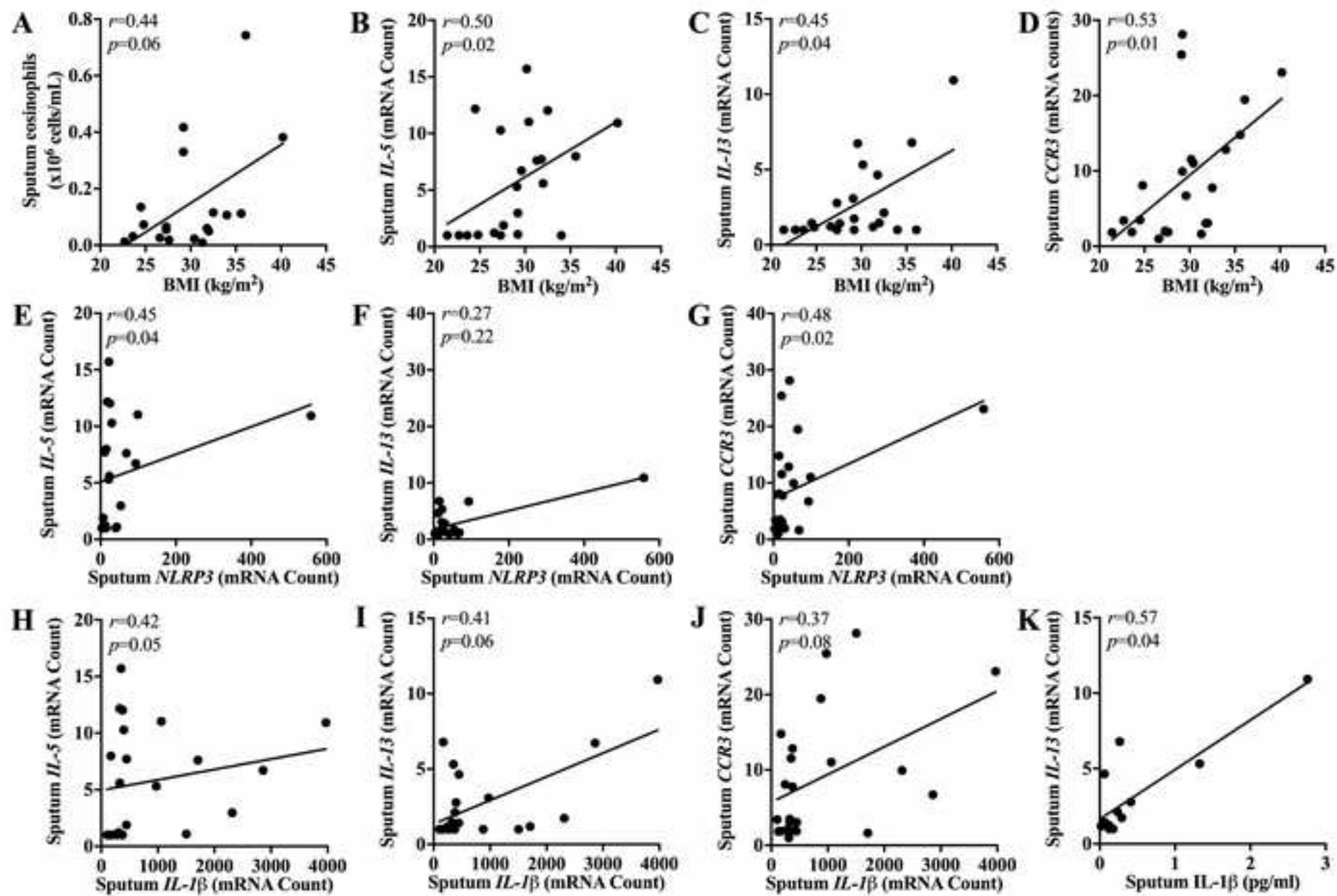
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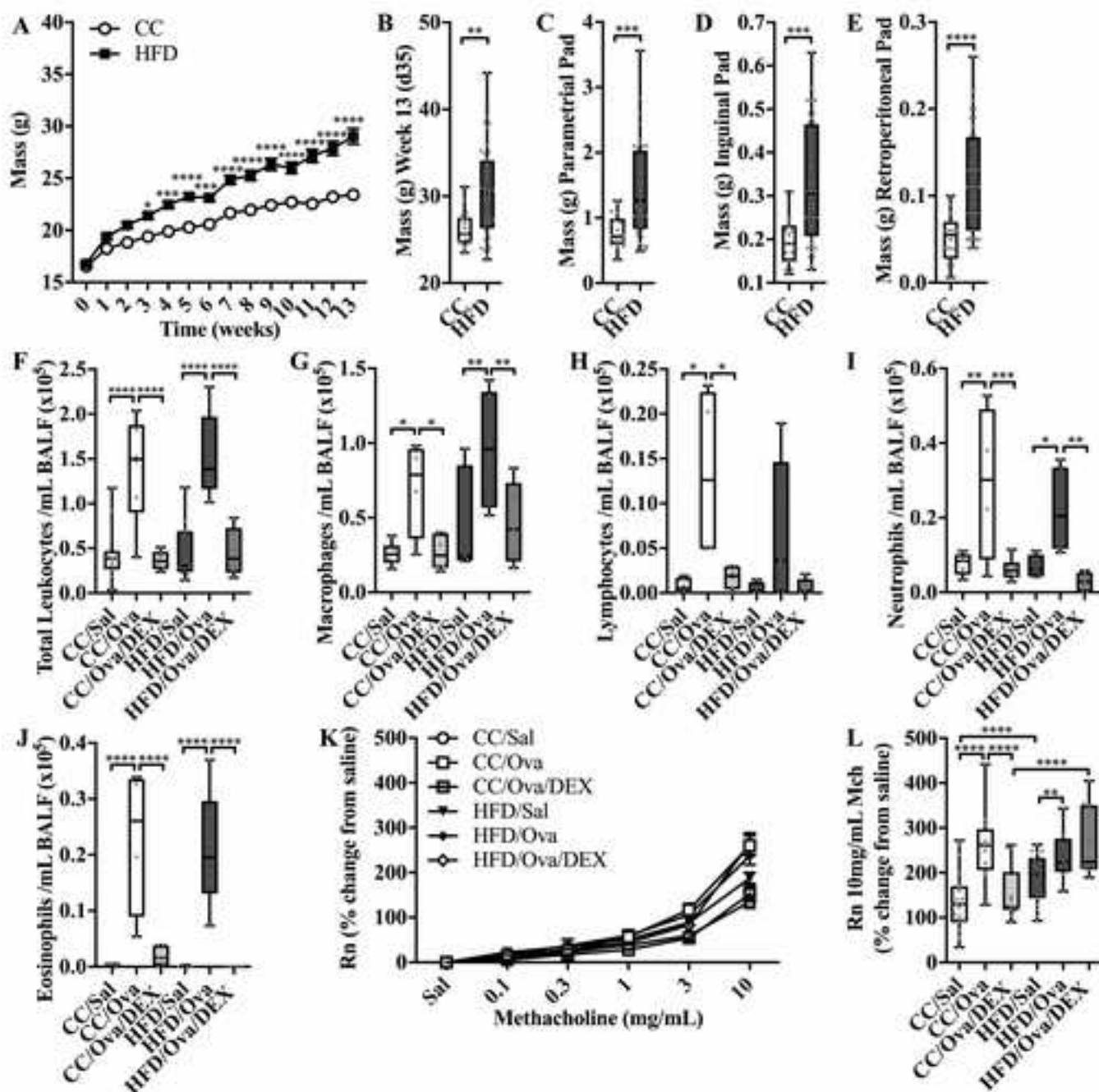
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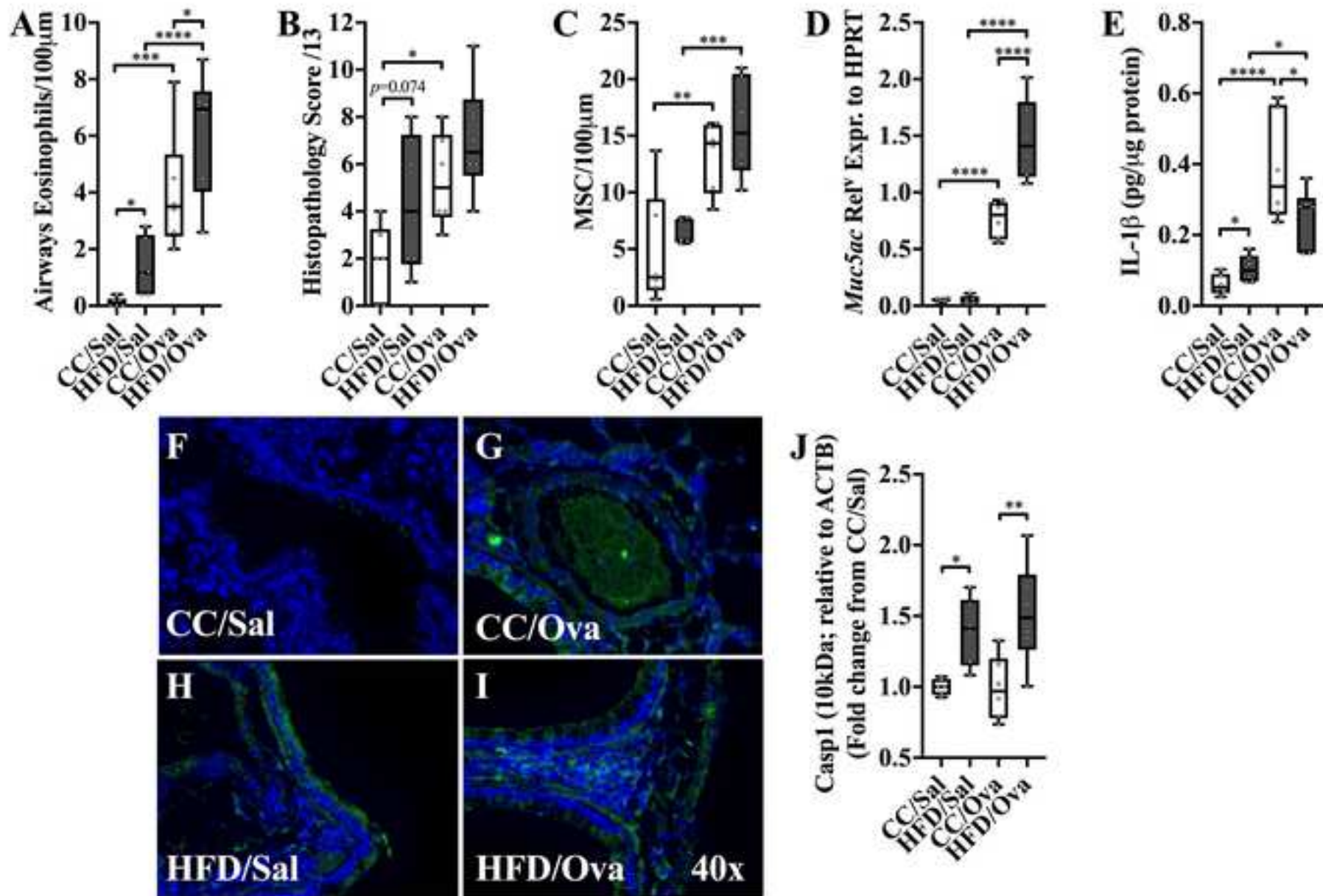
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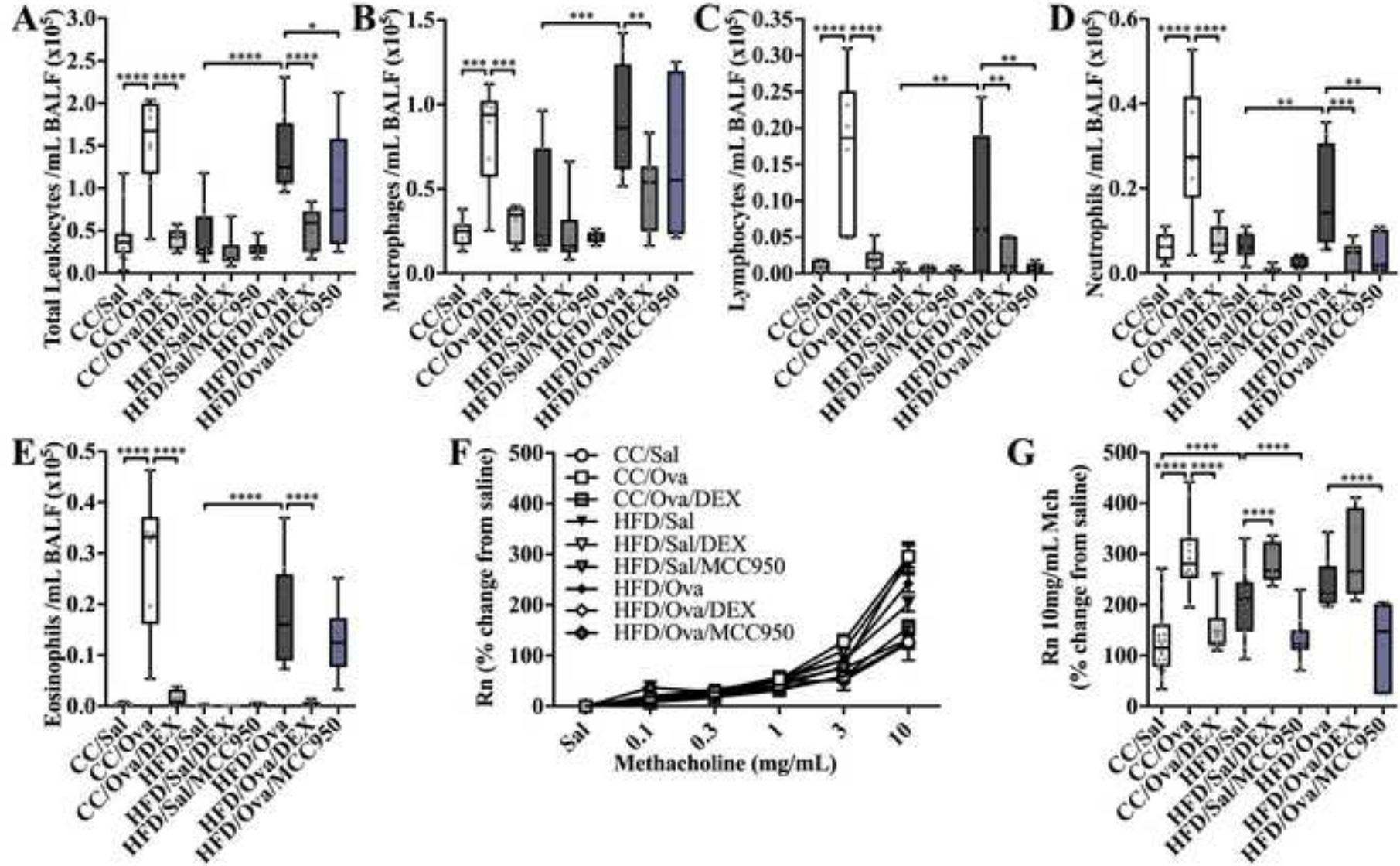
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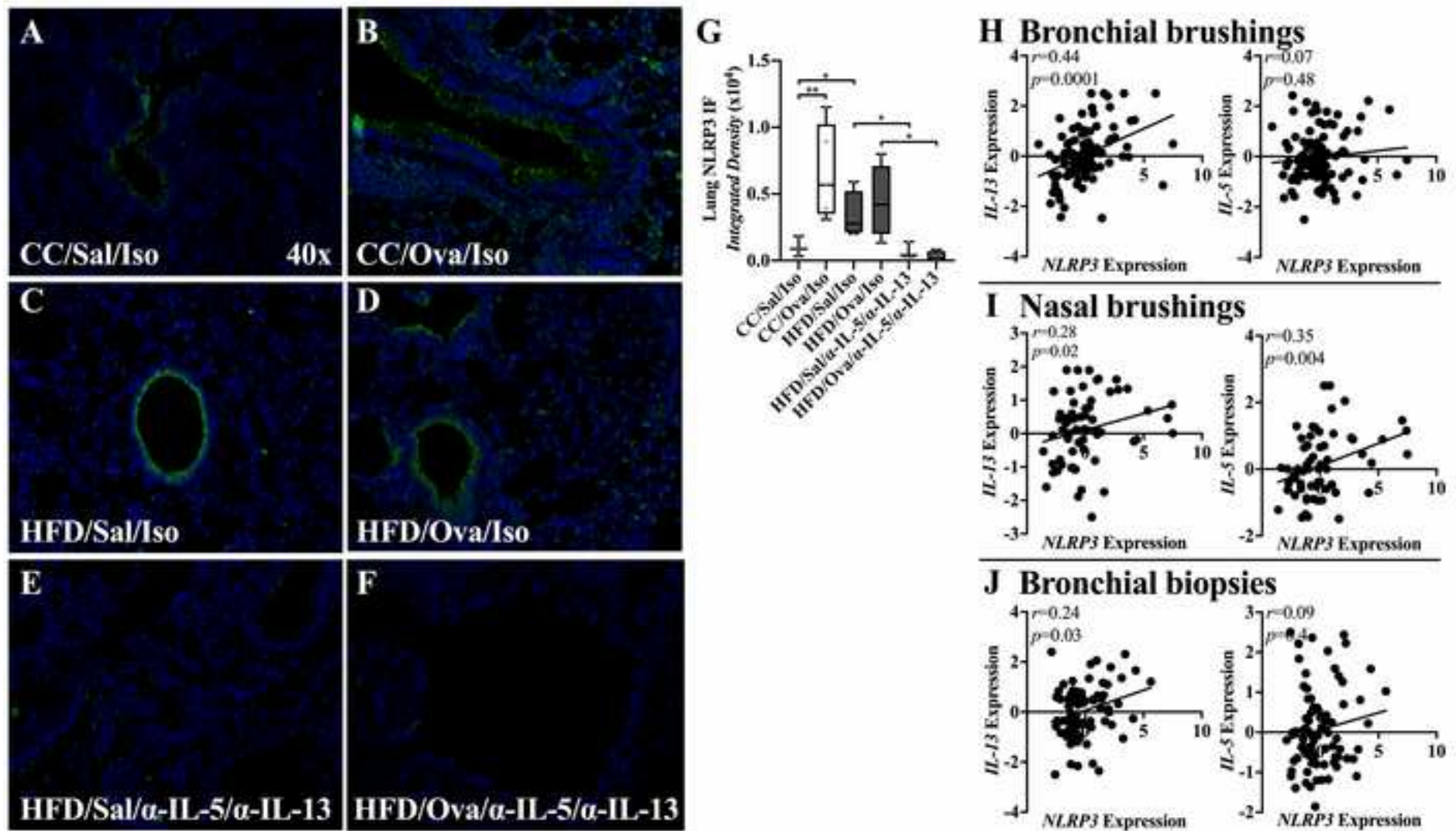
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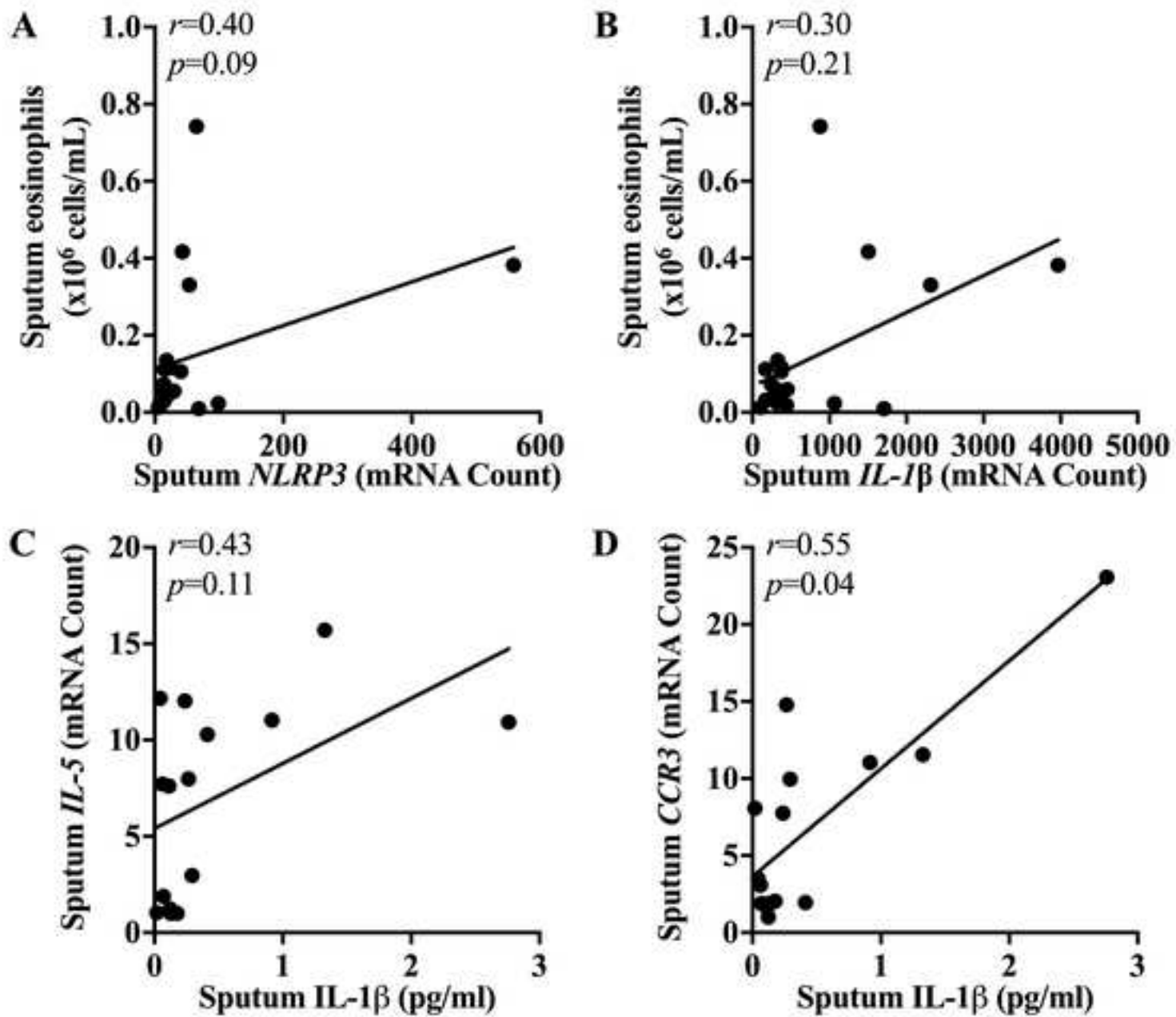
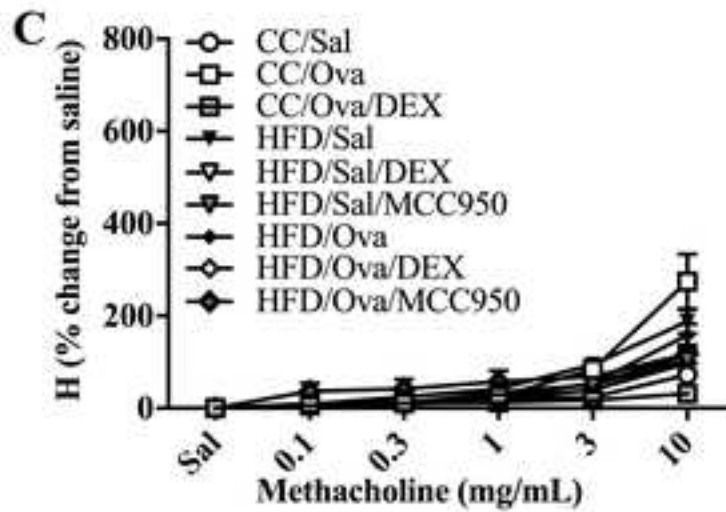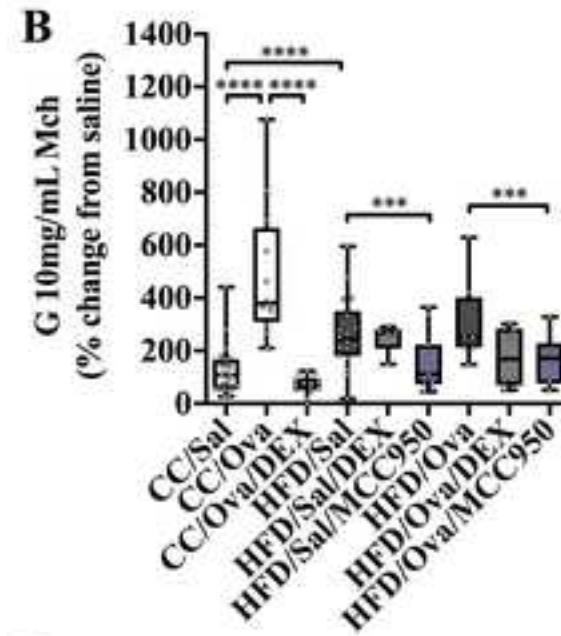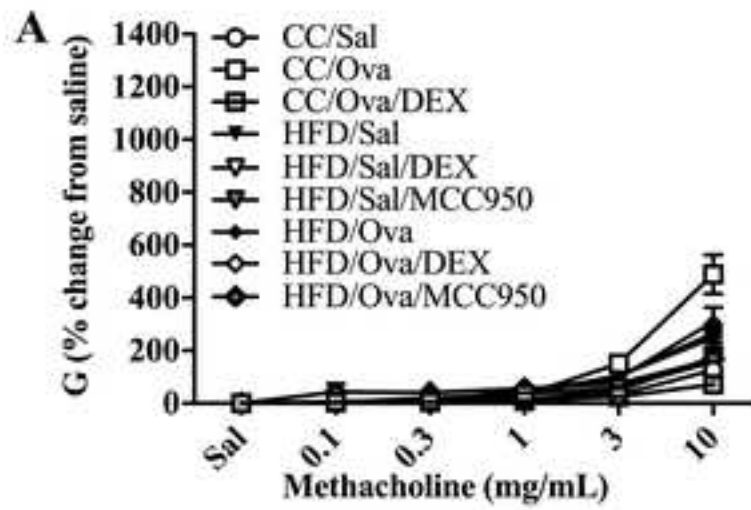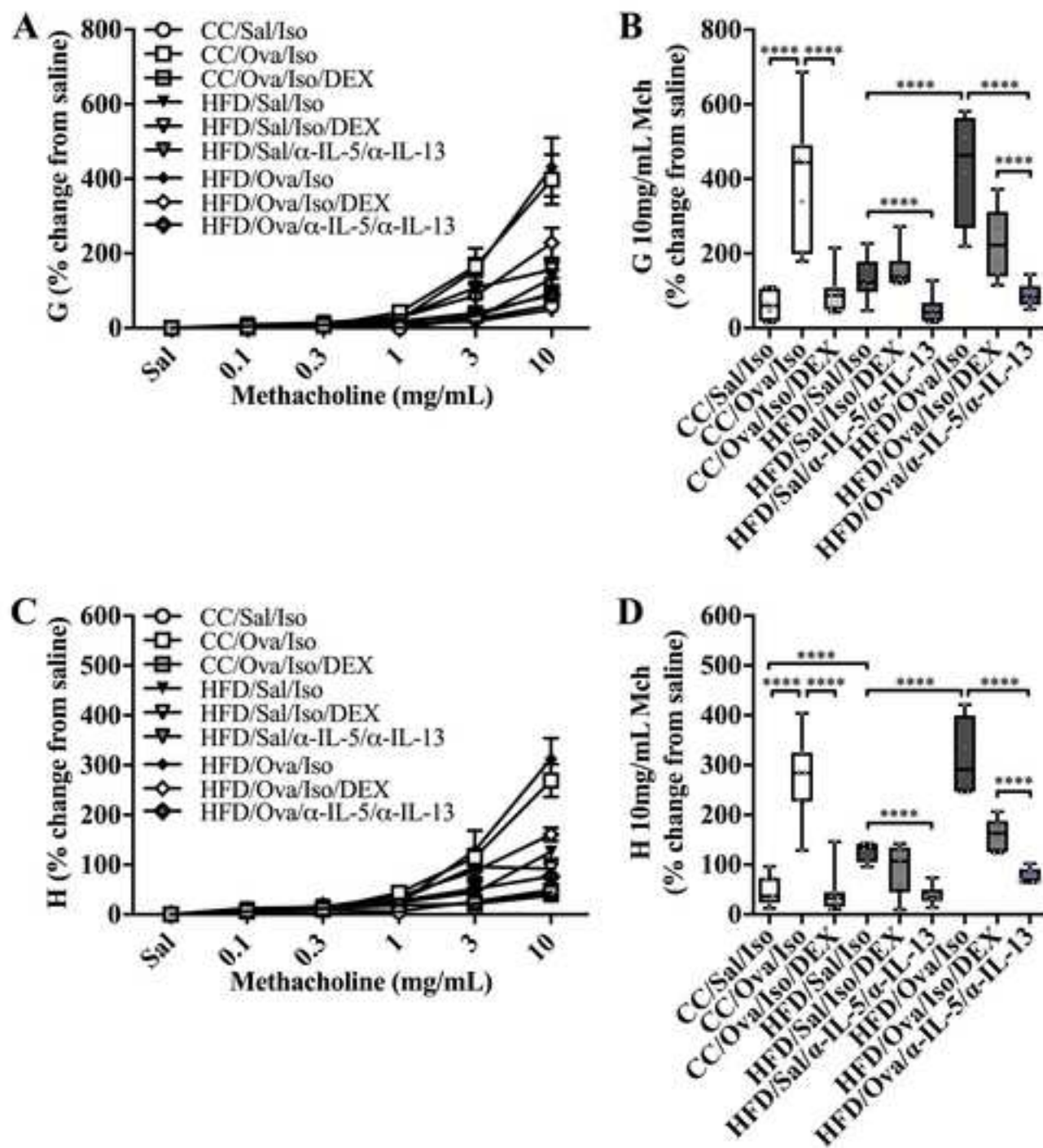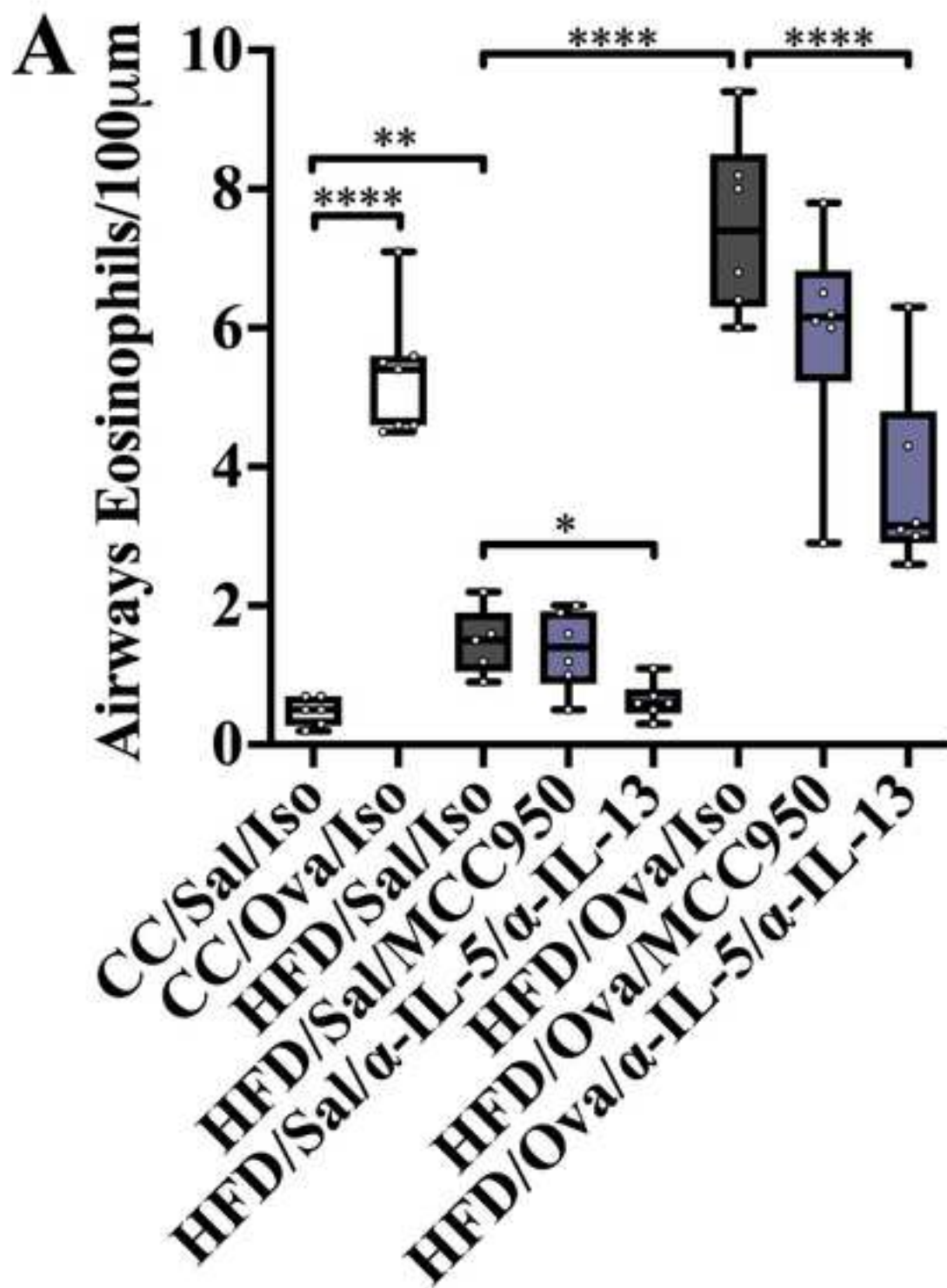Supplementary Figure 1.
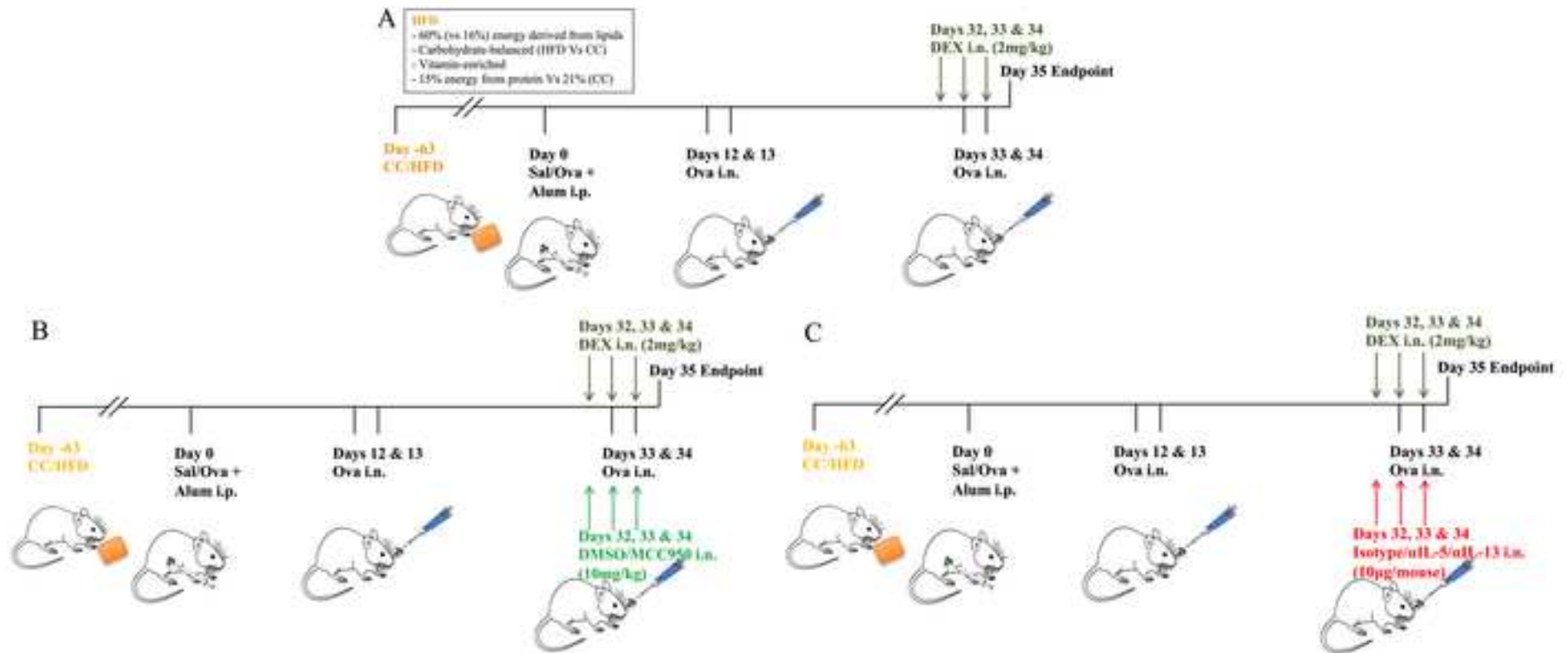
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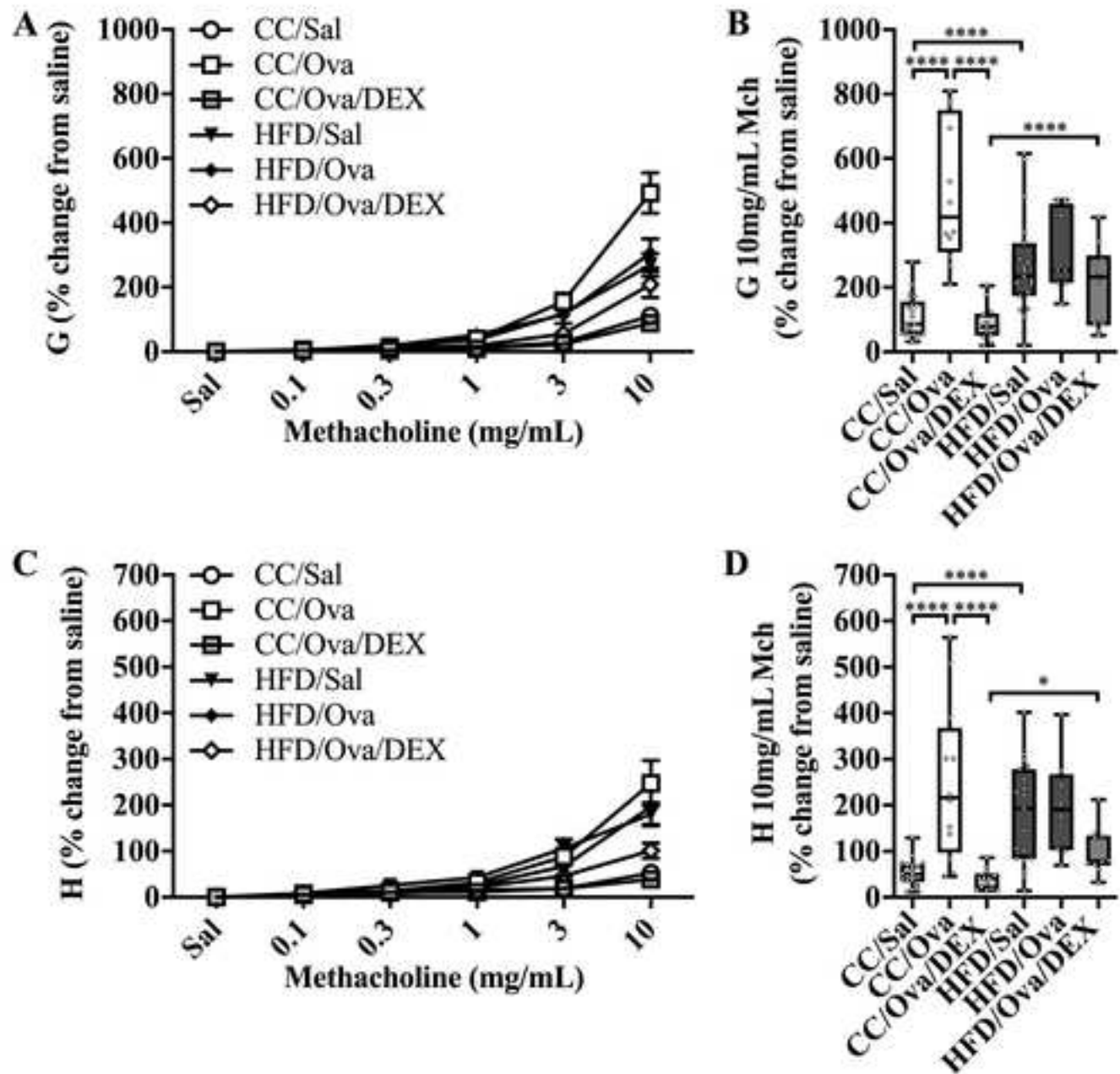
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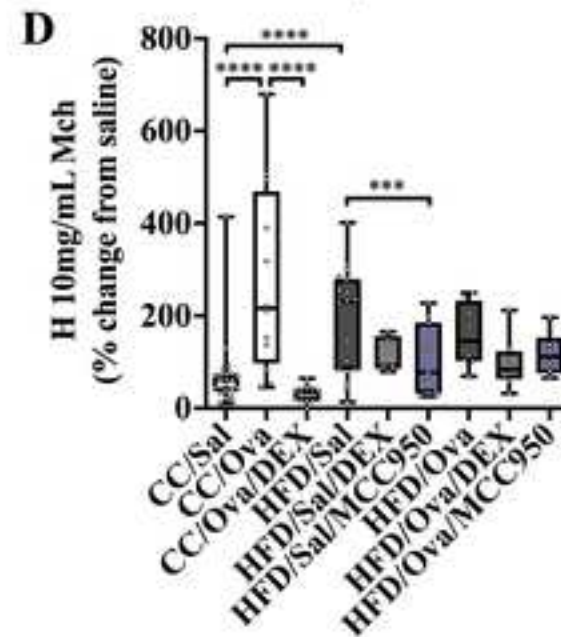
Supplementary Figure 4.

Supplementary Figure 5.

Supplementary Figure 6.

1 **Relationship between type 2 cytokine and inflammasome responses in obesity-associated**

2 **asthma**

3

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33 ~~Author contributions: JWP, RYK, PMH & JCH wrote the manuscript and prepared the figures.~~

34 ~~JWP, RYK, PMH & JCH conceived and designed the studies. JWP, RYK, CD, ACB, BER,~~

35 ~~JRM, ORC, MKA, MRS & JCH performed and validated the *in vivo* experimental studies.~~

36 ~~HAS, BSB, KJB, PGG & LGW collected, analysed and validated clinical data and provided~~

37 ~~intellectual input on obesity-associated asthma. IMA, YG, & NZK provided access to, and~~

38 ~~analysis of, data collected as part of the U-BIOPRED Study Group. LAO, AABR & MAC~~

39 ~~synthesised the NLRP3 inhibitor for *in vivo* experimental studies and provided intellectual~~

40 ~~input on role of NLRP3-associated inflammatory responses. All authors read, edited and~~

41 ~~approved the final manuscript.~~

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54 **Running title**: Type-2 cytokines and inflammasomes in obese asthma

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58 This article has an online data supplement, which is accessible from this issue's table of content

59 at the Online Repository at www.jacionline.org

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Abstract

Background: Obesity is a risk factor for asthma and obese asthmatics are more likely to have severe, steroid-insensitive disease. How obesity affects the pathogenesis and severity of asthma is poorly understood. Roles for increased inflammasome-mediated neutrophilic responses, type-2 immunity and eosinophilic inflammation have been described.

Objective: To investigate how obesity affects the pathogenesis and severity of asthma and identify effective therapies for obesity-associated disease.

Methods: We assessed associations between body mass index and inflammasome responses with type-2 immune responses in the sputum of 25 subjects with asthma. Functional roles for NLRP3 inflammasome and type-2 cytokine responses in driving key features of disease were examined in experimental high fat diet-induced obesity and asthma.

Results: Body mass index and inflammasome responses positively correlate with increased IL-5 and IL-13 expression, and C-C chemokine receptor type 3 expression in the sputum of subjects with asthma. High fat diet-induced obesity results in steroid-insensitive airway hyper-responsiveness in both the presence and absence of experimental asthma. High fat diet-induced obesity is also associated with increased NLRP3 inflammasome responses and eosinophilic inflammation in airway tissue, but not the lumen in experimental asthma. Inhibition of NLRP3 inflammasome responses reduces steroid-insensitive airway hyper-responsiveness but has no effect on IL-5 or IL-13 responses in experimental asthma. Depletion of IL-5 and IL-13 reduces obesity-induced NLRP3 inflammasome responses and steroid-insensitive airway hyper-responsiveness in experimental asthma.

Conclusion: We show a relationship between type-2 cytokine and NLRP3 inflammasome responses in obesity-associated asthma, highlighting the potential utility of type-2 cytokine-targeted biologics and inflammasome inhibitors.

Abstract word length: 250

Key messages:

- ~~Obesity is a risk factor for asthma and obese asthmatics are more likely to have severe, disease.~~ How obesity affects the pathogenesis and severity of asthma is poorly understood.

- Using ~~a complementary combination of~~ clinical and experimental studies, we highlight a novel ~~mechanistic~~ link between increased type-2 and NLRP3 inflammasome responses in the airways in obesity-associated ~~disease~~severe asthma and ~~.~~

- ~~Importantly, we highlight~~ the therapeutic potential of targeting type-2 cytokine and/or NLRP3 inflammasome responses ~~in obesity-associated, severe asthma~~.

Capsule summary:

Using a complementary combination of clinical and experimental studies, we show a relationship between type-2 cytokine and NLRP3 inflammasome responses in obesity-associated asthma and highlight the potential utility of type-2 cytokine-targeted biologics and inflammasome inhibitors.

Key Words: Asthma, Obesity, IL-5, IL-13, NLRP3 inflammasomes

Abbreviations:

AHR: Airway hyper-responsiveness

Alum: Aluminium hydroxide

BMI: Body mass index

CC: Control chow

111 CCR: Chemokine receptor type 3

112 DEX: Dexamethasone

113 FEV_1: Forced expiratory volume over one second

114 HFD: High fat diet

115 i.n.: Intranasal

116 i.p.: Intraperitoneal

117 Iso: Isotype

118 NOD: Nucleotide-binding oligomerization domain

119 NLR: NOD-like receptor

120 NLRP: NLR family, pyrin domain-containing

121 Ova: Ovalbumin

122 Sal: Saline

123

Introduction

Obesity is highly prevalent, affecting between 25-40% of the populations of the US, UK, and Australia (1). This high prevalence places a major burden on healthcare systems and is associated with many high burden diseases, such as cardiovascular disease and diabetes. Obesity is linked to the pathogenesis and/or increased severity of respiratory diseases, notably asthma. Obesity is associated with airway hyper-responsiveness (AHR) in some studies (2) and increases the risk of developing asthma, and asthma prevalence is higher in obese compared to lean individuals with the disparity greatest in women (3, 4). Increased weight gain and obesity precedes asthma development, particularly females, suggesting that these factors can play a causal role in disease pathogenesis (5-8). Importantly, studies show that obese asthmatics are more likely to have severe, steroid-insensitive disease and large multi-centre clustering analyses in both the US and Europe have identified a unique subtype of severe asthmatics that are obese and predominantly female (9-13). Collectively, these data suggest that obesity has roles in both the pathogenesis and increased severity of asthma, however, how obesity affects disease remains poorly understood. An improved understanding of the complex interactions that occur between obesity and inflammatory processes that underpin asthma is needed to enable the identification of effective therapies, particularly for obesity-associated, severe, steroid-insensitive forms of disease.

We recently showed that body mass index (BMI) correlates with increased expression of components of the nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family, pyrin domain-containing (NLRP)3 inflammasome (14), which is a multimeric protein complex that plays critical roles in innate immune signalling (15, 16) . Critically, we and others have shown that increased NLRP3 inflammasome responses have important roles in severe, neutrophilic steroid-insensitive asthma (15-18). Thus, increased BMI and NLRP3 inflammasome responses may drive neutrophil-enriched inflammation in the sputum as well as

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149   steroid-insensitivity in obesity-associated disease (19-21). It has also been shown that obesity

150   is associated with increased type-2 immune responses and eosinophilic inflammation in the

151   airway tissue (22-24). It is likely that these seemingly disparate findings reflect the complex

152   nature of the associations between obesity in adult- *versus* early-onset, and atopic *versus* non-

153   atopic asthma as well as differences in inflammatory responses in the airways tissue compared

154   to sputum (23, 25).

155   Increasing the understanding of how obesity affects both NLRP3 inflammasome and

156   type 2 responses in the airways in the absence and presence of asthma, and the role these

157   responses play in disease pathogenesis and severity, may identify effective therapeutic

158   strategies for obesity-associated, severe, steroid-insensitive asthma. In this study, we use a

159   combination of clinical analyses and mouse models of obesity and experimental asthma to

160   show a relationship between obesity-induced type-2 cytokine and NLRP3 inflammasome

161   responses in the airways and that these may play a role the pathogenesis and severity of steroid-

162   insensitive disease.

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Methods

Study Approvals

All procedures were performed with approval from the University of Newcastle Human and Animal Ethics committees.

Human data: subject characterization and sputum collection and processing

Baseline data was collected from 23 adults with stable asthma, who were participating in dietary intervention trials (*see* **Table E1** in the online supplement) (14, 26, 27). Subjects were recruited from ambulatory care clinics at John Hunter Hospital, Newcastle, Australia. Asthma was defined by physician diagnosis. Stable asthma was defined as no exacerbation, respiratory tract infection, or oral corticosteroid use in the past 4 weeks. Skin prick allergy tests determined atopic status. Subjects fasted overnight, and asthma medications were withheld (short-acting bronchodilators, 6 hours; long-acting bronchodilators and inhaled corticosteroids, 24 hours). Blood was collected, and spirometry and sputum induction were performed during hypertonic saline challenge. Lower respiratory tract sputum portions were selected and dispersed with dithiothreitol (26, 27). Differential sputum cell counts, RNA extraction, reverse transcription and gene expression were performed and analysed as previously described (14). Sputum supernatant IL-1β concentrations were analysed by ELISA DuoSet® (R&D Systems, Minneapolis, Minnesota, USA).

Experimental studies; Murine models of high fat diet (HFD)-induced obesity and experimental asthma with corticosteroid, NLRP3 inflammasome inhibitor and anti(α)-IL-5 (α-IL-5) and α-IL-13 treatment; Assessment of adiposity, AHR, airway lumen and tissue inflammatory cell and mucus secreting cell numbers, histopathology and gene expression and protein levels in lung tissues.

6

Murine models of experimental HFD-induced obesity and ovalbumin (Ova)-induced asthma were superimposed to investigate the impact of obesity on lung disease. Intranasal treatment with dexamethasone (DEX), MCC950, or α-IL-5 and α-IL-13 monoclonal antibodies was used to assess the effects of corticosteroids, NLRP3 inflammasomes and type-2 cytokine responses in obesity-induced disease, respectively. Airway inflammation, AHR, RNA and protein analyses, were determined as previously described and as in the online supplement (17, 28-30).

Statistics

Comparisons between two groups were performed using unpaired Student's t tests or a nonparametric equivalent as appropriate. Comparisons between multiple groups were performed using a one-way analysis of variance and an appropriate post-test or a nonparametric equivalent, as appropriate. Lung function data were assessed using a two-way analysis of variance with an appropriate post-test. Correlation analyses of sputum data were made using Spearman rank correlation. Analyses were performed using GraphPad Prism Software (San Diego, California, USA).

Results

BMI and NLRP3 inflammasome/IL-1β responses correlate with type-2 immune responses in human asthma

To assess the clinical relationships between obesity, NLRP3 inflammasome and type-2 responses and extend our previous findings (14, 17), we correlated BMI and NLRP3/IL-1β responses with the numbers of eosinophils and IL-5, IL-13 and C-C chemokine receptor type (CCR)3 gene expression in the sputum of asthma patients (*see* **Table E1** in the online supplement) (14). BMI positively correlates with the absolute numbers of sputum eosinophils (r=0.44; p=0.06), and IL-5 (r=0.50; p=0.02), IL-13 (r=0.45; p=0.04) and CCR3 (r=0.53; p=0.01) mRNA expression (**Figure 1A–D**). Absolute numbers of sputum eosinophils also trended towards a statistically significant positive correlation with NLRP3 mRNA expression (r=0.40; p=0.09) but not with IL-1β mRNA expression (r=0.30; p=0.21) (**Figure E1A and B**). Furthermore, IL-5 mRNA expression positively correlates with NLRP3 (r=0.45; p=0.04) and IL-1β (r=0.42; p=0.05) expression but not sputum IL-1β protein levels (r=0.43; p=0.11) (**Figure E1C**), and IL-13 expression positively correlates with IL-1β protein levels (r=0.57; p=0.04) (**Figure 1E–K**). CCR3 expression positively correlates with both NLRP3 (r=0.48; p=0.02) (**Figure 1G**) and sputum IL-1β (r=0.55; p=0.04) protein levels (**Figure E1D**). These data demonstrate potential clinical relationships between obesity, NLRP3 inflammasome and type-2 responses in the airways of asthmatics and extend our previous findings that show roles for NLRP3 inflammasomes in both severe, steroid-insensitive and obesity-associated asthma.

HFD induces obesity

We next established a murine model of HFD-induced obesity to determine functional relationships between obesity, NLRP3 inflammasome and type-2 cytokine responses in obesity-associated disease. BALB/c mice were fed a HFD or control chow (CC) diet for 13

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249 weeks (*see* **Figure E2** in the online supplement). Mice fed a HFD have significant increases in

250 total body mass from weeks 3-13 compared to mice fed a CC diet (**Figure 2A and B**). This

251 involved substantial increases in the mass of parametrial (81.14% increase), inguinal (65.23%

252 increase) and retroperitoneal (127.50% increase) fat pads when compared to mice fed a CC

253 diet (**Figure 2C-E**).

254

255 **HFD-induced obesity promotes steroid-insensitive AHR**

256 We next examined the effects of HFD-induced obesity on airway inflammation and AHR in

257 the presence and absence of Ova-induced experimental asthma. Mice were fed a HFD or CC

258 diet and after 9-weeks were systemically sensitized to Ova by intraperitoneal (i.p.) injection of

259 Ova in aluminium hydroxide (alum) (*see* **Figure E2A** in the online supplement). On days

260 (d)12-13, and 33-34, mice were intranasally (i.n.) challenged with Ova to induce and

261 recapitulate experimental asthma (17). Non-allergic controls were sham-sensitized with an i.p.

262 injection of saline (Sal) and alum and were treated i.n. with Ova.

263 HFD-induced obesity had no significant effect on the numbers of total leukocytes,

264 macrophages, neutrophils or eosinophils in bronchoalveolar lavage fluid (BALF) in the

265 absence (CC/Sal *vs* HFD/Sal) or presence (CC/Ova *vs* HFD/Ova) of Ova-induced experimental

266 asthma (**Figure 2F-J**). Inflammatory cell numbers were also sensitive to i.n. treatment (d32–

267 34) with the corticosteroid dexamethasone (DEX) in both lean (CC/Ova *vs* CC/Ova/DEX) and

268 obese (HFD/Ova *vs* HFD/Ova/DEX) mice with Ova-induced experimental asthma.

269 Importantly, HFD-induced obesity induces AHR in the absence of experimental asthma

270 (CC/Sal *vs* HFD/Sal) and, unlike in lean mice (CC/Ova *vs* CC/Ova/DEX), AHR in obese mice

271 is not suppressed by DEX treatment in experimental asthma (HFD/Ova *vs* HFD/Ova/DEX;

272 **Figure 2K and L**). Similar effects of HFD-induced obesity were observed in terms of tissue

273 damping and elastance (**Figure E3**).

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274   These data demonstrate that whilst our murine model of HFD-induced obesity does not

275 have a significant effect on the numbers of inflammatory cells in the airway lumen, obesity

276 alone induces AHR in the absence of experimental asthma and steroid-insensitive AHR when

277 superimposed with Ova-induced disease.

278

Obesity increases tissue eosinophil numbers and NLRP3 inflammasome responses in the

280 **absence and presence of experimental asthma**

281 We next examined inflammatory cell responses in the lung tissues of obese mice to understand

282 how obesity induces the effects observed on AHR (**Figure 2K and L**). HFD-induced obesity

283 increases the number of eosinophils in airway tissue in the absence (CC/Sal *vs* HFD/Sal) and

284 presence (CC/Ova *vs* HFD/Ova) of Ova-induced experimental asthma (**Figure 3A**). Whilst

285 obesity did not significantly increase the magnitude of inflammation in the lung tissues

286 (histopathology score) during experimental asthma (CC/Ova *vs* HFD/Ova), HFD-induced

287 obesity alone (CC/Sal *vs* HFD/Sal; Figure 3B) trended towards increasing inflammatory score

288 (p=0.074). We also show that whilst HFD-induced obesity did not affect the numbers of mucus

289 secreting cells in the airways in the absence or presence of experimental asthma compared to

290 CC diet-fed controls (**Figure 3C**), obese mice with Ova-induced experimental asthma had

291 increased lung *Muc5ac* expression, indicating increased mucus responses (**Figure 3D**).

292   We next investigated how obesity affects NLRP3 inflammasome responses by

293 assessing the levels of IL-1β, NLRP3 and active caspase-1 in lung tissues. HFD-induced

294 obesity increases IL-1β levels and NLRP3 staining in the lungs in the absence of Ova-induced

295 experimental asthma (CC/Sal *vs* HFD/Sal; **Figure 3E and H**). Interestingly, the levels of IL-

296 1β are lower, and NLRP3 staining similar, in HFD-fed obese mice with experimental asthma

297 compared to CC diet-fed controls (CC/Ova *vs* HFD/Ova; **Figure 3E, G and I**). However, most

298 importantly, we show that HFD-induced obesity increases the levels of active caspase-1 in lung

299 tissues, indicating that obesity increases inflammasome activation in the lungs in the absence

300 and presence of experimental asthma (**Figure 3J**).

301   These data show that whilst HFD-induced obesity induces subtly different effects in the

302 absence and presence of experimental asthma, obesity increases eosinophil numbers in the

303 airways tissue in association with increased IL-1β, NLRP3 and caspase-1 responses. This

304 indicates that obesity increases both eosinophilic inflammation and NLRP3 inflammasome

305 activity in the lung tissues irrespective of asthma status.

306

307 **NLRP3 inflammasome inhibition suppresses obesity-induced, steroid-insensitive AHR**

308 We next determined whether increased inflammasome responses have roles in obesity-induced,

309 steroid-insensitive AHR. HFD-fed mice with or without Ova-induced experimental asthma

310 were treated i.n. (d32-34) with the highly specific NLRP3 inflammasome inhibitor, MCC950

311 (HFD/Sal/MCC950, HFD/Ova/MCC950), or DEX (HFD/Sal/DEX, HFD/Ova/DEX; *see*

312 **Figure E2B** in the online supplement). The effects of treatment on airway inflammation and

313 AHR were assessed compared to CC diet-fed controls with and without DEX treatment. We

314 show that treatment with MCC950 reduced total leukocyte, lymphocyte, and neutrophil

315 numbers in BALF in obese mice with Ova-induced experimental asthma, compared to

316 untreated controls on a HFD (**Figure 4A-E**). MCC950 treatment had no statistically significant

317 effect on macrophage or eosinophil numbers in BALF in any of the groups with HFD-induced

318 obesity although there were trends to a reduction (**Figure 4B and E**). Importantly, we show

319 that treatment with the NLRP3 inflammasome inhibitor, MCC950, but not the corticosteroid,

320 DEX, completely suppresses AHR in the absence and presence of Ova-induced experimental

321 asthma in mice with HFD-induced obesity (**Figure 4F and G**). Similar effects of HFD-induced

322 obesity were observed in terms of tissue damping and elastance (**Figure E4**) and MCC950

323 treatment had similar suppressive effects on tissue damping in obese mice in the absence and

11

324 presence of Ova-induced experimental asthma. However, MCC950 treatment only suppressed

325 tissue elastance in obese mice in the absence of Ova-induced experimental asthma.

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327 **Treatment with α-IL-5 and α-IL-13 suppresses obesity-induced, steroid-insensitive AHR**

328 **and NLRP3 inflammasome responses**

329 Our findings demonstrate that NLRP3 inflammasome responses play an important role in the

330 pathogenesis of steroid-insensitive AHR that is observed in obese mice. Interestingly, we show

331 that treatment with MCC950 has no suppressive effects on lung IL-5 or IL-13 protein levels

332 (**Figure 5A and B**). To determine whether a functional relationship exists between type-2

333 cytokine and NLRP3 inflammasome responses we next assessed the effects of IL-5 and IL-13

334 depletion on inflammation, AHR and NLRP3 inflammasome responses in HFD-induced

335 obesity. HFD-fed obese mice with and without Ova-induced experimental asthma were treated

336 i.n. with a combination of α-IL-5 and α-IL-13 (HFD/Sal/α-IL-5/α-IL-13, HFD/Ova/α-IL-5/α-

337 IL-13), or isotype control (Iso) monoclonal antibodies with or without DEX (HFD/Sal/Iso,

338 HFD/Sal/Iso/DEX,   HFD/Ova/Iso, HFD/Ova/Iso/DEX; *see* **Figure E2C** in the online

339 supplement) and the effects of treatment on airway inflammation and AHR assessed compared

340 to CC diet-fed controls with or without DEX treatment.

341   Treatment with α-IL-5 and α-IL-13 reduces total leukocyte, macrophage, lymphocyte,

342 neutrophil and eosinophil numbers in BALF in obese mice with experimental asthma (**Figure**

343 **5C-G**). Importantly, treatment with α-IL-5 and α-IL-13, but not the corticosteroid, DEX,

344 completely suppresses AHR that is induced in the absence and presence of Ova-induced

345 experimental asthma in mice with HFD-induced obesity (**Figure 5H and I**, and **Figure E5**).

346   To determine the effects of depletion of IL-5 and IL-13 on NLRP3 inflammasome

347 responses in obesity induced disease, we next assessed the effects of α-IL-5/α-IL-13 treatment

348 on NLRP3 levels in lung histological sections from HFD-fed obese mice with or without Ova-

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induced experimental asthma. Significantly, α-IL-5/α-IL-13 treatment completely suppresses increased NLRP3-positive staining observed in lung tissues of obese mice with or without Ova-induced experimental asthma (**Figure 6A-G**). Interestingly, treatment with α-IL-5/α-IL-13, but not MCC950, suppresses airways eosinophils in HFD-fed obese mice in the absence or presence of experimental asthma (**Figure E6**). This is the first study to demonstrate functional links between type-2 cytokine and NLRP3 inflammasome responses in airways tissue.

To support our experimental findings, we interrogated data from the U-BIOPRED initiative and also show that IL-13 expression correlates with NLRP3 expression in bronchial and nasal brushings, and bronchial biopsies, in asthmatics (**Figure 6H-J**). Furthermore, IL-5 expression correlates with NLRP3 expression in nasal brushings, but not bronchial brushing or biopsies, in asthmatics.

Discussion

Extensive evidence shows that obesity has an important role in both the pathogenesis and severity of asthma and other respiratory diseases. This is likely due to the complex relationships between age, asthma and atopic status of the individual during which obesity occurs. The prevalence of asthma is increased in obese children and adults (31). Whilst it has been suggested that the increased prevalence of obesity in asthma patients may be due to reduced physical activity and/or other factors associated with asthma, studies also show that obesity often precedes asthma development (5-8). Obese asthmatics are more likely to be non-atopic and have more severe forms of disease suggesting that obesity promotes different phenotypes of asthma (13, 32, 33). Furthermore, bariatric surgery and weight loss improves respiratory symptoms in obese individuals (33-35). These findings suggest that obesity plays important roles in both the development and modification of asthma. In this study, we performed a series of clinical and experimental studies that examined the effects of obesity on immune responses in the lungs in the absence or presence of asthma to identify key drivers that link obesity and disease.

We previously reported associations between BMI and NLRP3 inflammasome responses and neutrophils in the sputum of asthmatics (14). Here, we extend these findings to show that BMI is also associated with increased type-2 cytokine expression, and trends towards correlation with the total numbers of eosinophils in the sputum of asthmatics. We also show a strong association between BMI and increased CCR3 expression. Since CCR3 is highly expressed by eosinophils, and since it has important roles in eosinophil chemoattraction, activation and mediator release, this finding supports a potential link between obesity and increased eosinophil infiltration and priming in the lungs (36, 37). We also show that NLRP3 and/or IL-1β responses are associated with increased type-2 cytokine and CCR3 expression, which highlights a potential link between type-2 immunity and NLRP3 inflammasome

398 responses in obesity-associated asthma. Interestingly, we also show that IL-5 and IL-13

399 expression strongly correlate with NLRP3 expression in nasal brushings in a different cohort

400 of asthma patients, and that IL-13 expression also correlates with NLRP3 expression in

401 bronchial brushings and biopsies in this cohort. These data further support a relationship

402 between type-2 immunity and inflammasome responses in lower and upper airway mucosa in

403 asthma.

404   We next established a murine model of HFD-induced obesity in BALB/c mice to assess

405 how obesity affects immune responses in the lung. We demonstrate that BALB/c mice fed a

406 HFD display greater weight gain (>16%) associated with increased adiposity (>128% in

407 retroorbital adipose tissue) compared to mice on a CC diet. Significantly, we show that HFD-

408 induced obesity results in the development of AHR in the absence of experimental asthma,

409 which further supports a role for obesity alone in inducing respiratory disease in the absence

410 of allergic asthma (12, 13, 38, 39). We show that obesity-induced AHR is associated with

411 increased NLRP3 inflammasome responses in the lungs of mice and that AHR is suppressed

412 by intranasal administration of the NLRP3 inflammasome-specific inhibitor MCC950. Our

413 findings are supported by a previous study that showed that obese mice develop spontaneous

414 AHR in the absence of experimental asthma, which did not occur in NLRP3-deficient mice

415 (40). Given that obesity is associated with increased inflammasome responses in the lung as

416 well as adipose tissues and systemically, our findings provide new evidence that obesity-

417 induced NLRP3 inflammasome responses in the airways play a critical role in disease

418 pathogenesis (17, 39).

419   We also show that obesity-induced NLRP3 inflammasome and IL-1β responses are

420 associated with concomitant increases in IL-5 and IL-13 protein levels in the lungs of mice.

421 This supports our observation of a link between inflammasome and type-2 immune responses

422 in the airways in obesity-associated disease in human subjects. Several studies report that the

423   NLRP3 inflammasome plays a critical role in the breaking of tolerance to antigen which is

424   required for the induction of allergic responses in murine models of experimental asthma (41,

425   42). Here, we show that MCC950 treatment has no effect on the levels of type-2 cytokines in

426   the lungs of mice, however, treatment with α-IL-5 and α-IL-13 completely ablates NLRP3-

427   positive staining in lung tissues. Our data suggest that type-2 responses can drive increased

428   inflammasome activation in the lung that promotes AHR. Interestingly, we show that MCC950,

429   which protects against obesity-associated steroid-insensitive AHR but does not suppress T2

430   cytokines, has no effect on obesity-associated increases in tissue eosinophil numbers (Figure

431   E6). Furthermore, treatment with α-IL-5 and α-IL-13, which decreases obesity-associated

432   NLRP3 responses and steroid-insensitive AHR, suppresses tissue eosinophil numbers in the

433   absence or presence of AAD (Figure E6). These data demonstrate that tissue accumulation of

434   eosinophils and/or the release of eosinophil-associated inflammatory mediators, as a feature of

435   increased T2 immune responses, are associated with increased T2 cytokine-induced NLRP3

436   responses, and that increased NLRP3 responses are not the driver of eosinophilic inflammation

437   in the airways tissue of obese mice. Furthermore, our data suggests that suppression of type-2

438   responses &/or inhibition of the NLRP3 inflammasome is sufficient to restore AHR to basal

439   levels, suggesting that AHR is primarily driven by aberrant immune responses rather than

440   altered lung mechanics associated with obesity (43).

441   Significantly, we show that obese mice have increased eosinophil numbers in the

442   airway tissue both in the absence or presence of experimental asthma. This agrees with clinical

443   data showing increased eosinophil numbers in the airway wall of obese individuals (12, 13,

444   33). Increased type-2 cytokine responses and eosinophilic inflammation in the airway tissue of

445   obese mice suggests that obesity may induce increased type-2 cytokine responses in the lung

446   that increases the homing of eosinophils to the airway tissues and that this can occur with or

447   without the presence of asthma. Our findings are supported by a recent study showing that

16

448 obesity promotes increased type-2 cytokine responses and eosinophilic inflammation in the

449 oesophagus in a murine model of eosinophilic oesophagitis (44). That study showed that

450 obesity alone was associated with increased eosinophilic inflammation in the lung and gut.

451 Thus, obesity may also play a role in the induction of type-2 immunity and eosinophilic

452 inflammatory responses in diseases of other mucosal sites.

453   Importantly, our findings provide insights into how obesity may modify asthma to

454 promote more severe forms of the disease. Severe, steroid-insensitive asthma is a heterogenous

455 disease with many phenotypes now recognized that are underpinned and/or associated with

456 different immunopathological processes. Large cohort clinical studies of adult asthmatics, such

457 as the European U-BIOPRED and the US SARP program, have stratified patients with

458 moderate to severe asthma based on clinico-physiologic parameters and tissue 'omics analyses

459 (12, 13). Both initiatives have identified unique severe asthma cohorts, which are associated

460 with obesity. Typically, these patients develop asthma in adulthood, are more likely to

461 experience more exacerbations and hospitalizations, and be on higher doses of inhaled steroids

462 (12, 13). However, the mechanisms that drive severe forms of obesity-associated asthma are

463 unclear. Previous studies utilized models of HFD-induced obesity to examine the effects on

464 airway disease (39, 45-49). Unfortunately, these studies did not examine the effect of obesity

465 on steroid responses in the airways, which is a critical factor in assessing severe asthma. In this

466 study, we show that obesity drives AHR that is steroid-insensitive in experimental asthma.

467 Interestingly, we also show that obesity does not affect intraluminal airway inflammatory cells

468 in Ova-induced experimental asthma, and that corticosteroid treatment does not affect AHR in

469 obese mice despite suppressing intraluminal inflammatory cell numbers in this T-helper type

470 2 (Th2) cell-mediated model. Together, these findings suggest that obesity likely drives innate

471 responses in the airways that are independent of the classical Th2 pathways that are associated

472 with atopic asthma. Importantly, we also show that increased NLRP3 inflammasome and/or

17

type-2 cytokine responses, that we and others have shown to be increased in obesity-associated severe disease (14, 23, 50), may be therapeutically targeted in the lung to suppress obesity-induced, steroid-insensitive disease. Whilst we show increased body weight and adiposity in our model of high fat diet-induced obesity, a limitation of this study is that we did not examine hyperglycemia and high cholesterol as other, common manifestations of obesity that are often observed in humans. Given the links between altered metabolism and regulation of immune responses, such indices and their role in driving increased T2 immunity and altered lung physiology would be interesting to follow up in these models in future studies.

In conclusion, we show that obesity increases NLRP3 inflammasome and type-2 cytokine responses in the lung and promotes steroid-insensitive AHR in both the absence and presence of experimental asthma. We also show that therapeutic targeting of either NLRP3 inflammasomes or type-2 cytokines can suppress obesity-induced, steroid-insensitive AHR. Importantly, we show that type-2 cytokine and NLRP3 inflammasome responses correlate with one another in the airways in clinical and experimental asthma, and that suppressing type-2 cytokine responses suppresses NLRP3 inflammasome responses in experimental disease. To our knowledge, these data are the first to provide a potential mechanistic link between increased type-2 and NLRP3 inflammasome responses that have been reported in obese asthmatics in the literature. Importantly, these data highlight the therapeutic potential of targeting type-2 cytokine and/or NLRP3 inflammasome responses in obesity-associated disease.

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Figure 1. Type-2 immune responses correlate with body mass index (BMI) and nucleotide-binding oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3)/IL-1β responses in human asthma. (*A*) Sputum eosinophil absolute number (per mL), and sputum (mRNA) expression of (*B*) IL-5, (*C*) IL-13, and (*D*) C-C motif chemokine receptor 3 (CCR3), correlate with BMI (kg/m^2) in a population of subjects with stable asthma (*n*=23, described previously (14)). Sputum (mRNA) expression of NLRP3 correlated with that of (*E*) IL-5, (*F*) IL-13, and (*G*) CCR3. Sputum (mRNA) expression of IL-1β correlated with that of (*H*) IL-5, (*I*) IL-13, and (*J*) CCR3. Sputum (protein) levels of IL-1β correlated with sputum (mRNA) expression of (*K*) IL-13. Associations for each comparison are expressed as Spearman rank correlation coefficient (Spearman rho; *r*) with *p* values.

Figure 2. High fat diet (HFD) exposure induces obesity that promotes steroid-insensitive airway hyperresponsiveness (AHR). Wild-type female BALB/c mice were fed either a HFD or control chow (CC) diet for 13 weeks and (*A* and *B*) whole body mass was measured weekly. The mass of major white adipose pads ([*C*] parametrial, [*D*] inguinal, and [*E*] retroperitoneal) was determined at 13 weeks (2 experiments; *n*=24-40). Total leukocytes (*F*), macrophages (*G*), lymphocytes (*H*), neutrophils (*I*), and eosinophils (*J*) were enumerated in bronchoalveolar lavage fluid (BALF) on day 35 of the study protocol (*see* **Figure E2A** in the online supplement) in HFD- and CC-fed groups with ovalbumin (Ova)-induced experimental asthma with or without steroid (dexamethasone [DEX]) treatment compared to non-allergic controls (Sal) (2 experiments; *n*=6-12). AHR in terms of airway resistance in response to increasing doses of methacholine (Mch; *K*), and at the maximal dose of 10mg/mL Mch (*L*) was also determined in all groups on day 35 (≥2 experiments; *n*=10-21). Data in *A* and *K* are presented as means ± SEM. Data in *B-J* and *L* are presented as box (Q2 to Q3 with the median) and whisker (min to max). *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$. Rn = airway resistance.

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Figure 3. High fat diet (HFD)-induced obesity increases tissue eosinophil numbers and nucleotide-binding oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3) inflammasome responses in the absence and presence of experimental asthma. (*A*) Airway basement membrane-associated eosinophils, (*B*) histopathological scores for gross tissue inflammation, and (*C*) mucus secreting cells (MSCs) in the airways, were enumerated on day 35 of the study protocol (*see* **Figure E2A** in the online supplement) in lung histological sections from HFD- and control chow (CC)-fed groups with ovalbumin (Ova)-induced experimental asthma, with or without steroid (dexamethasone, DEX) treatment compared to non-allergic controls (Sal). Lung (*D*) mRNA expression of *Muc5ac*, and (*E*) protein levels of IL-1β. (*F-I*) Representative photomicrographs of NLRP3 immunofluorescence (Alexa Fluor® 488 with Hoechst 33342 nuclear counterstain) in lung histology sections. (*J*) Lung protein levels of CASP1 (10kDa) normalized to β-actin (ACTB; 42kDa) were determined by quantification of immunoblot by densitometry and are expressed as fold change from CC/SAL from one experiment; (*n*=5-6). Data are presented as box (Q2 to Q3 with the median) and whisker (min to max)means ± SEM. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$.

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Figure 4. Nucleotide-binding oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3) inhibition suppresses obesity-induced, steroid-insensitive airway hyperresponsiveness (AHR). (*A*) Total leukocytes, (*B*) macrophages, (*C*) lymphocytes, (*D*) neutrophils, and (*E*) eosinophils were enumerated in bronchoalveolar lavage fluid (BALF) on day 35 of the study protocol (*see* **Figure E2B** in online supplement) in high fat diet (HFD)- and control chow (CC)-fed groups with ovalbumin (Ova)-induced experimental asthma, with or without steroid (dexamethasone, DEX or MCC950) treatment compared with

703 non-allergic controls (Sal). AHR in terms of airway resistance in response to increasing doses

704 of methacholine (Mch; *F*), and the maximal dose of 10mg/mL Mch (*G*). Data in *A-E* and *G* are

705 presented as box (Q2 to Q3 with the median) and whisker (min to max), and d~~D~~ata in *F* are~~is~~

706 presented as means ± SEM from ≥2 experiments (*n*=6-22). *P<0.05; **P<0.01; ***P<0.001;

707 ****P<0.0001. Rn = airway resistance.

708

709 **Figure 5. Treatment with anti(α)-IL-5/α-IL-13 suppresses obesity-induced, steroid-**

710 **insensitive airway hyperresponsiveness (AHR).** Lung (*A*) IL-5 and (*B*) IL-13 protein levels

711 were measured by ELISA on day 35 of the study protocol (*see* **Figure E2B** in the online

712 supplement) in high fat diet (HFD)- and control chow (CC)-fed groups with ovalbumin (Ova)-

713 induced experimental asthma, with or without MCC950 treatment compared to non-allergic

714 control subjects (Sal) (2 experiments; *n*=5-6). (*C*) Total leukocytes, (*D*) macrophages, (*E*)

715 lymphocytes, (*F*) neutrophils, and (*G*) eosinophils were enumerated in bronchoalveolar lavage

716 fluid (BALF) on day 35 of the study protocol (*see* **Figure E2C** in the online supplement) in

717 HFD- and CC-fed groups with Ova-induced experimental asthma, with or without steroid

718 (dexamethasone, DEX) or α-IL-5/α-IL-13 or isotype (Iso) antibody treatment compared to non-

719 allergic controls (Sal). AHR in terms of airway resistance in response to increasing doses of

720 methacholine (Mch; *H*), or at the maximal dose of 10mg/mL Mch (*I*). Data in *A-G* and *I* are

721 presented as box (Q2 to Q3 with the median) and whisker (min to max), and d~~D~~ata in *H* ~~are~~is

722 presented as means ± SEM from 2 experiments (*n*=4-8). *P<0.05; **P<0.01; ***P<0.001;

723 ****P<0.0001. Rn = airway resistance.

724

725 **Figure 6. Treatment with anti(α)-IL-5/α-IL-13 suppresses nucleotide-binding**

726 **oligomerization domain–like receptor family, pyrin domain–containing 3 (NLRP3)**

727 **inflammasome responses in obesity-induced experimental asthma, and type-2 cytokine**

responses are associated with NLRP3 responses in the airways in human asthma. (*A-F*) Representative photomicrographs of NLRP3 immunofluorescence (IF; Alexa Fluor® 488 with Hoechst 33342 nuclear counterstain) in lung histological sections on day 35 of the study protocol (*see* **Figure E2C** in the online supplement) in high fat diet (HFD)- and control chow (CC)-fed groups with ovalbumin (Ova)-induced experimental asthma, with or without α-IL-5/α-IL-13 or isotype (Iso) antibody treatment compared to non-allergic controls (Sal). (*G*) Quantification of NLRP3 IF (Integrated Density) in lung histological sections (representative images in *A-F*; data are presented as box [Q2 to Q3 with the median] and whisker [min to max]). Correlations between NLRP3 and IL-13 and IL-5 mRNA expression in (*H*) bronchial and (*I*) nasal brushings, and (*J*) bronchial biopsies, in a population of subjects with stable asthma (data collected as part of the U-BIOPRED Study).