

ORIGINAL ARTICLE

Experimental Models of Allergic Disease

Sex differences in the influence of obesity on a murine model of allergic lung inflammation

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Funding information

Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP/BRAZIL), Grant/Award Number: 2015/18997-7 and 2016/22722-6

Abstract

Background: Despite the overwhelming evidence showing the influence of sex or obesity in the development of respiratory diseases in humans and animals, the mechanisms by which these combined two factors influence allergic asthma are not well understood.

Objective: We have investigated the interaction between sex and weight gain in an experimental model of lung allergic inflammation induced by chicken egg ovalbumin (OVA) in mice.

Methods: Animals were fed a high-fat diet for 8 weeks and then sensitized and challenged with OVA.

Results: Our results demonstrate that in comparison with males, high-fat diet (HFD) allergic female mice exhibit a reduction in the number of leucocytes in the lung lumen when challenged with OVA and, in contrast, an accumulation of these cells in the lung tissue. In addition, we also observed that allergic HFD female mice presented a robust lung remodelling in comparison with HFD males, evidenced by higher deposition of collagen in the airways and TGF- β in lung fluid. Measuring epithelial adhesion molecule expression, we observed that female mice presented a significantly lower expression of CD103 than males in BAL cells, regardless of the diet. Similarly, HFD female mice express lower levels of EpCAM in lung tissue in comparison with males and lean females. Levels of A20/TNFAIP3 expression in lung tissue demonstrated that HFD female mice express lower levels of these regulatory factors than all the other groups. However, this reduction was not accompanied by an increase in activated NF- κ B.

Conclusions: Our results present evidence that the interaction between sex and weight gain alters the progression of allergic asthma in mice with females developing airway remodelling at a much earlier stage than males.

Clinical Relevance: These data may contribute to a better understanding of the clinical differences in the development and severity of allergic asthma observed between men and women of reproductive age.

1 | INTRODUCTION

Sex differences in asthma have been well described in the literature. Several reports have shown that asthma is more prevalent in boys than in girls but this status is inverted during puberty, indicating an important role for sex hormones in the development of this disease.¹ Similarly, the influence of obesity has also been documented with studies indicating that body weight is an important risk factor in asthma.² However, despite the overwhelming evidence showing the influence of sex or obesity in the development of respiratory diseases in humans, the mechanisms by which these two factors interact to influence asthma is not well understood. To date, the vast majority of publications in this field are longitudinal or cross-sectional studies of human populations.^{3–6} Some reports have shown that nearly 75 percent of emergency room visits for asthma have been among obese individuals,⁷ and several cross-sectional studies have found an increase in the prevalence of asthma among obese patients.⁴ More importantly, obesity appears to interfere with the efficacy of standard therapies of asthma,⁸ raising concerns about whether treatment of these patients is adequate. In addition, it has become increasingly clear that obesity as an important risk factor in the onset and worsening of asthma seems to be particularly higher among women.³ Several studies point towards an association between weight gain and asthma in females, but not in males,^{9–11} although some of the largest studies have not confirmed this association.¹² It is plausible that an increased association in women could be due to female sex hormones^{13,14} or that the effects of adiposity are greater in women simply because they have a higher proportion of body fat than men, but the reasons for the apparent sex difference observed in many studies are unclear.¹⁵

Sex differences in the development of allergic inflammation in the lung and obesity have also been demonstrated in murine models. Experimental studies have shown that female allergic mice develop a more robust eosinophilic inflammatory response to ovalbumin and also a higher amount of airway remodelling compared with male mice or ovariectomized females.^{16–18} Mice present sex differences in both the acquisition of weight gain and distribution of excess fat stores due to genetic factors.¹⁹ Sex differences in obesity, adiposity and insulin resistance have also been observed in animal models of metabolic syndrome,^{20,21} suggesting that the development of obesity is influenced by sex in mice as it is in humans.

In this study, we sought to investigate the interaction between weight gain and sex in an experimental model of allergic inflammation in the lung induced by chicken egg ovalbumin (OVA) in mice. More specifically, we have investigated the influence of sex and weight on leucocyte migration, adhesion molecule expression and airway remodelling in standard chow and high-fat diet fed male and female mice.

2 | METHODS

Additional information of the methodology used in these experiments can be found in the Supplemental Section.

2.1 | Animals and induction of experimental obesity

Four-week-old male and female mice (C57BL/6) (Envigo, UK) were acclimatized for 2 weeks with conventional diet and water *ad libitum* before the onset of the hyperlipidic diet. HFD group animals were fed for 8 weeks with a hyperlipidic diet (HFD—high-fat diet) (SDS diet, Cat. Numb. 824054) composed of 60% fat, 20% protein and 20% of carbohydrates. SC group animals were fed with conventional diet for the same period (SC—standard chow) (PicoLab Rodent Diet 20, Cat: 50503) composed of 5% fat, 20% protein, 5% fibre, 6% ash, 60% nitrogen-free extract and 10% moisture. The effectiveness of the diet was measured weekly by weight measurement and after 8 weeks by body weight gain, serum triglycerides, glucose and total cholesterol levels. At the end of the experiment, we also measured perigonadal fat weight and serum levels of leptin. Experiments were approved by the Home Office under The Animals (Scientific Procedures) Act (1986) and by the Ethics Committee of King's College London.

2.2 | Experimental design

Eight groups were evaluated:

1. OVA-sensitized and OVA-challenged standard chow male (male SC OVA)
2. OVA-sensitized and OVA-challenged high-fat diet male (male HFD OVA)
3. OVA-sensitized and OVA-challenged standard chow female (female SC OVA)
4. OVA-sensitized and OVA-challenged standard chow fed female (female HFD OVA)
5. Non-sensitized and OVA-challenged standard show fed male (male SC Sham)
6. Non-sensitized and OVA-challenged high-fat diet fed male (male HFD Sham)
7. Non-sensitized and OVA-challenged standard chow fed female (female SC Sham)
8. Non-sensitized and OVA-challenged high-fat diet female (female HFD Sham)

2.3 | Sensitization and challenge to chicken egg albumin (OVA)

At week 6 of the diet, female and male mice were immunized intraperitoneally three times, with a 5-day interval between injections, with 30 µg of OVA (type V; Sigma Chemical Co.) adsorbed to a saturated solution of aluminium hydroxide (Sanofi, Brazil). Control animals (Sham groups) received vehicle only. On days 14, 15 and 16 after the first immunization, all animals were challenged 3 times, once daily with a 3% solution of OVA for 20 minutes.

2.4 | Bronchoalveolar alveolar lavage (BAL)

Twenty-four hours after the last OVA challenge, mice were anaesthetized with urethane (2 g/kg i.p.; Sigma Gillingham, UK), a cannula inserted into the exposed trachea, and three 0.5 mL aliquots of sterile saline were injected into the lungs. The total number of cells in the lavage fluid was counted with an improved Neubauer haemocytometer. For differential cell counts, cytospin preparations were stained with Diff Quick (DADE Behring) and cells were counted using standard morphological criteria.

2.5 | Analysis of lung homogenates

Fragments of the lungs were removed and cut into small pieces. The fragments were placed in a falcon tube with 10 mL of RPMI 1640 medium enriched with enzymes Liberase (2 µg/mL, Roche) and DNase I Type IV (25 µL/mL, Sigma); the mixture was then homogenized at 37°C until complete dissociation. Red cells were lysed and any remaining cells enumerated manually as described above. In order to minimize differences due to samples size, we divided the cell count by grams of tissue collected.

2.6 | Lung histology

Pulmonary tissue integrity and inflammation levels were measured by histology using haematoxylin/eosin (Sigma-Aldrich). The lungs were fixed in 4% formalin and then cut into 5 µm slices and stained. The specific visualization of eosinophils was made using the Luna staining specific for eosinophils, as described elsewhere (Fisher Scientific).²² With this method, eosinophils stained strongly in red and were imaged using a light microscope.

2.7 | Cytokines, IgE and hormone determination

TGF-β1 (Mouse TGF-β1 DuoSet ELISA—R&D Cat DY1679-05), leptin (Mouse Leptin ELISA—Millipore Cod EZML-82K), triglycerides GOD/PAP (LaborLab 1770290), glucose GOD/PAP (LaborLab 1770130) and total cholesterol GOD/PAP (LaborLab 1770080) were measured in BAL and serum, respectively, following the instructions of the manufacturers.

2.8 | Collagen deposition

Collagen deposition in lung tissue was determined by histological staining using the Masson's trichrome method (Sigma-Aldrich). The presence of collagen fibres, stained in blue, was determined by optical microscopy, and their density was assessed by Image-Pro (v.2000) software.

2.9 | Immunohistochemistry

Histological sections were incubated with anti-TNFAIP3 (InsightTech bs-2803R-B) and anti-NF-κB p65 phospho S536 (Abcam Cat: ab86299). After washing, the tissue was incubated with the secondary antibody (Sigma, UK. Cat: SAB4600006) for 1 hour at 37°C. For quantification, five areas of interest per animal were photographed using a 20× objective and % of area of positive staining was quantified using Image-Pro Plus software, v5.5.0.29.

2.10 | Immunofluorescence (IF)

Bronchoalveolar alveolar lavage cells were attached to a microscope slide by cytospin, fixed with cold acetone and stored at -80°C. Cells were incubated with anti-CD103 (Clone 2E7 CAT 121407 BioLegend). Frozen lung tissue slices were incubated with anti-EpCAM (Clone G8.8 CAT 118201 BioLegend). Secondary antibody was added (Alexa-Fluor 594, CAT A11007, Life Technologies). All areas emitting fluorescence (regardless of intensity) were analysed and photographed using a 40× objective. The result is the product of both fluorescence intensity and fluorescent area, according to the following equation (CTCF = arbitrary units: CTCF = leucocyte integrated density-(leucocyte area × background value). Fluorescence was quantified using ImageJ, v 1.45s (NIH).

2.11 | Statistical analysis

Statistical analysis of data was performed by GraphPad Prism version 7. The statistical tests used were as follows: Student's *t* test, one-way ANOVA and two-way ANOVA with Tukey post-test, depending on the type of result analysed. All values with *P* < .05 were considered statistically significant according to visual demonstration: **P* < .05; ***P* < .01; ****P* < .001 and *****P* < .0001.

3 | RESULTS

Additional results can be found in the Supplemental Section.

3.1 | Experimental model of obesity

After 8 weeks of a high-fat diet, male and female mice presented an average 36% and 27% increase in body weight in comparison with their standard chow fed counterparts, respectively (Figure 1A). More importantly, mice fed a high-fat diet presented a significant increase in perigonadal fat accumulation in comparison with lean mice (Figure 1C). Metabolic markers like leptin, glucose, triglycerides and total cholesterol were also significantly increased after 8 weeks of a HFD (Figure 1D-G).

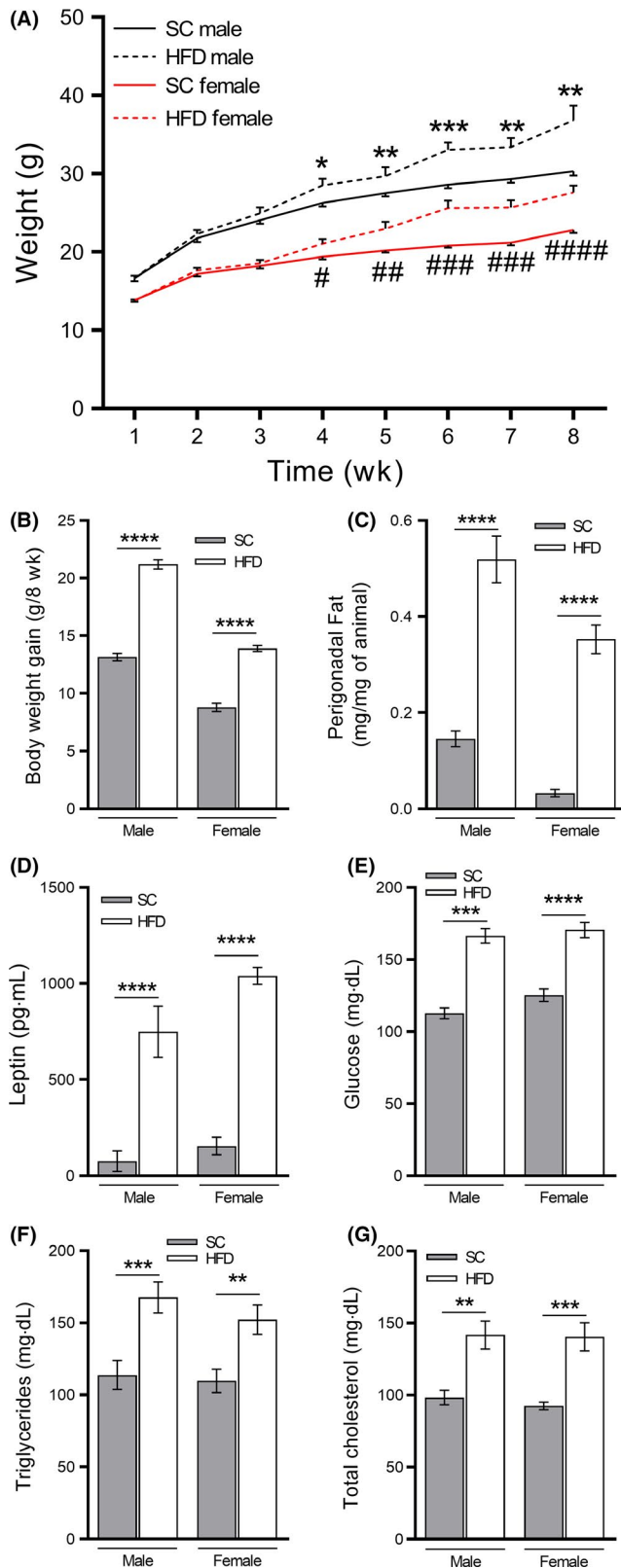


FIGURE 1 Metabolic panel of male and female C57Bl/6 mice fed for 8 wk with standard chow (SC) or high-fat (HFD) diets. Weight was measured weekly (A). At the end of the diet, we measured body weight gain (B), the perigonadal fat (C), serum levels of leptin (D), glucose (E), triglycerides (F) and total cholesterol (G). The results are expressed as Mean $\pm$ SEM of 10 animals per group. Significant differences among groups were analysed by Student *t* test. **P* < .05; ***P* < .01; ****P* < .001; *****P* < .0001

with Sham-immunized animals 24 hours after the last OVA challenge (Figure 2). Standard chow fed female mice presented a significantly higher number of total leucocytes in BAL in comparison with SC male mice (Figure 2A). This was reflected by a significant increase in BAL macrophages, neutrophils and eosinophils in comparison with female Sham-immunized animals and SC OVA males (Figure 2B-D).

High-fat diet male mice presented a significant increase in total leucocyte numbers in BAL fluid in comparison with male lean OVA and HFD control Sham-immunized mice (Figure 2A). HFD male mice also presented a significantly higher number of eosinophils in BAL fluid in comparison with SC OVA mice and control Sham mice (Figure 2D). In contrast, we did not observe a similar increase in total leucocyte numbers in HFD female mice. In fact, female HFD mice presented with a significant decrease in the number of leucocytes and eosinophils in BAL fluid in comparison with female SC OVA and male HFD OVA (Figure 2A). Female HFD mice also presented a significant decrease in eosinophils in BAL in comparison with female SC OVA and HFD male OVA group (Figure 2D).

More detail of these data, separated by sex and corrected by weight, can be found in the Supplemental Section, Figure S1 and S2.

3.3 | Leucocytes in lung tissue

Analysis of the number of leucocytes in the lung tissue revealed that male SC OVA mice had a significant accumulation of leucocytes in comparison with SC Sham group (Figure 3A). Female SC mice presented a significantly higher number of total leucocytes in response to OVA in comparison with all other groups, following the same trend observed in BAL fluid (Figure 3A). The differential analysis of leucocytes in the lung tissue demonstrated that female SC mice had a significantly higher number of macrophages, neutrophils and eosinophils in comparison with female SC Sham and male SC OVA group (Figure 3B-D).

High-fat diet male OVA mice presented a significant increase in total leucocytes in lung tissue in comparison with HFD Sham-immunized, but lower when compared to lean OVA mice (Figure 3A). Interestingly, HFD female Sham-sensitized animals presented a constitutive and significantly higher number of leucocytes in the lung tissue in comparison with SC Sham female mice that was not modified by the sensitization to OVA (Figure 3A). This increased number of leucocytes observed in the lung of HFD female mice is mainly reflected by a significantly higher number of neutrophils, macrophages and eosinophils in the lung in comparison with female SC Sham mice and male mice (Figure 3C).

3.2 | Leucocytes in BAL fluid

Our results confirm that immunization and challenge with OVA induced a significant increase in the total number of leucocytes in BAL fluid, reflected by significantly higher numbers of eosinophils in comparison

FIGURE 2 Effect of 8 wk of a standard chow (SC) or high-fat (HFD) diets on total and differential leucocyte count in bronchoalveolar lavage (BAL) on male and female C57Bl/6 mice sensitized and challenged with OVA. The intergroup differences on total leucocyte count in BAL of males and females in (A). The differential count for males and for females evidencing the intergroup differences for macrophages (B), neutrophils (C) and eosinophils (D). The results are expressed as Mean $\pm$ SEM of five animals per group. Significant differences among groups were analysed by two-way ANOVA, followed by Tukey post-test. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$

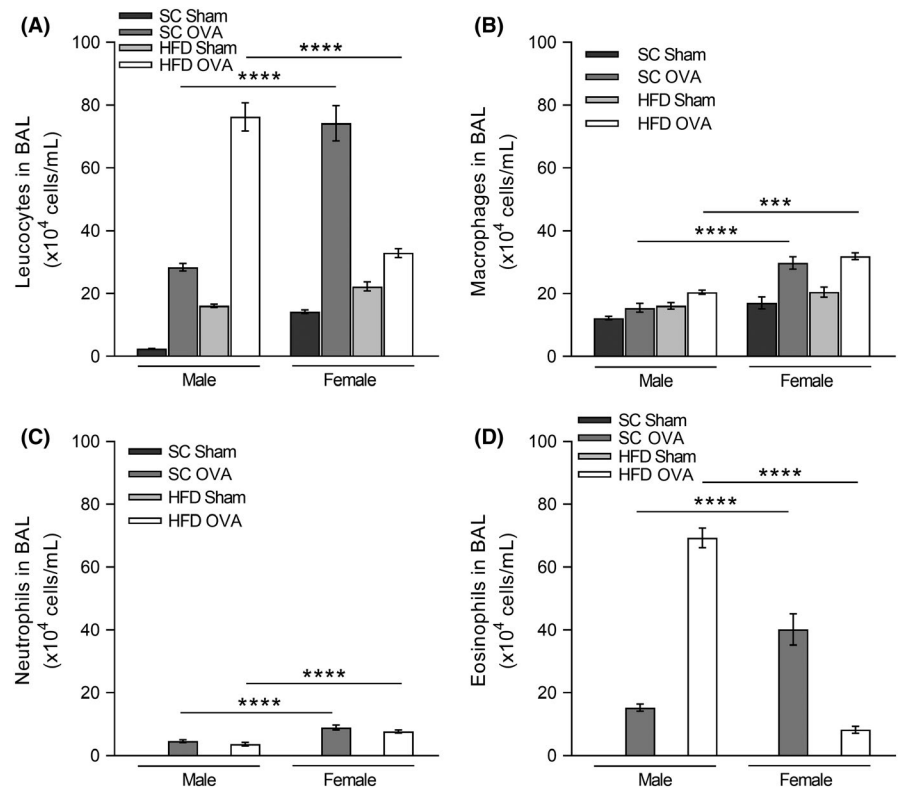
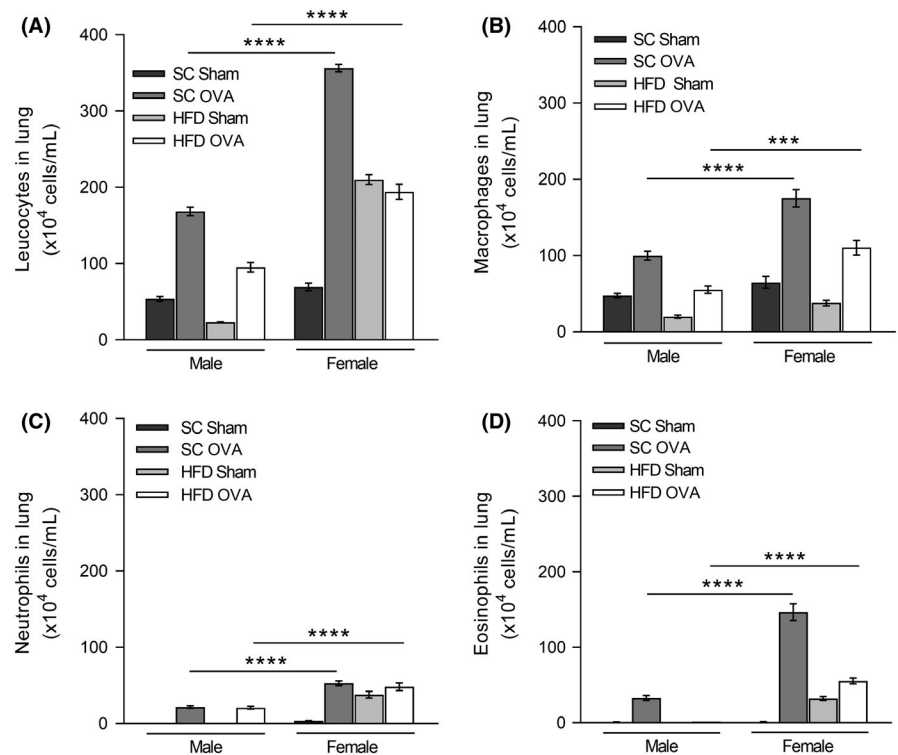


FIGURE 3 Effect of 8 wk of a standard chow (SC) or high-fat (HFD) diets on total and differential leucocyte count in lung tissue on male and female C57Bl/6 mice sensitized and challenged with OVA. The intergroup differences on total leucocyte count in lung tissue of males and females in (A). The differential count for males and for females evidencing the intergroup differences for macrophages (B), neutrophils (C) and eosinophils (D). The results are expressed as Mean $\pm$ SEM of five animals per group. Significant differences among groups were analysed by two-way ANOVA, followed by Tukey post-test. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$



More detail of these data, separated by sex and corrected by weight, can be found in the Supplemental Section, Figure S3 and S4.

The degree of pulmonary inflammation can be observed in Figure 4 by the analysis of haematoxylin/eosin (Figure 4A) and Luna staining for eosinophils (Figure 4B).

3.4 | Airway remodelling

To clarify whether the differences in leucocyte numbers between sexes would affect airway remodelling parameters, we measured collagen deposition around the airways and TGF- β 1 release into

BAL fluid. Our results show that in male mice, there are no significant differences in collagen deposition around the airways between HFD and SC OVA groups (Figure 5A-B). However, we observed a significant increase in collagen deposition in HFD OVA female mice when compared to HFD OVA males (Figure 5A-B). These results were mirrored by the levels of TGF- β 1 in BAL fluid, with HFD female OVA animals presenting significantly higher levels of this cytokine in comparison with HFD male OVA mice (Figure 5C).

3.5 | Expression of Adhesion molecules

To better understand the movement of leucocytes between lung tissue and lung lumen, we investigated the effect of a high-fat diet on

the expression of adhesion molecules associated with the epithelium. We measured the expression of CD103 on BAL cells and the transepithelial cell marker EpCAM in lung tissue. These results are depicted in Figure 5. Results show a significant increase in EpCAM in the lungs of OVA HFD male mice when compared to SC OVA mice (Figure 6A-B). In contrast, in OVA HFD female mice we observed a reduction in the expression of this adhesion molecule in the lung in comparison with lean females (Figure 6A-B). Similarly, the analysis of the expression of CD103 on the surface of BAL cells collected from male HFD mice demonstrated a significant reduction on its expression in comparison with SC mice. Female mice express significantly lower levels of this protein in comparison with males regardless of the diet (Figure 6C).

3.6 | Markers of inflammation

In the last step of our study, we investigated the expression of two important markers of inflammation in the lung tissue. We measured the expression of the A20/TNFAIP3 and activated NF- κ B (Figure 7). Independently of the diet, the expression of A20 protein was reduced in the lungs of all OVA groups compared to their respective Sham groups. HFD Sham animals presented a trend of reduction in the expression of A20 when compared to lean Sham animals, but this decrease only reached statistical significance in HFD females (Figure 7A).

The expression of phosphorylated NF- κ B in the lung tissue of lean mice revealed that, as expected, OVA-sensitized mice expressed a higher level of this nuclear factor in both male and female lean mice in comparison with Sham-immunized mice (Figure 7B). Lean female OVA mice demonstrated a statically higher increase in the activation of activated NF- κ B in comparison with Sham-sensitized and male mice. However, we did not observe an increase in the expression of this nuclear factor in HFD OVA male or OVA female mice in comparison with their Sham-sensitized counterparts (Figure 7B).

4 | DISCUSSION

In this study, we investigated the interaction between sex and weight in a model of allergic airway inflammation in mice. Using an acute model of lung inflammation induced by OVA, we demonstrated sex differences in the response to OVA that are significantly influenced by weight gain. In comparison with males, HFD allergic females exhibited a reduction in the number of leucocytes in BAL when challenged with OVA and, in contrast, an accumulation of these cells in the lung tissue. On the other hand, we also observed that allergic HFD female mice presented with a more robust lung remodelling than allergic HFD males, evidenced by higher deposition of collagen in the airways and higher levels of TGF- β in lung fluid and also a significantly higher levels of IgE in their serum (Figure S3). We did not observe significant differences in collagen deposition or TGF- β levels between SC and HFD males nor between SC females and SC males suggesting that in our model, airway remodelling is

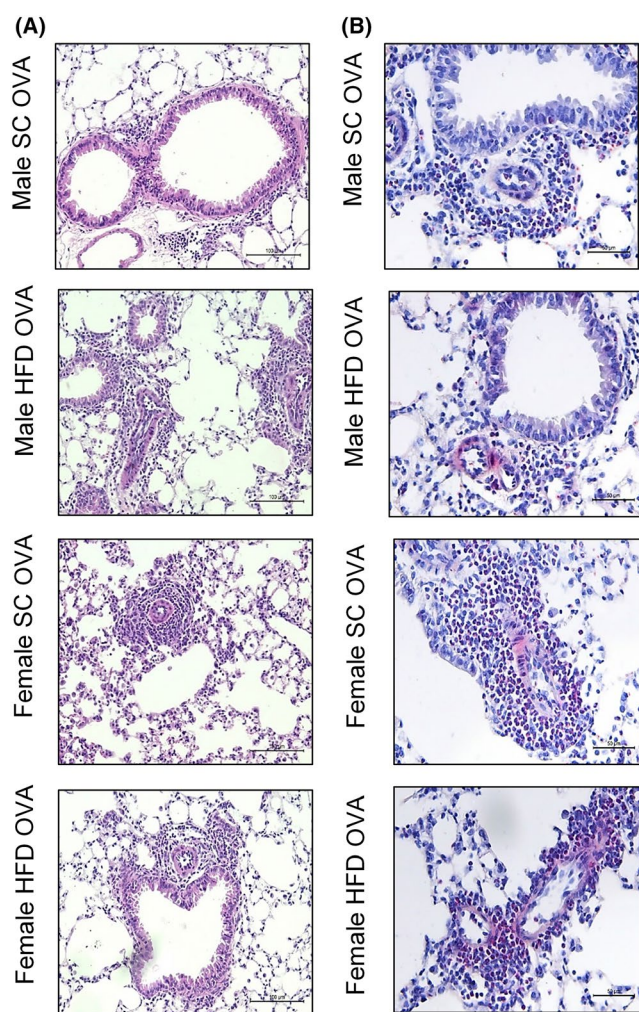
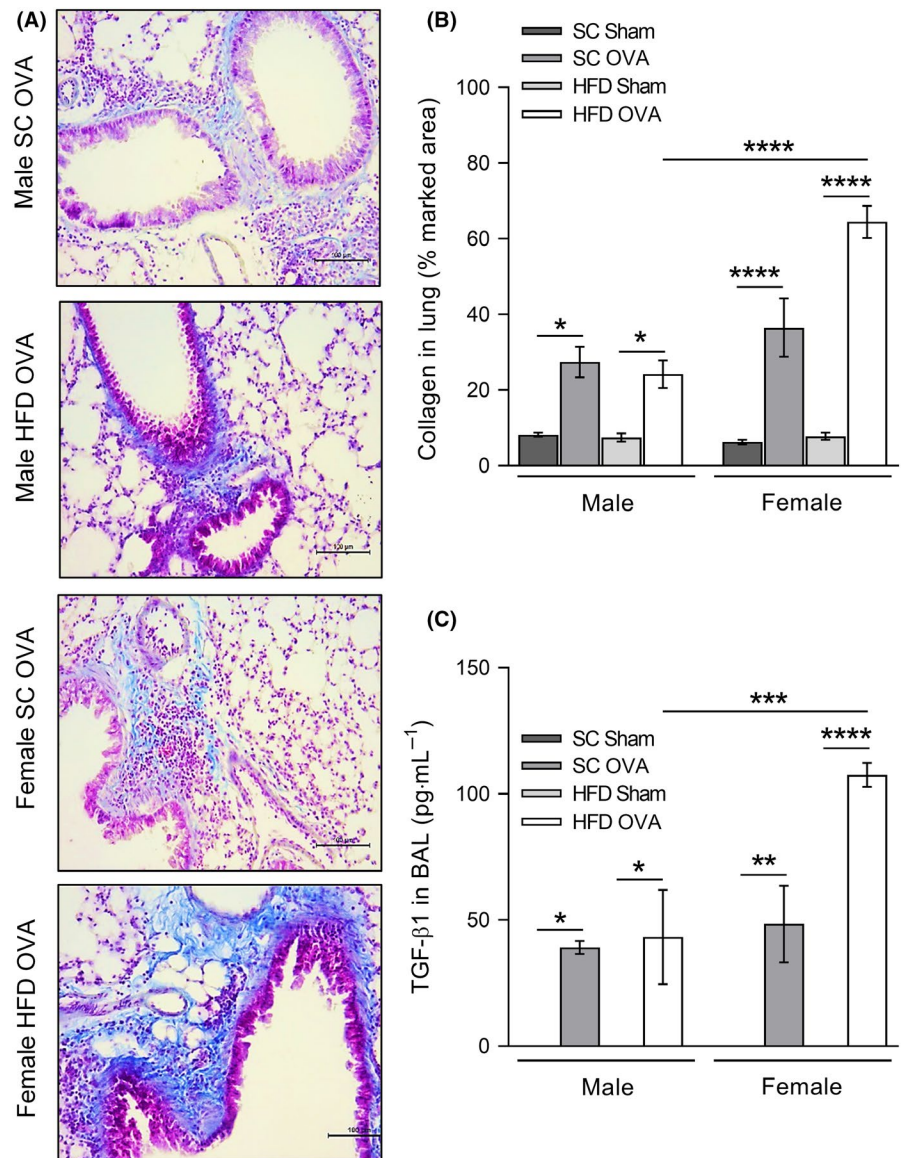


FIGURE 4 Effect of 8 wk of a standard chow (SC) or high-fat (HFD) diets on pulmonary inflammation on male and female mice fed with standard chow (SC) or high-fat (HFD) diets for 8 wk. In (A), general pulmonary histopathology stained by haematoxylin/eosin. In (B), pulmonary histopathology stained by Luna evidencing eosinophils in red. The photomicrographs refer to the OVA groups (sensitized and challenged to OVA). The scale has 100 μ m, the magnification is 20 $\times$, and the section has 5 μ m

FIGURE 5 Measurement of the presence of collagen in the peri-bronchoalveolar space and the presence of fibrotic agent TGF- β 1 in male and female mice fed with standard chow (SC) or high-fat (HFD) diets for 8 wk. In (A), pulmonary histopathology stained by Masson's trichrome for evidence of collagen fibres (in blue). In (B), transposition of the marked area in histopathology. In (C), production of TGF- β 1 measured by ELISA in bronchoalveolar lavage. The photomicrographs refer to the OVA groups (sensitized and challenged to OVA). The scale has 100 μ m, the magnification is 20 $\times$, and the section has 5 μ m. The results are expressed as Mean $\pm$ SEM of 4-5 animals per group. Significant differences among groups were analysed by two-way ANOVA, followed by Tukey post-test. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$



not affected independently by sex or by weight gain but rather by the interaction between these two factors. These findings are in line with what has been described in the literature where several studies have reported a sex difference in obesity and asthma^{9,23} with an association often found to be more significant in females than males.^{11,24} These observations have been frequently linked to the effect of female sex hormones in allergic asthma that have been well documented by us and other authors.^{17,18,25-29} In this regard, it has been shown that variations in female sex hormones during puberty, pregnancy or menopause can have a profound effect on asthma symptoms and severity.^{1,2}

Even though most part of the experimental studies found in the literature regarding allergic inflammation do not make comparisons between sexes, an attenuated eosinophilic response to OVA has been observed in HFD female mice, attributed to a lower production of eotaxin/CCL11.^{30,31} Other authors have observed similar results in male mice 24 hours after the last OVA challenge, but not at 48 hours, suggesting that diet-induced obesity delays the transit of

leucocytes through the airway epithelium into the airway lumen by an imbalance in TH2 cytokines.³² In clinical studies, analysing 131 patients with severe asthma, the authors have reported that airway submucosal eosinophil numbers were higher in obese subjects compared to a lean group, but there was no association between the numbers of eosinophils in sputum or peripheral blood and body mass index, suggesting a delayed transit of these cells through the epithelium into the airways.³³

Interestingly, the only cell type that was significantly modified by the HFD diet in the lung tissue of OVA males or OVA females was eosinophils. We did not observe significant differences in the number of neutrophils and macrophages between diets within the same sex. Eosinophils are the predominant effector cells in allergic inflammatory diseases and tissue eosinophilia is a hallmark of bronchial asthma and our results confirm this observation. Other experimental studies have described an increased production of eosinophils in the bone marrow of obese mice after the last OVA challenge.³² Clinically, an increased eosinophilic activity

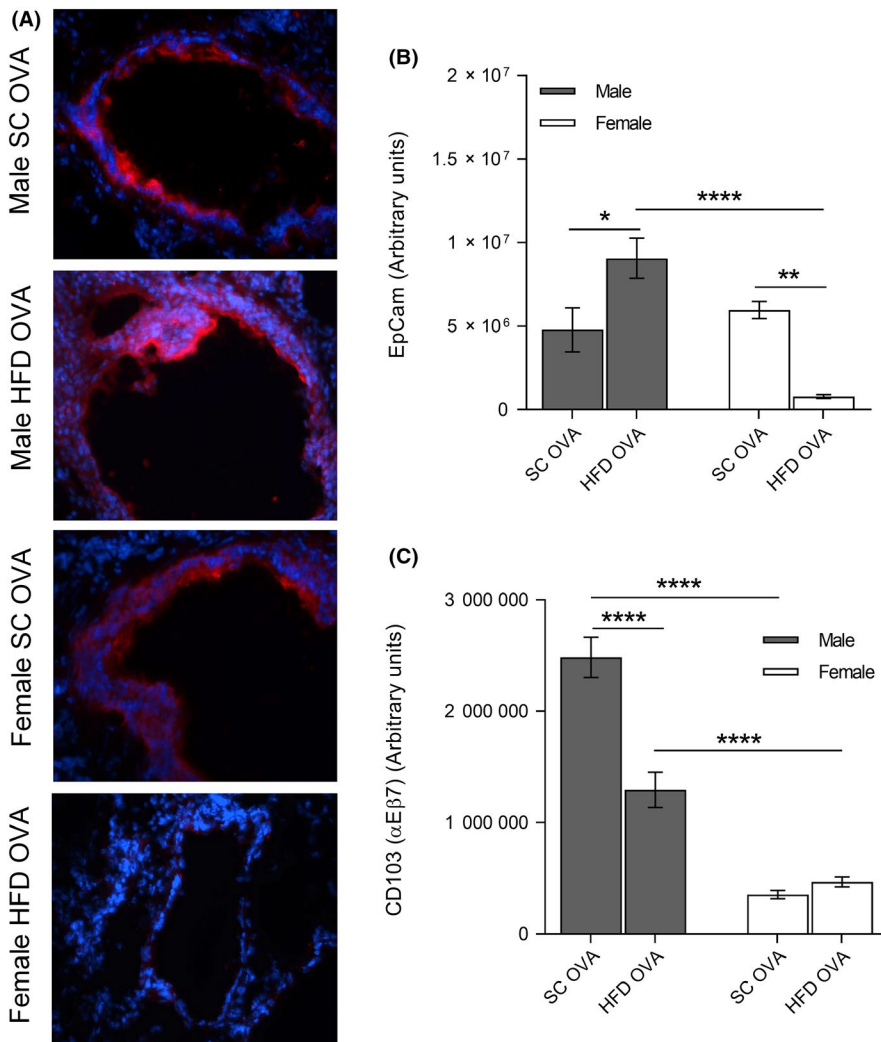


FIGURE 6 Expression of cell migration molecules in lung tissue and in bronchoalveolar lavage leucocytes from male and female mice fed with standard chow (SC) or high-fat (HFD) diets for 8 wk. In (A), expression of EpCAM in the bronchiolar epithelium by immunofluorescence. In (B), transposition of the labelled area into immunofluorescence. In (C), expression of CD103 in immunofluorescence bronchoalveolar lavage leucocytes. The photomicrographs refer to the OVA groups (sensitized and challenged to OVA). The scale has 100 μ m, the magnification is 20 $\times$, and the section has 5 μ m. The results are expressed as Mean $\pm$ SEM of 4-5 animals per group. Significant differences among groups were analysed by two-way ANOVA, followed by Tukey post-test. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$

(chemotaxis and adhesion) has been described in atopic asthmatic obese children and adolescent compared to non-obese and non-asthmatic volunteers, associated with high serum leptin and TNF- α levels.³⁴ In our study, we did not measure airway inflammation at later time-points or a longer period of diet so we cannot rule out the possibility that the attenuated migration of leucocytes to the lung lumen observed in HFD female mice is in fact a delayed response to OVA. However, the fact that we did not observe the same attenuation in HFD males suggests that this result cannot be attributed solely to the diet. Similarly, we cannot rule out significant changes in other cell populations in HFD mice at other time-points, as other authors have described before.³¹ More recently, Schroder et al have also demonstrated that female mice fed a 12-week HFD diet are protected against a lung allergic response to OVA in comparison with lean mice. The authors suggested that this is an effect related to the short-term of the diet and not to the obesogenic phenotype per se. The authors reported that HFD female mice presented a reduced airway hyperresponsiveness, airway inflammation, diminished Th1/TH17 but unchanged TH2 differentiation.³⁵ These results are in line with our observations, where females also presented an attenuated inflammatory

response to OVA after an even shorter high-fat diet of 8 weeks. However, the authors did not compare their observations to animals fed a HFD for a longer period or to male mice in order to confirm that the attenuation observed in their study is indeed due to the short length of the diet and not to the sex of the animals. This is very important when we consider that in our study, we do not see the same results in male mice fed the same diet and for the same period of time than the females, suggesting that the effect of a shorter diet may be sex-dependent.

The inability of leucocytes to cross the epithelial barrier towards the lung lumen when HFD female mice are challenged with an allergen is an indication that an impaired cell recruitment may be occurring. Many of the adhesion molecules involved in the pathology of allergic airway inflammation are expressed in both epithelial and endothelial cells with a few molecules more closely related to the crossing of leucocytes through the epithelium and to its integrity. Among them, we can point out EpCAM and CD103.^{36,37} CD103 is an integrin protein that is expressed widely on intraepithelial lymphocytes, T cells and regulatory cells.³⁸ It binds to E-cadherin in the epithelium, and it has been demonstrated to be highly expressed in lungs following allergen challenge with either OVA or house dust

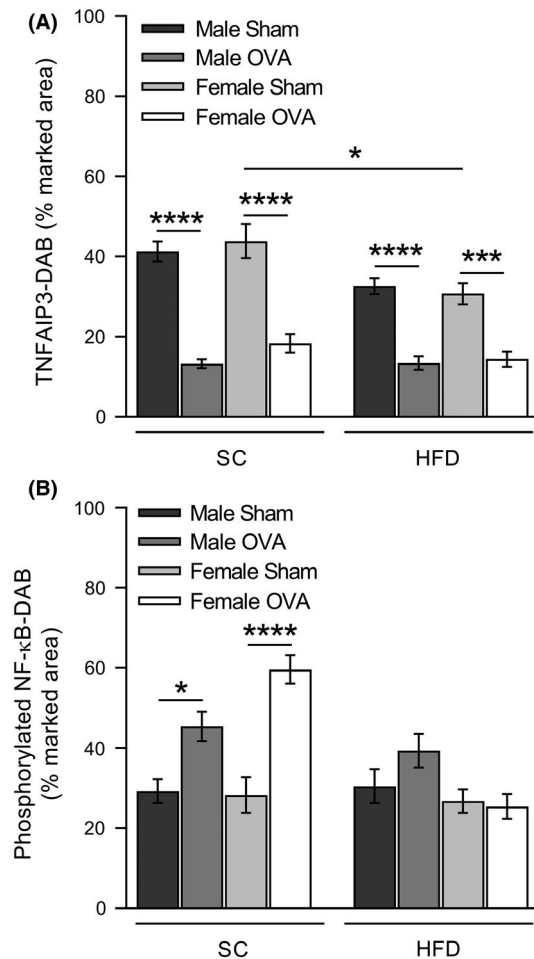


FIGURE 7 Involvement of NF-κB and its repressor, A20 protein, in pulmonary inflammation of male and female mice fed with standard chow (SC) or high-fat (HFD) diets for 8 wk. In (A) and (B), expression of protein A20 (TNFAIP3) and activated NF-κB, respectively, in lung tissue measured by immunohistochemistry. The results are expressed as Mean ± SEM of 4-5 animals per group. Significant differences among groups were analysed by two-way ANOVA, followed by Tukey post-test. * $P < .05$; ** $P < .01$; *** $P < .001$; **** $P < .0001$

mite (HDM).³⁷ The expression of CD103 has also been demonstrated in cells present in BAL fluid and lung homogenates, and the absence of CD103 worsens the migration of leucocytes into the lung and prolongs the response to the allergen, suggesting that CD103 is crucial in regulating the severity of airway inflammation in asthma and may have some important role in the resolution of an allergic inflammatory response.³⁷ Our results support these observations as we have demonstrated that CD103 is constitutively inhibited in BAL cells of females, which may explain why female mice present a higher accumulation of inflammatory cells in lung tissue, regardless of the diet. Similarly, we observed a significant inhibition of the expression of CD103 in HFD OVA male mice that may partly explain the worsening of their response to OVA in comparison with lean mice. Further experiments are necessary to determine whether the low expression of this molecule in HFD mice has any effect on the resolution of airway inflammation.

However, CD103 alone does not explain the differences observed between HFD males and females regarding the number of cells in the BAL fluid. Therefore, we investigated the role of the epithelial cell-adhesion molecule (EpCAM), an adhesion molecule that is important in the preservation of the integrity of the epithelium. It has also been described as having a stimulatory effect on cell migration in vitro.³⁹ EpCAM is a transmembrane glycoprotein that has been described in the lower respiratory tract, the trachea, bronchi, bronchioles and alveoli.^{40,41} In our study, we have found that the expression of EpCAM is not significantly different between lean males and lean females exposed to OVA, suggesting that in its expression is not regulated by sex hormones. However, in obesity this dynamic is significantly altered, and we observed an increase in the expression of this protein in the airways of HFD male mice and a significant inhibition in HFD female mice when exposed to the allergen, in comparison with their lean counterparts. These observations agree with what we observed regarding leucocyte migration through the epithelial layer in these animals, suggesting that the interaction between obesity and sex alters the expression of EpCAM in the epithelium of HFD female mice, contributing to their impaired movement of cells to the lung lumen and possibly altering the integrity of their epithelium.

Another important aspect observed in this study is the constitutive accumulation of leucocytes in the lung tissue of female HFD mice that is not significantly altered by sensitization and exposure to OVA. This observation suggests a pre-inflammatory status in the lung of these animals that is not present in the lung of HFD males. In addition, the accumulation of neutrophils in the lung of HFD Sham females pointed out to a inflammatory pathway that is not restricted to a TH2 profile. In this regard, data in the literature have suggested that persistent inflammation and airway remodelling in asthma are linked to NF-κB activation.⁴² NF-κB is a critical transcription factor that is activated in the airway epithelium of human asthmatics and mice after allergic stimulation, directing high-level transcription of many cytokines, adhesion molecules and other proinflammatory proteins.⁴²⁻⁴⁴ Inhibitors of NF-κB activation diminish the influx of inflammatory cells and reduce the airway responsiveness induced by the allergen as well as the expression of some adhesion molecules.⁴⁵ Among endogenous inhibitors of NF-κB, A20 has been suggested to be an important modulator of the inflammatory response in allergic asthma. The A20 ubiquitin protein, also known as TNFAIP3, is a zinc-finger protein that inhibits NF-κB activation by deubiquitinating key signalling pathways downstream of TLR-4, IL-1 and the TNF family of receptors.⁴⁶ It has been demonstrated that A20 is an important regulatory molecule in allergic asthma and mRNA levels of A20 measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) were shown to be significantly reduced in epithelial cells obtained from subjects with mild or severe asthma when compared with healthy controls, findings confirmed with protein levels.⁴⁷ However, despite the evidence of the role of A20 in allergic responses, little is known regarding its expression in obesity. Some reports in the literature have shown A20 expression is decreased in HFD mice leading to an increase in cardiac dysfunction.⁴⁸ In our

study, we show that the expression of this ubiquitin is decreased in HFD mice in comparison with lean animals, but this was statistically significant only in females, suggesting a pre-inflammatory status in the lung of these animals. Even though we did detect a further decrease in the levels of A20 in OVA HFD females in comparison with the Sham group, we did not observe an increase in the expression of activated NF- κ B in these animals. These results suggest that in obesity, an A20 independent pathway of activation/deactivation of NF- κ B may be involved. In this regard, several other endogenous molecules that negatively regulate the activation or activity of NF- κ B have been identified. These molecules include CYLD, cyPG15-deoxy-12,14-prostaglandin J2, Foxj1, Twist proteins and -arrestins.⁴⁹

In summary, our results present evidence that the interaction between sex and weight gain alters the progression of allergic asthma in mice with females developing airway remodelling at a much earlier stage than males. These data may contribute to a better understanding of the clinical differences in the development and severity of allergic asthma observed between men and women in reproductive age.

ACKNOWLEDGEMENTS

We would like to thank the King's College London and the Sackler Institute of Pulmonary Pharmacology for the institutional support, Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP/BRAZIL) for the financial support by the Process 2016/22722-6 and Dr Wothan Tavares de Lima for his important contribution to the discussion of the results.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: e-Lacerda RR, Anh  GF, Page CP, Riffo-Vasquez Y. Sex differences in the influence of obesity on a murine model of allergic lung inflammation. *Clin Exp Allergy*. 2019;00:1-11. <https://doi.org/10.1111/cea.13541>

SEX DIFFERENCES IN THE INFLUENCE OF OBESITY ON A MURINE MODEL OF ALLERGIC LUNG INFLAMMATION

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Supplemental Information second review

1 Methods

1.1 Pulmonary homogenate analysis

A fragment of lung was removed and broken into small pieces. The fragments were placed in a falcon tube with 10 mL of RPMI 1640 medium enriched with enzymes Liberase (2µg/mL) and DNase I Type IV (25µl/mL), the mixture was homogenized at 37 °C for 45 minutes or until complete dissociation. Cells suspension was filtered and centrifuged at 10 °C (300 G for 5 minutes). 1 mL of red blood cell lysis buffer was added and mixed for 1 minute followed by the addition of 10 mL of saline. The total number of cells in the suspension was counted with an improved Neubauer haemocytometer. For differential cell counts, cytopsin preparations were stained with Diff Quick (DADE Behring, Germany) and cells were counted using standard morphological criteria.

1.2 Lung histology

Pulmonary tissue integrity and inflammation level were measured by histology using Haematoxylin/Eosin and Luna staining. The lungs were fixed in 10% formalin for 72 hours, washed in saline solution, immersed in liquid paraffin and immobilized on a histological cassette. Cuts of 5 µm were produced with a microtome. Haematoxylin/Eosin staining was

performed after dewaxing tissue in xylene for 10 minutes followed by hydration in decreasing baths from ethanol, to water. The tissue was in contact with haematoxylin for 3 minutes followed by wash in water and 8 dives in a differentiator (methanol). After differentiation, the tissue was immersed in eosin for 15 seconds followed by a wash in water. The slides were dehydrated by rising with ethanol, diaphanized with xylol and mounted in Entellan for optical microscopy.

The specific visualization of eosinophils was made by Luna staining. The tissue was dewaxed and hydrated as described above. It was immersed in a solution of Ferric Haematoxylin and Biebrich Scarlet for 8 minutes. After washing with water, the slides were dipped 8 times in acid alcohol followed by washing in water. The slides were immersed in a lithium carbonate solution for 5 times, dehydrated, diaphanized and resin-assembled. Eosinophils, stained strongly in red, were observed under a microscope.

1.3 Collagen deposition

Collagen deposition in lung tissue was determined by histological staining using the Masson's Trichrome method (Sigma, UK). Dewaxed and hydrated tissue was immersed in mordent solution for 15 min at 56 °C with subsequent washing in water. The slides were immersed in ferric haematoxylin for 5 minutes followed by another water wash, and were in contact with acidic solution by Biebrich Scarlet for 5 min, followed by phosphotungen / phosphomolybdic acid solution for 5 min and then, in aniline solution for a further 5 min. The slides were then differentiated by immersion in acetic acid 1% aqueous solution for 2 minutes, followed by the dehydration step. The presence of collagen fibers, stained in blue, was determined by optical microscopy and their density by was assessed by a software: ImagePro (v.2000).

1.4 Immunohistochemistry (IHC)

Histological sections were hydrated in decreasing washings of ethanol to water and had their antigens recovered by steam in sodium citrate buffer pH 6. Endogenous peroxidase was inactivated in the tissue with an ethanol solution containing 3% hydrogen peroxide, followed by permeabilization with PBS + BSA (1% + 0.5%) and Tween for 15 minutes, and blocking with PBS + 1% BSA for 1 hour. Primary antibodies (A20/TNFAIP3 at concentration 1:200 and $\text{fNF-}\kappa\text{B}$ at 1:1000 concentration) were diluted in a blocking solution and incubated with the tissue overnight at 4 °C (Anti-TNFAIP3 – InsightTech bs-2803R-B and Anti-NF- κB p65 phospho S536 – Abcam Cod ab86299). After washing, the tissue was incubated with the secondary antibody (1:200 concentration) for 1 hour at 37 °C. After washing, the tissue was incubated with an amplifier antibody for 30 minutes and then with the substrate (DAB + H_2O_2) for 10 minutes. After contrast with haematoxylin, the sections were dehydrated in increasing solutions of ethanol and mounted on coverslips for optical microscope observation.

1.5 Immunofluorescence (IF)

BAL samples by cytopspin, were fixed with acetone and stored at -80 °C. Slides were washed with PBS + 0.01% Tween for 10 minutes, permeabilized with PBS + 0.5% Tween for 30 minutes and blocked with PBST + 1% BSA for 30 minutes. Cells were incubated with the primary antibodies for 2 hours (1:50 CD103 - Clone 2E7 Cat 121407 Biolegend), washed, and then the secondary antibody was added (1:250, Alexa-Fluor 594, cat A11007, Life Technologies, UK) and incubated for 1 hour at 37 °C, while protected from light. After washing, slides were immersed in DAPI (1:3000 concentration) for cell contrast and mounted for fluorescence microscopy observation. EP-Cam (1:250, Clone G8.8 Cat 118201 Biolegend) levels were determined in lung tissue. Lungs were perfused with OCT 10% and frozen by

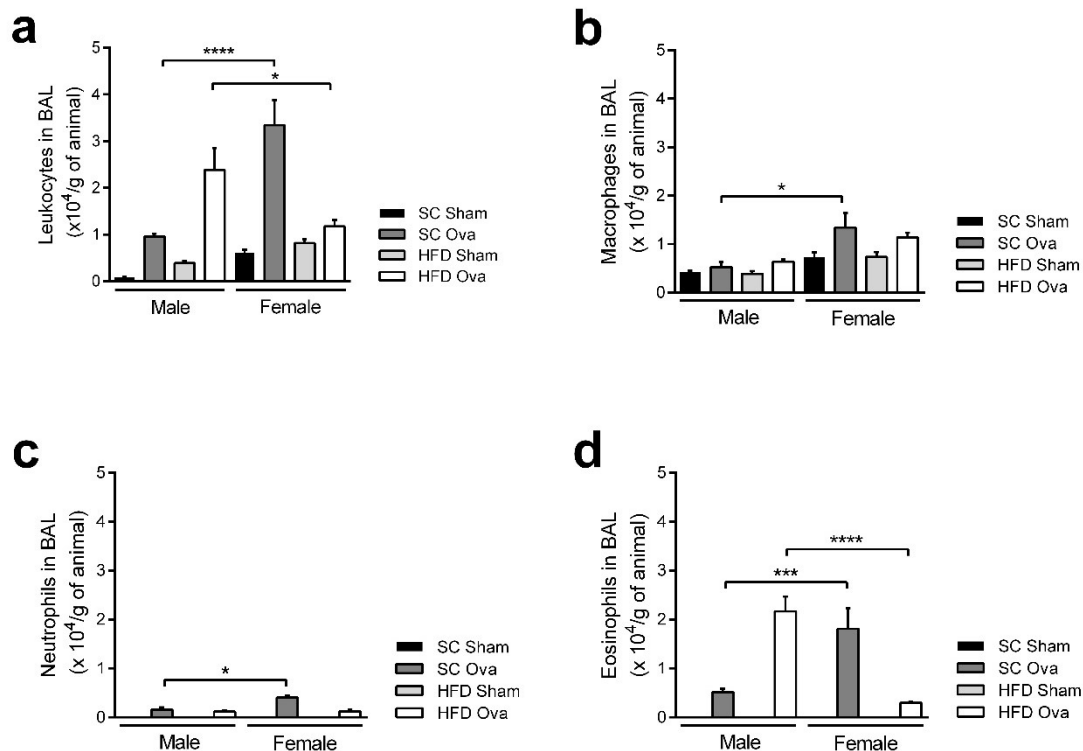
cryopreservation on OCT with dry ice. Fluorescence was quantified using Image J, v 1.45s (NIH, USA).

2 Results

Supplementary figure 1. Effect of 8 weeks of a standard chow (SC) or high fat (HFD) diets on total and differential leukocytes count in bronchoalveolar lavage (BAL) on male and female C57Bl/6 mice sensitized and challenged with OVA. Intergroup Differences corrected by weight:

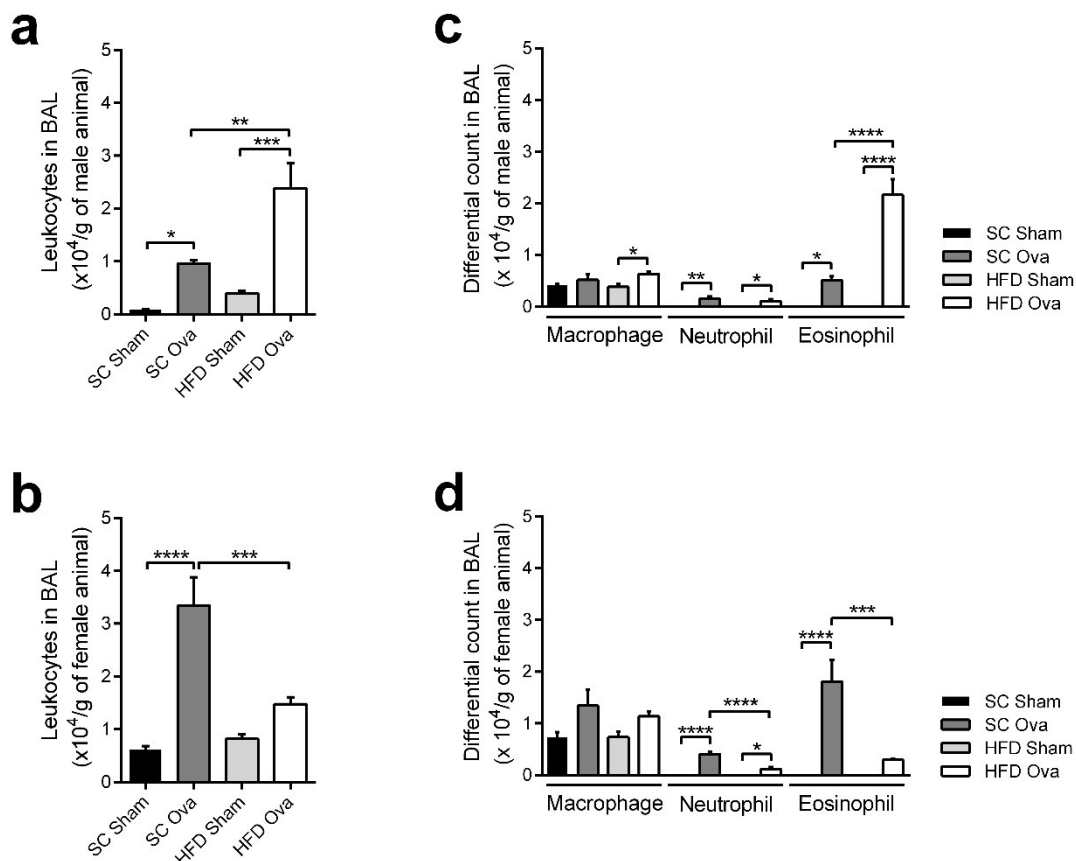
Total leukocytes count in BAL of males and females in (a). The differential count for males and for females evidencing the intergroup differences for macrophages (b), neutrophils (c) and eosinophils (d). The results are expressed as Mean $\pm$ SEM of 5 animals per group. Significant differences among groups were analysed by two-way ANOVA, followed by Tukey post-test.

* $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$.



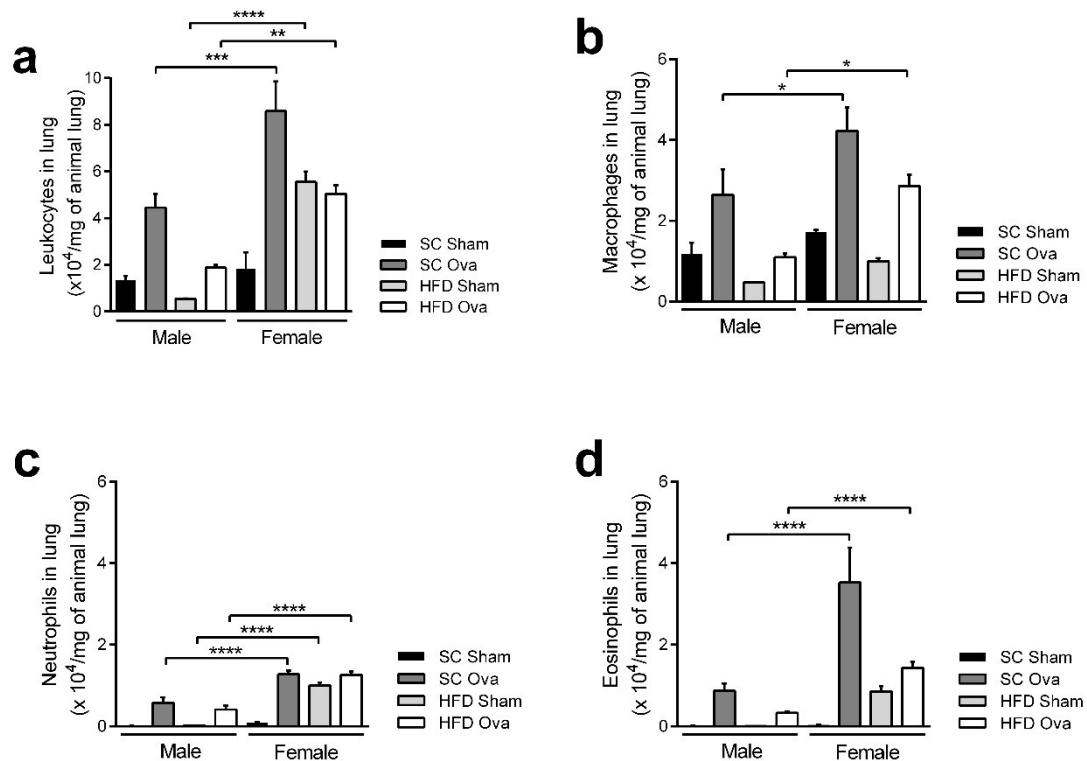
Supplementary figure 2. Effect of 8 weeks of a standard chow (SC) or high fat (HFD) diets on total and differential leukocytes count in bronchoalveolar lavage (BAL) on male and female C57Bl/6 mice sensitized and challenged with OVA- Intragroup differences corrected by weight:

Total leukocytes count in BAL in male is in (a) and female in (b). The differential count for males is in (c) and for females in (d) evidencing the intragroup differences. The results are expressed as Mean $\pm$ SEM of 4-5 animals per group. Significant differences among groups were analysed by two-way ANOVA, followed by Tukey post-test. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$.



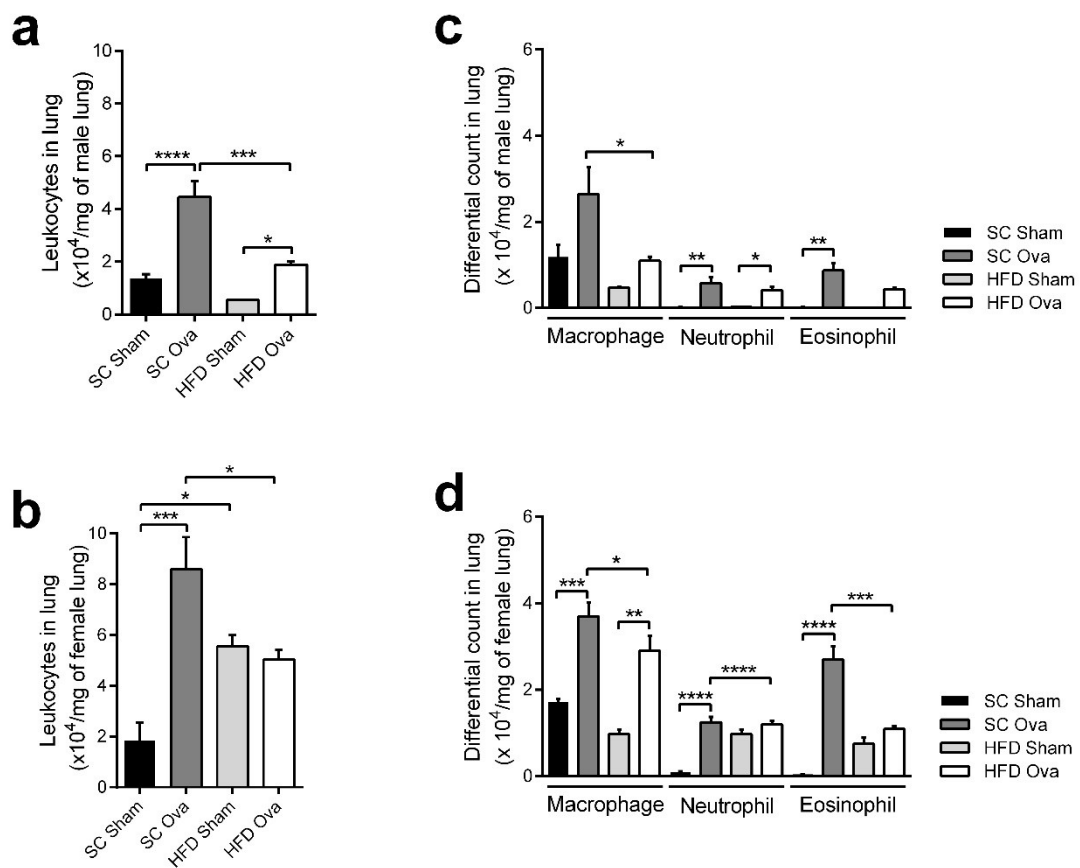
Supplemental figure 3. Effect of 8 weeks of a standard chow (SC) or high fat (HFD) diets on total and differential leukocytes count in lung tissue on male and female C57Bl/6 mice sensitized and challenged with OVA. Intergroup Differences corrected by weight:

Total leukocytes count in lung tissue of males and females in (a). The differential count for males and for females evidencing the intergroup differences for macrophages (b), neutrophils (c) and eosinophils (d). The results are expressed as Mean $\pm$ SEM of 5 animals per group. Significant differences among groups were analysed by two-way ANOVA, followed by Tukey post-test. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$.



Supplementary figure 4. Effect of 8 weeks of a standard chow (SC) or high fat (HFD) diets on total and differential leukocytes count in lung tissue on male and female C57Bl/6 mice sensitized and challenged with OVA. Intragroup differences corrected by weight:

Total leukocytes count in lung tissue in male is in (a) and female in (b). The differential count for males is in (c) and for females in (d) evidencing the intragroup differences. The results are expressed as Mean $\pm$ SEM of 4-5 animals per group. Significant differences among groups were analysed by two-way ANOVA, followed by Tukey post-test. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$.



Supplementary figure 5. Effect of 8 weeks of a standard chow (SC) or high fat (HFD) diets on serum IgE production on male and female C57Bl/6 mice sensitized and challenged with OVA.

The results are expressed as Mean $\pm$ SEM of 4-5 animals per group. Significant differences among groups were analysed by two-way ANOVA, followed by Tukey post-test. * $p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$.

