



Leukotriene-mediated sex dimorphism in murine asthma-like features during allergen sensitization

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ABSTRACT

The incidence and severity of asthma preponderate in women versus men. Leukotrienes (LTs) are lipid mediators involved in asthma pathogenesis, and sex disparities in LT biosynthesis and anti-LT pharmacology in inflammation have recently emerged. Here, we report on sex dimorphism in LT production during allergen sensitization and its correlation to lung function. While high plasma levels of IgE, as sensitization index, were elevated in both sexes, LT levels increased only in lungs of female ovalbumin-sensitized BALB/c mice. Sex-dependent elevated LT levels strictly correlated to an enhanced airway hyperreactivity, pulmonary inflammation and mast cell infiltration/activation in female mice. Importantly, this sex bias was coupled to superior therapeutic efficacy of different types of clinically used LT modifiers like zileuton, MK886 and montelukast in female animals. Our findings reveal sex-dependent LT production as a basic mechanism of sex dimorphism in allergic asthma, and suggest that women might benefit more from anti-LT asthma therapy.

1. Introduction

Asthma is a clinically heterogeneous disorder characterized by airway hyperreactivity, chronic inflammation and mucus production. The onset and progression results from genetic susceptibility, allergens, comorbidities and viral triggers [1,2]. Asthma has a higher incidence in adult females [3] and the prevalence and severity of asthma coincides with key phases in the reproductive life stage of women. It has been demonstrated that hormonal fluctuation during the menstrual cycle results in a phase-specific worsening of disease severity [4–6]. Moreover, women seem to experience more asthma symptoms than men and to use more rescue medications with a negative impact on life quality

[3]. Conversely, asthma during puberty is more prevalent in boys compared to girls [5]. Similarly, sex difference with preponderance in females versus males was observed in several experimental animal models of asthma [7–10].

LTs are a class of immunomodulating lipid mediators that are rapidly synthesized in response to various stimuli via the 5-lipoxygenase (5-LOX) pathway. After release from phospholipids by cytosolic phospholipase A₂, arachidonic acid (AA) is delivered to 5-LOX by the nuclear membrane-bound 5-LOX-activating protein (FLAP) for conversion to 5-hydro(pero)xyeicosatetraenoic acid and then to LTA₄. LTA₄ can be metabolized to LTB₄ by LTA₄ hydrolase or to the cysteinyl-containing LTs (cys-LTs) C₄, D₄ and E₄ by LTC₄ synthase [11]. Both, LTB₄ and cys-

Abbreviations: 5-LOX, 5-lipoxygenase; AA, arachidonic acid; ELISA, enzyme-linked immunosorbent assay; FLAP, 5-LOX-activating protein; H&E, Hematoxylin and eosin; MK, MK886; MS, montelukast; OVA, ovalbumin; PAS, periodic acid–Schiff; ZIL, zileuton

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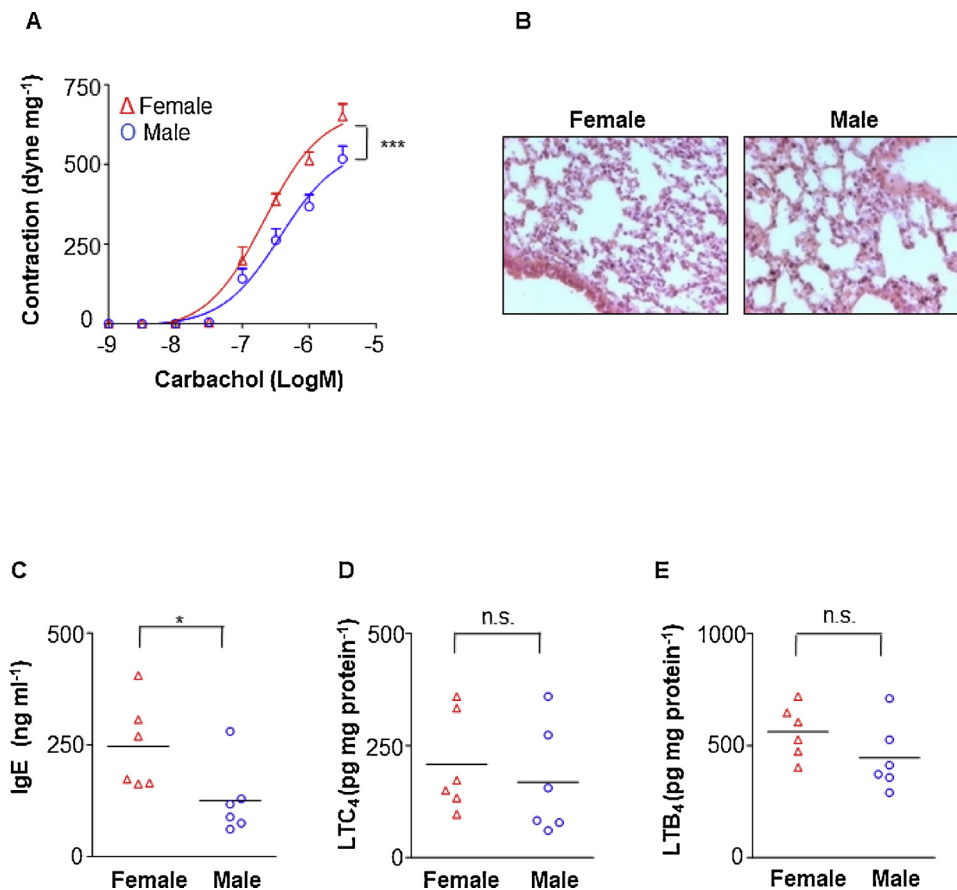


Fig. 1. Sex differences in lung function of non-sensitized mice. Lungs, bronchi and blood were harvested from naïve (untreated) BALB/c mice of both sexes. A) Bronchial reactivity to carbachol *in vitro*, B) lung histology using H&E staining, C) plasma IgE levels as well as D) LTC₄ and E) LTB₄ pulmonary levels were evaluated in female and male non-sensitized BALB/c mice. Values represent means $\pm$ S.E.M (A) and mean with single data (C–E); $n = 6$ mice for each group. Significance is represented by * $P < 0.05$ and *** $P < 0.001$, n.s., not significant ($P > 0.05$). Two-way ANOVA plus Bonferroni (A) and two-tailed Student *t*-test (C–E).

LTs play an important pro-inflammatory role in the early- and late-phase of asthmatic response [12].

Recently, sex has emerged as a key variable in the regulation of the 5-LOX pathway. It has been shown that female mice or human cells produce higher amounts of LTs [13–17]. Such sex difference in LT production may play an important role in the development of sex-biased inflammatory responses; many of which dominate in females [15,16]. During inflammatory reactions androgens affect 5-LOX regulatory cellular events, such as 5-LOX subcellular localization, phospholipase D activity, and the assembly of the 5-LOX/FLAP complex, with consequent lower LT biosynthesis in males versus females [13,14,16].

Here, we demonstrate that a significant increased LT formation occurs in female BALB/c mice as compared to males during allergen sensitization, an important risk factor for the development of asthma [18]. This sex-related LT generation translates into differences in bronchial hyperreactivity, lung inflammation and mast cell recruitment/activation in female versus male mice. Moreover, different types of LT modifiers, that is, the cys-LT receptor antagonist montelukast (MS), the 5-LOX inhibitor zileuton (ZIL), and the FLAP inhibitor MK886 (MK) protected female mice more efficiently against allergen sensitization and pathological lung functions than male animals. All together, our findings show a role for LTs in sex dimorphism in asthma and imply a superior efficacy of anti-LTs in asthma therapy of women versus men.

2. Materials and methods

2.1. Animals

Adult male and female BALB/c and C57BL/6 mice (8–9 weeks old, males 24–27 g and females 18–22 g, Charles River, Calco, Italy) were housed in the animal care facility of the Department of Pharmacy of the

University of Naples, Italy in a controlled environment (temperature $21 \pm 2^\circ\text{C}$ and humidity $60 \pm 10\%$) and provided with standard rodent chow and water. All animals were allowed to acclimate for four days prior to experiments and were subjected to 12 h light – 12 h dark schedule. Each mouse was assigned an identity number and randomized to different groups so that all experiments were carried out in a blinded manner. Mice were divided into equal-sized groups ($n = 6$ per group and per cage) not predetermined by a statistical method but by previous experimental data in this murine sensitization model [19–21]. Five animals were used for the Periodic acid–Schiff (PAS; Sigma-Aldrich, Milan, Italy) staining and lung resistance evaluation. Experiments were conducted during the light phase.

The experimental procedures were approved by the Italian Ministry according to International and National law and policies (EU Directive 2010/63/ EU and Italian DL 26/2014 for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept). Animal studies are reported in compliance with the ARRIVE guidelines [22]

2.2. Ovalbumin-sensitization and experimental protocol

Balb/c and C57BL/6 mice are commonly used to study asthma-like features *in vivo* and to investigate the underlying molecular and cellular mechanisms of allergen sensitization [19–21,23,24]. Adult male and female mice were treated subcutaneously (s.c.) with 0.4 mL (100 μg) of ovalbumin (OVA) complexed with alum (13 mg mL^{-1}) (OVA group) at day 0 and 7. This experimental procedure, without challenge in the airways, induces in the mice an increase in plasma IgE levels and this effect is by itself sufficient to induce a significant increase in bronchial hyperreactivity and pulmonary inflammation, typical hallmarks of asthma [19–21]. A group of animals was treated s.c. with saline (vehicle group). Animals were killed at selected time points by overdose of enflurane and lungs, bronchi and blood collected. At all

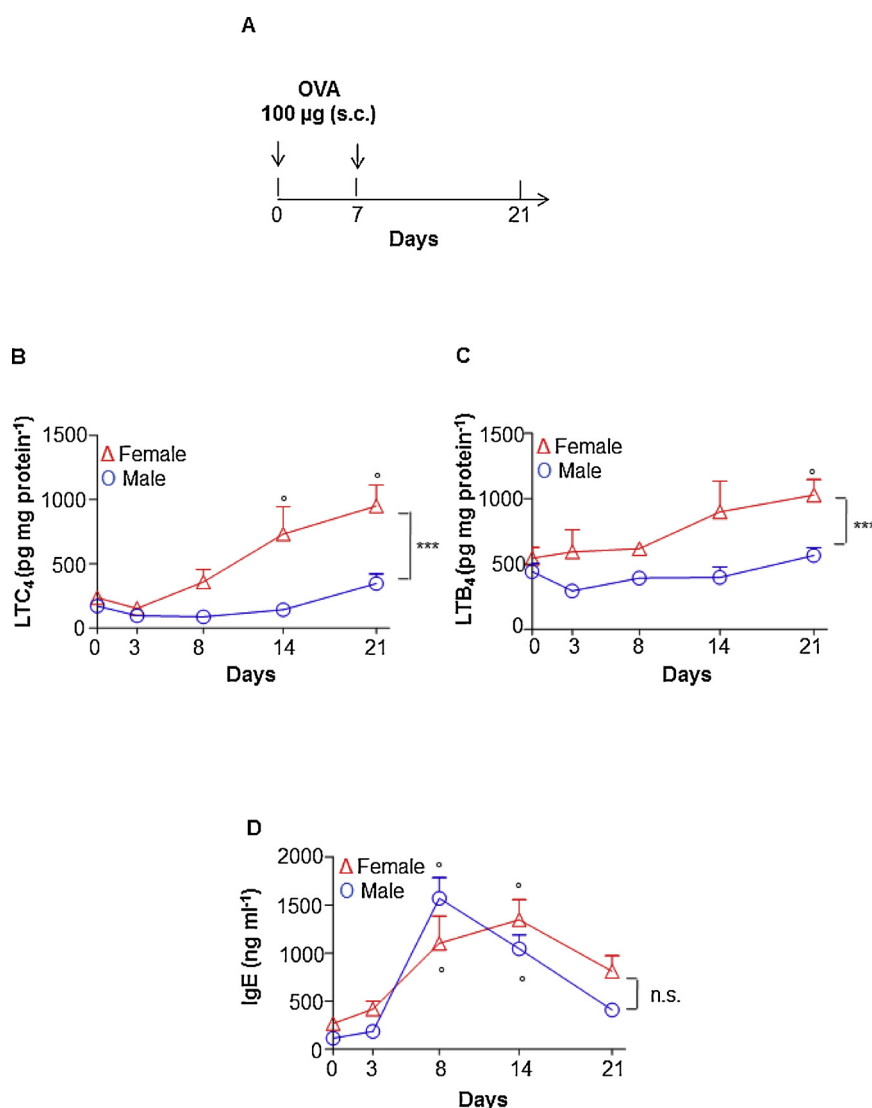


Fig. 2. Sex dimorphism in LTC₄ and LTB₄ levels during allergen sensitization. A) Female and male BALB/c mice received subcutaneous administration of OVA complexed with alum on day 0 and day 7. Mice were sacrificed at selected time points and pulmonary levels of B) LTC₄ and C) LTB₄ as well as D) plasma levels of IgE were measured. Values represent means $\pm$ S.E.M; $n = 6$ mice for each group. Significance is represented by * $P < 0.05$ vs. day 0 (one-way ANOVA plus Bonferroni); n.s., not significant ($P > 0.05$) and *** $P < 0.001$ Two-way ANOVA plus Bonferroni.

selected time points the data of vehicle groups were similar to the data obtained at day 0 (data not shown).

In another set of experiments animals were treated intraperitoneally (i.p.) with MS (1 mg kg⁻¹), 30 min before each OVA injection and 3 times per week until day 21. Similarly, mice were treated with ZIL (35 mg kg⁻¹) and MK (0.1 mg kg⁻¹) or vehicle (0.5 mL DMSO 2%) 30 min before each OVA injection and at 14 day.

2.3. Measurement of LT, IL-13 and PGD₂ levels

Lungs were isolated and digested with 1U mL⁻¹ collagenase (Sigma Aldrich, Milan, Italy). The homogenate was centrifuged (4 °C, 6000 $\times$ g, 10 min). Total protein concentration was measured using Bradford's method. The levels of LTC₄ and LTB₄ (Cayman Chemical, BertinPharma, Montigny Le Bretonneux, France) as well as IL-13 (Affymetrix eBioscience San Diego, CA) were measured with commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions. For PGD₂ measurement, lung homogenates (20 µL) were diluted with PBS to 1 mL. Then, 200 ng PGB₁ were added as internal standard and samples were frozen for 2 h at -20°C . Samples were centrifuged at 800 $\times$ g, 15 min at 4 °C and

obtained supernatants were diluted with the equal amounts of PBS and 25 µL mL⁻¹ of 1 N HCl. PGs were extracted by solid phase extraction and determined by liquid chromatography tandem mass spectrometry as described previously [25]. The levels of LTs, IL-13 and PGD₂ are expressed as pg mg⁻¹ of total proteins.

2.4. Measurement of plasma IgE levels

Blood was collected by intracardiac puncture using citrate as anticoagulant. Then plasma was obtained by centrifugation at 800 $\times$ g at 4 °C for 10 min and immediately frozen at -80°C . Total IgE levels were measured by means of ELISA using matched antibody pairs (BD Biosciences Pharmingen San Jose, CA).

2.5. Measurement of airway hyperreactivity

Main bronchi were rapidly dissected and cleaned from fat and connective tissue. Rings of 1–2 mm length were cut and mounted in 2.5 mL organ baths containing Krebs solution, at 37 °C, oxygenated (95% O₂ and 5% CO₂), and connected to an isometric force transducer (type 7006, Ugo Basile, Comerio, Italy) associated to a Powerlab 800

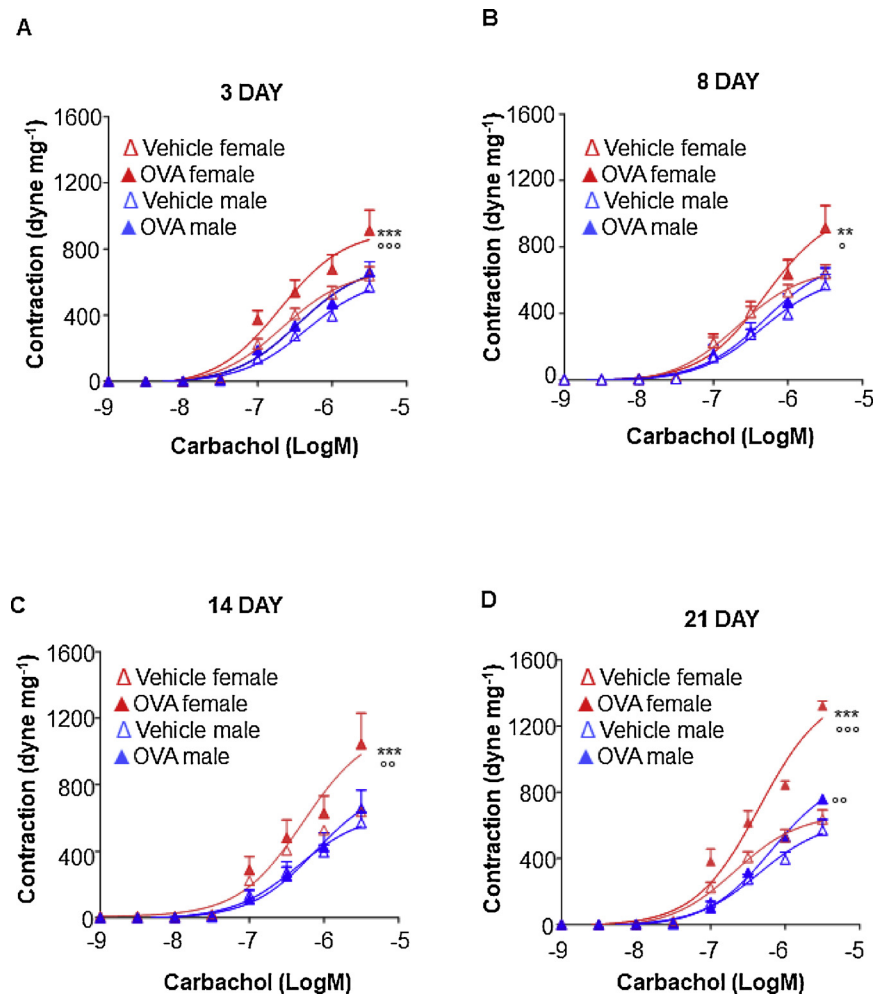


Fig. 3. Allergen sensitization induces sex differences in airway hyperreactivity. Female and male BALB/c mice received subcutaneous administration of saline (vehicle) or OVA complexed with alum on day 0 and 7. Bronchi were harvested from animals killed after A) 3 days, B) 8 days, C) 14 days and D) 21 days upon OVA sensitization, and subsequently bronchial reactivity to carbachol was assessed *in vitro*. Values represent means $\pm$ S.E.M; $n = 6$ mice for each group. Significance is represented by ** $P < 0.01$ and *** $P < 0.001$ vs. male mice; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. vehicle. Two-way ANOVA plus Bonferroni.

(AD Instruments). Rings were initially stretched until a resting tension of 0.5 g and allowed to equilibrate for at least 30 min. In each experiment bronchial rings were previously challenged with acetylcholine (10^{-6} M) until a reproducible response curve was obtained. Subsequently, after tissue washing, a cumulative concentration response curve to carbachol (10^{-9} – 3×10^{-6} M) was performed. Results were expressed as dyne per mg tissue.

Lung function was assessed using an isolated and perfused mouse lung model [26]. Lungs were perfused in a non-recirculating fashion through the pulmonary artery at constant flow of 1 mL min^{-1} resulting in a pulmonary artery pressure of 2–3 cm H₂O. The perfusion medium used was RPMI 1640 lacking phenol red (37 °C). The lungs were ventilated by negative pressure (–3 and –9 cm H₂O) with 90 breath min⁻¹. Every 5 min a hyperinflation (–20 cm H₂O) was performed. Artificial thorax chamber pressure was measured with a differential pressure transducer (Validyne DP 45-24) and airflow velocity was monitored with pneumotachograph tube connected to a differential pressure transducer (Validyne DP 45-15). The lungs were respired with humidified air. The arterial pressure was continuously monitored by means of pressure transducer (IsotecHealthdyne), which was connected with the cannula ending in the pulmonary artery. All data were transmitted to a computer and analysed with Pulmodyn software (Hugo Sachs Elektronik, March Hugstetten, Germany). Data were analysed through the following formula: $P = V \cdot C^{-1} + RL \cdot dV/dt^{-1}$, where P is chamber pressure, C pulmonary compliance, V tidal volume, RL airway

resistance. Successively, the airway resistance value registered was corrected for the resistance of the pneumotachometer and the tracheal cannula of $0.6 \text{ cm H}_2\text{O s mL}^{-1}$. Lungs were perfused and ventilated for 45 min without any treatment in order to obtain baseline state. Subsequently, lungs were challenged with carbachol. Dose-response curves for carbachol were performed by administration of 50 μL bolus of each dose, followed by intervals of 15 min in which lungs were perfused with buffer only.

2.6. Lung tissue histology

Left lung lobes harvested from female and male mice were paraffin-embedded and 7- μm cryosections were cut. Hematoxylin and eosin (H&E) staining was performed. Periodic acid-Schiff (PAS; Sigma-Aldrich, Milan, Italy) staining was performed according to the manufacturer's instructions to detect glycoprotein [27,28]. PAS-stained cryosections were analyzed for the presence of goblet cells (PAS + cells) along the bronchial basement membrane by means of imaging software (Zeiss, Germany). PAS + cryosections were graded with scores 0 to 4 to describe low to severe lung inflammation as follows: 0: < 5%; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: < 75% positive staining/total lung area.

2.7. Immunohistochemistry

Left lung lobes harvested from female and male mice were fixed and

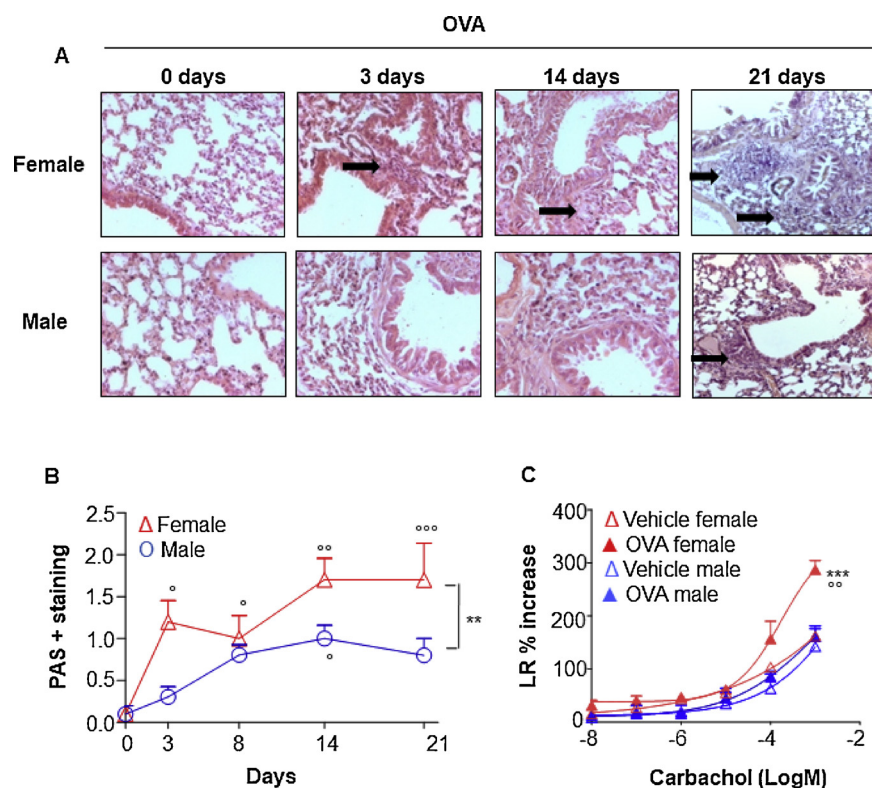


Fig. 4. Sex differences in allergic pulmonary inflammation. Female and male BALB/c mice received subcutaneous administration of OVA complexed with alum on day 0 and 7. Mice were killed after OVA-sensitization at the indicated time points. A) Lung cryosections were stained for PAS: black arrows in the picture indicate cellular infiltration. B) PAS + cryosections were graded with scores of 0–4 to describe low to severe lung inflammation as follows: 0: < 5%; 1: 5–25%; 2: 25–50%; 3: 50–75%; 4: < 75% positive staining/total lung area. C) Lung resistance (LR) was evaluated 21 days after OVA sensitization. Values represent means $\pm$ S.E.M; $n = 5$ mice for each group. Significance is represented by $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ and $^{\circ\circ\circ}P < 0.001$ vs. day 0 (one-way ANOVA plus Bonferroni); $^{\circ}P < 0.01$ vs. vehicle female (C); $^{\circ\circ}P < 0.01$ and $^{\circ\circ\circ}P < 0.001$ Two-way ANOVA plus Bonferroni.

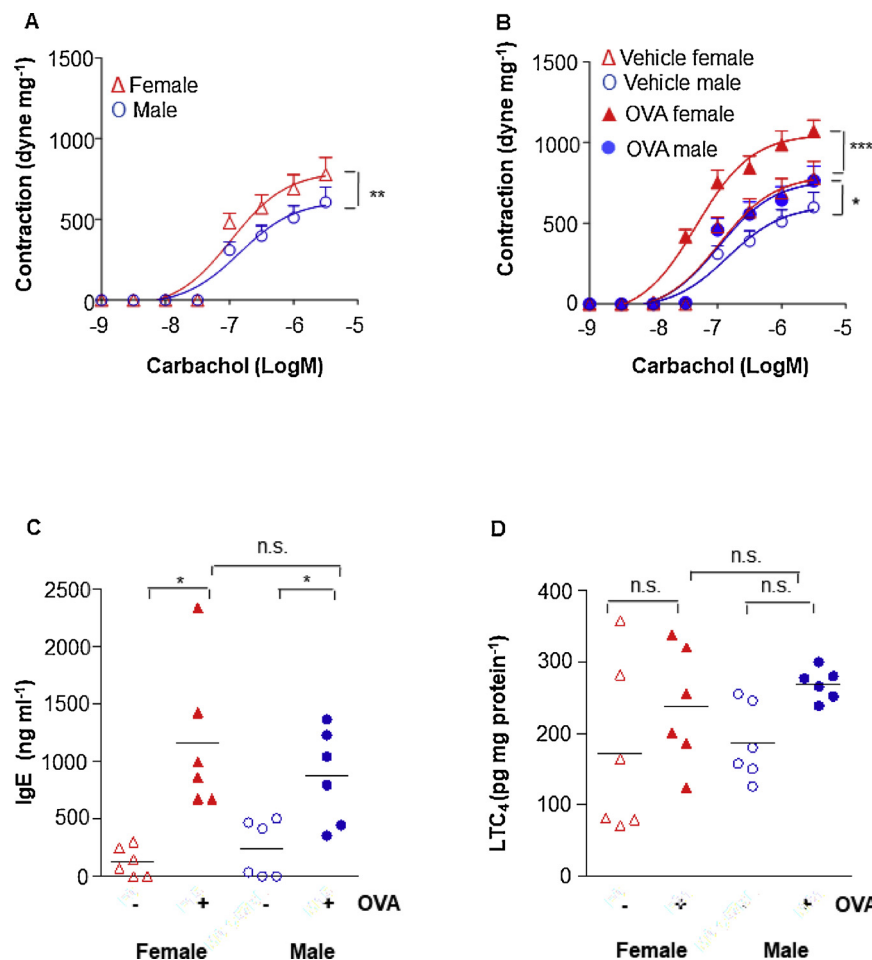


Fig. 5. Sex differences in airway hyperreactivity are LT-independent in C57BL/6 mice. A) Bronchial reactivity in non-sensitized female and male C57BL/6 mice. Female and male mice received subcutaneous administration of OVA complexed with alum or of saline (vehicle) on day 0 and 7. Lungs, bronchi and blood were harvested from the animals killed 15 days after treatment. B) Bronchial reactivity to carbachol in vitro, C) plasma IgE levels and D) LTC₄ pulmonary levels in vehicle- and OVA-treated mice. Values represent means $\pm$ S.E.M (A and B) or mean with single data (C and D); $n = 6$ mice for each group. Significance is represented by $^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ and $^{\circ\circ\circ}P < 0.001$; n.s., not significant ($P > 0.05$). Two-way ANOVA plus Bonferroni (A and B) and one-way ANOVA plus Bonferroni (C and D).

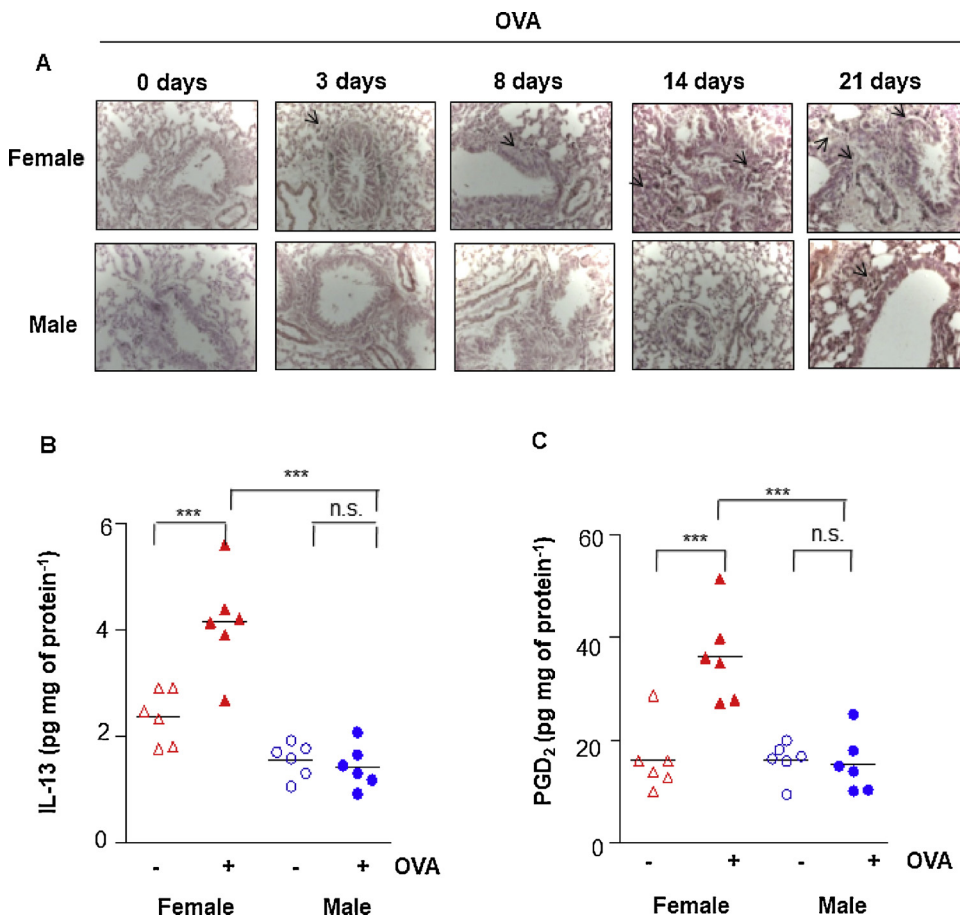


Fig. 6. Sex differences in mast cell recruitment and activation. Female and male BALB/c mice received subcutaneous administration of OVA complexed with alum on day 0 and 7. A) Mice were killed at selected time points following OVA administration and tryptase lung immunohistochemistry was evaluated (please refer to black arrows in the pictures to indicate typical brown spots). Pictures are representative of $n = 6$ mice in each group. Mice were killed 15 days after OVA sensitization and B) IL-13 and C) PGD₂ pulmonary levels were evaluated. Values represent mean with single data; $n = 6$ mice in each group. Significance is represented by *** $P < 0.001$; n.s., not significant ($P > 0.05$). One-way ANOVA plus Bonferroni.

7 μ m cryosections were cut. Anti-mast cell tryptase (1:250, AbCam, UK) or isotype control anti-rabbit IgG were used. The diamino-benzidine acid system was used to detect complexes and visualized by means of Zeiss microscope (Germany).

2.8. Statistical analysis

The results are expressed as mean $\pm$ S.E.M of the mean of n observations, where n represents the number of animals. Statistical evaluation was performed by two-tailed Student t -test for single comparisons or by one-way or two-way ANOVA using GraphPad InStat (Graphpad Software Inc., San Diego, CA) followed by a Bonferroni post-hoc test for multiple comparisons, respectively. *Post hoc* tests were run only when F achieved $P < 0.05$ and there was no significant variance in homogeneity.

A P value < 0.05 was used to define statistically significant differences between mean values.

2.9. Materials

The compounds used here were provided by the following suppliers: OVA by Sigma Aldrich, Milan, Italy, MS and zil by Sequoia Research Products, United Kingdom and MK by Cayman Chemical, BertinPharma, Montigny Le Bretonneux, France.

3. Results

3.1. Sex bias in bronchial reactivity is LT-independent in non-sensitized mice

Female mice are more prone to develop key asthma-like features

[7,8,10]. These differences have been in part coupled to differences in pulmonary structure. Moreover, sex differences in lung anatomy have been also reported in humans [29]. We first evaluated bronchial reactivity *in vitro* to carbachol and analyzed pulmonary levels of LTs as well as IgE levels in the plasma of naïve (non-sensitized) male and female BALB/c mice. Bronchial reactivity to carbachol in female non-sensitized mice was higher than that of male counterparts (Fig. 1A). The superior reactivity was coupled to an increased bronchial thickness (Fig. 1B) and to higher plasma IgE levels (Fig. 1C) in females compared to male mice. However, the pulmonary levels of LTC₄ (Fig. 1D) and LTB₄ (Fig. 1E) did not differ between the sexes.

3.2. Sex differences in allergen sensitization and related pulmonary LT production

LTs and other 5-LOX products play a key role in the development of lung inflammation and allergy [11,12]. In order to assess sex differences during allergen sensitization related to LTs, mice were treated s.c. with OVA complexed with alum on day 0 and day 7 and then sacrificed after 3, 8, 14 and 21 days (Fig. 2A). OVA sensitization induced a temporal and significant increase in pulmonary LTC₄ (Fig. 2B) and LTB₄ (Fig. 2C) levels in female mice. A trend to increase, although not significant, in pulmonary LT levels was observed in male animals (Fig. 2B, C). Importantly, IgE plasma levels increased in both sexes following OVA sensitization confirming the efficiency of sensitization (Fig. 2D). These data lead us to hypothesize that sex-specific LT formation might be causative for the sex dimorphism in murine asthma-like features following allergen sensitization.

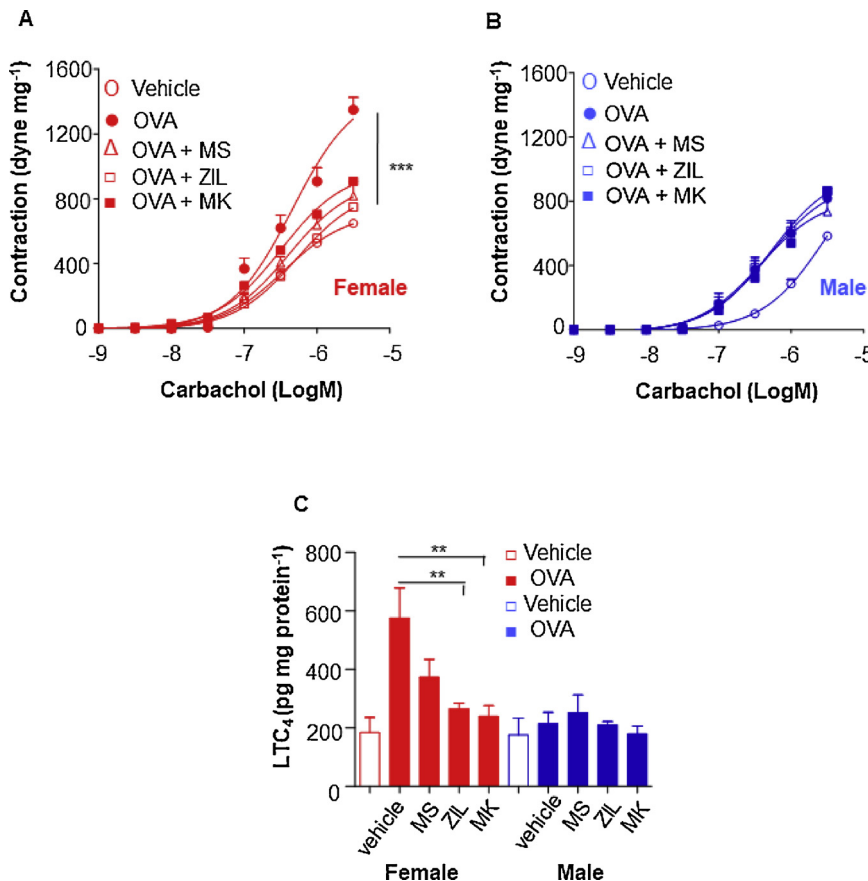


Fig. 7. Sex-dependent efficiency of LT-modifying drugs on airway hyperreactivity and LTC₄ formation. Adult female and male BALB/c mice received subcutaneous administration of OVA complexed with alum or of saline (vehicle) on day 0 and 7. Mice were pretreated i.p. with MS (1 mg/kg, 30 min before each OVA administration and 3 times per week), ZIL (35 mg/kg, 30 min before each OVA administration and at day 14), MK (0.1 mg/kg, 30 min before each OVA administration and at day 14) or vehicle. Bronchial reactivity to carbachol in A) female and B) male mice as well as C) LTC₄ pulmonary levels in female and male mice were evaluated 21 days after OVA sensitization. Values represent means $\pm$ S.E.M; $n = 6$ mice in each group. Significance is represented by $**P < 0.01$ and $***P < 0.001$. Two-way ANOVA plus Bonferroni (A and B) and one-way ANOVA plus Bonferroni (C).

3.3. Sex bias in LT production correlates to airway hyperreactivity

OVA sensitization by itself induces a significant increase in bronchial reactivity in female mice [19–21]. Here we demonstrate that a sex-related differences in bronchial hyperreactivity occurs following OVA sensitization. In particular bronchi harvested from females were more reactive than those from male mice at all selected time points (Fig. 3A–D).

3.4. Sex-bias in allergic pulmonary inflammation

Besides airway hyperreactivity, LTs contribute to another key asthma-like feature such as pulmonary inflammation [30]. OVA-sensitized female BALB/c mice exhibited a marked airway hyperplasia associated with higher cell pulmonary infiltration (Fig. 4A) as well as higher mucus production (Fig. 4B) as compared to males. In addition, the response was faster in onset and significantly higher in female mice (Fig. 4A, B). Accordingly, OVA administration induced a more robust increase in the lung resistance (LR) to carbachol in female mice as compared to male animals (Fig. 4C).

3.5. Sex dimorphism in LT production depends on a Th2 environment

Chronic allergic airway inflammation and the link to asthmatic disease are related to aberrant Th2 responses towards harmless environmental allergens [3]. Moreover, a link between LTs and Th2 immune responses in inflammation-related diseases is evident [31,32]. To assess the role of a Th2 environment in LT-mediated effects on airways, we next investigated C57BL/6 mice, a low Th2 responder strain [23,24]. As observed in BALB/c mice (Fig. 1A), carbachol-induced bronchial contractions were significantly more pronounced in female non-sensitized C57BL/6 mice compared to males (Fig. 5A). In addition,

OVA sensitization of C57BL/6 mice promoted bronchial hyperreactivity (Fig. 5B) and IgE increase (Fig. 5C) in both sexes. Conversely, OVA sensitization did not induce significant pulmonary LT production in both sexes of C57BL/6 mice (Fig. 5D), further supporting an influence of a Th2 environment in LT-mediated effects.

3.6. Allergen sensitization causes sex differences in pulmonary mast cell recruitment/activation

Mast cells are the major source of LTs in asthma [33,34]. Mast cell recruitment in the lungs of OVA-sensitized female BALB/c mice was found to be faster in onset and significantly more pronounced as compared to males. Thus, tryptase positive staining was more marked (brown spots) at the level of the basal membrane and parenchyma of the lung in female mice at day 3 post OVA sensitization (Fig. 6A). Male mice showed significant positive staining only at day 21 (Fig. 6A). Accordingly, IL-13, a typical asthma Th2-like cytokine mainly mast cell-derived [35], was almost doubled in the lung of OVA-sensitized versus non-sensitized female mice (Fig. 6B). Such increase in IL-13 was absent in male animals (Fig. 6B). A similar pattern was observed for levels of PGD₂ (Fig. 6C), which is a COX-derived lipid mediator considered as index of mast cell activation [36].

3.7. Sex-dependent therapeutic efficacy of LT-modifying drugs

Recently we demonstrated a sex bias in the efficiency of clinically relevant LT biosynthesis inhibitors caused by androgens [16], and for the cys-LT1 antagonist montelukast a higher efficacy in reducing asthma morbidity in girls was evident in a randomized, double-blind, placebo-controlled, 45-day study [37]. Therefore, we studied the efficiency of different types of LT modifiers in OVA-sensitized male and female BALB/c mice. The mice were treated with MS (1 mg kg⁻¹), the

5-LOX inhibitor ZIL (35 mg kg^{-1}) or the FLAP inhibitor MK (0.1 mg kg^{-1}). All investigated LT modifiers significantly inhibited OVA-induced bronchial hyperreactivity in female (Fig. 7A), but not in male animals (Fig. 7B). The reduced bronchial hyperreactivity in female mice was associated with significant inhibition of LTC₄ production in ZIL- and MK-treated mice (Fig. 7C), whereas in male mice they failed in this respect (Fig. 7C).

4. Discussion

Here, we demonstrate that sex-specific LT formation is causative for the sex dimorphism in murine asthma-like features following allergen sensitization. OVA sensitization of female BALB/c mice led to significant more pronounced bronchial hyperreactivity and pulmonary inflammation as compared to male counterparts. This sex bias well correlated to higher pulmonary LT levels in females versus males. This sex dimorphism in LT production seemingly depends on a Th2 environment, commonly associated to asthma pathology. Finally, different types of anti-LT drugs were effective in females, only. They reduced LT levels and this effect was coupled to an inhibition of bronchial hyperreactivity. Our findings might help to further explain why women suffer more often and more severely from allergen-induced asthma than men and suggest that females might have better benefit from anti-LT therapy than males.

The knowledge of the biological basis of sex differences has been often insufficient to support the inclusion of sex as a variable in pharmacological studies. Research over the past 20 years have considerably advanced our understanding in the pathology of asthma at the genetic, molecular and cellular level. In this context, sex differences became also clinically relevant and have been linked to immunological factors and/or to differences in the sex-specific responses to environmental or occupational exposures [3].

Here, we evaluated a potential sex-related contribution of LTs to allergen sensitization, an important risk factor for the development of asthma [38]. In fact, a detailed knowledge of the mechanisms underlying the allergen sensitization is mandatory for an appropriate diagnostic and preventive approach to asthma disease in both sexes. In the last years asthma incidence rapidly has increased suggesting that many factors (i.e. environmental), in addition to genetic components, are involved [3]. Thus, a more deep knowledge of allergy-promoting factors is essential to control the increased asthma risk [38].

Our data show that sex-related differences in lung function were already evident in non-sensitized mice. Indeed, in naïve (non-sensitized) mice bronchial reactivity in response to carbachol was more pronounced in female versus male BALB/c mice. Moreover, the pulmonary levels of LTs did not differ between sexes in physiological conditions. Conversely, this sex-related airway reactivity was coupled to higher plasma IgE and an increased bronchial thickness in females BALB/c mice in respect to males. Thus, this sex difference seems to be ascribed to structural differences in airways. Accordingly, anatomical sex differences in lung have been also reported in humans [29].

Upon sensitization a further significant difference in bronchial reactivity between sexes occurred. A higher pulmonary inflammatory response was evident in females as opposite to males. Although plasma IgE levels similarly increased in both sexes, significant differences in LT production occurred in sensitized BALB/c in favor of female mice. In male mice although a trend to increase in pulmonary LT levels occurred, this was not significant. Thus, sex-specific LT formation is causative for sex dimorphism in murine asthma-like features and suggests different molecular and cellular mechanisms underlying sensitization between males and females.

Chronic allergic airway inflammation and the link to asthmatic disease are related to aberrant Th2 response towards harmless environmental allergens [3]. Of interest, LTs are known to contribute to mount Th2 immune responses in inflammation-based diseases [32,39]. Indeed, female mice, deficient in LTC₄ synthase and thus unable to

produce cys-LTs, were shown to lack Th2 type inflammatory reactions following antigen challenge [32]. To further assess the role of the immune environment in LT-driving effects on airways, we utilized another mouse strain, i.e. C57BL/6 mice, a low Th2 responder strain [23,24]. Airway reactivity was higher in non-sensitized C57BL/6 female mice compared to males. The pattern observed was the same of BALB/c mice (high Th2 responder strain). This finding confirms the anatomical differences between the two sexes and independently from the strain. When C57BL/6 mice were sensitized the bronchial hyperreactivity was significantly more pronounced in females compared to males. The only difference between the two strains resides in the lack of an increase in LT levels in both sexes of C57BL/6-sensitized mice. Therefore, sex differences in LT production might constitute the key cellular checkpoint in the Th2 response in female mice and it is a necessary condition to develop asthma like features such as bronchial hyperreactivity and pulmonary inflammation.

Mast cell is the major cellular source of LTs [33,34] and resides in peripheral tissues to respond quickly to environmental stimuli contributing to Th2 response [33]. The mast cell plays an important role in the process of sensitization to allergen [40]. Indeed, a systemic exposure to allergens results in both the production of IgE and changes in mast cell number, tissue and phenotype [41]. Binding of IgE to FcεRI on mast cells sensitizes these cells to respond to specific antigens and can also enhance pro-inflammatory mediator production and cell recruitment [42]. All these events explain the development of bronchial hyperactivity and pulmonary inflammation following sensitization also in absence of an allergen airway challenge. Here, we show that the higher LT production in sensitized female BALB/c mice strongly correlates to increased pulmonary mast cell recruitment/activation coupled to elevated lung levels of PGD₂ and IL-13, an index of mast cell activation [35,36]. Thus, sex-related LT release from mast cells during sensitization may account for sexual dimorphism in allergen-induced asthma.

LT modifiers have been proven effective to reduce bronchoconstriction and airway inflammation in preclinical studies [43–45], however, their therapeutic efficacy in patients appears partially contradictory [46]. In clinical trials a subset of asthmatic patients, defined as high LT producers, responded better to Montelukast [47] and zileuton [48], thereby implying that the LT level is a predictor of the response to anti-LTs [49]. However, the sex of this subset of asthmatic patients was not mentioned. Note that female humans and rodents as well as isolated cells thereof produce more LTs than in male counterparts [13–16], and FLAP inhibitors and novel-type 5-LOX inhibitors display higher potencies in female cells or animals versus males [16]. Along these lines, in our experimental model all different types of LT-modifiers (MS, ZIL and MK) inhibited bronchial hyperreactivity selectively in female BALB/c mice in which allergen sensitization increased LT production. Therefore, sex-dependent pulmonary LT levels, together with genetic polymorphisms and environmental factors [3,38], might contribute to asthma phenotype identification and to asthmatic patient stratification.

5. Conclusions

In conclusion, our results demonstrate that sex differences in LT production are evident during allergen sensitization in Th2-prone mice, with relevance for development of asthma like features that significantly differ between sexes. Female subjects produce more LTs after allergen sensitization with consequently more pronounced asthma-like features, reflecting the preponderance of asthma frequency and severity in women. Notably, our findings have also major implications for anti-LT therapy in asthma, which implies sex-tailored medication with potentially better benefit of anti-LTs for women.

Declarations of interest

None.

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Author contributions

A. Rossi and F. Roviezzo designed, performed, and analyzed the data and contribute to write the manuscript; R. Sorrentino performed lung tissue histology and immunohistochemistry; M. A. Riemma and I. Cerquia performed animal studies, evaluated bronchial reactivity and cytokine determination; R. Bilancia performed animal studies and LT evaluation; G. Spaziano prepared lung tissue for histology and evaluated lung resistance; F. Troisi and S. Pace evaluated PGD₂ levels; A. Pinto analyzed experiments; B. D'Agostino, O. Werz and G. Cirino analyzed experiments and wrote the manuscript.

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