Pharmacological Research 87 (2014) 1–7

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

In vivo sex differences in leukotriene biosynthesis in zymosan-induced peritonitis

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ARTICLE INFO

Article history:
Received 19 February 2014
Received in revised form 14 May 2014
Accepted 21 May 2014

Chemical compounds studied in this article:
Leukotriene B_4 (PubMed CID: 5280492)
Leukotriene C_4 (PubMed CID: 5283121)
Zymosan (PubMed CID: 11375554)

Keywords:
Leukotrienes
Peritonitis
Sex differences
Zymosan

ABSTRACT

Leukotrienes (LTs) are 5-lipoxygenase (5-LO) metabolites which are implicated in sex-dependent inflammatory diseases (asthma, autoimmune diseases, *etc.*). We have recently reported sex differences in LT biosynthesis in *in vitro* models such as human whole blood, neutrophils and monocytes, due to down-regulation of 5-LO product formation by androgens. Here we present evidences for sex differences in LT synthesis and related inflammatory reactions in an *in vivo* model of inflammation (mouse zymosan-induced peritonitis). On the cellular level, differential 5-LO subcellular compartmentalization in peritoneal macrophages (PM) from male and female mice might be the basis for these differences. Sex differences in vascular permeability and neutrophil recruitment (cell number and myeloperoxidase activity) into peritoneal cavity were evident upon intraperitoneal zymosan injection, with more prominent responses in female mice. This was accompanied by higher levels of LTC_4 and LTB_4 in peritoneal exudates of female compared to male mice. Interestingly, LT peritoneal levels in orchidectomized mice were higher than in sham male mice. In accordance with the *in vivo* results, LT formation in stimulated PM from female mice was higher than in male PM, accompanied by alterations in 5-LO subcellular localization. The increased formation of LTC_4 in incubations of PM from orchidectomized mice confirms a role of sex hormones. In conclusion, sex differences observed in LT biosynthesis during peritonitis *in vivo* may be related, at least in part, to a variant 5-LO localization in PM from male and female mice.

Introduction

Leukotrienes (LTs) are biologically active lipid mediators with key roles in inflammation [1], including regulation of vascular permeability and of leukocyte extravasation and migration [2,3]. LTs derive from arachidonic acid (AA) through the action of 5-lipoxygenase (5-LO), which converts AA to LTA_4, representing the first step in the synthesis of all LTs [4]. In fact, LTA_4 can be hydrolyzed to the chemotactic agent LTB_4 by LTA_4 hydrolase or conjugated with reduced glutathione by LTC_4 synthase to form LTC_4. LTC_4 is then metabolized by sequential proteolytic hydrolysis to LTD_4 and LTE_4, which are together known as cysteinyl-LTs (cys-LTs) with vascular actions. LT biosynthesis is regulated by different mechanisms and among them post-translational modifications of 5-LO, including phosphorylation at serine residues, Ca^{2+} binding, interactions with certain phospholipids and glycerides, and interactions with 5-LO-activating protein and coactosin-like protein [4]. Also, LT synthesis is strongly influenced by the intracellular localization of 5-LO and the amount of trafficable enzyme. Thus, 5-LO is a soluble protein in the cytosol or nucleoplasm of resting cells, and translocates to perinuclear membranes when intracellular Ca^{2+} levels increase or/and after its phosphorylation by mitogen-activated protein kinase (MAPK) [3,5–7].

LTs are implicated in a range of inflammatory diseases, often presenting sex-related differences in the incidence and/or the

Abbreviations: 5-LO, 5-lipoxygenase; AA, arachidonic acid; cys-LTs, cysteinyl-leukotrienes; DMEM, Dulbecco's modified Eagle's medium; EIA, enzyme immunoassay; ERK, extracellular signal-regulated kinases; LTs, leukotrienes; MAPKs, mitogen-activated protein kinases; MPO, myeloperoxidase; ORCH, orchidectomized; PM, peritoneal macrophages; PMSF, phenylmethylsulfonyl, fluoride; PG, prostaglandin.

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http://dx.doi.org/10.1016/j.phrs.2014.05.011
1043-6618/

course, such as asthma, allergic rhinitis, and autoimmune diseases [8–10]. Interestingly, the sex has emerged as a fundamental variable for the LT pathway and we have recently reported sex differences in LT biosynthesis in *in vitro* models such as human whole blood, neutrophils [11] and monocytes [12]. Thus, male sex hormones repressed LT biosynthesis in human neutrophils by affecting 5-LO localization *via* differential activation of extracellular signal-regulated kinase (ERK) [11]. Also, sex-related differences in LT biosynthesis were observed in human monocytes where, *in vitro*, testosterone caused a repression of phospholipase D, resulting in impaired 5-LO product biosynthesis due to lack of activating diacylglyceride [12]. Moreover, data from animal models showing sex-specific attenuation of atheroma formation in dual 12-/15- and 5-LO knock-out mice [13] and exclusive protection of female mice from platelet-activating factor-induced shock after knocking out of the LTB_4 receptor 1 [14] suggested a major contribution of LTs in the pathophysiology of females. Nevertheless no *in vivo* data about sex-dependent LT biosynthesis are available.

Here, we investigated whether the sex influences the production of LTs *in vivo*, using zymosan-induced peritonitis as experimental model. This model displays all classical signs of acute inflammation, including increased vascular permeability, edema, leukocyte influx and release of inflammatory mediators. We show for the first time, that LT production is sex-biased *in vivo* and significantly higher in the peritoneum of female mice challenged with zymosan. The data indicate that this bias can be traced back to sex differences in the LT-synthetic capacities of peritoneal macrophages (PM) and are accompanied by sex-specific 5-LO subcellular localization.

Materials and methods

Materials

Enzyme immunoassay (EIA) kits were from Cayman Chemical Company (Aurogene, Rome, Italy). [^{3}H-PGE_2] was from PerkinElmer Life Sciences (Milan, Italy). All other reagents and compounds were obtained from Sigma–Aldrich (Milan, Italy).

Animals

Male and female CD-1 mice (8–9 weeks old, Charles River, Calco, Italy) were housed in a controlled environment ($21 \pm 2\,^\circ C$) 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. Experiments were conducted during the light phase.

To investigate the impact of sex hormones, male mice were orchidectomized (ORCH group) and allowed to recover for 5 weeks. This time period ensures that sex hormones have been metabolized and are no longer in the blood, and it is sufficient to allow the turnover of immune cells generated under the influence of this reproductive hormone [15,16]. In particular, orchidectomies were performed through scrotal incision. Sham groups were performed through the similar procedures, except that the gonads were not removed. Plasma testosterone was measured with a commercially available EIA (Cayman Chemical Company). The experimental protocols were approved by the Animal Care Committee of the University of Naples Federico II, in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116/92) as well as with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986).

Zymosan-induced peritonitis

Peritonitis was induced by i.p. injection (0.5 mL) of zymosan ($2\,mg\,mL^{-1}$ in saline, boiled and washed; Sigma, Milan, Italy). At selected time points after zymosan injection (0, 15, 30, 60, 120, 180 and 240 min), mice were euthanized and peritoneal exudates were collected using 2 mL of PBS. The cells were counted using a light microscope in a Burker's chamber after vital trypan blue staining. Exudates were centrifuged at $20{,}000 \times g$ for 20 min and supernatants and cell pellets were collected and frozen at $-80\,^\circ C$ for measurements of eicosanoids and myeloperoxidase (MPO) activity, respectively. The peritoneal exudate levels of LTC_4 and LTB_4 were measured by EIA kits according to manufacturer's instructions (Cayman Chemical; Aurogene, Rome, Italy) and expressed as $ng\,mL^{-1}$.

Vascular permeability

Evans blue dye ($40\,mg\,kg^{-1}$, 0.3 mL; Sigma, Milan, Italy) was injected into the tail vain followed by an *i.p.* injection of zymosan (0.5 mL; $2\,mg\,mL^{-1}$). After 0, 15, 30, 60, 120, 180 and 240 min, mice were euthanized and peritoneal exudates were collected using PBS ($0.1\,mL\,g^{-1}$ of body weight). After centrifugation, supernatants (diluted 1:3) were analyzed for Evans Blue bound to plasma albumin by reading at 610 nm in a plate reader (MultiskanGo, Thermo Scientific).

MPO assay

The use of MPO as an index of neutrophil infiltration is well documented [17]. Cell pellets were disrupted by sonication in 2 mL PBS (50 mM, pH 6) with 0.5% hexadecyltrimethylammonium bromide, freeze-thawed three times and centrifuged ($32{,}000 \times g$, 20 min) to collect supernatants which were used in MPO assay, performed as described by Bradley et al. [17]. Briefly, 20 μl samples were added to a 96 well plate and the reaction was initiated by the addition of 0.2 mL of assay buffer containing $0.167\,mg\,mL^{-1}$ of *o*-dianisidine and 0.0005% hydrogen peroxide. The rate of change of absorbance was monitored in kinetic mode by a plate reader (IMarkmicroplate Reader, Bio-Rad, Segrate, Milan, Italy). Levels of MPO in samples were determined from the calibration curve using human neutrophil MPO as the reference standard. The levels of MPO were expressed as $U\,mL^{-1}$.

Peritoneal macrophages

Resident PM were obtained by lavage of the peritoneal cavity of mice with 7 mL of cold Dulbecco's modified Eagle's medium (DMEM) with heparin ($5\,U\,mL^{-1}$). PM were then centrifuged at $500 \times g$ 4 °C for 5 min, resuspended at 1×10^7 cells mL^{-1} and 0.5 mL of cell suspension were added to a 15 mL conical polypropylene tube for each experimental condition. Cells were then incubated at 37 °C with ionophore A23187 (4.5 mL, final concentration 2.5 μM, 15 min), zymosan (4.5 mL, final concentration 13 particles per cell, 30 min) or vehicle (0.25% dimethyl sulfoxide in DMEM for A23187 and DMEM for zymosan). EDTA at a final concentration of 4 mM was added at the end of incubation time to A23187-stimulated cells. All samples were then centrifuged at $500 \times g$ at 4 °C for 5 min. Supernatants were collected and frozen at $-80\,^\circ C$ for measurements of LTC_4 and prostaglandin (PG)E_2 by EIA (Cayman Chemical Company; Aurogene, Rome, Italy) and radioimmunoassay (PerkinElmer Life Sciences, Milan, Italy; Sigma, Milan, Italy) respectively. The cell pellets were washed by resuspending in ice-cold PBS, centrifuged and collected and frozen at $-80\,^\circ C$ for 5-LO evaluation.

Cell disruption and fractionation

Total cell lysates were prepared by lysis for 15 min at 4 °C with lysis buffer (20 mM HEPES, pH 7.6, 1.5 mM $MgCl_2$, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM phenylmethanesulfonyl fluoride (PMSF), $15\,\mu g\,mL^{-1}$ soybean trypsin inhibitor, $3\,\mu g\,mL^{-1}$ pepstatin A, $2\,\mu g\,mL^{-1}$ leupeptin, 1% Nonidet P-40, 20% glycerol,

50 mM NaF). Lysates were centrifuged (12,000 × *g*, 15 min 4 °C), the supernatants were collected and stored at −80 °C.

Subcellular fractionation was carried out by mild-detergent lysis (0.1% NP40) according to Werz and colleagues [6]. Isolated PM were suspended in ice-cold NP-40-lysis buffer (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, 0.1%NP-40, 1 mM PMSF, 60 μg mL^{-1} soybean trypsin inhibitor and 10 μg mL^{-1} leupeptin), vortexed (3× 5 s), kept on ice for 10 min, and centrifuged (1250 × *g*, 10 min, 4 C). Resultant supernatants (non-nuclear fractions) were transferred to a new tube whereas the pellets (nuclear fractions) were resuspended in ice-cold relaxation buffer (50 mM Tris–HCl, pH 7.4,250 mM sucrose, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM PMSF, 60 μg mL^{-1} soybean trypsin inhibitor, 10 μg mL^{-1} leupeptin). Both nuclear and non-nuclear fractions were centrifuged (1250 × *g*, 10 min, 4 °C) for further purification. Lysis of cells and integrity of nuclei were confirmed by light microscopy with trypan blue exclusion. Nuclei in relaxation buffer were disrupted by sonication (3 × 5 s).

Analysis of 5-LO from cellular fractions by SDS/PAGE and Western blotting

Protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad Laboratories, Segrate, Milan, Italy). The samples were mixed with 4× gel loading buffer (250 mM Tris, 8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 10 mg mL^{-1} of bromophenol blue), heated for 6 min at 95 °C. Each sample was loaded and analyzed by electrophoresis on a 10% SDS–polyacrylamide gel. Correct loading of the gel and transfer of proteins to the nitrocellulose membrane was confirmed by Ponceau staining. After electroblot to nitrocellulose membrane (Whatman Protran, Dassel, Germany), membranes were blocked with 0.05% PBS/Tween containing 5% BSA for 2 h at room temperature. Membranes were washed and then incubated with primary antibody overnight at 4 °C. 5-LO (AK-7, 1551, affinity purified) and β-actin antibodies were used as 1:1000 dilution. After the incubation, the membranes were washed six times with 0.1% PBS-Tween and were incubated for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies diluted 1:10,000 in 0.1% PBS-Tween containing 5% non-fat dry milk. The membranes were washed and protein bands were detected by an enhanced chemiluminescence system.

Statistical analysis

The results are expressed as mean ± S.E.M. of the mean of *n* observations, where *n* represents the number of animals or number of experiments performed in different days. The results were analyzed by Student *t* test. A *p*-value less than 0.05 was considered significant.

Results

Zymosan-induced peritonitis in male and female mice

Sex differences in vascular permeability and cellular influx into peritoneum

Increased vascular permeability is one of the primary steps in initiating inflammation leading to plasma leakage and swelling [18]. Intraperitoneal injection of zymosan induced an increase of vascular permeability that reached its maximum after 60 min, with a similar time-course in male and female mice (Fig. 1A). However, significant sex differences were observed in the amount of dye extravasation, and higher vascular permeability was observed in female *vs* male mice, particularly after 30 and 60 min ($p = 0.077$ at 15 min; $p < 0.05$ at 30 and 60 min; Fig. 1A).

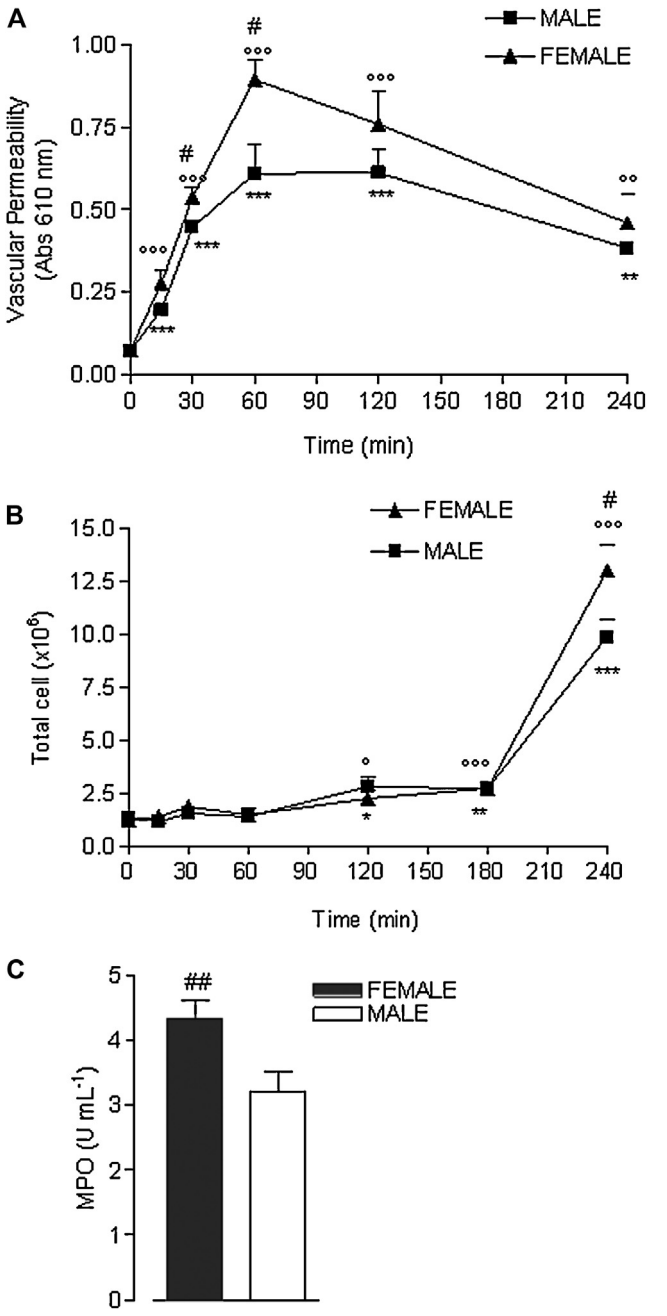

Fig. 1. Sex-dependent differences in vascular permeability (A) peritoneal cell influx (B) and MPO activity (C) in zymosan-induced peritonitis in mice. Mice were treated with zymosan (*i.p.*) and sacrificed at the indicated time points. Time zero (T0) corresponded to untreated mice. Values represent means ± S.E.M.; (A) $n = 8$ mice; (B) $n = 10$ mice/T0; 15 min; $n = 13$ mice/30 min; $n = 13$–15 mice/60 min; $n = 8$ mice/120 min; 180 min for cell influx; $n = 16$ mice/240 min; (C) $n = 12$ mice/240 min.° $p < 0.05$;°° $p < 0.01$;°°° $p < 0.001$ female *vs* female T0; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ male *vs* male T0; # $p < 0.05$; ## $p < 0.01$ female *vs* male.

The extravasated plasma contains first-line chemotactic factors (*e.g.*, LTs) that mediate subsequent neutrophil influx, leading to their accumulation in the inflamed tissue. In fact, zymosan induced a slight cellular influx of neutrophils into mouse peritoneum that was significant 120 min after zymosan injection ($p < 0.05$ for male and female mice) and was substantial at 240 min in both genders ($p < 0.001$ for male and female mice) (Fig. 1B). Interestingly, the number of peritoneal cells was significantly higher in female than in male mice at 240 min ($p < 0.05$), which was accompanied by a

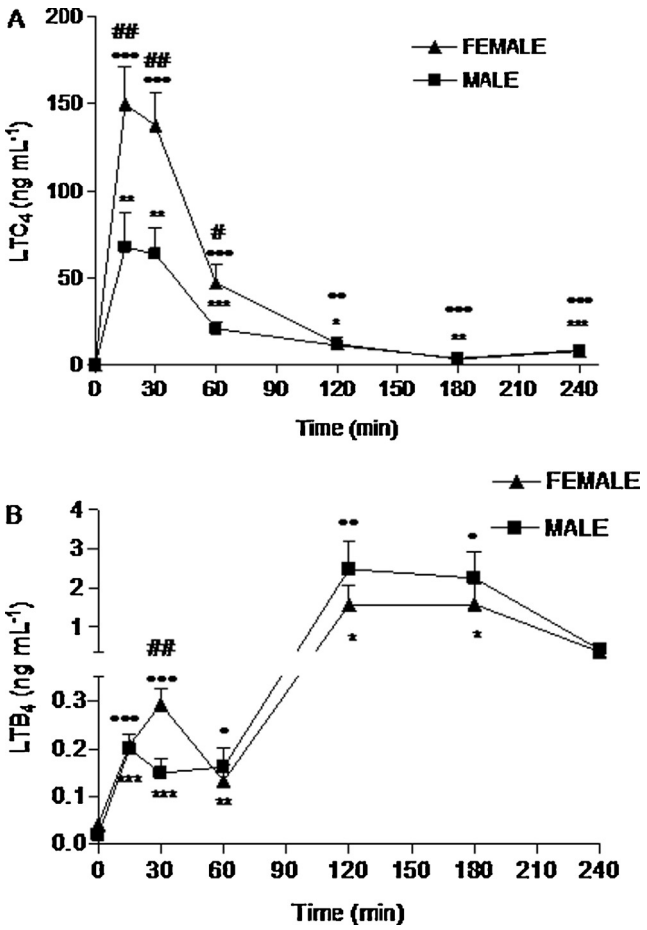

Fig. 2. Sex-dependent differences in LT biosynthesis in zymosan-induced peritonitis in mice. (A, B) Mice were treated with zymosan (*i.p.*) and sacrificed at the selected time points. Time zero (T0) corresponded to untreated mice. Values represent means ± S.E.M.; $n = 8$ mice/T0; $n = 14$–15 mice/15 min; $n = 11$–14 mice/30 min; $n = 13$–14 mice/60 min; $n = 8$ mice/120 min, 180 min and 240 min.°° $p < 0.01$;°°° $p < 0.001$ female *vs* female T0; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ male *vs* male T0; # $p < 0.05$ female *vs* male at time 60 min; ## $p < 0.01$ female time 15 and 30 *vs* male time 15 and 30.

higher MPO activity (a neutrophil marker) in female mice (Fig. 1C; $p < 0.01$).

Sex differences in LT production

Zymosan injection resulted in a marked production of LTs in the peritoneal exudates of male and female mice. In particular, the amount of LTC_4, the main 5-LO metabolite in this experimental model [18,19], rapidly increased within 15–30 min after zymosan injection, and then sharply decreased at 60 min (Fig. 2A). Though similar kinetics were observed in both genders, LTC_4 levels were significantly higher in female mice (15–30 min, $p < 0.01$; 60 min $p < 0.05$), resulting in an absolute difference of about 2–3 fold (Fig. 2A). LTB_4 levels were much lower as compared to LTC_4 and showed a biphasic trend, but also a significant sex-dependent difference. In fact, a slight but significant early increase was observed in both genders ($p < 0.001$), with a peak at 15 min after peritonitis induction in male and at 30 min in female, where levels were significantly higher than in males ($p < 0.01$). No sex difference was observed in the late LTB_4 increase (120–180 min) (Fig. 2B).

Effects of orchidectomy on LT production

To determine the relative impact of sex hormones on LT production in zymosan-induced peritonitis, gonads from adult male mice were surgically removed. This procedure significantly decreased testosterone plasma levels in ORCH mice that were similar to levels in female mice (Table 1). Compared with sham male mice, which have higher testosterone levels in respect to ORCH mice, gonadectomy caused a significant ($p < 0.05$) increase of LTC_4 (Fig. 3A) and LTB_4 (Fig. 3B) production at 15 and 30 min after zymosan injection, respectively. It is important to underline that the LT levels in ORCH mice were similar to LT levels in female mice (Fig. 3A and B), implying a negative role of testosterone on LT production.

Table 1
Plasma testosterone levels (ng mL^{-1}).

Male	2.00 ± 0.7
ORCH	0.050 ± 0.006*
Female	0.055 ± 0.008*

Values represent mean ± S.E.M.; $n = 9$–10 mice/group.
* $p < 0.05$ *vs* male sham.

Resident peritoneal macrophages from male and female mice

Sex differences in LT production

It has been reported that LTC_4 is the main 5-LO-derived LT synthesized by resident PM in zymosan-induced peritonitis [18,19]. Thus, we analyzed whether the observed sex differences in LTC_4 production *in vivo* could be traced back to isolated PM *in vitro*. To this aim resident PM were collected from adult male and female mice at 4 °C to minimize cellular modification (according to [11]) and stimulated *in vitro* in suspension with zymosan (13 particles cell^{-1} corresponding to *in vivo* condition; 30 min, 37 °C) or ionophore A23187 (2.5 μM; 15 min, 37 °C). Biosynthesis of LTC_4 in PM from female mice (isolated and treated side-by-side with male PM) stimulated with zymosan ($p < 0.01$; Fig. 4A) or with A23187 ($p < 0.05$; Fig. 4B) was significantly higher than in PM from male mice, which is in line with the data obtained in the *in vivo* model.

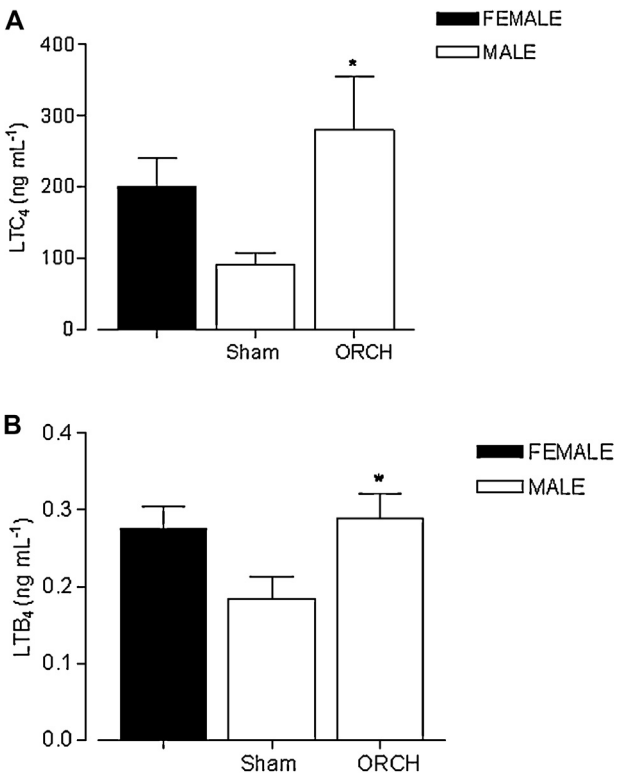

Fig. 3. Effects of orchidectomy on LT biosynthesis in zymosan-induced peritonitis in mice. ORCH and sham-operated mice were sacrificed at 15 (LTC_4, A) and 30 min (LTB_4, B) after zymosan (*i.p.*) treatment, respectively. Values represent mean ± S.E.M.; $n = 8$–9 mice/time. * $p < 0.05$ ORCH *vs* male sham.

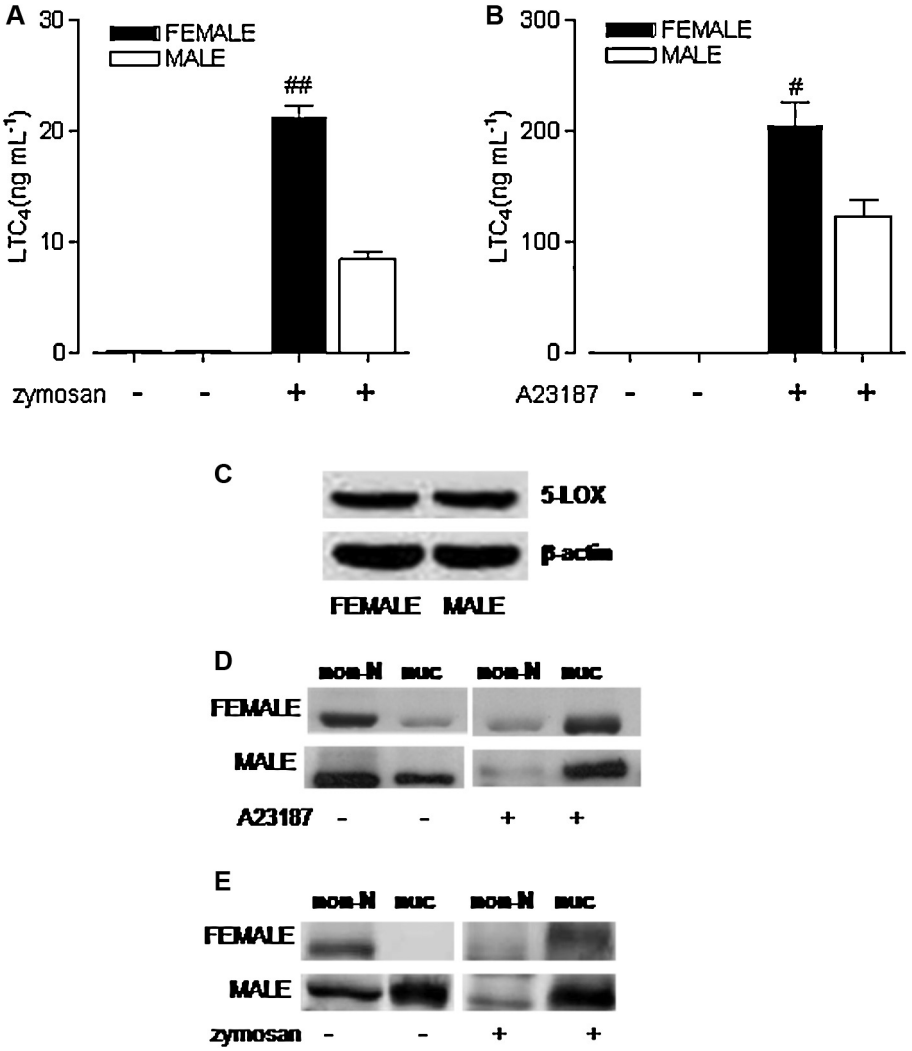

Fig. 4. Sex-dependent differences in LTC_4 biosynthesis in mouse peritoneal macrophages. LTC_4 formation in resident PM induced by (A) zymosan (13 particles cell^{-1}; 30 min, 37 °C) or (B) A23187 (2.5 μM; 15 min, 37 °C). Data are expressed as means ± S.E.M. from $n = 2$ (zymosan) or $n = 4$ (A23187) independent experiments performed in triplicates, each. $^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$ stimulated PM from female *vs* stimulated PM from male mice. (C) 5-LO protein expression in resident PM was analyzed by immunoblotting of total cell lysates. Results are representative of three independent experiments. (D, E) Subcellular localization of 5-LO in resident PM from female and male mice induced by zymosan (13 particles cell^{-1}; 30 min, 37 °C) or A23187 (2.5 μM; 15 min, 37 °C) analyzed by immunodetection of 5-LO in the nuclear (Nuc) and non-nuclear (Non-N) fractions of mild-detergent (0.1% Nonidet P-40)-lysed cells. Data are representative of 2–4 independent experiments.

Next, we analyzed if the difference in the LTC_4 production between activated female and male PM was accompanied by different 5-LO protein levels or subcellular localization and/or observed also for other AA metabolites relevant in PM (*e.g.*, PGE_2). Interestingly, while the absolute 5-LO protein levels were not different between genders (Fig. 4C), a difference in 5-LO subcellular localization was observed (Fig. 4D and E). In fact, 5-LO was found mainly in the non-nuclear fraction of resting cells from female mice whereas in PM from male mice (isolated and treated side-by-side with female PM) 5-LO was detected in both the non-nuclear and in the nuclear fraction of resting cells, indicating a sex-dependent localization of the 5-LO enzyme. When PM were stimulated with A23187 (2.5 μM; 15 min, 37 °C, Fig. 4D) or zymosan (13 particles cell^{-1}; 30 min, 37 °C, Fig. 4E), translocation of cytoplasmic 5-LO to the nuclear fraction was observed in both genders (Fig. 4D and E).

Importantly, the biosynthesis of cyclooxygenase-derived AA metabolite PGE_2 did not vary between female and male after zymosan (13 particles cell^{-1}; 30 min, 37 °C) (Fig. 5A) or A23187 (2.5 μM; 15 min, 37 °C) (Fig. 5B) stimulation, also suggesting that the sex-difference in LT synthesis was specific for LTs.

Effects of sex hormones on LT production

To assess the role of orchidectomy on LT production in PM from adult male mice, we finally analyzed the LTC_4 levels in PM isolated from ORCH mice. Compared with PM isolated from sham male mice, ORCH caused a significant ($p < 0.05$) increase of LTC_4 biosynthesis after stimulation of cells with A23187 (2.5 μM; 15 min, 37 °C) (Fig. 6). Interestingly, the *in vitro* treatment of PM with testosterone (100 nM; 30 min, 37 °C), 5α-dihydrotestosterone (10 nM; 30 min, 37 °C), or 17β-estradiol (100 nM; 30 min, 37 °C) did not affect LTC_4 generation in PM from male mice (data not shown) suggesting that the LT sex difference is seemingly caused by the *in vivo* actions of male reproductive steroid hormones.

Discussion

Inflammatory disorders are often characterized by sex-related differences, but there is only a limited knowledge on the mechanisms and pathways involved. Here, we demonstrate that the biosynthesis of the pro-inflammatory LTs is sex-biased *in vivo*, by the use of a well-established model of acute inflammation, that is, zymosan-induced peritonitis in mice [18–20]. Thus, we

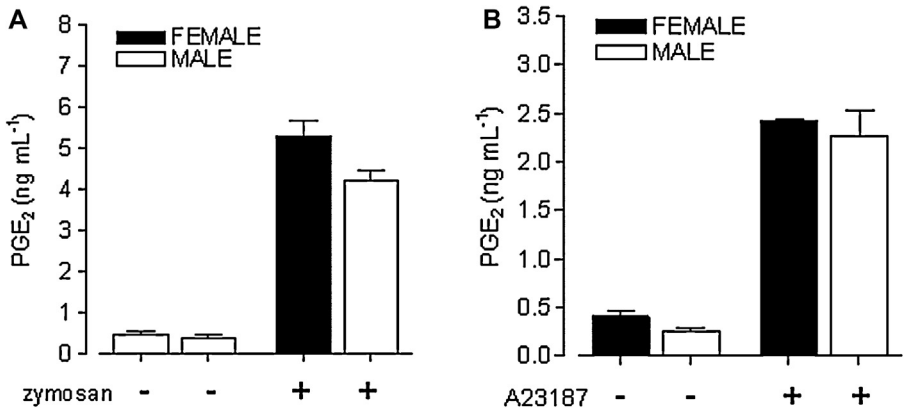

Fig. 5. PGE_2 biosynthesis in peritoneal macrophages from male and female mice. PGE_2 formation in resident PM induced by (A) zymosan (13 particles $cell^{-1}$; 30 min, 37 °C) or (B) A23187 (2.5 μM; 15 min, 37 °C). Data are expressed as means ± S.E.M. from $n = 2$ (zymosan) $n = 4$ (A23187) independent experiments performed in triplicates, each.

showed that the levels of LTC_4 and LTB_4 were significantly higher in the inflamed peritoneum of female than male mice, which was followed and/or accompanied by sex differences in relevant inflammation parameters. In fact, higher vascular permeability and neutrophil extravasation were observed in females. Finally, the analysis of isolated PM allowed the identification of sex-related differences in LT formation at the cellular level, accompanied by a differential subcellular localization of 5-LO, which was in agreement with previous findings *in vitro* in human cells [11]. Taking together, our data suggest LTs and their production as critical factors for sex-related differences in inflammation, with *in vivo* relevance in determining the amplitude of the acute response and possible significance in inflammatory diseases.

Increased vascular permeability is one of the primary steps involved in initiating the inflammatory process and is a prerequisite for the leakage of plasma into the inflamed tissue. The *i.p.* injection of zymosan in mice induced an inflammatory reaction characterized by early vascular permeability and steady increase of protein leakage into the peritoneum in the first 60 min. Interestingly, these events were more robust in female than in male mice, implying a more pronounced inflammatory response in female animals. Vascular permeability represents, however, a highly regulated multistep process mediated by inflammatory mediators, and cys-LTs are particularly recognized as potent vascular-regulating agents through the interaction with the $cysLT_1$ receptor [21,22].

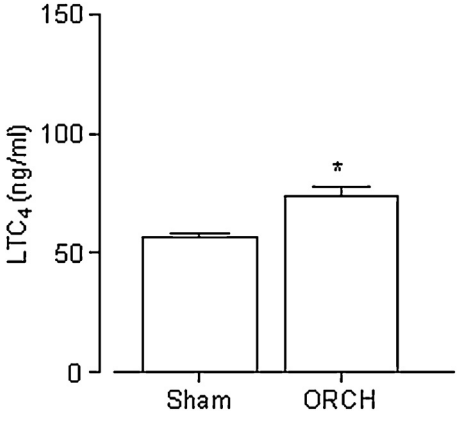

Fig. 6. Effects of orchidectomy on LTC_4 production in stimulated-peritoneal macrophages from male mice. LTC_4 formation in resident PM from orchidectomized (ORCH) and sham-operated mice induced by A23187 (2.5 μM; 15 min, 37 °C). Data are expressed as means ± S.E.M. from $n = 3$ independent experiments performed in triplicates, each. * $p < 0.05$, stimulated PM from ORCH mice *vs* stimulated PM from sham male mice.

We observed that LTC_4 levels rapidly raised after zymosan injection (peaking at 15–30 min) and were significantly higher in female mice, suggesting that the sex differences in vascular permeability could be due, at least in part, to differential LT biosynthesis.

Previous studies showed that the dominant peritoneal leukocyte population in the early phase of the inflammatory response in CD1 mice are resident macrophages (about 95%, [20]), which are known to mainly synthesize LTC_4 as 5-LO products [18,19]. In fact, it has been reported that macrophage depletion caused a profound decrease of LTC_4 production and vascular permeability in zymosan-induced peritonitis [18]. It is important to underline that resident peritoneal cells consist of a mixture of cell types including macrophages, granulocytes and lymphocytes, but the proportion of these cell types can vary. In fact, the number and type of resident peritoneal cells depends on the mouse strain utilized. For example, in outbred CD1 mice resident cells are predominately macrophages (95% [20]), while in inbred C57BL/6 mice the peritoneal cells are macrophages, T lymphocytes and B lymphocytes [16], reflecting a probable different inflammatory mediator production and subsequent chemotaxis, as observed in the different time-course of cell infiltration in CD1 [20, present data] and C57BL/6 mice [16]. Though small sex differences have been reported in the number of resident leukocytes (macrophages, T lymphocytes and B lymphocytes) in C57BL/6 mice [16], we neither observed a significant sex bias in the amount of peritoneal cells in untreated CD-1 mice nor within the first 180 min after zymosan injection. In particular, under our experimental conditions and according to the findings by Doherty and colleagues [20], zymosan injection induced a slight peritoneum cellular influx of neutrophils until 180 min in CD1 mice, a phenomenon that was very pronounced in C57BL/6 mice [16]. In contrast to CD1 mice, in the inbred strain the peak of cell infiltration occurred at 180 min with higher number of cells in male mice with respect to female mice. Instead, cell numbers as well as MPO activity in the CD1 mouse peritoneum (which is an index of neutrophil infiltration) were significantly higher in female than in male mice after 240 min, suggesting that chemotaxis and/or migration of neutrophils was superior in female *vs* male animals challenged with zymosan.

Interestingly, the extravasation and accumulation of neutrophils appeared as a delayed event as compared to the rapid protein leakage and might be attributable to slow migration of neutrophils from the vascular system [20,23,24], leading to predominance of neutrophils in the peritoneum only at 240 min after zymosan injection, as observed by others before [20]. In contrast to the substantial amount of rapidly synthesized LTC_4, the concomitant levels of LTB_4 were about 500-fold lower (<0.3 ng mL^{-1}) and, though increased in a biphasic manner with a delayed peak

(120–180 min), were still minute (<3 ng mL^{-1}) as compared to LTC_4 at a later time point. Note that neutrophils produce much more LTB_4 than LTC_4 [11]. Conclusively, we suggest that the LTC_4 peak in the early phase (15 and 30 min) is produced by peritoneum resident macrophages while the delayed peak (120–180 min) in LTB_4 synthesis is supported by accumulating neutrophils. Because the levels of chemotactic LTB_4 in female animals were twice as high as in male mice the more sustained neutrophil influx in female animals may be explainable.

Androgens were previously shown to suppress LT biosynthesis in human monocytes [12] and neutrophils [11] leading to lower LT-synthetic capacities of cells from males. Androgen-dependent suppression of LT biosynthesis during zymosan-induced peritonitis is reflected by increased levels of LTC_4 and LTB_4 found in male mice after orchidectomy which are similar to female mice and which we suggest to be related to the lack of 5-LO-suppressive androgens. It is important to underline that LT levels as well as testosterone levels were similar in male ORCH and female mice.

As mentioned above, resident PM are seemingly responsible for LT production in zymosan-induced peritonitis. Interestingly, a sex difference in LTC_4 production in PM, activated with different stimuli such as zymosan and A23187, was observed also *in vitro*, supporting that differential regulation of 5-LO product synthesis in the cell may account for the *in vivo* bias of LT levels. Interestingly, protein 5-LO levels were not different between males and females, but sex-differences were observed for 5-LO subcellular localization in line with our previous observation *in vitro* in human neutrophils [11]. In particular, differences in basal 5-LO localization (*i.e.*, higher levels of nuclear 5-LO in unstimulated PM from males) resulted in a reduced trafficable 5-LO protein in stimulated male PM, correlating to lower 5-LO product synthesis *vs* female cells. Higher LTC_4 synthetic capacity of PM from male ORCH mice was observed, suggesting a prevalent role of androgens as responsible factors causing sex differences in LT biosynthesis in PM, according to our previous findings in neutrophils [11] and monocytes [12]. On the other hand, the *in vitro* treatment of PM with testosterone failed to modulate LT biosynthesis, which could be related to characteristics acquired in this specific cell type during cell differentiation. Note that the levels of the cyclooxygenase-derived PGE_2 were instead equal in PM from male and female mice, indicating selective bias on the 5-LO pathway within eicosanoid biosynthesis.

Conclusions

In conclusion our data reveal significant sex differences in the biosynthesis of LTs in mouse zymosan-induced peritonitis, which were associated with variant inflammatory responses. On the cellular level, differential 5-LO subcellular compartmentalization in PM from male and female mice might be the basis for these differences. Notably, tissue-resident macrophages represent the first line of defense against invading pathogens and activate immunological and inflammatory responses [25]. Therefore, the data presented here add significant insights into the understanding of basic sex differences in mounting inflammatory responses, with possible therapeutic implications for a sex-tailored treatment of related disorders.

Acknowledgements

A.R., C.P., S.P., O.W. and L.S designed research; A.R., C.P. and S.P. performed research; A.R., C.P., S.P., O.R., O.W. and L.S. analyzed data; and A.R., C.P. and L.S. wrote the paper.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2014.05.011.

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