Up-regulation of prostaglandin biosynthesis by leukotriene C_4 in elicited mice peritoneal macrophages activated with lipopolysaccharide/interferon-γ

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Abstract: Leukotrienes (LT) and prostaglandins (PG) are proinflammatory mediators generated by the conversion of arachidonic acid via 5-lipoxygenase (5-LO) and cyclooxygenase (COX) pathways. It has long been proposed that the inhibition of the 5-LO could enhance the COX pathway leading to an increased PG generation. We have found that in in vitro models of inflammation, such as mice-elicited peritoneal macrophages activated with lipopolysaccharide (LPS)/interferon-γ (IFN-γ), the deletion of the gene encoding for 5-LO or the enzyme activity inhibition corresponded to a negative modulation of the COX pathway. Moreover, exogenously added LTC_4, but not LTD_4, LTE_4, and LTB_4, was able to increase PG production in stimulated cells from 5-LO wild-type and knockout mice. LTC_4 was not able to induce COX-2 expression by itself but rather potentiated the action of LPS/IFN-γ through the extracellular signal-regulated kinase-1/2 activation, as demonstrated by the use of a specific mitogen-activated protein kinase (MAPK) kinase inhibitor. The LT-induced increase in PG generation, as well as MAPK activation, was dependent by a specific ligand-receptor interaction, as demonstrated by the use of a cys-LT1 receptor antagonist, although also a direct action of the antagonist used, on PG generation, cannot be excluded. Thus, the balance between COX and 5-LO metabolites could be of great importance in controlling macrophage functions and consequently, inflammation and tumor promotion. *J. Leukoc. Biol.* 78: 985–991; 2005.

Key Words: *eicosanoids · cyclooxygenase 2 · 5-lipoxygenase · mitogen-activated protein kinase*

INTRODUCTION

The leukotrienes (LT), LTB_4 and cysteinyl LT (cys-LT; LTC_4, LTD_4, and LTE_4), are metabolic products of arachidonic acid (AA) via the 5-lipoxygenase (5-LO) pathway [1]. The biologic activities of LT suggest that they are mediators of acute inflammatory and immediate hypersensitivity responses. The cys-LT and LTB_4 increase the adhesion of leukocytes to endothelial cells [2]; LTC_4 causes contraction of endothelial cells, resulting in increased permeability of postcapillary venules, and LTB_4 is a potent chemotactic factor for neutrophils [3]. Moreover, they seem to be involved in the regulation of inflammatory cell functions, such as macrophages. These cells, as a result of their phagocytosing and secretory functions, are important effector cells and because of their interaction with lymphocytes, are also involved in the surveillance of the immune system cells [4] and play a role in host defense against tumor cells. Moreover, macrophages are a major component of the leukocyte infiltrate of tumors. Tumor-associated macrophages have a complex, dual function in their interactions with neoplastic cells, and experimental evidences suggest that they are part of inflammatory circuits that promote tumor progression [5, 6]. Activated macrophages express many bioactive molecules, including eicosanoids [LT and prostaglandins (PG)] [7]. The biosynthesis of prostanoids and LT depends on AA availability, as AA is the common substrate. It has long been proposed that the inhibition of the 5-LO pathway could enhance the cyclooxygenase (COX) pathway leading to an increased PG generation. This is of particular interest, as the concentration of PG, especially PGE_2, is increased in inflammation as well as in different tumor types [8, 9]. However, contrasting results have been reported. In fact, some reports failed to show this hypothetical AA shunt [10, 11]. Conversely, it has been shown that the metabolic products of the COX pathway, especially PGE_2, inhibit LT biosynthesis by neutrophils [12].

Thus, the comprehension of the mutual regulation of these proinflammatory mediators seems to be of particular importance in the evolution of acute to chronic inflammation and in the involvement of the latter in tumor development and progression.

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The aim of this study was to investigate the role of 5-LO in PG production by the use of 5-LO knockout (5-LOKO) mice and the correspondent littermate in in vitro models of inflammation, such as elicited mice peritoneal macrophages activated with lipopolysaccharide (LPS)/interferon-γ (IFN-γ).

MATERIALS AND METHODS

Materials

LTC_4, LTD_4, LTE_4, and LTB_4 were obtained from SPIBIO (Paris, France). The PGE_2 antibody was a gift from Prof. Giovanni Ciabattoni (University of Chieti, Italy), and cys-LT antibody was obtained from Sigma-Aldrich (Milan, Italy). [^{3}H-PGE_2], [^{3}H-6-keto-$PGF_{1\alpha}$], and [^{3}H-LTC_4] were from PerkinElmer Life Sciences (Milan, Italy). Zileuton was obtained from Sequoia Research Products (Oxford, UK). All other reagents and compounds used were obtained from Sigma-Aldrich. Stock solutions of test compounds (zileuton, LTB_4, LTC_4, LTD_4, LTE_4, ICI204.219, and PD98059) were prepared in ethanol; an equivalent amount of ethanol was included in control samples.

Animals

Mice with a targeted disruption of the 5-lipoxygenase gene (5-LOKO) and littermate wild-type controls (5-LOWT) were purchased from Jackson Laboratories (Harlan Nossan, Italy). All mice used for studies were male, 4–5 weeks old, weighing 20–22 g. The animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations regarding protection of animals used for experimental and other scientific purposes (D.M. 116192) as well as with the European Economic Community regulations (O.J. of E.C. L 358/1 12/18/1986).

Peritoneal macrophages

Peritoneal macrophages were elicited by intraperitoneal (i.p.) injection of 2 ml 4% sterile thioglycolate (TG) medium (Sigma-Aldrich). After 3 days, mice were killed, and macrophages were harvested as described [13]. The peritoneal macrophages were grown in Dulbecco's modified Eagle's medium, supplemented with 2 mM glutamine, 25 mM Hepes, penicillin (100 U/ml), streptomycin (100 μg/ml), 10% fetal bovine serum (FBS), and 1.2% sodium pyruvate (BioWhittaker, Europe). Cells were plated on tissue-culture plates for 3 h, and then, nonadherent cells were removed by washing with sterile phosphate-buffered saline (PBS). Immediately before the experiments, culture medium was replaced by fresh medium without FBS to avoid interference with radioimmunoassay (RIA) [14]. The peritoneal macrophages were treated with various concentrations of LPS *Escherichia coli* (Serotype 0111:B4) and IFN-γ in the presence or absence of test compounds. Incubation media were used for eicosanoid measurements by RIA. Quantification of the cys-LT was performed by the use of RIA from Sigma-Aldrich, whereas PGE_2 and 6-keto-$PGF_{1\alpha}$ were quantified as described previously [15]. The levels of prostanoids and cys-LT were expressed as ng/ml. Cells were used for Western blot analysis.

Mitochondrial respiration

Cell respiration was assessed by the mithochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan [16]. After stimulation with LPS/IFN-γ in the absence or presence of the 5-LO inhibitor for 24 h, cells in 96-well plates were incubated with MTT (0.2 mg/ml) for 1 h. Culture medium was removed by aspiration, and the cells were solubilized in dimethyl sulfoxide (0.1 ml). The extent of reduction of MTT to formazan within cells was quantitated by the measurement of optical density at 550 μm.

Western blot analysis

Cells were washed in cold PBS and lysed for 10 min at 4°C with 1 ml lysis buffer [50 mM Tris, pH 7.4, 0.5% Nonidet P-40, 0.01% sodium dodecyl sulfate (SDS)] containing complete protease inhibitor cocktail (Roche, Mannheim, Germany). Lysates from adherent cells were collected by scraping and centrifuged at 12,000 *g* for 15 min at 4°C. The supernatants were collected, and protein concentration in cell lysates was determined by Bio-Rad protein assay (Bio-Rad, Richmond CA), and 70 μg total protein from each sample was analyzed. Proteins were separated by a 12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred on nitro-cellulose membrane [Hybond enhanced chemiluminescence (ECL) nitrocellulose, Amsherman, Rainham, UK]. The membrane was blocked with 0.1% Tris-buffered saline-Tween containing 5% nonfat milk for 1 h at room temperature. After the blocking, the membranes were incubated with the relative primary antibody overnight at 4°C. Mouse monoclonal antibodies anti-phosphorylated extracellular signal-regulated protein kianse (pERK)-1/2 and anti-ERK-2 were diluted, 1:1000 in 0.1% PBS-Tween, 0.5% bovine serum albumin (BSA; Santa Cruz Biotechnology, CA), whereas rabbit polyclonal antibodies anti-pAKT and anti-AKT (Cell Signaling Technology Inc., Beverly, MA) were diluted 1:2000. Rabbit polyclonal antibody anti-COX-2 and mouse monoclonal tubulin (Santa Cruz Biotechnology) antibody were diluted 1:1000 in 0.1% PBS-Tween, 5% BSA. After the incubation, the membranes were washed six times with 0.1% PBS-Tween and were incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Bio-Rad), diluted 1:2000 in 0.2% PBS-Tween; the membranes were washed; and protein bands were detected by an ECL system (Amersham Pharmacia, Little Chalfont, UK). Densitometric analysis was performed with a Fluor S quantitative imaging system (Bio-Rad).

Statistical analysis

The results are expressed as mean ± SEM of n observations, where n represents the number of experiments performed in different days. Triplicate wells were used for the various treatment conditions. The results were analyzed by one-way ANOVA followed by a Bonferroni post-hoc test for multiple comparisons. A P value less than 0.05 was considered significant.

RESULTS

PG production by peritoneal macrophages from 5-LOKO and 5-LOWT mice

To verify if the deletion of the 5-LO gene or enzyme inhibition could affect PG generation in elicited peritoneal macrophages activated with LPS/IFN-γ, we incubated the cells from 5-LOWT and 5-LOKO mice with LPS (0.1 μg/ml) and IFN-γ (1 U/ml) for 24 h. Unstimulated peritoneal macrophages from 5-LOWT or 5-LOKO mice produced only small amounts, not significantly different, of PGE_2 and 6-keto-$PGF_{1\alpha}$. Activation of the cells with LPS (0.1 μg/ml) and IFN-γ (1 U/ml) for 24 h significantly increased PGE_2 and 6-keto-$PGF_{1\alpha}$ ($P<0.001$) production in peritoneal macrophages from 5-LOWT and 5-LOKO mice. However, the increase observed in 5-LOKO animals was significantly ($P<0.001$) lower for PGE_2 and 6-keto-$PGF_{1\alpha}$ with respect to what was observed in macrophages from 5-LOWT mice (**Fig. 1, A** and **B**). In the incubation media of the cells from WT animals, we could only detect cys-LTs and not LTB_4 by the unstimulated and stimulated cells. The release of cys-LT was detectable only at 18 and 24 h (0.38±0.05 and 0.33±0.06 ng/ml, respectively); in fact, at 2 and 4 h the release was below the detection limit of the assay (<0.078 ng/ml). The LT production from unstimulated and stimulated cells from 5-LOKO animals was undetectable.

As 5-LO gene deletion decreased PG generation, we investigated if the inhibition of the enzyme activity produced similar results. To this aim, we measured PGE_2 and 6-keto-$PGF_{1\alpha}$ production by elicited peritoneal macrophages from 5-LOWT mice activated with LPS (0.1 μg/ml) and IFN-γ (1 U/ml) for 24 h in the absence or presence of the 5-LO inhibitor zileuton. Similarly to what was observed with 5-LO gene deletion, the

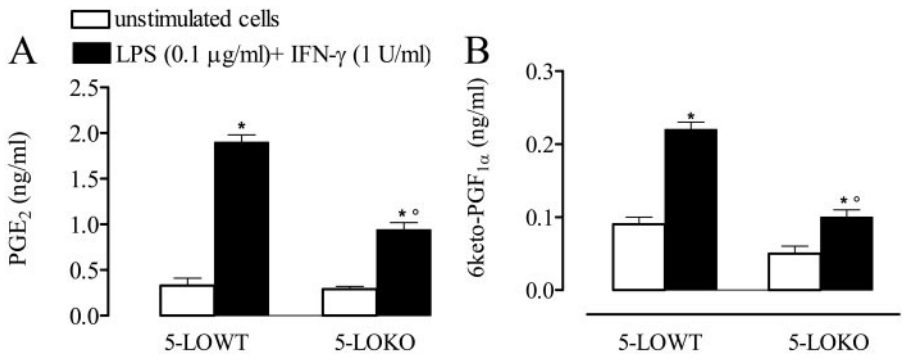

Fig. 1. PG production by peritoneal macrophages from 5-LOKO and 5-LOWT mice. Peritoneal macrophages were elicited by i.p. injection of 2 ml 4% sterile TG medium. After 3 days, mice were killed, and macrophages were harvested. Cells were plated on 24-multi-well plates for 3 h, and nonadherent cells were removed by washing with sterile PBS. Immediately before the experiments, culture medium was replaced by fresh medium without FBS to avoid interference with RIA. Peritoneal macrophages from 5-LOWT and 5-LOKO mice were treated subsequently for 24 h with LPS (0.1 μg/ml) and IFN-γ (1 U/ml). PGE_2 (A) and 6-keto-$PGF_{1\alpha}$ (B) levels were quantified in medium from cell incubation without prior extraction or purification by RIA. Data are expressed as means ± SEM from three separate experiments performed in triplicate. *, $P <$ 0.001, versus unstimulated cells; ○, $P < 0.001$, versus 5-LOWT LPS/IFN-γ.

inhibition of enzyme activity by 30 μM of the well-known 5-LO inhibitor, zileuton, significantly decreased PGE_2 and 6-keto-$PGF_{1\alpha}$ generation ($P<0.001$). However, the inhibition observed in the presence of the 5-LO inhibitor was more pronounced with respect to what was observed following gene deletion. In fact, at 100 μM zileuton, which completely reduced cys-LT biosynthesis by ~98%, the observed inhibition was 93% and 85% for PGE_2 and 6-keto-$PGF_{1\alpha}$, respectively (**Fig. 2, A** and **B**). The inhibition was not correlated to a toxic effect of the compound, as demonstrated by cell viability (data not shown).

Effect of exogenous LTC_4 on PG production by peritoneal macrophages

In the light of the above reported results, we tested the effect of increasing concentration of exogenous LTC_4 (0.001–1 μM) on PGE_2 and 6-keto-$PGF_{1\alpha}$ generation by elicited peritoneal macrophages from 5-LOWT mice activated with LPS (0.1 μg/ml) plus IFN-γ (1 U/ml) for 24 h. LTC_4 (1 μM) was unable to affect PGE_2 and 6-keto-$PGF_{1\alpha}$ production by unstimulated cells (0.39±0.003 ng/ml, 0.09±0.001 ng/ml and 0.32±0.02 ng/ml, and 0.1±0.01 ng/ml in the absence or presence of LTC_4, respectively), whereas LTC_4 concentration dependently increased PGE_2 and 6-keto-$PGF_{1\alpha}$ biosynthesis in stimulated cells from 5-LOWT mice (**Fig. 3, A** and **B**). The described action of LTC_4 was also observed in 5-LOKO mice. In fact, exogenously added LTC_4 (1 μM) induced an increase in prostanoid production by stimulated cells of the same extent observed in WT animals (seven to eight times). The cys-LT1 receptor antagonist ICI 204.219 completely reversed the action of LTC_4 in 5-LOWT and 5-LOKO mice (**Fig. 4, A** and **B**), although a direct action of the antagonist on PG generation cannot be excluded (data not shown). It is interesting that the action of the exogenous LTC_4 in WT animals was dependent on the effect of the stimulus used. In fact, at the concentration of LPS (0.1 μg/ml) plus IFN-γ (1 U/ml), the exogenously added LTC_4 (1 μM) induced an increase in PGE_2 and 6-keto-$PGF_{1\alpha}$ production of eight to nine and six times, respectively (Fig. 4, A and B); at LPS (10 μg/ml) plus IFN-γ (100 U/ml), of approximately three and two times, respectively (**Fig. 5, A** and **B**); and finally, no effect was observed at LPS (100 μg/ml) plus IFN-γ (1000 U/ml; Fig. 5, A and B). LTB_4, LTD_4, and LTE_4 did not produce any effect (data not shown).

Effect of exogenous LTC_4 on activation of mitogen-activated protein kinase (MAPK) signal transduction pathway and COX-2 expression in peritoneal macrophages from 5-LOWT mice

To investigate whether the increase in prostanoid production, observed in our experimental conditions, corresponded to an

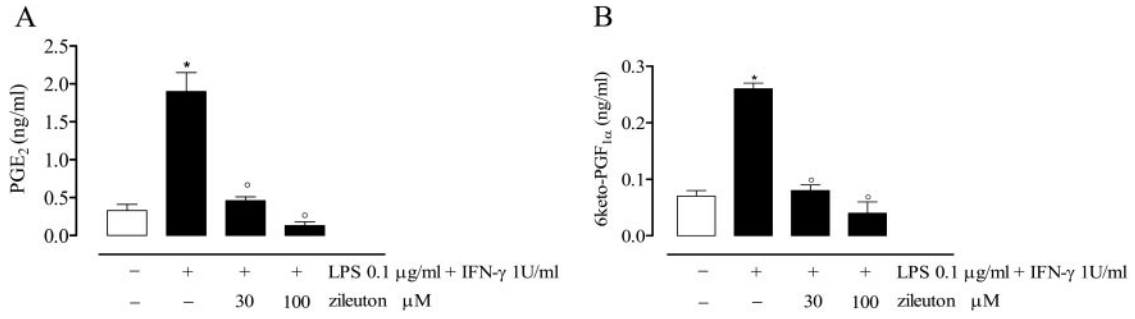

Fig. 2. Effect of zileuton on PG production by peritoneal macrophages from 5-LOWT mice. Peritoneal macrophages were elicited by i.p. injection of 2 ml 4% sterile TG medium. After 3 days, mice were killed, and macrophages were harvested. Cells were plated on 24-multi-well plates for 3 h, and nonadherent cells were removed by washing with sterile PBS. Immediately before the experiments, culture medium was replaced by fresh medium without FBS to avoid interference with RIA. Peritoneal macrophages from 5-LOWT were treated subsequently for 24 h with LPS (0.1 μg/ml) and IFN-γ (1 U/ml) in the absence or presence of the 5-LO inhibitor zileuton (30–100 μM). PGE_2 (A) and 6-keto-$PGF_{1\alpha}$ (B) levels were quantified in medium from cell incubation without prior extraction or purification by RIA. Data are expressed as means ± SEM from three separate experiments performed in triplicate. *, $P < 0.001$, versus unstimulated cells; ○, $P < 0.001$, versus 5-LOWT LPS/IFN-γ.

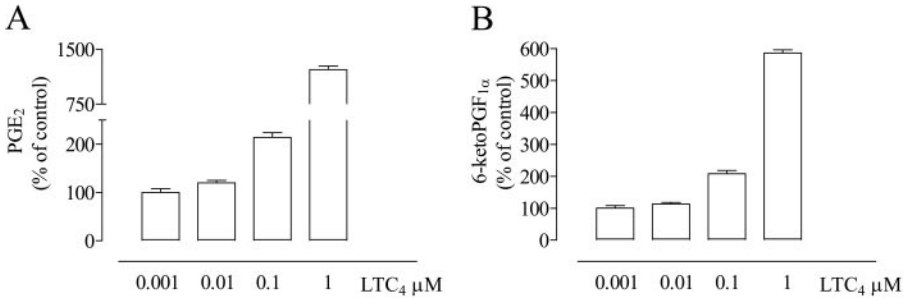

Fig. 3. Effect of increasing concentration of exogenous LTC_4 on PGE_2 and 6-keto-$PGF_{1\alpha}$ production by peritoneal macrophages from 5-LOWT mice stimulated with LPS/IFN-γ. Peritoneal macrophages were elicited by i.p. injection of 2 ml 4% sterile TG medium. After 3 days, mice were killed, and macrophages were harvested. Cells were plated on 24-multi-well plates for 3 h, and nonadherent cells were removed by washing with sterile PBS. Immediately before the experiments, culture medium was replaced by fresh medium without FBS to avoid interference with RIA. Peritoneal macrophages were treated subsequently for 24 h with LPS (0.1 μg/ml) and IFN-γ (1 U/ml) in the presence of increasing concentration of LTC_4 (0.001–1 μM). Prostanoids were quantified in medium from cell incubation without prior extraction or purification by RIA. Data are expressed as percent increase of prostanoid production with respect to control (LPS/IFN-γ only) in three separate experiments performed in triplicate.

activation of signal transduction pathways involved in the regulation of COX-2 expression, we analyzed the activation of phosphatidylinositol-3 kinase (PI-3K) and MAPK pathways after 24 h treatment with LPS (0.1 μg/ml)/IFN-γ (1 U/ml) in the presence or absence of 1 μM LTC_4. As shown in **Figure 6A**, stimulation of the cells in the presence of LTC_4 induced an activation of the MAPK pathway of approximately two times over the basal activation induced by LPS/IFN-γ only. The basal and the LTC_4-induced MAPK activation were abolished completely in the presence of the MAPK kinase inhibitor 2′amino-3-methoxyflavone (PD98059; 40 μM; Fig. 6A). No activation of the PI-3K pathway was observed (data not shown). To investigate if MAPK activation was LTC_4 receptor-mediated, we performed the same experiment in the presence of a cys-LT1 receptor antagonist (ICI 204.219). As shown in Figure 6B, the activation of MAPK over LPS/IFN-γ-induced basal level was inhibited by 1 μM ICI 204.219. To demonstrate that LTC_4-induced PG increase was a result of an up-regulation of COX-2, following the activation of the MAPK pathway, we analyzed the COX-2 expression after 24 h activation of the cells in the presence or absence of LTC_4. As shown in **Figure 7**, we observed an increase two to three times over the LPS/IFN-γ-induced basal level, which corresponded to an increase of PG levels.

DISCUSSION

We have found that in an in vitro model of inflammation, such as TG-elicited mice peritoneal macrophages activated with LPS/IFN-γ, the modulation of the 5-LO pathway, by gene deletion or enzyme activity inhibition, corresponded to a parallel modulation of the COX pathway. However, it must be pointed out that the modulation observed by 5-LO inhibition was more pronounced with respect to what was produced by 5-LO gene deletion. This could be a result of compensatory effects on other genes or other unknown actions of zileuton. However, other mechanisms affecting the activation of the cells cannot be ruled out. Thus, the suppression or inhibition of the 5-LO pathway in TG-elicited activated macrophages does not result in an increase of PGE_2 or 6-keto-$PGF_{1\alpha}$ generation, related to the shunting of the common substrate AA to the COX pathway, as previously postulated in these cells but conversely, results in an inhibition of the prostanoid biosynthesis. No increase, but rather a decrease of PG biosynthesis following 5-LO inhibition, was also observed in the same cell type following activation with LPS, according to Hubbard and Erickson [10]; however, the results were neither explained nor investigated further. Previous data by Feuerstein et al. [17] showed that in elicited rat peritoneal macrophages, exogenous LTC_4 induced PG release in unstimulated cells with a similar pattern with respect to LPS. However, the concentration of LPS was not reported nor was the action of LTC_4 on LPS activation. We could not observe any action of exogenous LTC_4 on PG release from unstimulated cells. In contrast to what was reported by Feuerstein et al. [17], our results show that the engagement of the LPS pathway is needed for the increase of PG biosynthesis induced by LT. Thus, the LPS "sensing apparatus" seems to be crucial.

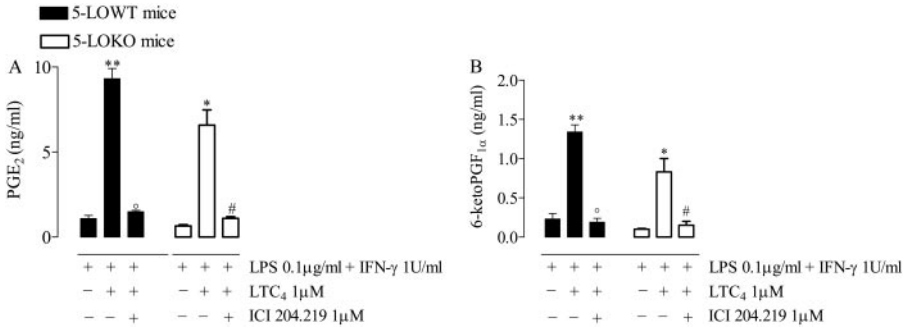

Fig. 4. Effect of exogenous LTC_4 on PGE_2 and 6-keto-$PGF_{1\alpha}$ production by peritoneal macrophages from 5-LOWT and 5-LOKO mice after stimulation with LPS and IFN-γ. Peritoneal macrophages were elicited by i.p. injection of 2 ml 4% sterile TG medium. After 3 days, mice were killed, and macrophages were harvested. Cells were plated on 24-multi-well plates for 3 h, and nonadherent cells were removed by washing with sterile PBS. Immediately before the experiments, culture medium was replaced by fresh medium without FBS to avoid interference with RIA. The peritoneal macrophages from 5-LOWT and 5-LOKO mice were subsequently treated for 24 h with LPS (0.1 μg/ml) plus IFN-γ (10 U/ml) in the presence or absence of 1 μM LTC_4 and cys-LT1 receptor antagonist ICI 204.219 (1 μM). PGE_2 (A) and 6-keto-$PGF_{1\alpha}$ (B) were quantified in medium from cell incubation without prior extraction or purification by RIA. Data are expressed as means ± SEM from three separate experiments performed in triplicate. **, $P < 0.001$; *, $P < 0.01$, versus LPS/IFN-γ alone; ○, $P < 0.001$, versus LPS/IFN-γ plus LTC_4; #, $P < 0.01$, versus LPS/IFN-γ plus LTC_4.

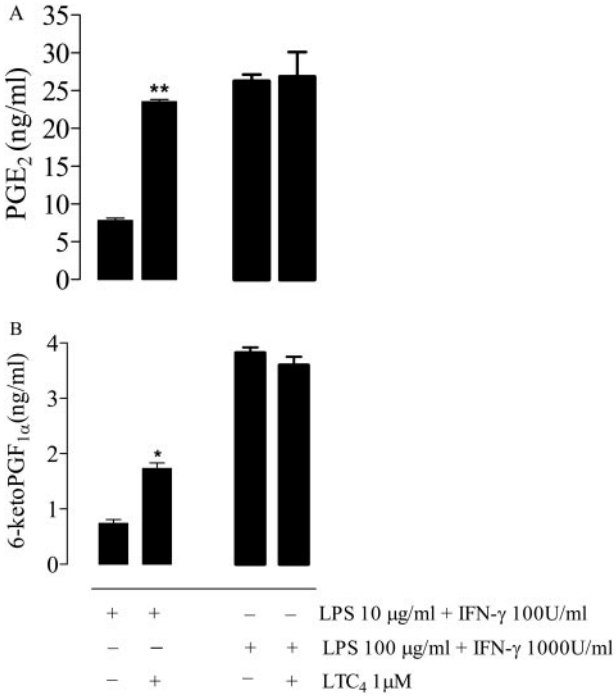

Fig. 5. Effect of exogenous LTC_4 on PGE_2 and 6-keto-$PGF_{1\alpha}$ production by peritoneal macrophages from 5-LOWT mice after stimulation with increasing concentration of LPS and IFN-γ. Peritoneal macrophages were elicited by i.p. injection of 2 ml 4% sterile TG medium. After 3 days, mice were killed, and macrophages were harvested. Cells were plated on 24-multi-well plates for 3 h, and nonadherent cells were removed by washing with sterile PBS. Immediately before the experiments, culture medium was replaced by fresh medium without FBS to avoid interference with RIA. The peritoneal macrophages from 5-LOWT mice were subsequently treated for 24 h with increasing concentration of LPS and IFN-γ in the presence or absence of 1 μM LTC_4. PGE_2 (A) and 6-keto-$PGF_{1\alpha}$ (B) were quantified in medium from cell incubation without prior extraction or purification by RIA. Data are expressed as means ± SEM from three separate experiments performed in triplicate. **, $P < 0.001$; *, $P < 0.01$, versus LPS/IFN-γ alone.

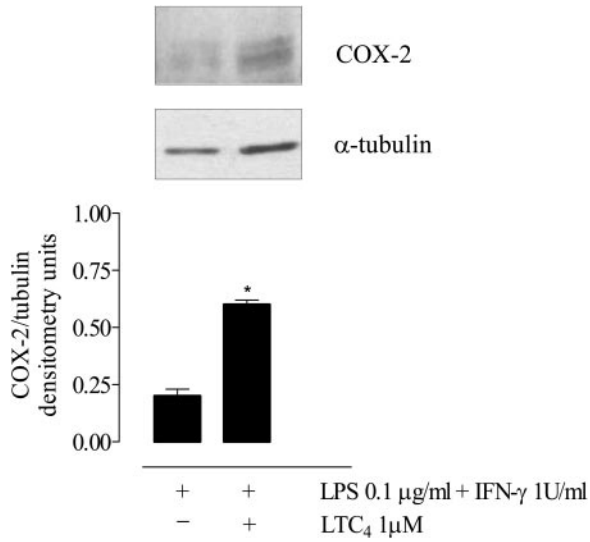

Fig. 7. Effect of exogenous LTC_4 on COX-2 expression in peritoneal macrophages from 5-LOWT mice stimulated with LPS/IFN-γ. Peritoneal macrophages were elicited by i.p. injection of 2 ml 4% sterile TG medium. After 3 days, mice were killed, and macrophages were harvested. Cells were plated on 24-multi-well plates for 3 h, and nonadherent cells were removed by washing with sterile PBS. Peritoneal macrophages were subsequently treated for 24 h with LPS (0.1 μg/ml) and IFN-γ (1 U/ml) in the presence or absence of 1 μM LTC_4. After 24 h, the cells were lysed, and the proteins were separated by SDS-PAGE and immunoblotted with an antibody specific for COX-2 and tubuline. The illustrated blots are representative of at least three separate experiments. Data are expressed as means ± SEM of three experiments for each group. *, $P < 0.001$, versus control (LPS/IFN-γ only).

The modulation of PG generation by 5-LO was specifically dependent on LTC_4, as LTD_4, LTE_4, and LTB_4 were not active. It must be pointed out that exogenously added LTC_4 also potentiated PG biosynthesis in stimulated macrophages from

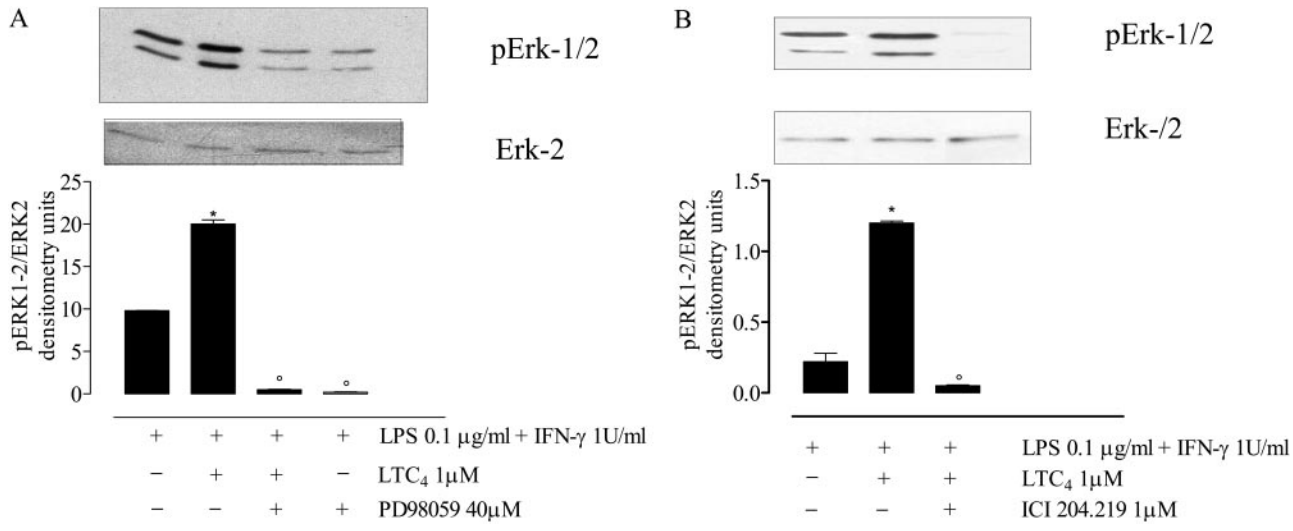

Fig. 6. Effect of exogenous LTC_4 on the activation of MAPK signal-transduction pathway in peritoneal macrophages from 5-LOWT mice stimulated with LPS/IFN-γ. Peritoneal macrophages were elicited by i.p. injection of 2 ml 4% sterile TG medium. After 3 days, mice were killed, and macrophages were harvested. Cells were plated on 24-multi-well plates for 3 h, and nonadherent cells were removed by washing with sterile PBS. Peritoneal macrophages were subsequently treated for 24 h with LPS (0.1 μg/ml) and IFN-γ (1 U/ml; A) in the presence or absence of the MAPK kinase (MEK) inhibitor PD98059 (40 μM) and (B) in the presence or absence of 1 μM LTC_4 and cys-LT1 receptor antagonist ICI 204.219 (1 μM). After 24 h, the cells were lysed, and the proteins were separated by SDS-PAGE and immunoblotted with an antibody specific for pERK-1/2 and ERK-2. The illustrated blots are representative of at least three separate experiments. Data are expressed as means ± SEM of three experiments for each group. *, $P < 0.001$, versus control (LPS/IFN-γ only), and ○, $P < 0.001$, versus LPS/IFN-γ plus LTC_4.

5-LOKO mice. It is interesting that in our experimental conditions, the cells were able to release, after 18 and 24 h of activation, only cys-LTs. This is in agreement with previous data reported by Hubbard and Erickson [10]. Moreover, our results show that inflammatory mediators display a certain degree of specificity depending on cell types. This is in agreement with what was shown in other cell types, such as intestinal epithelial cells, where only LTB_4 and LTD_4 but not LTC_4 positively modulate PG biosynthesis [18]. However, our results do not exclude that other 5-LO products, such as 5-hydroperoxyeicosatetraenoic acid, 5-hydroxyeicosatetraenoic acid, or eicosatetraenoic acid 5-oxo-6,8,11,14-HETE, could affect PG generation. The LT-induced increase in PG generation, observed in peritoneal macrophages, seems to be dependent by a specific ligand-receptor interaction, as demonstrated by the use of a cys-LT1 receptor antagonist (ICI 204.219), although also, a direct action of the antagonist used on PG generation cannot be excluded. Moreover, the finding that LTC_4 was not able to affect PG generation in unstimulated cells suggests that this action could be specific for PG generated by COX-2. In fact, as reported by Brock et al. [19], unstimulated peritoneal macrophages produce PG from COX-1 activity, whereas PGE_2 and PGI_2 generation, following activation with LPS, depends on COX-2 activity. An action on PGE-2 synthase can be excluded, as the same results were obtained for 6-keto-$PGF_{1\alpha}$. Further support for this assumption is provided by the observation that the increased PG generation, induced by the cys-LT, correlated with an up-regulation of COX-2 expression. This is of particular interest, as COX-2 up-regulation is present in different tumor types. The signal transduction mechanism involved in this up-regulation is related to the activation of the MAPK, a family of protein serine/threonine kinases, which includes the ERK-1 and ERK-2 [20]. In particular, the MAPK activation observed in our study is correlated with the phosphorilation of Erk-1/2 through the activation of the cys-LT1 receptor, as demonstrated by the use of the specific Mek inhibitor PD98059 and the cys-LT1 receptor antagonist ICI 204.219, respectively. It is well known that among the multiple signal pathways activated in macrophages upon LPS exposure, there is the MAPK/ERK1/2. Thus, the potentiation by LTC_4 of LPS activity could also be related to a stimulation of this signaling pathway. Further support for this hypothesis is the LPS concentration dependence of the LTC_4 action. In fact, we showed that at a concentration of LPS, being a maximal stimulus for PG production, the LTC_4 did not produce any effect, whereas at LPS concentration, giving a submaximal stimulus or half-maximal stimulus, we observed a graduated response. MAPK are involved in the regulation of cellular proliferation [20]. Recently, it has been shown that in intestinal epithelial cells and in colon cancer cells, LTD_4 strongly increase COX-2 expression thorough the cys-LT1 receptor via the MAPK Erk-1/2 pathway [21, 22]. Thus, in these cell types, cys-LTs seem to promote cell proliferation through the up-regulation of COX-2 via the MAPK Erk-1/2 pathway. It is interesting to note that in a tumor type, such as colorectal adenocarcinomas, there are high levels of cys-LT1 receptor and COX-2 [23]. Moreover, some studies have shown that increased COX-2 expression in colon cancer originates mainly from interstitial cells (i.e., macrophages), whereas little COX-2 expression was found in epithelial cancer cells [24]. As described for COX-2, 5-LO also seems to participate in the regulation of cell proliferation, and 5-LO is expressed by a broad variety of cancer cells [25]. In light of our and other findings [20, 23, 24], it is evident that there is a mutual interaction between the COX and 5-LO pathways. In fact, if 5-LO positively modulates the COX pathway, PG, especially PGE_2, via its EP4 receptor, negatively regulates 5-LO translocation and activation through a complex series of events [19]. As COX-2 and 5-LO seem to have redundant functions, the balance between COX and 5-LO metabolites seems to be of great importance in controlling macrophage functions, inflammation, and tumor promotion.

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