Pulmonary epithelial cancer cells and their exosomes metabolize myeloid cell-derived leukotriene C₄ to leukotriene D₄

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Abstract Leukotrienes (LTs) play major roles in lung immune responses, and LTD₄ is the most potent agonist for cysteinyll LT1, leading to bronchoconstriction and tissue remodeling. Here, we studied LT crosstalk between myeloid cells and pulmonary epithelial cells. Monocytic cells (Mono Mac 6 cell line, primary dendritic cells) and eosinophils produced primarily LTC₄. In coincubations of these myeloid cells and epithelial cells, LTD₄ became a prominent product. LTC₄ released from the myeloid cells was further transformed by the epithelial cells in a transcellular manner. Formation of LTD₄ was rapid when catalyzed by γ-glutamyl transpeptidase (GGT₁) in the A549 epithelial lung cancer cell line, but considerably slower when catalyzed by GGT₅ in primary bronchial epithelial cells. When A549 cells were cultured in the presence of IL-1β, GGT₁ expression increased about 2-fold. Also exosomes from A549 cells contained GGT₁ and augmented LTD₄ formation. Serine-borate complex (SBC), an inhibitor of GGT, inhibited conversion of LTC₄ to LTD₄. Unexpectedly, SBC also upregulated translocation of 5-lipoxygenase (LO) to the nucleus in Mono Mac 6 cells, and 5-LO activity. Our results demonstrate an active role for epithelial cells in biosynthesis of LTD₄, which may be of particular relevance in the lung.

Supplementary key words arachidonic acid • eicosanoid • inflammation • cancer • lung • macrophage/monocytes • extracellular vesicles • γ-glutamyl transpeptidase • 5-lipoxygenase

Leukotrienes (LTs) are mediators of inflammation (1) formed by immune cells in response to pathogens or danger signals. When cells are stimulated, cytosolic phospholipase A₂ α (cPLA₂α) and 5-lipoxygenase (LO) migrate to the nuclear membrane, arachidonic acid (AA) is released from nuclear membrane phospholipids, and LTA₄ is produced (2). Efficient translocation and activation of 5-LO requires two scaffold proteins, 5-LO activating protein (FLAP) and coactosin-like protein (CLP) (3). LTA₄ is then further metabolized by cytosolic LTA₄ hydrolase to LTB₄, or by LTC₄ synthase (LTC₄S) to LTC₄. LTC₄S is primarily located at the nuclear membrane together with FLAP, and both proteins are members of the family of membrane bound proteins involved in eicosanoid and glutathione metabolism (MAPEG). LTC₄ and its metabolites, LTD₄ and LTE₄, are jointly referred to as the cysteinyll LTs (CysLTs). CysLTs elicit edema formation, mucus secretion, and smooth muscle contraction, as well as eosinophil trafficking and tissue remodeling, and thus contribute to symptoms in several chronic inflammatory diseases, such as asthma (4). There are at least three different receptors for the CysLTs; in the lung, bronchoconstriction and other effects are mediated via CysLT1. LTD₄ has a 10- to 50-fold higher potency for activation of CysLT1 compared with LTC₄, while LTE₄ is the least active (5, 6). Thus, metabolism of LTC₄ to LTD₄ may have a considerable impact on CysLT1-mediated effects, also depending on further conversion to LTE₄.

Abbreviations: AA, arachidonic acid; COPD, chronic obstructive pulmonary disease; CysLT, cysteinyll leukotriene; FLAP, 5-lipoxygenase activating protein; GGT, γ-glutamyl transpeptidase; LO, lipoxygenase; LT, leukotriene; LTC₄S, LTC₄ synthase; MDDC, monocyte-derived dendritic cell; MM6, Mono Mac 6; PBEC, primary bronchial epithelial cell; SBC, serine-borate complex.

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γ-Glutamyl transpeptidase (GGT)1 and GGT5 are cell surface enzymes that hydrolyze γ-glutamyl compounds, typically in glutathione metabolism, and also the conversion of LTC4 to LTD4 (7–10). In rat lung, GGT (probably GGT1) was found to be expressed in Clara cells of the bronchial epithelium and in alveolar type II epithelial cells. The cells were maintained within a range of 0.2–1 × 10^6 cells/ml. LTC4S activity in MDDCs, TGFβ was found in macrophages present in human tissues, most abundant in the lung (19).

Studies on human blood monocytes and peritoneal macrophages have shown that these cells produce mainly LTC4 and only small amounts of LTD4. This was found both in early work (20) and in recent lipidomic analyses (21–24). Likewise, eosinophils (25–27) and mast cells (28–32) produce mainly LTC4, but apparently no or only small amounts of LTD4. Here we show transcellular conversion of leukocyte-derived LTC4 to LTD4 by A549 lung cancer cells expressing both GGT1 and GGT5, and by normal human primary bronchial epithelial cells (PBECs) expressing GGT5. Epithelial cells have an active role in pathogenesis of asthma (33); our findings highlight the role of epithelium in biosynthesis of the bronchoconstrictor, LTD4.

**MATERIAL AND METHODS**

All materials were purchased from Sigma-Aldrich unless otherwise stated.

**Cells**

Mono Mac 6 (MM6) cells were cultured in RPMI 1640 medium with glutamine supplemented with 10% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 1× nonessential amino acids, and oxalacetic acid, sodium pyruvate, and insulin, as described (34). The cells were maintained within a range of 0.2–1 × 10^6 cells/ml.

A549 cells were cultured on 10 cm Falcon tissue culture dishes (nr 353005) in Ham’s F12 medium supplemented with 10% FBS, 100 mg/ml streptomycin, and 100 U/ml penicillin. IL-1β was added as indicated. Cells were split regularly before confluence, typically every 3–4 days.

Monocyte-derived dendritic cells (MDDCs) were differentiated from primary monocytes isolated from buffy coats of healthy human donors (Karolinska Hospital Blood Bank). PBMCs, obtained by Ficoll-Paque PREMIUM (GE Healthcare) gradient centrifugation, were seeded in multi-well cell culture plates for 2 h, allowing monocytes to adhere to plastic. The plates were washed twice with PBS to remove lymphocytes. To obtain MDDCs, monocytes were differentiated for 7 days in RPMI 1640 medium with glutamine supplemented with 10% FBS, 100 mg/ml streptomycin, 100 U/ml penicillin, 1× nonessential amino acids, 25 mM HEPES, and rhGM-CSF (10 ng/ml; Sigma) plus IL-4 (10 ng/ml, Invitrogen). On day 3 and day 6, cells were resupplied with the cytokines. To enhance LTC4S activity in MDDCs, TGFβ was added on day 0 at a final concentration of 2 ng/ml (35). Eosinophils were isolated from two female donors. Following ficoll gradient centrifugation, eosinophils were prepared from the PMNL fraction using an eosinophil isolation kit (Milteny Biotech, Bergisch Gladbach, Germany).

PBECs were harvested in connection with lobectomy, as earlier described (36) (ethical approval 99-357 from Karolinska Institutet research ethics committee North at Karolinska Hospital). The PBECs were cultured at a density of 1–2 × 10^6 on T75 tissue culture flasks (ThermoFisher Scientific, Waltham, MA) that were precoated with coating buffer [fibronectin (Gibco, Life Technologies, Paisley, UK), BSA fraction V, vitrogen 100 collagen, and PBS without Ca^{2+}/Mg^{2+}]. The cells were maintained in keratinocyte serum-free medium (Gibco) supplied with human recombinant epidermal growth factor (5 ng/ml; Gibco), bovine pituitary extract (50 μg/ml; Gibco), and penicillin and streptomycin antibiotics (BioWhittaker, Lonza, Basel, Switzerland). The cultures were kept in 5% CO2 at 37°C and medium was changed every second day. At confluence, the cells were detached by treatment with trypsin/EDTA solution (0.03/0.01% in PBS without Ca^{2+}/Mg^{2+}; Gibco). The number of cells was counted and the viability (>95%) was assessed using exclusion of trypan blue dye. The PBECs used in this study were at passage 5 and passage 6 from three different donors.

**Exosome isolation**

Exosomes were isolated as described (35), with minor changes. Briefly, A549 cells were grown to confluence for 48 h in complete medium with exosome-depleted FBS (centrifuged at 100,000 g ON) and IL-1β (5 ng/ml). The cell culture supernatant was collected (typically 400 ml) and centrifuged at 3,000 g for 30 min, 10,000 g for 30 min, and 100,000 g for 2 h. The pellet was washed in PBS and centrifuged again at 100,000 g for 2 h. Finally, the pellet was resuspended in 1 ml of PBS and protein concentration (0.5–1 mg/ml) was measured by Bradford assay (Bio-Rad protein reagent). The average yield was 2.8 μg exosomal protein per 10^6 A549 cells. Exosomes were further characterized by nanoparticle tracking analysis and FACS, as described (37).

**Timeline of cell culture and coculture**

MM6 and A549 cells were treated and cocultured as shown in Fig. 1. Briefly, on day 0, MM6 cells were seeded at a density of 0.2 × 10^6/ml and differentiation was started by addition of TGFβ (2 ng/ml) and 1,25-dihydroxyvitamin D3 (50 nM) (38). A549 cells were seeded at 0.5 × 10^6 cells per plate. On day 1, A549 cells were washed twice with PBS, and 10 ml of Ham’s F12 medium containing 2% FBS and IL-1β (1 ng/ml) was added to starve and stimulate the cells. On day 3, cocultures were started. The number of A549 cells on the separate counting plate was determined after detachment with trypsin/EDTA (typically 2–3 × 10^6).

**Fig. 1.** Timeline of MM6 and A549 cell treatments and coculture.
cells). Inspection under the microscope consistently indicated that similar cell numbers were present on the other A549 dishes in that experiment. Also, the MM6 cell count was determined on day 5 (typically 0.3–0.7 × 10^6 cells/ml). MM6 cell suspension was added to A549 dishes to achieve a 1:2 MM6:A549 ratio. Thus, around 2–3 ml of MM6 cell suspension was added to the medium on A549 dishes (starvation medium kept since day 1). On day 4, all cells were harvested. MM6 cells not subjected to coculture were thus kept in differentiation culture (with TGFβ + VD3) for 4 days, and A549 cells not subjected to coculture were grown in the presence of IL-1β for 3 days. Morphology and trypan blue exclusion indicated that A549 and MM6 cells were in good condition during coculture, there were no signs of reduced cell viability.

**Cell incubations**

On day 4, the MM6 cultures, A549 cultures, and MM6-A549 cocultures were collected. MM6 cells were counted and then centrifuged at 150 g for 5 min. The final pellet was resuspended in 0.5 ml PBS for incubations with LTA₄ or PGC buffer for incubations with A23187 (PGC is PBS containing 1 mg/ml glucose and 1 mM CaCl₂). A549 cells were washed twice with PBS and then detached using trypsin/EDTA. After counting, the cells were centrifuged at 150 g for 5 min and the final pellet was resuspended in 0.5 ml PBS or PGC. Coclurures were detached by scraping and centrifuged at 150 g for 5 min. The final pellet was resuspended in 0.5 ml PBS or PGC. The coincubations (0.5 ml) contained 2–3 × 10⁶ A549 cells and 1–1.5 × 10⁶ MM6 cells.

Cells were incubated with Ca²⁺ ionophore A23187 at two different conditions. In condition 1, cells were pretreated with 100 nM PMA for 10 min at 37°C and subsequently incubated with 5 μM A23187 for 10 min at 37°C. In condition 2, cells were incubated with 40 μM AA together with 5 μM A23187 for 10 min at 37°C. The amount of ethanol (solvent for A23187, AA) did not exceed 0.2% (v/v). The reaction was stopped by adding 0.5 ml of methanol containing internal standards (normally 250 pmol PGB₂ and 250 pmol 17-OH-C₂₂:₄, kind gifts from Mats Hamberg, Karolinska Institutet) and kept on ice or at −20°C for at least 1 h. For the coincubations, formation of eicosanoids is given per million of MM6 cells present.

Cells were incubated with LTA₄ (20 μM) for 5 min at 37°C. LTA₄ was added in ethanol (1–2 μl). The reactions were stopped as described above. For LTA₄ incubations of primary leukocytes together with A549 cells, the incubation time was 15 min.

**Analysis of LTs and 5-HETE**

After precipitation of proteins, the samples were centrifuged at 10,000 g for 10 min at 4°C and supernatants (approximately 1 ml) collected and added to 2 ml citrate/phosphate buffer (0.1 and 0.2 mM, respectively, pH 5.6). Extraction was performed using a C₁₈ column (Supelco, Bellefonte, PA) preconditioned with 3 ml methanol and 1 ml water. After application of the 3 ml sample, the column was washed with 1 ml water and finally oxylipins were eluted with 300 μl methanol followed by 300 μl water. The methanol and water eluates were pooled and aliquots analyzed by reverse-phase HPLC. In test extractions of mock samples (known amounts of PGB₂ and LTC₄ added to PBS buffer), the recovery of PGB₂ was 85% and of LTC₄ was 83%. HPLC was performed with a C₁₈ column [Phenomenex Luna, 5 μC₂₈(2) 100A, 2 × 150 mm] eluted at a flow rate of 0.8 ml/min. For LT analysis, the mobile phase was water:methanol:acetonitrile:acetic acid (43:27:30:0.1, pH 5.6) adjusted with NH₄Cl and UV absorbance at 280 nm was monitored. For analysis of the less polar mono-HETEs, the mobile phase was water:acetonitrile:acetic acid (40:60:0.1) and UV absorbance at 255 nm was monitored. LTs and 5-HETE were quantitated from peak areas and extinction coefficients in relation to the internal standards (PGB₂ for LTs; 17-OH-C₂₂:₄ for 5-HETE).

**Conversion of LTC₄ to LTD₄ by A549 cells**

A549 cells were harvested on day 4 (Fig. 1). After centrifugation (150 g for 4 min), around 16 × 10⁶ cells were resuspended in 3 ml PBS and incubated with 0.36–0.56 μM LTC₄ (Cayman Chemicals). At the indicated intervals (1, 2, 3, 5, 15, and 30 min), aliquots of 0.5 ml containing 2.7 × 10⁵ cells were removed and added to a tube containing 0.5 ml methanol and 125 pmol of PGB₂ in order to stop the reaction. Extraction and analysis were performed as described above. Two incubation series were performed in each experiment.

**GGT1 inhibition with serine-borate complex**

Serine-borate complex (SBC) is a competitive inhibitor of GGT1 (39). Cells in 0.5 ml of PBS or PGC buffer were treated with 20 μl of a solution of equimolar L-serine (0.25 M) and sodium borate (0.25 M) to give a concentration of 10 mM SBC. Cells were treated with SBC for 5–10 min at 37°C before incubations with LTA₄ or PMA plus A23187.

**SDS-PAGE and Western blotting**

SDS-PAGE and Western blotting were performed essentially as described (34) using Bio-Rad 4–20% ready-made gels and nitrocellulose membranes (Amershams). The following antibodies were used: in-house antisera (rabbit polyclonal) against 5-LO (1:3,000 dilution), FLAP (1:300 dilution), LTA₄H (1:2,500 dilution), LTC₄S (1:750 dilution), and mPGES-1 (1:3,000 dilution). A mouse monoclonal against GGT1 (used at 1:500 dilution) was from Santa Cruz Biotechnology, Santa Cruz, CA. A rabbit polyclonal against GGT5 (used at 1:200 dilution) was from Abcam, Cambridge, UK. A peroxidase-conjugated primary antibody against β-actin (1:2,000 dilution) and peroxidase-conjugated secondary antibodies (1:5,000 dilution) were from Sigma. Protein bands were detected by enhanced chemiluminescence; after scanning, relative amounts were calculated with ImageJ software.

**Subcellular fractionation by detergent lysis**

MM6 cells, differentiated with TGFβ and VD3 for 96 h, were subjected to subcellular fractionation after NP-40 lysis, as described (34). Histone H4, a nuclear protein, was used as marker for correct fractionation.

**Immunofluorescence microscopy**

Differentiated MM6 cells were harvested and resuspended in 1 ml PGC buffer. Cells (1–2 × 10⁶) were resuspended in 1 ml PGC buffer and stimulated, in order, with SBC (10 mM), PMA (100 nM), and A23187 (5 μM) for 10 min, 10 min, and 5 min, respectively. Unstimulated control cells received only vehicle. Samples were immediately chilled on ice for 5 min and centrifuged onto glass slides at 4°C. The cells were then fixed with methanol at −20°C for 4 min and washed four times with PBS. Samples were blocked with PBS containing 1% BSA and 0.1% Tween-20 for 1 h at room temperature and incubated with anti-5-LO antibody overnight at 4°C. Then samples were washed four times with PBS, incubated with Cy3 goat anti-rabbit IgG (Invitrogen) for 1 h at room temperature, and washed five times with PBS. The samples were coated with two drops of aqueous antifade gel mounting medium (Vector Laboratories) with DAPI under coverslips. The fluorescent signal was observed with an Olympus FluoView FV1000 microscope system.

**Data analysis**

Student’s t-test was performed, *P < 0.05* was considered statistically significant.
RESULTS

Formation of 5-LO products in coincubations of MM6 and A549 cells

MM6 cells were differentiated with TGFβ and VD3 for 72 h, which leads to upregulation of 5-LO (2), and A549 cells were starved and stimulated with IL-1β for 48 h. The cells were then combined and cocultured for 24 h before different incubations and determinations of LT and 5-HETE biosynthesis, see Fig. 1.

For MM6 cells, it was observed before that priming with PMA strongly upregulated ionophore-induced translocation of 5-LO, as well as product formation from endogenous AA (34). In such incubations of MM6 cells alone, the major 5-LO product was LTC4, closely followed by 5-HETE, see Fig. 2A. Only minute amounts of LTB4 and LTD4 were formed. When MM6 cells and A549 cells were cocultured (cell ratio 1:2) and incubated together (priming with 100 nM PMA for 10 min followed by 5 μM A23187 for 10 min), the major difference was that LTD4 also, but not LTE4, appeared (Fig. 2A). Concomitantly, the recovery of LTC4 was reduced (Fig. 2A). LTB4 was slightly increased; nevertheless, LTB4 was still a minor product. Formation of 5-HETE was similar as incubations of MM6 cells alone, and no other mono-HETEs appeared. Finally, when A549 cells alone were incubated with PMA plus A23187, no LTs or HETEs were detected.

In incubations of MM6 cells with ionophore A23187 (5 μM) together with exogenous AA (40 μM), formation of all 5-LO products was strongly increased compared with incubations with PMA plus ionophore (Fig. 2B). When MM6 cells and A549 cells were cocultured (cell ratio 1:2) and incubated together with A23187 plus exogenous AA, again LTD4 appeared, LTE4 was not observed, and LTB4

Fig. 2. Formation of 5-LO products in different incubation conditions. MM6 monocultures, A549 monocultures, and MM6/A549 cocultures (ratio 1:2) were prepared as described in the Materials and Methods (see also Fig 1). On day 4, cells were harvested and different incubations were performed. After solid phase extraction, LTs and 5-HETE were analyzed by HPLC. In coincubations, product formation is given as picomoles per million MM6 cells. A: Cells were primed with PMA (100 nM) for 10 min and then stimulated with ionophore A23187 (5 μM) for 10 min (n = 9). B: Cells were stimulated with ionophore A23187 (5 μM) and AA (40 μM) for 10 min (n = 5). C: Cells were incubated with LTA4 (20 μM) for 5 min (n = 10). Results are given as mean ± SE, Student’s t-test. n.s., not significant; ND, not detected.
formation was approximately the same as with MM6 cell monocultures. A clear difference compared with incubations of MM6 cells alone was that the yield of 5-HETE decreased considerably (Fig. 2B). Again, A549 cells alone did not produce LTs or HETEs.

Cells were also incubated with LTA₄ (20 μM). With MM6 cells alone, this gave the highest production of LTC₄, a very small amount of LTD₂, and a minor amount of LTB₁ (less than 10% of the LTC₄ formation) (Fig. 2C). In coincubations of MM6 cells and A549 cells, LTD₂ was produced and formation of LTB₁ now also increased. This was most probably due to LTA₄ hydrolase activity in A549 cells, as LTB₁ formation was also evident in the incubations of A549 cells alone with LTA₄ (Fig. 2C).

For increased formation of LTD₄, it was required to have MM6 and A549 cells together. In control experiments, MM6 cells were cultured with addition of conditioned medium from IL-1β-treated A549 cells. In subsequent incubations with PMA and ionophore, or with LTA₄, there was no increased LTD₄ production and no appreciable effect on the other LTs or 5-HETE. Also, when A549 cells were cultured with MM6 cell-conditioned medium (TGFβ and VD3 added), there was no effect on their capacity to transform LTC₄ to LTD₂ and there was no induced 5-LO activity (data not shown). In the incubations of A549 cells with LTA₄, one of the nonenzymatic LTA₄ hydrolysis products (12-epi-6-trans-LTB₄), particularly, was further metabolized to the 5-oxo derivative with UV maximum at 318 nm (40).

Expression of enzymes for LT biosynthesis in MM6 and A549 cells

MM6 cells were differentiated with TGFβ and VD3, and A549 cells were starved and stimulated with IL-1β. The 10,000 g supernatants from total cell lysates were analyzed by Western blots. The 5-LO, FLAP, and LTC₄S were expressed only in differentiated MM6 cells; these proteins were not detected in A549 cells (Fig. 3). On the other hand, GGT1 and mPGES-1 were only found in A549 cells; these proteins were not expressed in MM6 cells. For GGT1, the small subunit was analyzed and at least two bands were observed in the 20–25 kDa range, in accordance with previously observed glycosylation (41). Finally, LTA₄ hydrolase was about equally expressed in differentiated MM6 cells and in A549 cells. The exclusive expression of 5-LO and LTC₄S in MM6 cells, and of GGT1 in A549 cells, agrees with both cell types being required for biosynthesis of LTD₄.

Export of LTC₄ from myeloid cells

The relative amount of LTC₄ that was further converted to LTD₄ was quite different for the three different incubation conditions, see Fig. 2. In incubations with LTA₄, the highest amount of CysLT was formed (360 pmol/10⁶ MM6 cells) and, of this, about 37% was LTD₄. In incubations with A23187 and AA, 110 pmol CysLT was formed per 10⁶ MM6 cells and, of this, about 40% was LTD₄. Finally, in incubations with PMA plus A23187, 42 pmol CysLT was formed per 10⁶ MM6 cells and, of this, about 60% was LTD₄. Cell numbers and incubation times were the same in all these incubations. The result from the incubations with LTA₄ indicated a high capacity of the A549 cells to convert LTC₄ to LTD₄, which should be more than sufficient for all CysLT to be metabolized to LTD₄ in the incubations with ionophore plus AA and with PMA plus ionophore. However, this was not the case. This could depend on the release of LTC₄ from MM6 cells, which would determine conversion to LTD₄ by the A549 cells.

To investigate this, MM6 cells were incubated as described above, but before stopping the reactions, cells and media were separated by quick centrifugation and intracellular LTC₄ was analyzed (Table 1). For MM6 cells incubated with PMA and ionophore or with LTA₄, substantial shares of the LTC₄ formed remained associated to the cell fractions and would, thus, not be available for metabolism by A549 cells. The degree of extracellular LTC₄ (Table 1) agrees with the further conversions to LTD₄ in coincubations (compare Fig. 2). For MDDCs and eosinophils, the amount of extracellular LTC₄ was about 60%, seemingly compatible with the relative amounts of LTD₄ formed in coincubations (Fig. 6). It appears that export of LTC₄ from the myeloid cells is one factor determining LTD₄ formation in coincubations.

### Table 1. Release of LTC₄ from MM6 cells, MDDCs, and eosinophils

|                | Intracellular | Extracellular | Sum   |
|----------------|---------------|---------------|-------|
| MM6 incubated LTA₄ | 163 ± 21     | 90 ± 5        | 253   |
| MM6 incubated PMA + A23187 | 9 ± 1       | 17 ± 1        | 26    |
| MDDCs incubated LTA₄ | 25 ± 10      | 33 ± 7        | 56    |
| Eosinophils incubated LTA₄ | 28 ± 7       | 44 ± 12       | 72    |

Release of LTC₄ from MM6 cells, MDDCs, and eosinophils was analyzed after incubations with LTA₄ (20 μM) for 5 min at 37°C (n = 4–5). MM6 cells were incubated with PMA also (100 nM, 10 min) followed by A2387 (5 μM, 10 min) (n = 6). The incubations were stopped by centrifugation at 4°C (3 min at 1,000 g). Supernatants (extracellular) were rapidly removed and added to 0.5 ml methanol containing 250 pmol PGB₂, while the cell pellet was resuspended in 1 ml methanol/water (1:1), with 250 pmol PGB₂. After extraction, LTC₄ was analyzed by HPLC. Results are mean ± SE.
GGT1 in exosomes from A549 cells

Exosomes prepared from A549 cells were of the expected size (Fig. 5A) and carried the tetraspanins, CD63 and CD81, characteristic for exosomes (Fig. 5B). By Western blot, GGT1 was detected in both A549 cells and A549-derived exosomes (Fig. 5C). Conversion of LTA₄ was determined in coincubations of MM6 cells with exosomes from A549 cells. When cells and exosomes were mixed and incubated directly with LTA₄, the yield of LTD₄ increased (Fig. 5D). Exosomes were enriched from A549-conditioned medium, and it should be observed that in the final preparation the exosome concentration was increased considerably (>100-fold). We could not detect GGT1 activity in the control experiments of MM6 together with A549-conditioned medium. Furthermore, when MM6 cells were cultured with exosomes for a long time (24 h) before incubation with LTA₄, LTD₄ was not increased (data not shown), possibly indicating degradation of exosomal contents.

SBC inhibits formation of LTD₄ in MM6/A549 coincubations

GGT is inhibited by SBC (39) and SBC was 8-fold more potent to inhibit purified GGT1 ($K_i$ 0.5 mM) compared with purified GGT5 ($K_i$ 4.2 mM) (9). When MM6 and A549 cells were coincubated with LTA₄, both LTC₄ and LTD₄ were formed. In cells pretreated with SBC (10 mM, 10 min) before addition of LTA₄, formation of LTD₄ was undetectable (supplemental Fig. S1A). A similar result was obtained when

Conversion of exogenous LTC₄ to LTD₄ by A549 cells

When A549 cells (approximately $3 \times 10^6$ cells in 0.5 or 1 ml of PBS) were incubated with exogenous LTC₄ (0.36–0.56 μM) for 5 min, LTD₄ formation was 25–50 pmol/10⁶ cells. In all these incubations, LTC₄ was in excess; more than half of the recovered CysLT was LTD₄. Cells treated with IL-1β produced about 2-fold more LTD₄ compared with untreated cells (Fig. 4A), and in the Western blot, the GGT1 band intensity was increased (Fig. 4B, C). In a time course experiment, $35 \times 10^6$ cells (in 6.5 ml PBS) were incubated with 2.6 nmol of LTC₄ (0.4 μM). As shown in Fig. 4D, after 15 min almost half of the substrate had been converted and at 30 min about 60% of the CysLT was LTD₄. Practically no LTE₄ was formed; at 30 min, less than 1% of the CysLT was LTE₄.

The formation of LTD₄ from exogenous LTC₄ in these experiments (25–50 pmol/10⁶ A549 cells at 5 min, Fig. 4A, C) is in the same range as the formation of LTD₄ in coincubations of MM6 and A549 cells with LTA₄ (Fig. 2C). In the coincubations, the sum of CysLT produced per 10⁶ MM6 cells during 5 min was 360 pmol. Of this, 130 pmol was further converted to LTD₄ by $2 \times 10^6$ A549 cells (MM6 to A549 cell ratio 1:2). Thus, in the coincubations, the formation of LTD₄ was 65 pmol/10⁶ A549 cells. The similar yields of LTD₄ from exogenously added LTC₄ and from LTC₄ produced by MM6 cells indicates that there was no special role for MM6-A549 cell to cell interactions in the further metabolism of LTC₄ to LTD₄.
cells were stimulated with PMA plus ionophore A23187 (supplemental Fig. S1B). These observations support that, in the MM6/A549 coincubations, LTC₄ is converted to LTD₄ by GGT1. Interestingly, in the incubations with PMA plus A23187, SBC also had another effect: the total formation of CysLTs (LTC₄) increased in comparison to CysLTs in cells not receiving SBC (LTC₄ plus LTD₄). This was not observed for cells incubated with LTA₄.

**SBC upregulates 5-LO activity and translocation in MM6 cells**

To investigate the SBC-induced upregulation of CysLTs in cells incubated with PMA plus A23187, MM6 cells alone were pretreated with SBC for 5 min. When these cells were stimulated with PMA plus ionophore A23187, pretreatment with SBC increased formation of both LTC₄ and 5-HETE 2- to 3-fold (supplemental Fig. S2A, B). Human monocytes isolated from peripheral blood showed a similar increase in 5-HETE formation when pretreated with SBC (supplemental Fig. S2D). This indicated that SBC could upregulate 5-LO activity. One factor determining the activity of 5-LO in cells is translocation to the nuclear membrane. This was investigated first by subcellular fractionation. Thus, MM6 cells were lysed with NP-40 and 5-LO was analyzed in nuclear and nonnuclear fractions by Western blot. As shown in supplemental Fig. S2C, in unstimulated control cells, 5-LO was recovered in the soluble nonnuclear fraction, while stimulation with PMA plus A23187 resulted in 5-LO in both nuclear and nonnuclear fractions. When cells were pretreated with SBC (5 min) before stimulation with PMA plus A23187, the majority of 5-LO was recovered in the nuclear fraction. When MM6 cells were treated with SBC alone, there was no change in the subcellular localization of 5-LO (data not shown). MM6 cells were analyzed also by immunocytochemistry. As shown in supplemental Fig. S3, pretreatment with SBC seemed to give a more pronounced accumulation of 5-LO to the perinuclear membrane, as compared with stimulation with only PMA plus A23187. These findings indicate that SBC can prime the cells for more efficient translocation of 5-LO, possibly due to effects of GGT on cellular redox status.

**Formation of CysLT in coincubations of A549 cells with MDDCs and eosinophils**

To validate the results in primary cells, MDDCs and eosinophils were prepared from two donors each. These primary cells were mixed in suspension with scraped A549 cells (leukocyte to A549 ratios 1:2 to 1:4) and coincubated for 15 min with LTA₄ (20 nM). Prior to incubations, the A549 cells had been starved and stimulated with IL-1β for 72 h. As shown in Fig. 6, the dominating CysLT formed in the leukocytes alone was LTC₄, while in the coincubations, the yield of LTD₄ increased substantially. In the incubations with PMA plus A23187, SBC also had another effect: the total formation of CysLTs (LTC₄) increased in comparison to CysLTs in cells not receiving SBC (LTC₄ plus LTD₄). This was not observed for cells incubated with LTA₄.

![Fig. 5. GGT1 in exosomes from A549 cells treated with IL-1β. A: Size range of A549 exosomes determined by nanoparticle tracking analysis. B: A549-derived exosomes carry CD81 and CD63. Beads coated with CD63 or HLA-DR antibody were added to exosomes (corresponding to 5 μg of exosomal proteins) in 500 μl PBS and incubated overnight at room temperature. After washing, the beads were incubated with FITC-labeled CD81 antibody and subjected to FACS analysis. C: A549 cells were starved and stimulated with IL-1β. An aliquot (20 μg) of a 10,000 g supernatant (from total cell lysate) and exosomes corresponding to 16 μg of protein were analyzed for GGT1 by Western blot. D: Formation of LTD₄ in coincubations of MM6 cells (1 × 10⁷) and A549 exosomes (100 μg of exosomal protein) in 1 ml PBS. Incubated with LTA₄ (10 μM) for 15 min (n = 6).](image)

![Fig. 6. CysLT formation in coincubations of A549 cells with MDDCs and eosinophils. The primary cells were mixed in suspension with scraped A549 cells (leukocyte to A549 ratios 1:2 to 1:4) and coincubated for 15 min with LTA₄ (20 μM). Before the incubations, the A549 cells had been starved and stimulated with IL-1β for 72 h. As shown in Fig. 6, the dominating CysLT formed in the leukocytes alone was LTC₄, while in the coincubations, the yield of LTD₄ increased substantially. In the incubations of MDDCs and eosinophils (both known to express}
We determined LT biosynthesis in coincubations of monocytic cells (MM6, MDDCs) and eosinophils with epithelial A549 cells or primary epithelial cells. Our study confirms and extends a role for epithelial cells in LTD4 biosynthesis in the lung environment. Conversions of exogenous LTC4 by epithelial cells has been studied previously (43, 44), but to our knowledge, metabolism of leukocyte-derived LTC4 was not demonstrated before in coincubations of monocytic and epithelial cells. The non-small cell lung cancer cell line, A549, transformed leukocyte-derived LTC4 to LTD4 within minutes; while for normal PBECs, long incubation times (6 h) were required. The slow LTC4 to LTD4 conversion by PBECs is in accordance with these cells expressing GGT5, while A549 cells also express GGT1, which is considerably faster (9). Treatment with proinflammatory IL-1β upregulated the capacity of A549 cells for conversion of LTC4 to LTD4, and GGT1 was also present in exosomes from these cells.

GGT enzymes are present on epithelial cells in many tissues (7). GGT activity can also be expressed in leukocytes, e.g., in U937 cells (45), and accordingly, LTD4 was formed in short-term (A23187, 10 min) incubations of U937 cells differentiated with DMSO (46). LTD4 was also produced in RBL-1 cells (47). GGT1 was not detectable in MM6 cells, in line with the very small amounts of LTD4 in 15 min incubations of MM6 cells alone. This is in agreement with several
previous studies on human blood monocytes and human peritoneal macrophages using incubation times up to 60 min [see (20) and references therein]. Also, in more recent lipidomic analyses of mouse peritoneal macrophages (21) and RAW264.7 murine macrophages (22, 23), LTC4 and 5-HETE (but not LTD4) were major 5-LO products. In a recent study of macrophages, FACS-sorted from a mouse lung tumors, LTC4 was more than 10-fold more abundant than LTD4 (24). In all these studies, incubation times were at most 60 min. Likewise, eosinophils (25–27) and mast cells (28–32) produce mainly LTC4, but apparently no or only small amounts of LTD4. Interestingly, in mast cells cocultured with 3T3 fibroblasts [lacking GGT (48)], LTC4 was also the major CysLT produced (29). Our results with MM6, MDDCs, and eosinophils support the concept that LTC4 is the predominating CysLT formed in short-term (5–15 min) incubations of these cells. At the same time, LTD4 was formed in long-term incubations of MM6 cells, in agreement with these cells (Fig. 7), as human macrophages (19), expressing GGT5. However, in previous experiments with lung tissue, induced formation of CysLT including LTD4 was quick (10–15 min) [see (49)], suggesting involvement of GGT1. Thus, it appears that GGT1 can be expressed in normal human lung; candidates are Clara cells and alveolar type II epithelium, which were previously found to express GGT in rat lung (11, 12). The A549 cell line has alveolar type II-like characteristics (50). However, human PBECs, which differ from alveolar epithelium in several respects (36, 50), did not express GGT1 (Fig. 7C).

In normal human lung, the CysLT1 receptor was first observed in smooth muscle fibers and in interstitial macrophages (51). In bronchial biopsies from chronic obstructive pulmonary disease (COPD) patients, CysLT1 was expressed in mast cells, monocytes/macrophages, and neutrophils, and CysLT1 expression was associated with exacerbations of COPD (52). CysLT1 mediates bronchoconstriction (4) and, via CysLT1, LTD4 also upregulated expression of TGFβ (in A549 cells and in normal human bronchial epithelial cells) and furin (in monocyteic TH-1 cells), both implicated in airway remodeling (53, 54). Of the three CysLTs, LTD4 is the most efficient CysLT1 agonist, while LTE4 is the weakest (5, 6). Thus, further conversion of LTC4 to LTD4, but not to LTE4 (as shown here by A549 and PBECs), could have a considerable effect on all the effects mediated by CysLT1. However, in a study of human tracheal epithelial cells, conversion of LTD4 to LTE4 was found (43). This shows that CysLT metabolism can be different in epithelial (and other) cells from different anatomical sites. It can most probably also be influenced by other factors, such as cell differentiation states, inflammation, and disease.

LTB4 formation was not increased in coincubations of MM6 and A549 cells when stimulated with A23187 plus AA. In these coincubations, a similar minor amount of LTB4 was produced, as in MM6 cells alone. At the same time, A549 cells incubated with exogenous LTA4 produced substantial amounts of LTB4 (Fig. 2). Thus, monocyctic MM6 cells do not appear to export LTA4, in the manner shown before for neutrophils (55), but MM6 cells did release LTC4 (Table 1). In a previous study where A549 cells were coincubated with PMNL, CysLTs were formed, presumably by transcellular metabolism of LTA4 (56). However, in our incubations of A549 cells with LTA4, CysLT formation was negligible and LTC4S was not detectable in A549 cell extracts by Western blot. This may be attributed to A549 cell line heterogeneity (57).

We found previously that when MM6 cells were differentiated in the presence of zymosan, formation of PGE2 was induced, which in turn downregulated the activity of LTC4S (58). Thus, we hypothesized that, in coincultures (24 h), PGE2 produced by A549 cells might downregulate MM6 cell CysLT formation. The A549 cells had been starved and stimulated with IL-1β for 48 h, which upregulates PGE2 formation (58). However, as evident from the LTC4S incubations, the activity of LTC4S was not decreased. The concentration of PGE2 in medium from these cocultures was 3–5 nM, determined by LC-MS. This may not be sufficient to downregulate LTC4S in MM6 cells, because exogenous PGE2 (100 nM) reduced conversion of LTA4 to LTC4 only by half (58). Previously, treatment of macrophages with apoptotic cells led to downregulation of 5-LO pathways via mechanisms involving TGFβ and PGE2 (59).

MM6 and A549 cells have been combined before in coculture models of pulmonary inflammation; e.g., when cocultures of A549 and MM6 cells were exposed to ultrafine particles, cytokine release (IL-6 and IL-8) was increased in comparison to monocultures of the respective cells [see (50) and references therein]. Also, growth of mycobacteria in MM6 cells was reduced when MM6 were in coculture with A549 cells, which was attributed to A549-derived cytokines (particularly TNFα) (60). Our results on transcellular conversion of LTC4 to LTD4 are in line with an increased inflammatory response due to cell interactions and to interactions between myeloid cells and epithelium-derived exosomes. Exosomes have the potential to travel to distant sites and could possibly contribute to spreading of an inflammatory reaction to other sites in the body.

The coculture of MM6 cells with A549 cells and exosomes may also be considered as a model of cell interactions and transcellular LTD4 biosynthesis in a tumor milieu. LTs and the monohydroxy acid, 5-HETE, can have growth factor-like effects, promoting cancer cell growth and survival. Interestingly, quite different effects of LTC4 and LTD4 were published for intestinal epithelial cells and CaCo-2 cells; LTD4 promoted proliferation, while this was not found for LTC4 (61). This effect of LTD4 involved increased expression of COX-2 and PGE2 (62, 63). Accordingly, in a mouse model of lung cancer, the production of CysLTs and PGE2 increased considerably during tumor growth (24). In addition to GGT influencing cancer cell redox status (8), its role in LTD4 formation can be another tumor promoting effect of GGT. Thus, not only in pulmonary inflammation, but also in cancer, conversion of LTC4 to LTD4 by transcellular metabolism, possibly involving tumor-associated monocytic cells and epithelial cancer cells, may be important for the final LT effects and outcome.

The results suggest that pulmonary epithelium may be a source of LTD4 in the lung. Monocyctic cells (macrophages, dendritic cells), mast cells, and eosinophils are fundamental
players in pulmonary inflammation, with established roles in diseases such as asthma and COPD. These cells are major sources of CysLTs, but mainly LTC₄, as also observed in this study. This leads us to speculate that transcellular metabolism of LTC₄ involving healthy or cancerous pulmonary epithelium may contribute substantially to biosynthesis of LTD₄.

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