Original Articles

Exosomes and cells from lung cancer pleural exudates transform LTC4 to LTD4, promoting cell migration and survival via CysLT1

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ABSTRACT

Tumor-derived exosomes can modulate the cancer microenvironment and induce metastatic spread. Exosomes may carry enzymes for leukotriene (LT) biosynthesis, but the role of exosomal LTs has not been studied in cancer. We isolated exosomes and malignant cells from pleura exudates from 14 patients with non-small cell lung cancer. Lipidomic profiles, migration and apoptosis were determined. Both exosomes and primary cancer cells contained γ-glutamyl transpeptidase 1 (GGT-1) and avidly transformed exogenous LTC_4 to pro-tumorigenic LTD_4, for the cells to levels 100-fold above their endogenous CysLT production. This suggests that cancer cells promote their own survival via LTD_4 if supplied with LTC_4, which in the exudates was produced by monocytic cells. Furthermore, exosomes promoted migration of cancer cells, which was counteracted by the CysLT1 antagonist montelukast. Montelukast also induced apoptosis of cancer cells, and this was partially inhibited by exosomes. Our results demonstrate how cancer cells and exosomes, together with monocytic cells in lung cancer tissue, can produce high amounts of LTD_4, to stimulate cancer cell migration and survival. This suggests that part of the pro-metastatic effect of exosomes is mediated by the leukotriene machinery, further supporting the use of CysLT1 antagonists for lung cancer therapy.

1. Introduction

Persistent inflammation in the lungs is linked to higher risk for lung cancer; thus, mitigation of inflammation may be a preventive or therapeutic approach [1,2]. Leukotriene (LT) C_4 and its metabolites LTD_4 and LTE_4 (together referred to as Cysteinyl LTs, CysLTs) are inflammatory mediators derived from arachidonic acid in the 5-lipoxygenase (5-LO) pathway. They exert many of their functions through the CysLT1 receptor, which is expressed in pulmonary smooth muscle and interstitial macrophages. CysLTs contribute to cancer progression [3] and several observations support a pro-tumorigenic effect of LTD_4 via CysLT1 in colorectal cancer [4]. Montelukast is a CysLT1 receptor antagonist already used in asthma treatment. Interestingly, asthma patients treated with montelukast have a considerably lower risk to develop cancer [5]. In animal studies, montelukast increased survival

rates in a spontaneous metastasis model of Lewis lung carcinoma (LLC) [6] and delayed tumor growth [7]. Abrogation of ALOX5, the gene encoding 5-LO, reduced pro-tumorigenic effects of neutrophils in lung metastasis in a mouse breast cancer model, an effect sustained by LTB_4 and CysLTs [8]. On the other hand, deletion of 5-LO could also promote LLC primary tumor and metastasis [9] probably reflecting different effects of distinct macrophage populations in different stages of cancer progression [10].

Cancer cells promote low-grade inflammatory conditions in the surrounding milieu to support tumorigenesis [1]. The tumor microenvironment is influenced also *via* exosomes, nanovesicles which can be released by all cell types including cancer cells and immune cells, and carry RNA, proteins and other signaling molecules [11]. For example, myeloid derived suppressor cells release pro-angiogenic exosomes supporting lung metastasis [12]. Also, tumor derived exosomes could

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Common abbreviations list

5-LO	5-lipoxygenase
CysLT	cysteinyl leukotriene
CysLT1	cysteinyl leukotriene receptor 1
GGT	gamma glutamyl-transpeptidase
HETE	hydrohyeicosatetraenoic acid
LT	leukotriene
PG	prostaglandin

prepare the pre-metastatic niche via MET [13] and were found to activate TLR3 in alveolar epithelium to recruit neutrophils and promote lung metastasis [14].

We recently demonstrated that LTC_4 produced by monocytic cells was efficiently transformed to LTD_4 by γ-glutamyl transpeptidase-1 (GGT-1) in the lung cancer cell line A549 and their exosomes, suggesting that cancer cell-derived exosomes contribute to inflammation by amplifying the biosynthesis of this most potent CysLT1 agonist [15]. In this study, we extended these investigations to authentic exosomes and primary cells from cancer patients. Exosomes and cells were prepared from pleural exudates of 14 patients with lung cancer. We demonstrate that both cells and exosomes avidly transform LTC_4 to LTD_4, and furthermore that the pleural exudate derived exosomes promote migration and survival of primary cancer cells '*ex vivo*'. This highlights a role for exosomes in supporting tumor growth via leukotrienes, but also opens for investigations on reducing tumor burden by interfering with exosomes and/or leukotriene pathways.

2. Materials and methods

For detailed methods, see supplement.

2.1. Study subjects

Pleural exudates from 14 patients with stage IV non-small cell lung cancer were collected at the Thorax clinic, Karolinska Hospital. For patient data see Table 1 and Supplementary Table 1. All patients provided written consent, in agreement with ethical permit 2015/1584-31/4. Pleural exudates were kept at room temperature and processed within 1 h.

2.2. Exosome isolation and characterization

Pleural exudates were centrifuged at $300g \times 10$ min to pellet cells, again at $3000g \times 30$ min to remove cell debris, and stored at $-80\,°C$.

Pleural exudates were thawed overnight and exosomes isolated by differential centrifugation and filtration steps (Fig. S1A). For some experiments, exosomes were further purified by sucrose gradient centrifugation as described [16]. Exosome proteins were quantified by Bradford assay (Bio-Rad protein reagent). Exosomes were characterized by electron microscopy and nanoparticle tracking analysis.

2.3. Isolation and culture of malignant cells and monocytic cells from pleural exudates

Malignant cells were isolated from pleural exudates, principally as described [17]. The pleural exudate was centrifuged at $300g \times 10$ min to pellet cells. Cells were seeded in Falcon T25 flasks and grown at 37 °C and 5% CO_2 in RPMI 1640 + 10% FBS + penicillin (10U/ml) and streptomycin (100 μg/ml. Medium was replaced at least once a week. Within 3 weeks contaminating leukocytes died or were washed away, only malignant cells remained in cell culture as verified by microscopy. A549 cells were cultured in the same complete medium. Cells were split regularly before confluency, normally twice a week. Monocytes and macrophages in pleural exudate were isolated as described [18]. After Ficoll centrifugation cells in the mononuclear layer which adhered within 2 h were collected.

2.4. SDS-PAGE and western blotting

Cell lysates were prepared as described and mixed with 4x loading buffer prior to SDS-PAGE [15]. Exosomes were mixed directly with loading buffer and vortexed thoroughly 3×1 min. Approx. 40 μg of cellular protein and 10 μg of exosomal protein were loaded on Bio-Rad 4–20% ready-made gels and run on SDS-PAGE. After transfer, nitrocellulose membranes (Amersham) were incubated with antibodies: mouse anti-human CD81 (1:500 dilution, non-reducing conditions, BD Pharmingen), and GGT1 (1:500 dilution, Santa Cruz); rabbit anti-human CD44 (1:400, Santa Cruz), EGFR (1:500, Santa Cruz), Vimentin (1:400, Santa Cruz), and CysLT1 (1:500, Cayman). A peroxidase-conjugated primary antibody against β-actin (1:2000 dilution) and peroxidase-conjugated secondary antibodies (1:2000 to 4000 dilution) were from Sigma. Protein bands were detected by enhanced chemiluminescence with a Li-Cor scanner (Lincoln, NE, USA).

2.5. Migration assay

Pleural exudates-derived cancer cells from patient 12 or A549 cells (1×10^5 cells) were seeded in 24-well plates and let to adhere overnight. Next day cells were washed and starved for 16–18 h in 1% FBS medium. At this time confluence was reached, and cells were scratched

Table 1
List of patients. All patients had stage IV lung cancer, according to the staging classification for non-small cell lung cancer (NSCLC) from the International Association for the study of Lung Cancer. Malignant cells were isolated as described in methods, and cell lines were considered as established when cells survived at least one freeze/thaw cycle. The growth rate was described as fast when doubling time was between 1 and 3 days; slow when 5–10 days.

patient	gender	born	cancer form	stage	Established cell culture (growth rate)
P1	M	1946	adenocarcinoma	IV	No (no growth)
P2	F	1939	adenocarcinoma	IV	No (no growth)
P3	F	1960	Squamous cell carcinoma	IV	Yes (slow)
P4	F	1971	adenocarcinoma	IV	Yes (fast)
P5	M	1937	adenocarcinoma	IV	Yes (slow)
P6	F	1941	adenocarcinoma	IV	No (fungal contamination)
P7a/b	M	1980	adenocarcinoma	IV	No (fungal contamination)
P8	F	1936	adenocarcinoma	IV	No (fungal contamination)
P9	M	1946	adenocarcinoma	IV	No (fungal contamination)
P10	F	1935	adenocarcinoma	IV	No (fungal contamination)
P11	F	1936	adenocarcinoma	IV	No (no growth)
P12	M	1924	form of lung cancer uncertain	IV	Yes (fast)
P13	M	1939	adenocarcinoma	IV	Yes (slow)
P14	M	1953	adenocarcinoma	IV	Yes (fast)

with a 1 ml tip in the center of each well, washed with PBS x 3, and complete medium was added. Cells were stimulated with 7 different pleural exudate-exosome preparations (50 µg/ml), with montelukast (1 µM), or with exosomes plus montelukast. Pictures were taken directly after exosome stimulation, and at 24/48 h, with a Nikon TMS inverted microscope (10x magnification) and a Canon EOS camera. Cell-free area was determined by ImageJ software, results (% healing) means reduction of cell-free area relative to the corresponding control.

2.6. Cell viability and apoptosis assay

Cell viability was determined by MTT assay [19]. Annexin V/Propidium iodide assay was performed according to the manufacturer's instruction (Biolegend), using a LSRFortessa flow cytometer (Becton Dickinson). Data were analyzed in FlowJo (Treestar Inc).

2.7. Cell preparation and incubation for lipidomic analysis by LC-MS/MS

Cancer cells from four patients (0.6 to 1×10^6 cells) were seeded in six-well plates to adhere overnight. Next day cells were washed and starved for 16–18 h in 1% FBS medium, washed with PBS x 3, and re-suspended in complete medium. Cells from each patient were treated with two different pleural exudate-exosome preparations (50 µg/ml), diluted in RPMI. Control cells received plain RPMI. After 24 h, culture medium was collected, and analyzed for lipid mediators released from resting cells. Adherent cells were then covered with 2 ml PGC buffer and stimulated with arachidonic acid (40 µM) plus ionophore A23187 (5 µM) for 30 min. The incubations were stopped by ice-cold MeOH and stored at −20 °C overnight. Lipids were extracted and analyzed by LC-MS/MS as described [20,21,37].

2.8. Exosome and cell incubations, HPLC-UV analysis

Exosomes (corresponding to approximately 100 µg of exosomal proteins) in 0.1 ml PBS was incubated with 175 pmol LTC_4 (Cayman Chemicals). This concentration of LTC_4 (1.75 µM), which may be at the high end of possible pathophysiological conditions, gives saturating conditions. In the ensuing HPLC-UV analysis both LTD_4 and remaining substrate (LTC_4) were robustly detected. After 30 min reactions were stopped with MeOH containing internal standard, 100 pmol of PGB_2 (kind gift from Mats Hamberg, KI), and stored at −20 °C for 1 h to precipitate proteins, centrifuged at 10,000g for 15 min, and an aliquot of the supernatant was analyzed by HPLC. Pleural exudate-derived cancer cells, 1–3 x10^6 in 0.5 ml PBS, were incubated with 175 pmol LTC_4 for 30 min. Pleural exudate-derived macrophages (1.5 to 3×10^6 cells) were incubated in 2 ml PGC buffer and stimulated with A23187 (2.5 µM) for 10 min. Cell incubations were extracted and analyzed by HPLC [15].

2.9. Statistics

Groups were analyzed with GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA), by paired Student's *t*-test and 1-way ANOVA, Bonferroni or Dunn correction.

3. Results

3.1. Malignant pleural exudates contain exosomes and primary cancer cells

Vesicles isolated from pleural exudates from 14 lung cancer patients showed exosome characteristics; cup-shape morphology in transmission electron microscope imaging (Fig. 1A), expression of the exosomal marker CD81 (Fig. 1B), and a size distribution of 50–150 nm (Fig. 1C). The average yield of exosomal proteins was 9.6 µg/ml of pleural exudate (Fig. S1B). Primary lung cancer cells were obtained from 12 patients, and 6 of these could be established as cell cultures (Table 1). The cell morphologies resembled malignant epithelial cells rather than fibroblasts (Fig. 2A). Western blot demonstrated markers of malignancy including EGFR [22]; CD44, a stem cell marker present in cancer cells [23]; vimentin, highly expressed by invasive cancer cells [24]; and mPGES-1 [25], supporting a malignant phenotype. We also detected the leukotriene receptor CysLT1 in cancer cells from all patients (Fig. 2B).
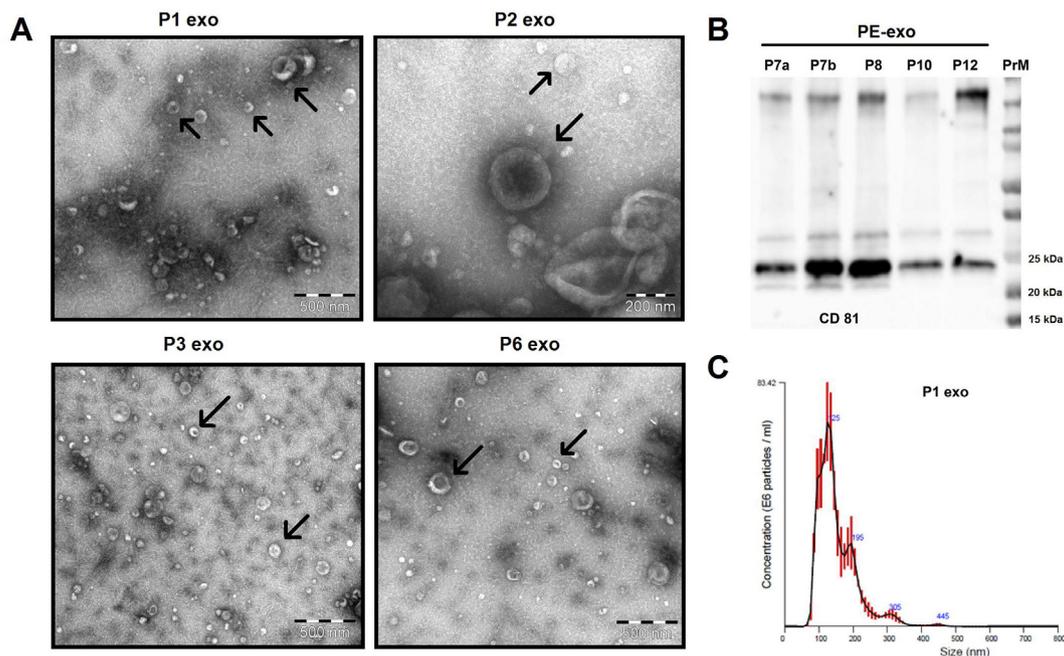
Fig. 1. Pleural exudates from lung cancer patients contain exosomes. We isolated exosomes by ultracentrifugation from pleural exudates of 14 patients with lung cancer. (A) Pleural exudate exosomes (indicated by arrows) possess the typical cup-shape, as verified by electron microscopy (n = 4). (B) 10 µg of exosomal proteins from 5 preparations were analyzed by Western blot for the tetraspanin CD81, an exosomal marker. Similar results were observed for all exosome preparations. (C) Pleural exudate exosomes were analyzed by nanoparticle tracking analysis to characterize the size distribution. Similar results were obtained for 4 other exosome preparations. The number associated with samples corresponds to the number of the patient. Exo = exosomes; P = patient; PE = pleural exudate.
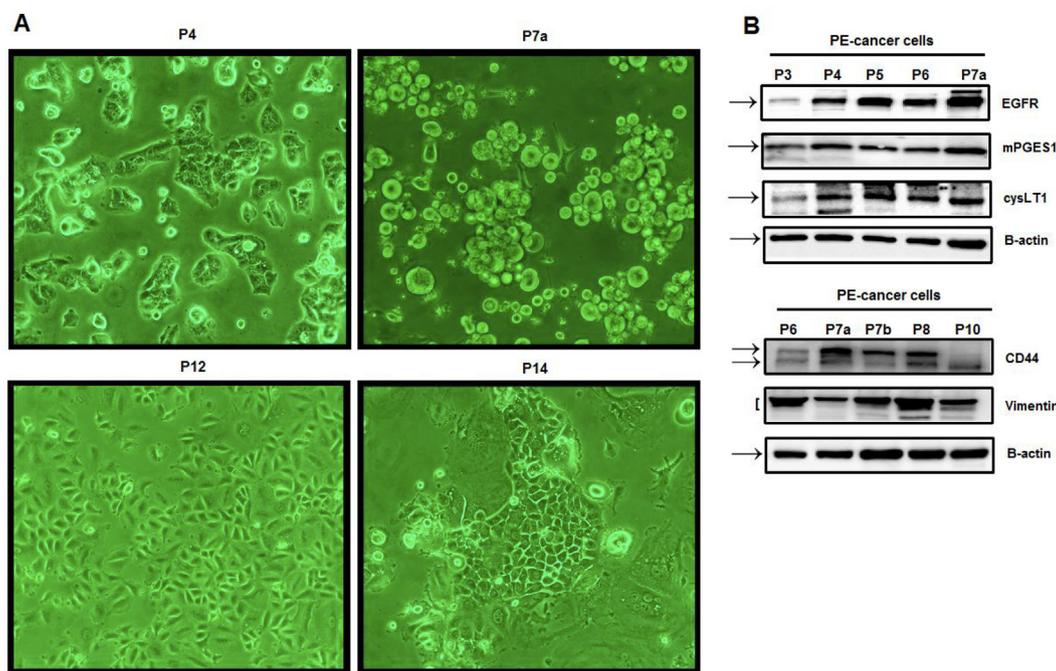
Fig. 2. Cancer cells from pleural exudates. We isolated cancer cells from pleural exudates for 12 lung cancer patients. (A) Light microscopy pictures (10x magnification), 4 representative donors are shown. (B) 40 μg of cancer cell lysates were analyzed by Western blot for EGFR, mPGES1, CysLT1, CD44 and vimentin. Similar results were obtained for cells from all patients. The number associated with samples corresponds to the number of the patient. P = patient; PE = pleural exudate.

3.2. Lipid mediators in pleural exudates

Lipid mediators in the original pleural exudates used to isolate the primary cancer cells and exosomes were analyzed by LC-MS/MS (Table S2). Pleural exudates, depleted of cells, contained similar amounts of CysLTs (65 pg/ml) and PGE_2 (22 pg/ml) (Fig. 3A, Table S2). LTE_4 was the dominating CysLT in pleural exudates. In addition, pleural exudates contained multiple lipid mediators from different polyunsaturated fatty acids, linoleic acid metabolites were the most abundant in pleural exudates, with 13-HODE showing the highest concentration (Table S2). High levels were also noted for the CYP products 17,18-DiHETE, 20-HETE and 19-HETE, and for the lipoxygenase product 12-HETE.

3.3. Lipid mediators produced by cancer cells

We also profiled lipid mediators in the cultured primary cancer cells (culture medium of cells in rest, as well as after incubations of cells with exogenous arachidonic acid and A23187 to stimulate eicosanoid formation) (Table S2).

Amongst the lipid mediators investigated, prostaglandins dominated: resting cells released mainly PGE_2, ~ 37 ng/10^6 (Fig. 3B) while the stimulated cells formed ~ 53 ng/10^6 cells of PGE_2 (Fig. 3C). The cancer cells could also produce CysLTs, but considerably less compared to prostaglandins: in resting conditions (cell culture medium) we

detected ~ 8 pg/10^6 cells of LTD_4 but no LTC_4. In the arachidonic acid and A23187 incubations, 57 pg/10^6 cells of LTD_4 and ~ 6 pg/10^6 cells LTC_4 was formed, suggesting that cancer cells efficiently convert LTC_4 to LTD_4.

To investigate possible effects of exosomes on eicosanoid formation, primary cancer cells from four patients were treated with two different pleural exudate exosome preparations each for 24 h, before collection of medium and stimulation of cells with arachidonic acid and A23187. Exosomes increased the formation of CysLTs, the mean absolute values for LTD_4 increased 3-fold in medium and 2-fold in incubations with arachidonic acid and A23187.(Fig. 4A). Treatment with exosomes increased formation of the resolvin precursor 17-HDoHE in the incubations, while CYP-derived 20-HETE and COX-derived TxB_2 and PGD_2 were reduced (Table S2). The pleural exudate-exosomes alone did not contain CysLTs or other eicosanoids (data not shown). These results show that pleural exudate contain exosomes with the capacity to modulate several eicosanoid metabolic pathways.

3.4. Pleural exudate-derived exosomes and malignant cells contain active GGT-1, with high capacity to transform exogenous LTC_4 to LTD_4

As exosomes induced an increase in LTD_4 formation, we investigated whether pleural exudate-derived exosomes contained the enzyme GGT-1. Indeed, they showed the presence of GGT-1 (Fig. 5A),
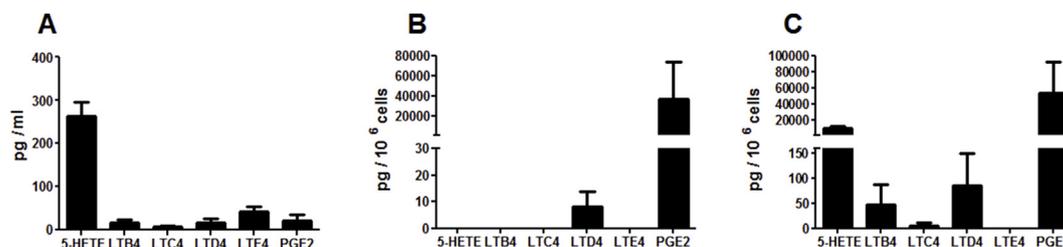
Fig. 3. 5-LO-products and PGE_2, data from Table S2. (A) 5-LO metabolites and PGE_2 in pleural exudates. (B) 5-LO products and PGE_2 released from resting cells. (C) Formation of 5-LO products and PGE_2 in cells stimulated with arachidonic acid + A23187. Results are shown as mean + SE (n = 4).
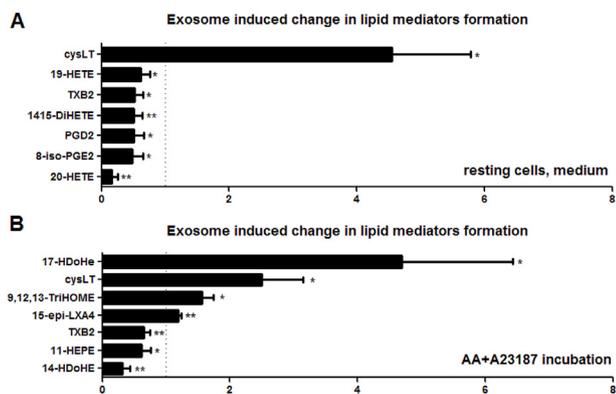
Fig. 4. Exosomes modulate formation of specific lipid mediators in cancer cells. After starving O/N, cancer cells from 4 patients were treated with two distinct exosome preparations each (50 μg exosomal protein/ml) or vehicle for 24 h. Formation of lipid mediators was analyzed by LC-MS/MS. Due to donor variability, the values obtained after exosome treatment were normalized for the values obtained after vehicle treatment and differences are shown as fold-change. (A) Lipid mediators changed by exosome treatment, in cell culture medium of resting cells. (B) Lipid mediators changed by exosome treatment, in cells incubated for 30 min with arachidonic acid (40 μM) plus Ca^{2+} ionophore A23187 (5 μM). Results are shown as fold change (mean + SE) compared to vehicle control, (n = 8). *P* values < 0.05 were considered significant. *P < 0.05, **P < 0.01.

and when exosomes were incubated with exogenous LTC_4, considerable amounts of LTD_4 were produced (Fig. 5B), suggesting a role for exosomes in favoring protumorigenic LTD_4 formation. Density gradient isolations confirmed that GGT-1 was indeed bound to the exosomal fraction and not just co-pelleted with exosomes during isolation (Fig. 5C).

Similarly, western blot analyses showed that nine of twelve primary cancer cell cultures expressed GGT-1 (Fig. 5D, Table S1). To test the effective enzymatic capacity of GGT-1, primary cancer cells were

incubated for 30 min with LTC_4, resulting in a rapid formation of LTD_4 (28 ± 5 ng/10^6 cells, Fig. 4E). This is almost 500-fold more compared to LTD_4 formation from LTC_4, produced within the cancer cells themselves (in response to Ca^{2+} ionophore plus exogenous arachidonic acid (Fig. 3, Table S2). This suggests that when LTC_4 can be provided to the cancer cells from another source this metabolic capacity can be highly exploited to favor tumorigenesis. Notably, also the exosomes from cultured cells of patients 1 and 3 contained GGT-1, although this enzyme was not detectable in cancer cells derived from these patients.

### 3.5. LT formation in monocytic cells from pleural exudates

In search for cell sources of LTC_4, we isolated monocytes/macrophages from six pleural exudates. The cells were incubated for 10 min with A23187, and extracts were analyzed by HPLC-UV. In these monocyte cultures, leukotrienes were formed from endogenous arachidonic acid (~ 35 ng/10^6 of LTC_4, 3.3 ng/10^6 of LTD_4 and 19.5 ng/10^6 of LTB_4, Fig. 6F). Thus, the monocytic cells had a considerably higher leukotriene biosynthesis capacity compared to the cancer cells, which strongly suggests monocytes to be the major source of LTC_4 in the pleural exudates.

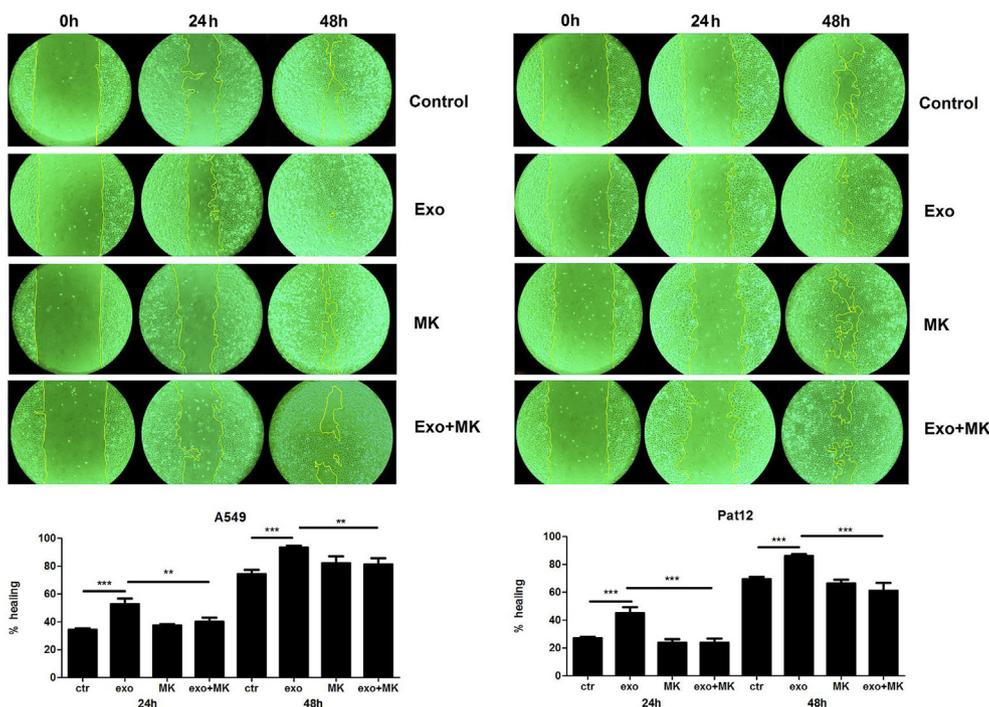### 3.6. Exosomes promote migration of cancer cells via CysLT1

Migratory capacity was tested in a wound healing assay. A549 cells and cancer cells from patient 12 (P12 cells) were stimulated with 7 different pleural exudate-derived exosome preparations, in presence or absence of 1 μM montelukast. Exosome treatment increased migration of both A549 and P12 cells, at 24 h and to a lesser extent at 48 h (Fig. 6). Montelukast almost abolished this effect, suggesting that CysLTs are involved in exosome-promoted migration of cancer cells.

3.7. CysLT1 antagonist montelukast promotes cancer cell death, an effect rescued by exosomes

Primary lung cancer cells were subjected to montelukast for 48 h,
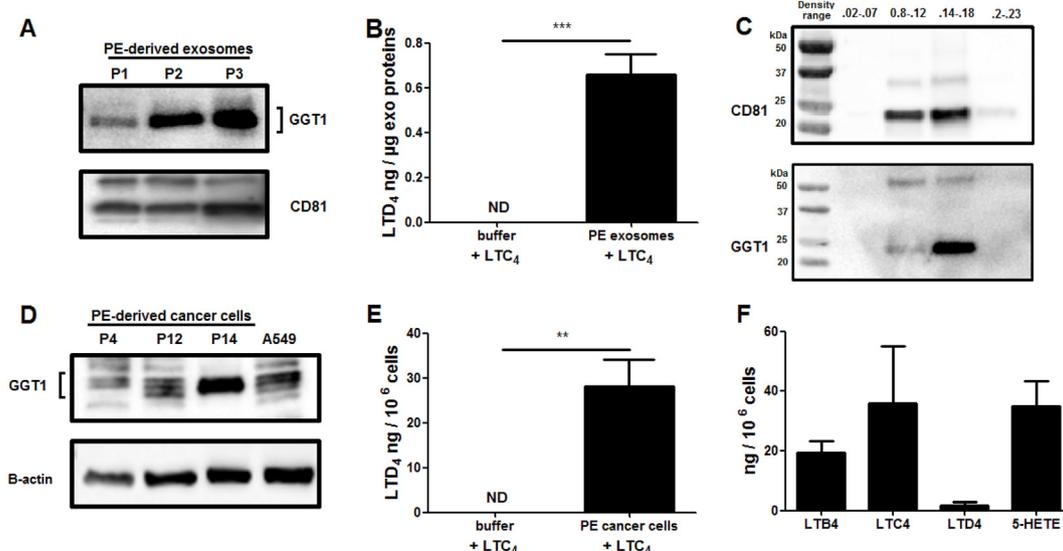
Fig. 5. Pleural exudate exosomes and cancer cells possess GGT-1 and convert LTC_4 to LTD_4. (A) 10 μg of exosomal proteins were analyzed by Western blot for GGT-1 and CD81. Similar results were obtained for all exosome preparations. (B) Exosomes (corresponding to 100 μg exosome proteins) were incubated with 175 pmol of LTC_4 for 30 min and LTD_4 formation was analyzed by HPLC. Results are shown as mean + SE (n = 11), ND = not detected. (C) One exosome preparation was further purified by sucrose gradient centrifugation, and fractions were analyzed by Western blot for CD81 and GGT-1. Similar results were obtained for another exosome preparation. (D) 40 μg of cancer cell lysates were analyzed by Western blot for GGT-1. A549 cell lysate was used as positive control. Similar results for GGT-1 were obtained for cells from 9 of the 12 patients. (E) Pleural exudate cancer cells ($1–3x10^6$) were incubated with 175 pmol of LTC_4 for 30 min and LTD_4 formation was analyzed by HPLC. Results are shown as mean + SE (n = 8), ND = not detected. (F) Formation of 5-LO products in pleural exudate monocytic cells in 10 min incubations with Ca^{2+} ionophore A23187 (2.5 μM). Results are shown as mean + SE (n = 6). P = patient; PE = pleural exudate. *P* values < 0.05 were considered significant. **P < 0.01, ***P < 0.001.

Fig. 6. Pleural exudate exosomes promote migration of cancer cells. Migration assay was performed for cells from patient 12 and A549 cells, in two independent experiments. Left, A549 cells, right, P12 cells. Both cell types were stimulated with vehicle (control), 7 different exosome preparations (50 µg exosomal protein/ml), montelukast 1 µM, or exosomes + montelukast. The cell-free area was determined by ImageJ. Results (% healing) means reduction of cell-free area relative to the corresponding control. Results are shown as mean + SE, (n = 7 exosome donors). Exo = exosomes; MK = montelukast. P values < 0.05 were considered significant. **P < 0.01, ***P < 0.001.

with a dose-dependent reduction of cell viability (Fig. 7A). Notably, 1 µM montelukast (used in scratch assay experiments) had no effect on viability. As found by trypan blue exclusion, montelukast from 5 µM induced cell death (Fig. 7B). Annexin V/PI staining indicated mainly early apoptosis (Fig. 7C and D). Montelukast also enhanced the cytotoxic effect of Adriamycin and Cisplatin (Fig. S2). Interestingly, when we combined exosomes and montelukast, we observed that exosomes reduced cell death, thus partially protecting the cancer cells from
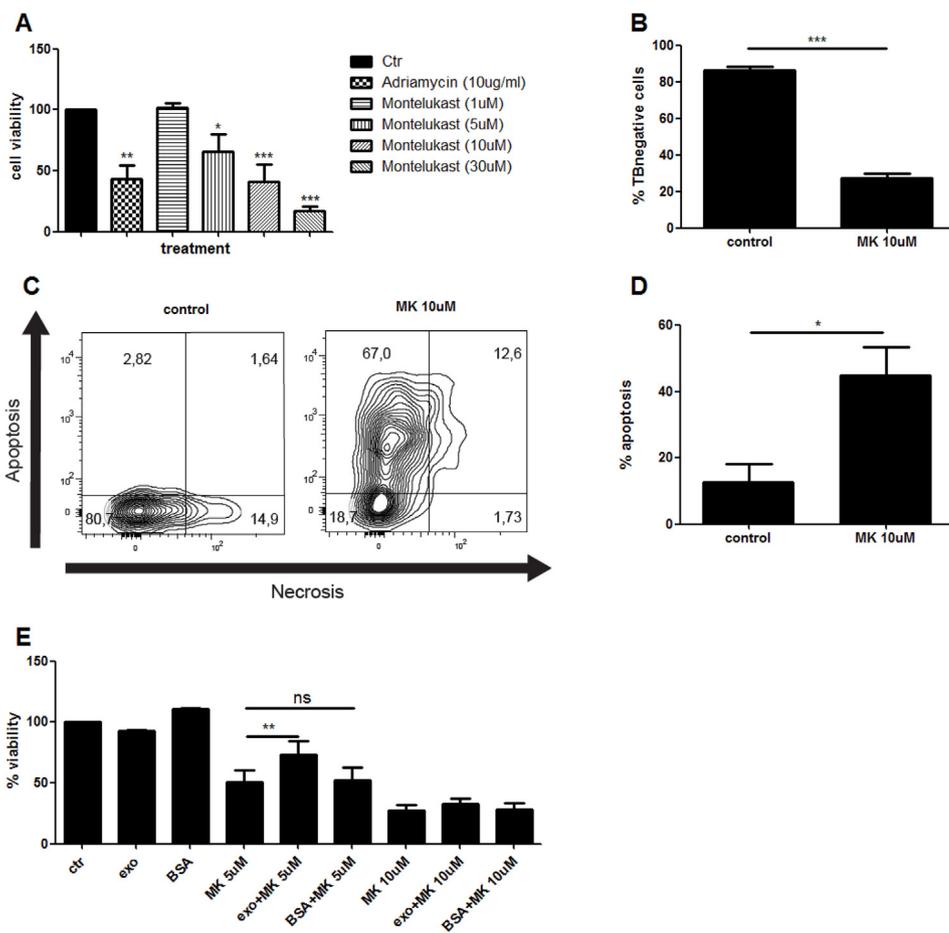
Fig. 7. Montelukast promotes apoptosis of cancer cells. Cells were treated with montelukast for 48 h. (A) Effect on viable cancer cell mass, MTT assay (n = 8). (B) Effect of montelukast (10 µM) on cancer cell viability measured by Trypan blue exclusion (n = 5). (C–D) Effect of montelukast (10 µM) on Annexin V/PI staining, indicating apoptosis. Results are shown as mean + SE, (n = 6). (E) Cancer cells from patient 12 were treated as indicated for 24 h, in triplicates. Exosomes were used at 50 µg exosomal protein/ml. BSA was used as control at 50 µg/ml. Cell viability was assessed by MTT assay. Results are shown as mean + SE, (n = 3 exosome donors). Exo = exosomes; MK = montelukast. P values < 0.05 were considered significant. *P < 0.05, **P < 0.01, ***P < 0.001.

apoptosis (Fig. 4E). We also tested the LTC_4 synthase inhibitor TK05 [26], which reduced cell viability in a dose dependent manner similar to that of montelukast (Fig. S3), which further underlines the role of cysLTs in lung cancer cell survival.

4. Discussion

The pleural cavity is a metastatic niche in lung cancer [27], and a fundamental step in metastasis is invasion of malignant cells. Exosomes promote migration of granulocytes [16] and chemotaxis of cancer cells [28]. Here we demonstrate a new mechanism for exosome mediated cancer cell migration. We show that exosomes prepared from lung cancer pleural exudates promoted migration of both A549 and primary cancer cells (Fig. 6), via cysLTs, as montelukast abolished this migration. Importantly, MTT assay revealed no increase in proliferation by exosomes, thus the effects seen in the scratch assay were due to migration and not to proliferation, specifically LTD_4, formed effectively via GGT-1.

In the pleural exudates, which reflect the *in vivo* conditions, the concentrations of CysLTs and PGE_2 were quite similar. However, when cancer cells were cultured for 24 h, both analyses of cell culture medium and cell incubations with Ca^{2+} ionophore showed low levels of CysLTs. In contrast, the predominating eicosanoid formed by cancer cells in culture was PGE_2. In view of our previous findings with A549 cells and their exosomes [15], we then incubated the cancer cells with exogenous LTC_4, to determine possible high capacity for LTD_4 biosynthesis. Indeed, in these incubations with LTC_4, the formation of pro-tumorigenic LTD_4 was in the same range as for the PG biosynthesis capacity. Thus, it appears possible that in the cancer tissue, formation of LTD_4 and PGE_2 could be of similar magnitudes, if another cell type provides sufficient amounts of LTC_4, for transcellular metabolism to LTD_4. Macrophages are abundant in malignant pleural exudates [18], particularly M2-primed macrophages have high capacity to produce LTC_4 [21]. We isolated monocytic cells (including macrophages) from six pleural exudates. When these were incubated with ionophore A23187, high levels of LTC_4 were formed, suggesting that monocytic cells may deliver large amounts of LTC_4 to cancer cells for further conversion to pro-tumorigenic LTD_4. Other cells producing LTC_4 which may be present in cancer tissue are mast cells and eosinophils [29]. Furthermore, pleural exudate exosomes contained the enzyme (GGT-1) converting LTC_4 to LTD_4, and their capacity for this conversion was in the same range as found before for other leukotrienes biosynthesis steps [16]. Thus, formation of LTD_4 in lung cancer tissue could be substantial.

In the *in vitro* assays of migration and survival of isolated cancer cells, the low levels of LTD_4 produced by the cancer cells alone were obviously sufficient for the observed migratory effects which were sensitive to montelukast. When the cancer cells were treated with pleural exudates-exosomes, CysLTs (particularly LTD_4) in cancer cell culture medium increased about 4-fold, indicating that the exosome effect on migration and survival was related to LTD_4 formation. This is also in agreement with our previous finding that BAL-derived exosomes of asthma patients increased CysLT formation in bronchial epithelial cells [30]. Within a tumor tissue containing multiple cell types which could metabolize and degrade LTs, a higher transcellular formation of LTD_4 should increase the potential of LTD_4-mediated effects on *in vivo* cancer cell motility and survival.

LTD_4 is the most potent agonist of CysLT1, the receptor antagonized by montelukast. CysLT1 expression is increased in several malignancies, including prostate, colon and breast cancer [4,31]. Here cancer cells from 12 patients expressed CysLT1 (Fig. 2), and died in the presence of montelukast (IC_{50} ~6 μM) mainly by apoptosis, in agreement with previous studies on prostate cancer [31], neuroblastoma [32], chronic myeloid leukemia cell lines [19] and lung cancer cell lines [7]. Ample evidence supports a pro-tumorigenic effect of LTD_4 but not LTC_4 in colorectal cancer, see Ref. [4] for review. Thus, conversion of LTC_4 to LTD_4 should be important, for the effect of CysLTs in cancer

progression. GGT-1 efficiently catalyzes this conversion, and over-expression of GGT-1 has been linked with several cancer types, including colorectal and lung cancer [33,34]. LTD_4 formation can also be catalyzed by GGT-5, which however is slower acting [15,35], so the GGT-1 in the exosomes is likely to contribute to rapid LTD_4 formation. In line with this, serum exosomes from patients with malignant prostate cancer possess higher GGT-1 activity compared to exosomes from patients with benign forms [36]. We here show here that exosomes and cells from stage IV lung cancer pleural exudate contained biologically relevant amounts of GGT-1, and promptly formed LTD_4 in incubations with LTC_4 (Fig. 5) underlining a potential role for exosomes in leukotriene-mediated cancer progression. This is further supported by our previous finding that exosomes from the non-small cell lung cancer cell line A549 catalyze LTD_4 formation [15].

In cancer, interactions between the transformed cancer cells and other cell types recruited to the tumor are important. Tumor associated macrophages provide cancer cells with a suitable low-grade inflammatory milieu including growth promoting factors. Our results suggest a novel pro-tumorigenic mechanism based on cell interaction: tumor associated macrophaghes provide LTC_4 which lung cancer cells and their exosomes convert to LTD_4. Via CysLT1 this promotes survival and migration of the cancer cells. This mode of formation of LTD_4 may occur also in other pulmonary inflammatory conditions, and for other cancer forms. However, we did not obtain pleural exudates from other inflammatory diseases, and healthy controls are technically and ethically unavailable. In any case, the high presence of exosomes in pleural exudates and their strong eicosanoid-mediated activity on cancer cells strongly suggest their relevance in cancer progression and metastasis.

Finally, both animal models and human studies indicate that targeting CysLT1 with the well tolerated drug montelukast, alone or combined with chemotherapeutic agents, has anti-tumor effects. Our results suggest that in lung cancer tissue, a substantial part of the LTD_4 formation may proceed via transcellular routes, depending on GGT-1 in cancer cells and their exosomes, leading to increased cancer cell migration and survival. We therefore propose further studies on using montelukast as a treatment to reduce the risk of lung cancer metastasis.

Disclosure of potential conflict of interest

S. Gabrielsson is part of the scientific advisory board of Anjarium Biosciences and has a patent (PCT/EP2010/003946) issued. The other authors declare no relevant conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.033.

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