Original article

Exosomes from human macrophages and dendritic cells contain enzymes for leukotriene biosynthesis and promote granulocyte migration

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Background: Leukotrienes (LTs) are potent proinflammatory lipid mediators with key roles in the pathogenesis of asthma and inflammation. Recently, nanovesicles (exosomes), released from macrophages and dendritic cells (DCs), have become increasingly appreciated as messengers in immunity.
Objective: We investigated whether exosomes from human macrophages, DCs, and plasma contain enzymes for LT biosynthesis and studied potential roles for exosomes in transcellular LT metabolism and granulocyte chemotaxis.
Methods: The presence of LT pathway enzymes and LT biosynthesis in exosomes and cells was analyzed by Western blot, immunoelectron microscopy, and enzyme activity assays. Surface marker expression was evaluated by flow cytometry, and granulocyte migration was assessed in a multiwell chemotaxis system.
Results: Exosomes from macrophages and DCs contain functional enzymes for LT biosynthesis. After incubation of intact cells with the LT biosynthesis intermediate LTA_4, LTB_4 was the major product of macrophages, whereas DCs primarily formed LTC_4. However, in exosomes from both cell types, LTC_4 was the predominant LTA_4 metabolite. Exosomal LTC_4 formation (per milligram protein) exceeded that of cells. In macrophages and DCs, TGF-β1 upregulated LTA_4 hydrolase along with increased LTB_4 formation also in the exosomes. Moreover, TGF-β1 modified the expression of surface marker proteins on cells and exosomes and reduced the exosome yield from macrophages. On Ca^{2+}-ionophore and arachidonic acid stimulation, exosomes produced chemotactic eicosanoids and induced granulocyte migration. Interestingly, active LTA_4 hydrolase and LTC_4 synthase were present also in exosomes from human plasma.
Conclusion: Our findings indicate that exosomes can contribute to inflammation by participation in LT biosynthesis and granulocyte recruitment. (J Allergy Clin Immunol 2010;■■■:■■■-■■■.)

Key words: *Antigen-presenting cells, eicosanoids, exosomes, inflammation, leukotrienes, TGF-β1*

Leukotrienes (LTs) are proinflammatory lipid mediators derived from arachidonic acid (AA) with roles in normal host defense and inflammatory disease.[1] LTB_4 elicits neutrophil chemotaxis and bacterial killing, whereas the cysteinyl LTs (cysLTs: LTC_4, LTD_4, and LTE_4) increase vascular permeability, airway mucus secretion, smooth muscle constriction, and eosinophil migration. Major sources of LTB_4 and cysLTs are various types of leukocytes.[2] During the first steps of LT biosynthesis, AA is liberated from the nuclear membrane by cytosolic phospholipase A_2 and oxygenated by 5-lipoxygenase (5-LO), resulting in formation of the instable intermediate LTA_4. LTA_4 serves as the substrate for LTA_4 hydrolase (LTA_4H) and LTC_4 synthase (LTC_4S), catalyzing its conversion into LTB_4 or LTC_4, respectively.[3] LTs play key roles in the pathogenesis of asthma, allergy, and chronic inflammation, and cysLT receptor antagonists are used in the treatment of allergic rhinitis and asthma.[2]

Cells of the monocyte lineage are both target cells and producers of eicosanoids.[4,5] Monocyte-derived dendritic cells (MDDCs) and monocyte-derived macrophages (MDMs) are commonly used *in vitro* models for dendritic cells (DCs) and macrophages. MDDCs and MDMs are generated from $CD14^+$ monocytes by culture in the presence of IL-4 and GM-CSF, or only GM-CSF, respectively.[6] A DC subset with high capacity for LT production is Langerhans cells (LCs) in the skin.[7] The pleiotropic cytokine TGF-β1 is crucial for the appearance of LCs in mice,[8] and TGF-β1 upregulated LT formation in DCs derived from human precursor cells.[9] In addition, TGF-β1, implicated in allergic inflammation and airway remodelling,[10,11] modified LC maturation in response to inflammatory stimuli.[12]

Recent studies have shown that DCs and macrophages secrete nanosized membrane vesicles (exosomes) with immunologic functions. Such vesicles, ranging in size from 40 to 100 nm, are released by various cell types as means of intercellular communication, and they are present in various biological fluids.[13] Generation of exosomes occurs either by inward budding of the late

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Abbreviations used

AA:	Arachidonic acid
APC:	Antigen-presenting cell
cysLT:	Cysteinyl leukotriene
DC:	Dendritic cell
FITC:	Fluorescein isothiocyanate
FLAP:	5-Lipoxygenase–activating protein
HETE:	Hydroxy eicosatetraenoic acid
KETE:	Keto eicosatetraenoic acid
LC:	Langerhans cell
5-LO:	5-Lipoxygenase
LT:	Leukotriene
LTA_4H:	Leukotriene A_4 hydrolase
LTC_4S:	Leukotriene C_4 synthase
MDDC:	Monocyte-derived dendritic cell
MDM:	Monocyte-derived macrophage
PMNL:	Polymorphonuclear leukocyte

endosomal membrane or by a mechanism involving ceramide to generate multivesicular bodies, which fuse with the plasma membrane, releasing exosomes into the extracellular space.[14,15] Antigen-presenting cell (APC)–derived exosomes contain MHC class II molecules and can, if loaded with antigen, elicit antigen-specific T-cell responses.[16] Moreover, exosomes from infected macrophages induced proinflammatory responses *in vitro* and *in vivo*.[17] Because of their immunostimulatory capacities, exosomes are being investigated as novel vaccine delivery vehicles.[18]

Leukotrienes and exosomes are implicated in similar immunologic settings, such as allergic reactions.[19] Thus, we investigated whether exosomes from human APCs contain the enzymes for LT biosynthesis and how exosomal LT metabolism compares to that of the parent cells. In this context, we studied effects of TGF-β1 as well as a potential role of exosomes for granulocyte migration.

METHODS

Cell culture of MDDCs and MDMs

Monocyte-derived dendritic cells and MDMs were generated from buffy coats of healthy human blood donors mainly as previously described.[6] PBMCs were isolated by centrifugation on a Ficoll-density gradient (GE Healthcare, Uppsala, Sweden). $CD14^+$ monocytes were isolated by magnetic bead separation and cultured for 6 days in the presence of the following cytokine combinations: GM-CSF, GM-CSF + TGF-β1, GM-CSF + IL-4, and GM-CSF + IL-4 + TGF-β1 (for a more detailed description, see this article's Methods section in the Online Repository at www.jacionline.org). Cells were harvested on day 6, and the culture supernatants were centrifuged at 3000*g*, 30 minutes, and kept at –80°C until exosome preparation.

Exosome preparation from culture supernatants from the 4 APC subtypes and from plasma

Cell culture supernatants were centrifuged at 100,000*g* for 1 hour 30 minutes. The pellets were resuspended and washed in PBS and centrifuged again (in total, 3 centrifugations). The final pellets were dissolved in PBS, and protein content was measured with a DC Protein Assay (BioRad, Hercules, CA.) according to the manufacturer's instructions. Plasma exosomes were prepared similarly as described.[20] Plasma from healthy blood donors was diluted 1:1 in PBS and spun twice at 11,600*g* for 45 minutes to remove aggregates. Exosomes were then pelleted at 140,000*g* for 2 hours, filtered through 0.22-μm filters (Advantec MFS, Inc, Dublin, Calif), and washed twice in PBS at 140,000*g* for 1 hour 30 minutes.

Phenotyping of MDDCs and MDMs and their exosomes

Antigen-presenting cells and exosomes were stained with fluorescein isothiocyanate (FITC)–labeled or phycoerythrin-labeled mAbs and phenotyped by flow cytometry using a FACSCalibur (BD Biosciences, San Jose, CA) as previously described.[21] For a more detailed description, see the Methods section in the Online Repository.

Sucrose gradient assay

Exosomes were layered on top of a continuous sucrose gradient from 0.25 mol/L sucrose/20 mmol/L HEPES (pH 7.0) and 2 mol/L sucrose/20 mmol/L HEPES (pH 7). Gradients were spun overnight at 80,000*g*. One-milliliter fractions were recovered, and density was determined by refraction index measurement. For tracking exosomes in the gradient, 100 μL each fraction was incubated with 0.5 μL anti–MHC-class II loaded Dynabeads (Dynal, Norway), and fluorescence-activated cell sorting analysis was performed for CD81, human leukocyte antigen (HLA)-DR, and corresponding isotype control antibodies in 1:20 dilutions. Fractions were then centrifuged for 1 hour 30 minutes at 100,000*g*, and pellets were resuspended in 50 μL PBS for Western blot WB analysis.

Immunonegative staining and electron microscopy

Immunonegative staining, using in-house primary rabbit anti-LTC_4-synthase antibodies, was performed as described.[22] Electron microscopy was performed by using a Leo 906 transmission electron microscope (Zeiss, Oberkochen, Germany) with camera and software from Morada (SiS System, Munster, Germany; see Methods in the Online Repository for a detailed description).

Western blotting

Western blotting for exosome or cell samples was performed by using the following primary antibodies: in-house antisera against LTA_4H, LTC_4S, 5-LO–activating protein (FLAP), and 5-LO and antibodies against HLA-DR and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Protein bands were visualized by using a peroxidase-conjugated secondary antibody and an *enhanced* chemiluminescence detection system (see Methods in the Online Repository for a detailed description).

Determination of enzymatic conversions of LTA_4 and AA

Cells or exosomes were incubated with LTA_4 or AA plus Ca^{2+} ionophore (A23187). Reactions were stopped by addition of methanol and cooling on ice. Samples were extracted via C18 cartridges (Supelco, Belfonte, Pa) and analyzed for LTs and other AA metabolites by reversed-phase HPLC or liquid chromatography-mass-spectrometry/mass-spectrometry (LC-MS/MS) as previously described[23,24] (see Methods in the Online Repository for detailed procedures).

Chemotaxis assay

Exosome suspensions (± AA and A23187) were transferred into the wells of a chemotaxis microplate, and polymorphonuclear leukocytes (PMNLs) were allowed to transmigrate for 1.5 hours. The number of migrated PMNLs was determined, measuring absorbance at 405 nm, and verified by flow cytometry (see Methods in the Online Repository for a detailed description).

Statistical analysis

Statistical analysis was performed by using Graphpad Prism version 4.03 for Windows (Graphpad Software Inc, San Diego, Calif). Statistical methods were Wilcoxon matched pairs (within 1 group) and unpaired Mann-Whitney test (between groups). A P value <.05 was considered statistically significant.

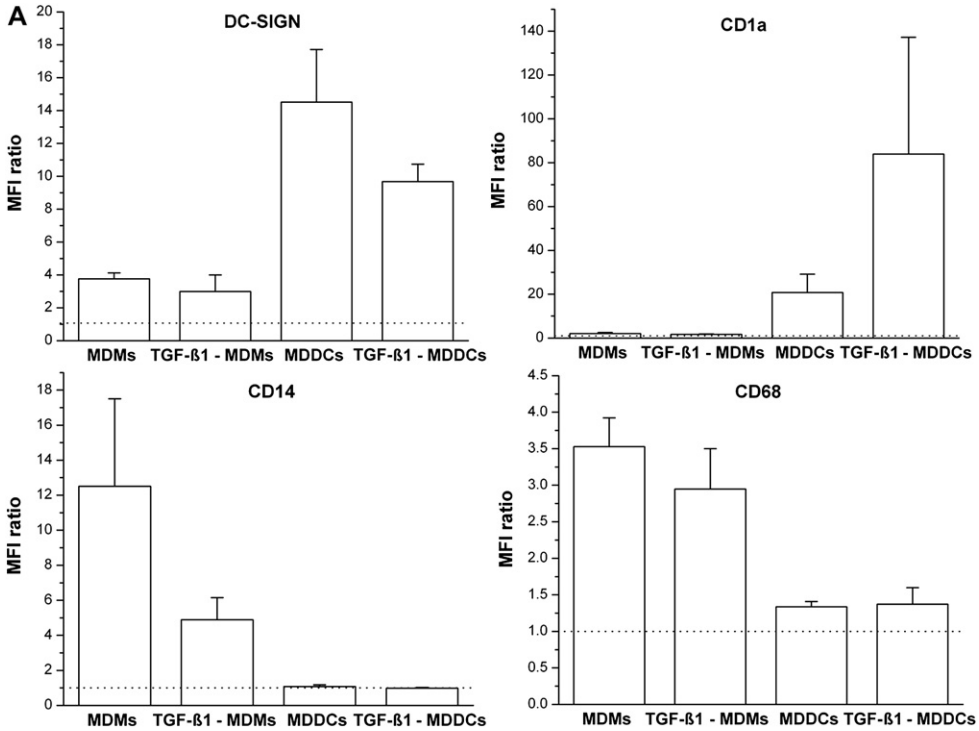

FIG 1. Cell surface proteins and exosome formation of 4 APC subtypes. Surface marker proteins on APCs **(A and B)** and exosomes *(B)* assessed by flow cytometry. Results are for the same healthy blood donors (n = 4). **C,** Exosome secretion is decreased in TGF-β1–MDMs versus MDMs. Amounts of exosomal protein per 10^6 cells are shown (n = 9). All data are means ± SEMs. *MFI*, Mean fluorescence intensity.

RESULTS

Presence of TGF-β1 during differentiation regulates expression of cell and exosome surface markers as well as exosome release

To verify the differentiation of monocytes into macrophages or DCs after cytokine treatment, cells were phenotyped using flow cytometry. In agreement with previous studies,[6,25] the DC markers CD1a and DC-SIGN were upregulated in GM-CSF/IL-4–treated cells, whereas higher levels of the macrophage/monocyte markers CD14 and CD68 were detected for GM-CSF–treated cells (Fig 1, *A*). TGF-β1 tended to downregulate levels of CD14, HLA-DR, CD40, inter-cellular adhesion molecule (ICAM-1) (CD54), CD63, and CD86 on MDMs, whereas CD1a in MDDCs was upregulated (Fig 1, *A and B*).

Exosomes exhibited different patterns of surface marker expression compared with their parent cells. For example, whereas CD63 and CD86 were downregulated on TGF-β1–MDMs compared with MDMs, no such differences were observed for MDM-exosomes (Fig 1, *B*). Only for ICAM-1, the exosomal levels followed the pattern seen for the cells.

Exosome production, determined as the amount of exosomal protein per million cells, differed between MDMs and MDDCs. MDMs tended to release larger amounts of exosomes compared with MDDCs (Fig 1, *C*), and TGF-β1 clearly reduced the amount of exosomal protein released from MDMs ($P = .011$).

Proteins of the leukotriene pathway are present in macrophage-derived and DC-derived exosomes

The presence of enzymes of the LT biosynthesis pathway in MDMs, MDDCs, and their exosomes was determined by WBs. To obtain sufficient amounts of protein, exosomes from the same cell subtypes were pooled from 3 donors.

Leukotriene C_4 synthase was present in exosomes derived from both MDMs and MDDCs (Fig 2, *A*). In most WBs, a pronounced band of about 36 kd appeared in addition to the LTC_4S monomer band at 18 kd. Also, the recombinant standard LTC_4S protein gave 2 bands, corresponding to monomer and dimer. Bands of similar intensities were detected for the different exosomes, and as observed for the corresponding cells (Fig 2, *A*), TGF-β1 did not change the LTC_4S protein amount.

In contrast, protein levels of LTA_4H varied considerably between the different cells and their exosomes. Exosomes derived from TGF-β1–MDMs contained the highest levels of LTA_4H, reflecting the properties of the parent cells. Substantial amounts of LTA_4H were also detected in exosomes from TGF-β1–MDDCs (Fig 2, *B*). In general, lower protein and mRNA levels of LTA_4H were observed for MDDCs compared with MDMs, and TGF-β1 increased LTA_4H expression in both MDMs and MDDCs (see this article's Fig E1 in the Online Repository at www.jacionline.org).

Exosomes from all 4 cell subtypes contained substantial amounts of FLAP, which was also present in the cell lysates (Fig 2, *B and C*). 5-LO protein levels were generally lower in exosomes than cells (Fig 2, *C*), and HLA-DR, previously shown to be abundant in exosomes from APCs,[26] was present in all exosome samples (Figs 2, *B and C*).

Pools of MDM exosomes from 3 donors were fractionated on a sucrose gradient, and the fractions were analyzed for LTA_4H, 5-LO, FLAP, and LTC_4S by WB and for HLA-DR and CD81 by flow cytometry. The largest amounts of all LT pathway

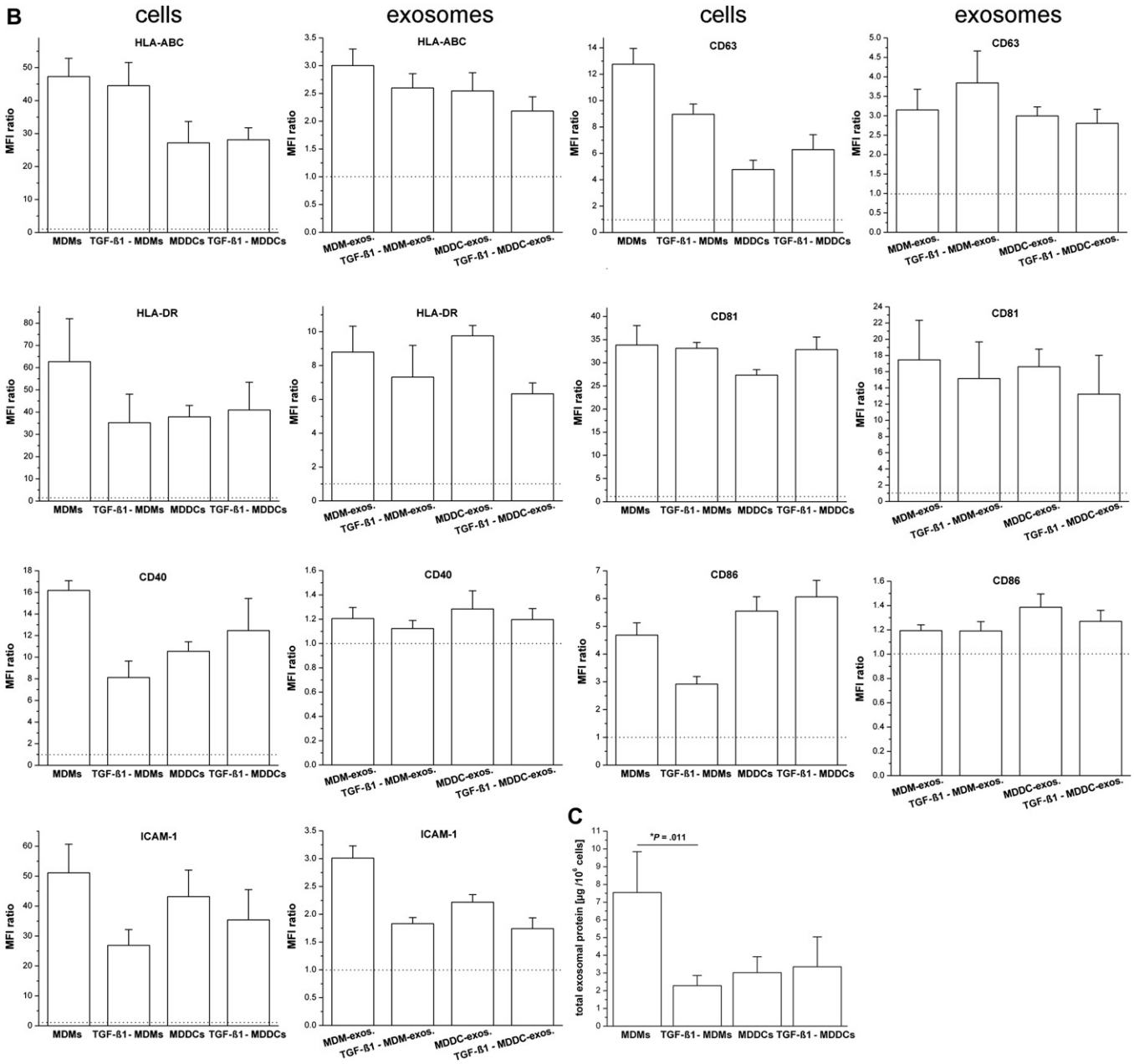

FIG 1. *(Continued)*

proteins overlapped with peaks for the typical exosomal proteins CD81 and HLA-DR, appearing in fractions with densities between 1.13 and 1.19 g/mL (Fig 3, *A-C*), which is in the expected density range for exosomes.[27]

Immunoelectron microscopy of LTC_4S in MDDC and MDM exosomes

To confirm further the presence of LTC_4S in exosomes, we performed immunoelectron microscopy. Immunogold labeling of MDDC and MDM exosomes indicated that LTC_4S was present in nanovesicles with a size around 100 nm, corresponding to exosomes (Fig 3, *D*).

APCs and their exosomes convert LTA_4 to leukotrienes B_4 and C_4; TGF-β1 during differentiation increases cellular LT production

To clarify whether APC-derived exosomes carried active LT producing enzymes, and to compare to LT production in the parent cells, cells and exosomes were incubated with LTA_4. The reverse phase (RP)-HPLC analysis showed that the major metabolite in MDMs was LTB_4, whereas MDDCs predominantly converted LTA_4 to LTC_4 (Fig 4, *A*). When MDMs were treated with TGF-β1, the yield of LTB_4 increased 3-fold to 4-fold ($P = .043$), whereas LTC_4 formation remained minute (Fig 4, *A*). In contrast, TGF-β1 treatment of MDDCs resulted in a significantly increased conversion of LTA_4 to LTC_4.

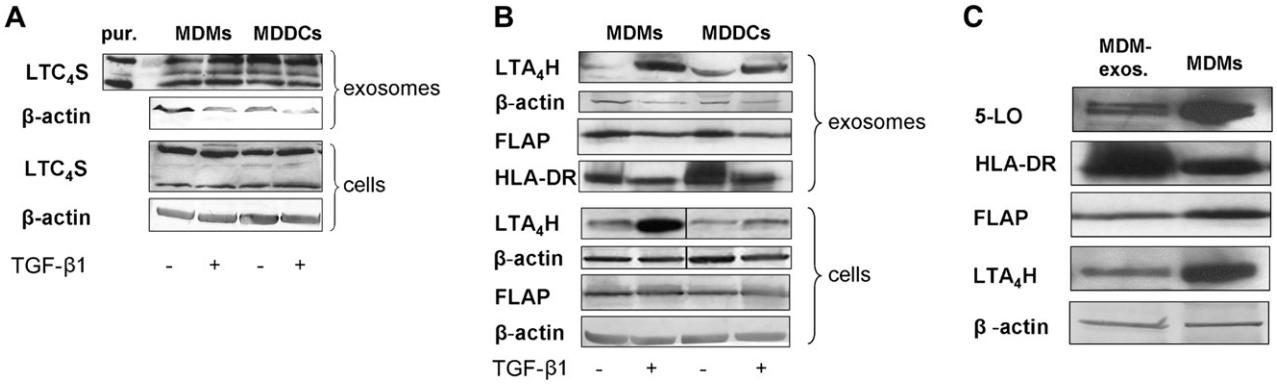

FIG 2. Enzymes of the LT pathway are present in APC derived exosomes. WBs for LTC_4 synthase, 50 μg total protein in exosome and cell samples; lane 1, purified recombinant LTC_4 synthase (100 ng) **(A)**. LTA_4H, FLAP and HLA-DR, 50 μg total protein in all samples **(B)**. 5-LO, HLA-DR, FLAP, and LTA_4H, 25 μg total protein in all samples **(C)**. For all proteins, similar results were obtained in at least three independent experiments.

Interestingly, exosomes from all 4 APC types had a pronounced capacity for conversion of LTA_4 to LTC_4 (Fig 4, *B*). Also in MDM exosomes, the major metabolite was LTC_4, although LTB_4 predominated in incubations of the corresponding cells (Fig 4, *A*). Thus, it appears that MDM exosomes are enriched in LTC_4S activity in relation to LTA_4H, which is in accordance with the WB analyses (Fig 2). The relative amounts of LTB_4 and LTC_4 formed by MDDC exosomes (Fig 4, *B*) were similar to the results for the corresponding cells (Fig 4, *A*). LTC_4 formation predominated, and LTA_4H activity was low. Plain exosomes did not contain detectable amounts of LTB_4 or LTC_4.

LT formation in co-incubations of PMNLs with MDM exosomes is higher than in PMNLs alone

Because PMNLs are a major source of endogenous LTA_4, we mixed freshly isolated PMNLs with MDM exosomes and triggered LTA_4 formation by addition of AA and Ca^{2+}-ionophore A23187. When exosomes alone were incubated with AA and A23187, LTB_4 or LTC_4 could not be detected (Fig 4, *C*). However, formation of LTC_4 and total LTs was significantly higher in mixtures of PMNLs and exosomes compared with PMNLs alone. Similar results were obtained with MDDC exosomes (data not shown).

Exosomes are weak sources of lipoxygenase products

When exosomes were incubated with AA and Ca^{2+}-ionophore A23187, the condition yielding maximum cellular 5-LO activity (see this article's Fig E2 in the Online Repository at www.jacionline.org), product formation was low. Peaks corresponding to 5–hydroxy eicosatetraenoic acid (HETE), 5–keto eicosatetraenoic acid (KETE), 15-HETE, 12-HETE, and 15-KETE could be observed, but the small amounts precluded quantification by HPLC with UV detection. LC-MS/MS analysis was performed after incubations of 2 pools of the most abundant exosomes (from MDMs). The following 5-LO products were detected: 5-HETE, 5-KETE, and 20-carboxy-LTB_4. In addition, the 15-lipoxygenase (15-LO) products 15-HETE, 15-KETE, and 12-HETE were found (for quantitative data, see Table I). Thus, the formation of 5-LO products from AA in exosomes is considerably lower than formation of LTs from LTA_4.

Exosomes can induce migration of granulocytes

Macrophages and DCs attract inflammatory cells by releasing chemotactic factors, including several eicosanoids, such as LTB_4 and 5-KETE. We therefore analyzed whether APC-derived exosomes could induce migration of PMNLs in a transwell experiment and whether stimulation with Ca^{2+}-ionophore and addition of AA could influence the chemotactic potential. PMNLs migrated to control wells containing PGC buffer (PBS with additions of glucose [1 mg/ml] and $CaCl_2$ [1 mM] without or with AA and A23187. However, the presence of MDM or MDDC exosomes resulted in a significantly increased PMNL migration (Fig 5). Exosomes alone (no AA, A23187) elicited PMNL migration, but less than exosomes together with AA and Ca^{2+}-ionophore ($P = .046$ for MDDC exosomes; Fig 5). These observations suggest that exosomes released chemotactic factors, including eicosanoids, which amplified PMNL chemotaxis.

Plasma exosomes from healthy individuals contain active LTA_4H and LTC_4S

To study whether nanovesicles, secreted *in vivo*, contain proteins of the LT pathway, we performed WBs for LTA_4H, LTC_4S, 5-LO, and FLAP on exosomes obtained from human plasma. Strong bands were observed for LTC_4S and FLAP in exosomes from all tested individuals (3 representatives shown in Fig 6, *A*). A diffuse signal was detected for LTA_4H, whereas 5-LO was barely detectable. Furthermore, in LTA_4 incubations, plasma exosomes produced both LTB_4 and LTC_4 (Fig 6, *B*), although in substantially lower yields compared with APC exosomes.

DISCUSSION

Here we show for the first time that exosomes are carriers of enzymes for LT biosynthesis. LTs have been widely appreciated as mediators of inflammation[28] and as modulators of APC functions.[4,29] However, regulation of LT-producing enzymes in APC subtypes has not received significant attention. Also, the lipid mediator repertoire of APC-derived exosomes, functioning as messengers in immunity, has not been studied before.

We show that exosomes from MDMs and MDDCs contain LTA_4H and LTC_4S, the downstream enzymes for LT biosynthesis, and metabolize LTA_4 to the proinflammatory LTs B_4 and C_4. We further demonstrate that TGF-β1 and IL-4, which together with

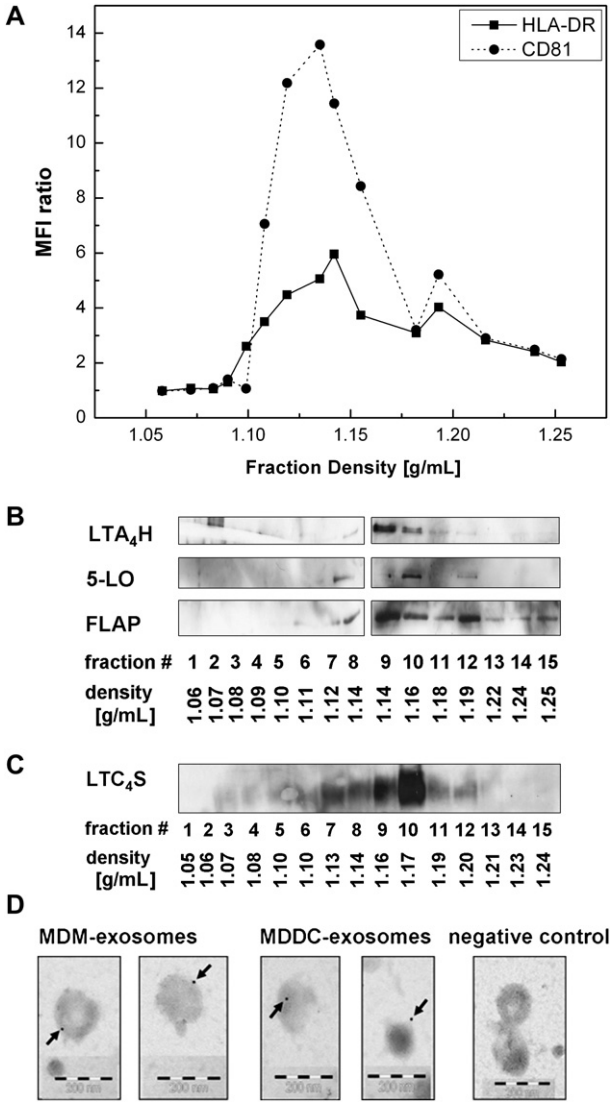

FIG 3. APC exosome markers and LT pathway proteins in exosome-enriched sucrose fractions; immunoelectron microscopy for LTC_4S in exosomes. Flow cytometry for HLA-DR and CD81 **(A)** and WBs for LTA_4H, 5-LO, and FLAP **(B)** and LTC_4S **(C)** in fractionated MDM vesicles. Results are representative of 3 separate experiments. Immunoelectron microscopy for LTC_4S in exosomes **(D)**; *arrows,* gold-labeled antibodies *(black dots); bars,* 200 nm. *MFI,* Mean fluorescence intensity.

GM-CSF are the key cytokines determining the APC phenotype, govern expression and activity of LT pathway enzymes in APCs and their exosomes. Prominent expression of CD1a and increased levels of 5-LO, as observed by Spanbroek et al,[7] indicate that the TGF-β1–MDDCs studied here had an LC-like phenotype. TGF-β1 changed the phenotype also of MDMs in conjunction with a considerably increased capacity to produce LTB_4 (Fig 4, A; Fig E2).

The monocyte-derived APCs are efficient producers of LTs and HETEs, although their biosynthetic capacities vary considerably between blood donors (Fig E2). After AA and ionophore incubation, TGF-β1–MDMs synthesize 1 to 1.5 nmol 5-LO products/10^6 cells, which is comparable to neutrophils (300-500 pmol 5-LO products/10^6 cells).[30] Also the capacities of APCs for enzymatic conversion of LTA_4 are high, ranging from 50 to 300 pmol/10^6 cells (100-600 pmol/mg cellular protein). Interestingly, APC-derived exosomes had even higher capacities for conversion of LTA_4, especially into LTC_4. Thus, in exosomes, about 2000 pmol LTC_4 was formed per milligram protein. On a protein basis, this is about 5 times higher than for the cell type with maximum LTC_4-producing capacity (TGF-β1–MDDCs, about 400 pmol/mg protein). Strikingly, MDMs with a low capacity for conversion of LTA_4 to LTC_4 (about 20 pmol/mg protein) secreted exosomes with high LTC_4S activity (1000-2000 pmol/mg protein). In WBs (Fig 2, *A*) exosomes were richer in LTC_4S compared with cells, but the differences were not as pronounced as for enzyme activity. This indicates that LTC_4S may become activated during the formation of exosomes. LTC_4S can be inhibitorily phosphorylated,[31] suggesting that the degree of phosphorylation of LTC_4S might change during exosome formation, resulting in increased activity. Indeed, we have observed that the higher LTC_4S activity in MDDCs versus MDMs can be ascribed to distinct posttranslational regulation (Esser J et al, unpublished data, November 2009).

Because LTC_4S is a membrane-bound enzyme and exosomes have an increased membrane to cytosol ratio compared with cells, it seems likely that more LTC_4S is sorted into the nanovesicles compared with the cytosolic LTA_4H. This may contribute to the high potency of exosomes for conversion of LTA_4 to LTC_4. Also, FLAP, a membrane associated protein in eicosanoid and glutathione metabolism (MAPEG) protein closely related to LTC_4S,[32] was highly abundant in all exosome samples. Interestingly, connections between lipid raft–associated proteins, exosomes, and FLAP have appeared.[33,34] Furthermore, in rat basophilic leukemia cell line 2H3 (RBL-2H3) cells, LTC_4S was in complex with FLAP.[35] Such a complex may also be present in the exosomes. Exosome-derived LTC_4 could contribute to all physiological effects described for this eicosanoid—for example, regarding the symptoms of asthma and rhinitis. One role of exosomal LTC_4, particular for APC function, may be to stimulate chemotaxis and migration of immature DCs to lymph nodes.[36]

The exosomes with the highest LTA_4H activity (1800 pmol/mg protein) were derived from TGF-β1–MDMs. On a protein basis, the LTA_4H activity in TGF-β1–MDM exosomes was about 7 times higher than in the corresponding cells. The discrepancy between these activity data and the WB results (Fig 2, *B*) might be explained by restriction of cellular LTA_4H activity by a mechanism that is absent in the exosomes and/or the possibility that LTA_4H in exosomes is more readily available for the LTA_4 substrate. In comparison, LTB_4 formation in human leukocyte homogenates (incubated with LTA_4) was 400 pmol/mg.[37] The different LTA_4 metabolism in APC-derived exosomes compared with their parent cells most likely results from protein sorting and changed enzyme regulation. In this context, it should be noted that we have compared cells, collected on day 6 of the differentiation schemes, with exosomes that were released from days 1 to 6 of culture. This might at least partly explain the observation that the differences in surface marker expression and LTA_4 metabolism were more pronounced between the APC subtypes than their exosomes (compare Figs 1B, 4). Nevertheless, we conclude that the high LTA_4 metabolic capacity of the exosomes, compared with the parent cells, reinforces the picture of exosomes as specifically released nanovesicles with potent messenger functions.

The presence of exosomes that carry active enzymes for LT biosynthesis in human plasma demonstrates that nanovesicles with the capacity for LT production are also released *in vivo*.

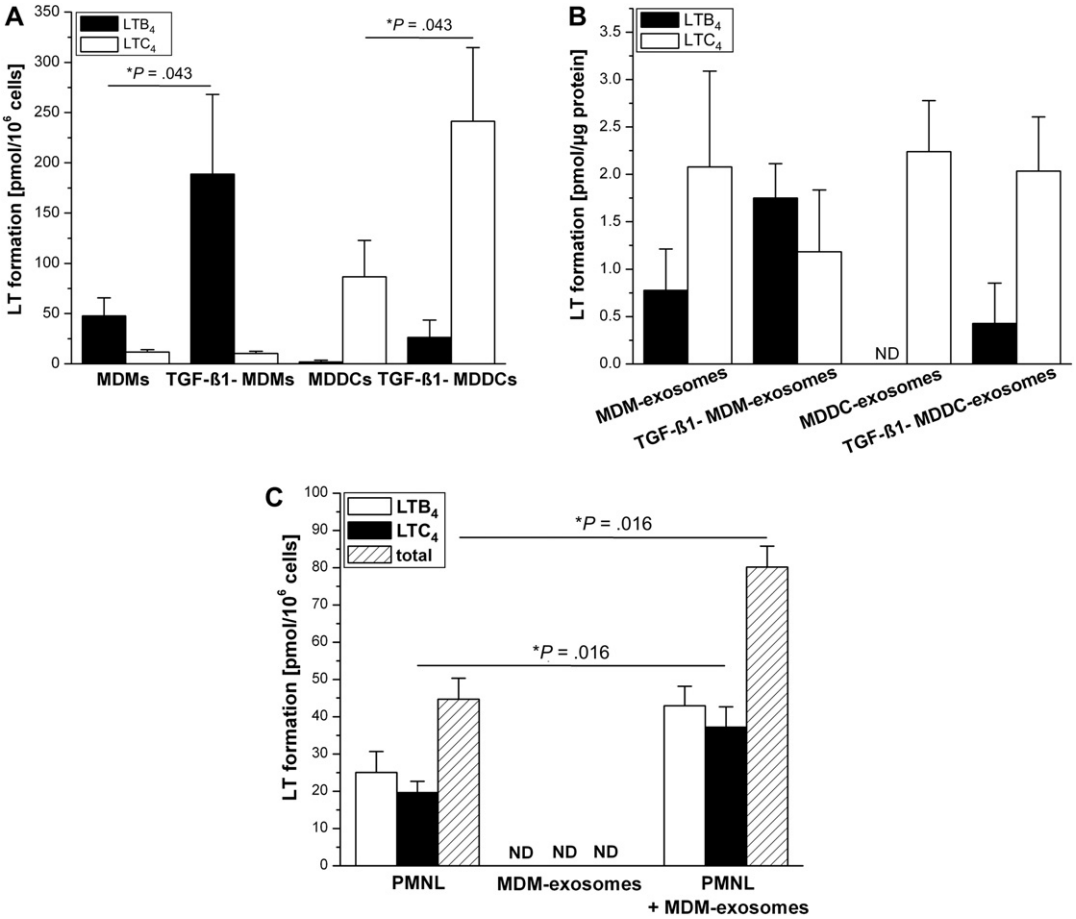

FIG 4. APCs and their exosomes convert LTA_4 to LTC_4 and LTB_4; TGFβ1 up-regulates LTB_4 production. LTA_4 conversions in APCs **(A)** and their exosomes **(B);** Mean ± SEM, n=5 donors of cells and n=3 pooled exosomes (each pool from 3 individuals); LT formation in co-incubations of MDM exosomes (50 μg) and PMNL (1×10^6 cells) stimulated with AA and A23187 **(C);** Mean ± SEM (n=7). *ND* = non detectable.

TABLE I. AA metabolites in MDM exosomes

AA metabolite	Amount (pmol/mg) Pool 1	Pool 2
5-HETE	6.9	21.1
5-KETE	5.0	66.9
20-carboxy-LTB_4	10.2	44.8
15-HETE	30.4	108.4
15-KETE	4.3	19.3
12-HETE	16.9	ND

ND, Not detected.
Exosomes from 2 pools with 3 healthy individuals in each were incubated with AA and A23187, and the extracted samples were analyzed by LC-MS/MS as described in the Methods section in the Online Repository. The results from the quantitative analysis (performed in duplicate) are given as mean of the duplicates in picomoles per milligram total exosomal protein.

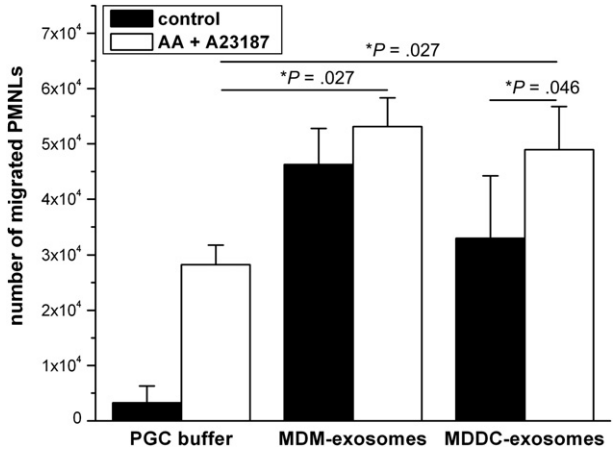

FIG 5. Migration of PMNLs to MDM and MDDC–derived exosomes. Suspensions of 1×10^5 PMNLs were added on top of the chemotaxis plate filter and allowed to migrate for 1.5 hour to PGC buffer controls or exosome suspensions (in the wells), without or with AA (40 μmol/L) and A23187 (2.5 μmol/L). Numbers of migrated cells presented as means ± SEMs (n = 6).

Plasma exosomes displayed much lower levels of HLA-DR and produced substantially less LTB_4 and LTC_4 than MDM and MDDC exosomes, indicating that they originate mainly from other cell types. Of considerable interest, recent results from our group indicate a high abundance of LTC_4S in exosomes from bronchoalveolar lavage fluid from patients with asthma (Torregrosa Paredes P, et al, unpublished data, February 2010).

In addition to the effects on LTA_4 metabolism, TGF-β1 affected exosome formation, particularly in MDMs. The amount of exosomes was reduced to less than one third in TGF-β1–MDMs

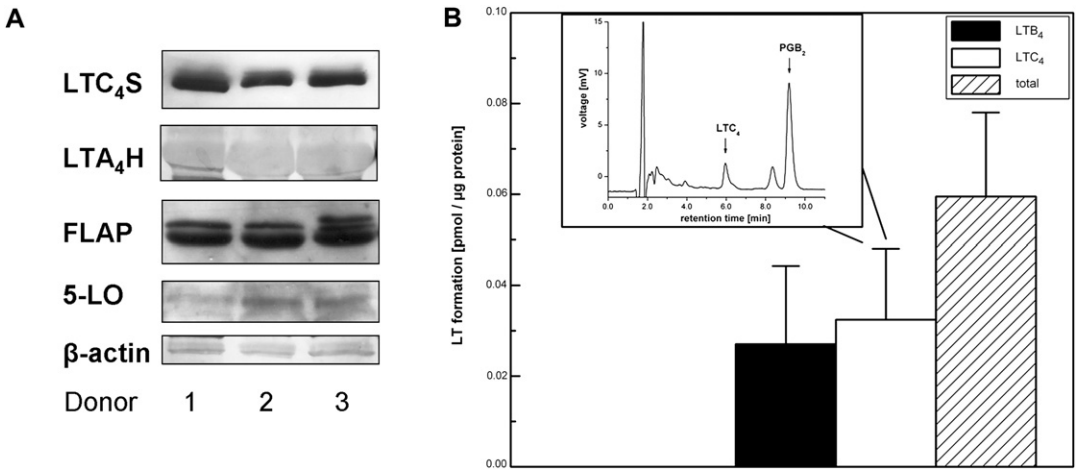

FIG 6. Plasma exosomes contain active LTA_4H and LTC_4S. WB analyses of LTA_4H, LTC_4S, FLAP, 5-LO, and β-actin (100 μg total protein; **A**) and LTA_4 conversions in plasma exosomes (500 μg protein used for incubations) from healthy individuals **(B)**; mean ± SEM (n = 3 [LTB_4], n = 2 [LTC_4]). Inserted small picture (in *B*): representative chromatogram for the LTC_4 analysis. *PGB_2*, prostaglandin B_2.

compared with MDMs, suggesting that TGF-β1 signaling affects the exosome formation process. Several rab protein guanosine triphosphate phosphatase (rabGTPases) are involved in the exosome release mechanism,[38] and regulatory networks between small guanosine triphosphate phosphatase (GTPases) and TGF-β1 signaling[39] might thus explain the TGF-β1 effects on exosome secretion.

In a chemotaxis assay, we could show that MDM and MDDC–derived exosomes induced PMNL migration. 5-KETE and the LTB_4 metabolite 20-carboxy-LTB_4 were detected in incubations of MDM exosomes with AA and A23187 (Table I). These compounds (5-KETE and LTB_4) are potent chemoattractants for eosinophils and neutrophils. However, probably a variety of chemotactic factors contributed to the induction of PMNL migration in our assay, and we cannot make any claims regarding the relative contribution of particular AA metabolites. Secretion of neutrophil chemotactic proteins by macrophage exosomes has been reported,[40] and enhanced PMNL migration was observed when exosomes alone were used as stimuli (Fig 5). Regardless of the nature of the chemotactic agents released from the nanovesicles, the results support the hypothesis that exosomes could aggravate inflammation by enhancing granulocyte migration.

The finding that nanovesicles can convert LTA_4 to LTs suggests an alternative route for transcellular metabolism, an important principle in eicosanoid biosynthesis. Activated neutrophils release large amounts of LTA_4, which by transcellular metabolism can be converted by other cells (platelets, endothelial cells, erythrocytes) expressing LTC_4 and LTB_4–producing enzymes.[41] At sites of inflammation, neutrophils are in proximity to macrophages and DCs. Our data suggest that LTA_4, secreted by ionophore-activated PMNLs, can be converted by APC-derived exosomes to LTs (Fig 4, *C*). Moreover, together with a recent report describing cysLT-mediated release of eosinophil granule content,[42] our findings open possibilities for a vesicle-based system for inflammatory mediator secretion.

In conclusion, this report is the first to demonstrate exosomal enzyme activity with a potential functional role in inflammation. The results indicate that, besides their antigen-presenting capacities,[13] APC-derived exosomes can participate in formation of LTs, inflammatory mediators functioning for example as chemotactic agents, and contribute to the pathogenesis of diseases such as asthma.

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Clinical implications: Exosomes from APCs contribute to LT formation and granulocyte recruitment, suggesting a role for these vesicles in the pathophysiology of chronic inflammatory diseases and their application as novel biomarkers.

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METHODS

Cell culture of MDDCs and MDMs

After the isolation of PBMCs by centrifugation on a Ficoll density gradient, $CD14^+$ monocytes were isolated in sorting buffer (PBS, 0.5% BSA [Sigma-Aldrich, St Louis, Mo], 2 mmol/L EDTA) by positive selection using α-CD14-magnetic bead sorting (MACS) bead kit (Miltenyi Biotech, Bergisch-Gladbach, Germany). Purity was assessed by flow cytometry using CD14-FITC antibodies (BD Biosciences, Bedford, Mass) and ranged from 75% to 96% (viability > 95%; n = 11). The cells were plated at 4×10^5 cells/mL complete medium (RPMI-1640; Hyclone Laboratories, South Logan, Utah), 10% heat-inactivated exosome-depleted FCS (Hyclone Laboratories),[E1] 25 μg/mL gentamycin (Gibco, Paisley, United Kingdom), 2 mmol/L L-glutamine (Hyclone Laboratories), 100 IU/mL penicillin with 100 μg/mL streptomycin (Hyclone Laboratories), and 50 μmol/L 2-ß-mercaptoethanol (Merck, Whitehouse Station, NJ) using 175 cm^2 culture flasks (BD Biosciences, Bedford, Mass). For generation of MDDCs, 1 ng/mL recombinant human (rh) IL-4 (Biosource, Camarillo, Calif) and 10 ng/mL rhGM-CSF (Biosource) were added to the medium; for MDM cultures, only rhGM-CSF (10 ng/mL) was added. TGF-β1 (purified from human platelets, a kind gift from Prof Dieter Steinhilber, University of Frankfurt) was supplied to the medium in a concentration of 2 ng/mL on the first day of culture to obtain TGF-β1–MDMs and TGF-β1–MDDCs. On day 3 of the culture, 50% of the medium was exchanged and resupplied with cytokines. On day 6, cells were harvested by scraping (in contrast with MDDCs and TGF-β1–MDMs, MDMs grew firmly adherent to the culture dishes), counted, and used for further experiments.

Phenotyping of MDDCs and MDMs and their exosomes

On day 6 of MDDC and MDM cultures, cells were phenotyped by using flow cytometry. The analysis was performed on a FACSCalibur (BD Biosciences) by using Cellquest software (BD Biosciences). Cells were blocked with 1% mouse serum (Dako Cytomation, Glostrup, Denmark) and stained with FITC-labeled mouse mAbs against human leukocyte antigen ABC (HLA-ABC), HLA-DR, CD14, CD40, CD63, CD80, CD81, CD83, CD86, DC-SIGN (all BD Biosciences), CD68 (Affinity Bioreagents, Rockford, Ill), and phycoerythrin-labeled mAbs against CD1a (Beckman Coulter, Fullerton, Calif) and CD54 (BD Biosciences), and their corresponding isotype controls. To phenotype exosomes, material corresponding to 10 μg total protein was loaded per 1 μl of anti-MHC-class II coated Dynabeads (clone HKB1, recognizing all MHC class II subtypes) (Dynal, Norway) overnight. After wash, surface markers on the exosomes were analysed using FITC-labeled mAbs against HLA-ABC, HLA-DR, CD40, CD63, CD81, CD86, and PE-labeled mAb against CD54 (BDBiosciences, San Jose, CA), and their corresponding isotype controls.[E2] A gate was set on single beads, and 5×10^3 events were collected per sample.

mRNA extraction and quantitative real-time PCR

Total RNA from the 4 APC subtypes was isolated by the TRIZOL method (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized from 500 ng RNA with the SuperScript II Reverse Transcriptase kit (Invitrogen) for RT-PCR with Oligo $d(T)_{16}$ primer (Applied Biosystems, Warrington, UK). Real-time PCR was performed with TaqMan reagents and an ABI Prism 7700 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. Normalizations were made to transcripts of human β-actin. The following primer/probe pairs were from Assay-on-Demand (Applied Biosystems): LTA_4H (assay ID, Hs00168505_m1) and β-actin (ACTB) (assay ID, Hs99999903_m1).

Western blotting

A total of 5×10^6 cells were resuspended in PBS (0.5 mL) containing complete protease inhibitor cocktail (Roche, Mannheim, Germany) and sonicated 3 times for 5 seconds. Samples were cooled on ice for 10 minutes and centrifuged for 10 minutes at 10,000*g*. The protein concentration of the supernatant (cell lysate) was determined in a Bradford Assay (Bio-Rad, Hercules, CA.) according to the manufacturer's instructions. Cell lysate or exosome material were mixed with 5x Lämmli buffer (containing 10% β-mercaptoethanol) and heated for 5 minutes at 95°C. Equal amounts of total protein were applied to each lane for SDS-PAGE, and rabbit in-house antisera were used in subsequent immunoblotting for LTA_4H, LTC_4S, FLAP, and 5-LO. Polyclonal antibodies against HLA-DR (rabbit) and β-actin (goat) were from Santa Cruz Biotechnology. The second antibody was peroxidase-conjugated goat antirabbit at 1:5000 dilution or alkaline-phosphatase–conjugated rabbit antigoat (1:1000 dilution) for β-actin (both Sigma, St Louis, MO). Protein bands were visualized with an enhanced chemiluminescence (ECL plus) detection system (GE Healthcare). Autoradiography exposure time (Hyperfilm ECL; GE Healthcare, Uppsala, Sweden) was 10 seconds to 5 minutes.

Determination of enzymatic conversions of LTA_4 and arachidonic acid

Leukotriene A_4 methyl ester was obtained from Biomol (Plymouth Meeting, Pa). LTA_4 was saponified in tetrahydrofuran with 1 mol/L LiOH (6% vol/vol) for 48 hour at 4°C.

A total of 2×10^6 cells or exosome material corresponding to 50 μg total protein were incubated for 5 minutes with 20 μmol/L LTA_4 or for 10 minutes with 80 μmol/L AA and 5 μmol/L Ca^{2+} ionophore (A23187; Sigma Aldrich, Germany) in 0.5 mL PBS or PGC buffer (PBS containing glucose [1 mg/mL], $CaCl_2$ [1 mmol/L]) at 37°C. Reactions were stopped by addition of methanol together with internal standards (250 pmol prostaglandin B_2 [PGB_2] and 250 pmol 17-OH-C22:4, kind gifts from Mats Hamberg, Karolinska Institutet) and chilled on ice. Samples were extracted on C18 columns (Supelco, Belfonte, Pa) at pH 5.6 and analyzed by reverse-phase HPLC for monohydroxy acids (5-HETE, 12-HETE, 15-HETE), LTB_4, LTC_4, and 5-KETE, and 15-KETE as previously described.[E3]

LC-MS/MS analysis

Oxylipin analysis was performed by using modifications of previously reported procedures.[E3] Glycerol (30 μL, 5% in ethanol) was added to the extracted samples from exosome incubations, and solvent was stripped via SpeedVac centrifugation (Heto Laboratory Equipment A/S, Copenhagen, Denmark). The remaining material was resuspended in 100 μL of a 400-nmol/L solution of 1-cyclohexyluriedo-3-dodecanoic acid in methanol, vortexed, and filtered at 0.1 μm using Amicon Ultrafree-MC durapore polyvinylidene fluoride (PVDF) filters (Millipore). Analytes were separated by reverse-phase ultraperformance liquid chromatography (Waters Corp, Milford, Mass) on a 1.7-μm Acquity BEH column (Waters, Milford, MA) as previously described.[E4] Oxylipins were detected by using negative mode electrospray ionization tandem quadrupole mass spectroscopy with previously published methods.[E4] Ionization and fragmentation energies for the reported oxylipins were optimized for analysis on an API 4000 QTrap (Applied Biosystems Inc, Foster City, CA).

Immunonegative staining and electron microscopy

An aliquot of 3 μL exosomes was added to a carbon-coated formvar grid for 5 minutes. The excess solution was soaked off by a filter paper, and the grid was placed on 20 μL drops of 2% gelatin, 2% BSA in 0.1 mol/L phosphate buffer for 10 minutes. Grids were then transferred to in-house primary rabbit anti-LTC_4-synthase antibodies diluted 1:50 for 1 hour 30 minutes and rinsed with 0.1 mol/L phosphate buffer containing 0.1% gelatin and 0.1% BSA. Bound antibodies were detected by protein A conjugated with 10-nm gold particles, rinsed in buffer, and fixed in 2% glutaraldehyde in 0.1 mol/L phosphate buffer for 5 minutes. The grids were washed 4 times for 1 minute in distilled water and negatively stained by 2% uranyl acetate for 10 seconds.

Chemotaxis assay

Polymorphonuclear leukocytes were isolated from whole blood (obtained from the same healthy donor) by Percoll centrifugation and resuspended in PGC + BSA (0.3%) to a concentration of 3.3×10^6 cells/mL. Exosome material from MDMs or MDDCs (25 μg total protein) was resuspended in 100 μL PGC with or without AA (40 μmol/L) and A23187 (2.5 μmol/L).

A total of 32 μL of the exosome samples was then directly transferred into the lower wells of the chemotaxis microplate (3 μm pore size; ChemoTx; NeuroProbe, Warwickshire, United Kingdom). After mounting the filter unit of the microplate, 30 μL of the PMNL suspension was pipetted on top of each filter, and migration was allowed for 1.5 hour at 37°C, 5% CO_2. Cells remaining on top of the filter were washed out, the filter unit was removed, and the number of migrated cells for each condition was determined in duplicate with a microplate reader (405 nm; Thermo Scientific, Waltham, Mass) by comparing with a standard curve for dilution series of PMNLs. For some experiments, migration of PMNL was verified by subsequent flow-cytometric analysis, using forward and side scatter, of the suspensions collected from the lower wells after the microplate readout, with a FACSCalibur (BD Biosciences).

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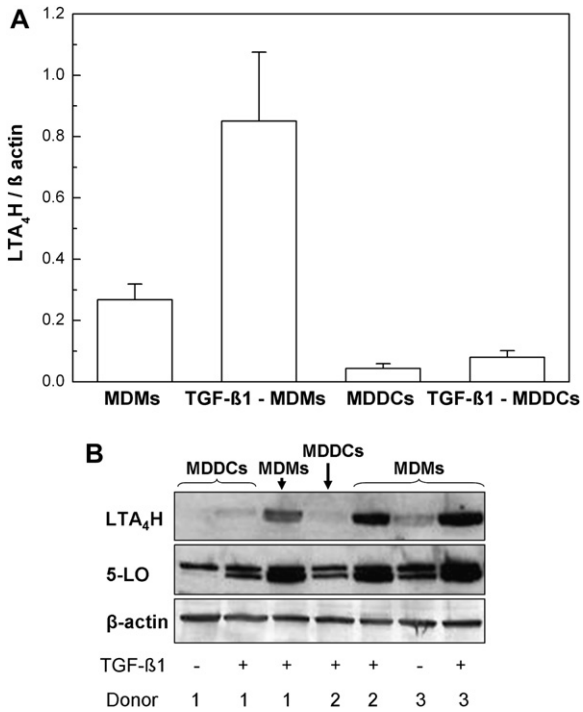

FIG E1. Expression of LTA_4H and 5-LO in APCs. LTA_4H mRNA levels (relative to β-actin) in MDMs and MDDCs (± TGF-β1; **A;** mean ± SEM (n=4). WB analysis of LTA_4H and 5-LO in cell lysates from APCs (± TGF-β1; 25 μg protein; **B;** lower band in 5-LO WB corresponds to 5-LO. Representative results of 4 independent experiments.

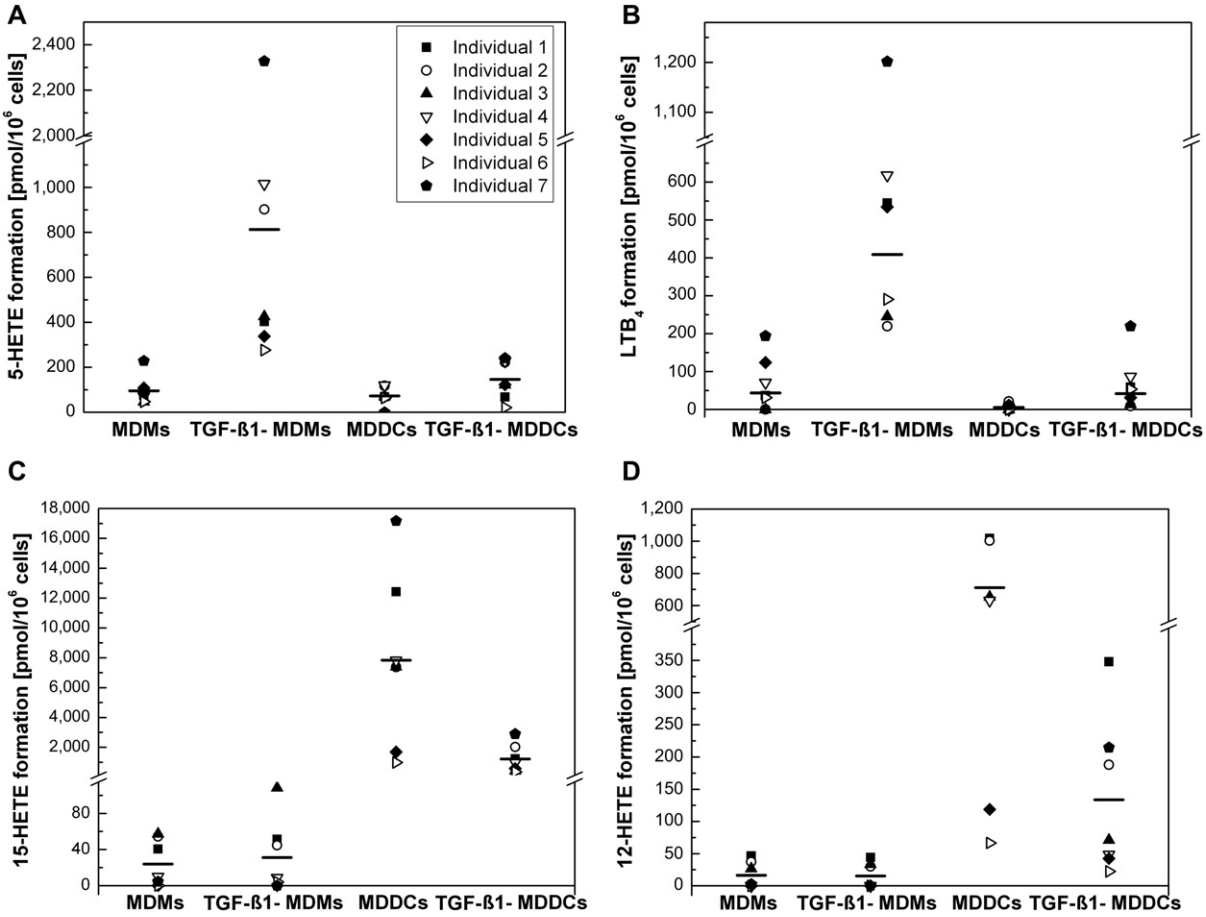

FIG E2. Biosynthesis of 5-LO and 15-LO products in APCs depends on the blood donor and is regulated by TGF-β1. Data from incubations of APCs with AA and A23187. Samples were analyzed by HPLC for 5-HETE **(A)**, LTB_4 **(B),** 15-HETE **(C)**, and 12-HETE **(D)**; data points for MDMs and MDDCs (±TGF-β1) derived in parallel from 7 donors.