Biosynthesis of eicosanoids and transcellular metabolism of leukotrienes in murine bone marrow cells

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Abstract **Leukotriene B_4 (LTB_4) biosynthesis by polymorphonuclear leukocytes (PMNs) is an important factor of inflammatory responses. PMNs also release LTA_4, an unstable intermediate that can be taken up by neighboring cells and metabolized into LTC_4. Most studies of LT synthesis have been carried out using human PMNs, but very little information is available about mouse PMNs. Mouse bone marrow PMNs were found to synthesize eicosanoids upon stimulation with A23187, fMLP, or zymosan. The major eicosanoids produced are LTB_4 and 5-hydroxyeicosatetraenoic acid, with some nonenzymatic products of LTA_4 hydrolysis. No cysteinyl leukotrienes were produced, in contrast to what was observed with human blood neutrophil preparations. Human megakaryoblast-like MEG-01 cells synthesized thromboxane B_2 and prostaglandin E_2 in response to A23187 but produced no 5-lipoxygenase (5-LO)-derived eicosanoids. When mouse bone marrow cells (mBMCs) and MEG-01 cells were stimulated during coincubation, LTC_4 and LTD_4 were produced. Mouse peritoneal macrophages from 5-LO-deficient mice were able to synthesize LTC_4 when incubated with mBMCs from wild-type mice, demonstrating transcellular exchange of LTA_4 from mBMCs into murine peritoneal macrophages. These data demonstrate that murine bone marrow PMNs are a valid model for the study of LT biosynthesis, which now offers the possibility to investigate specific biochemical pathways through the use of transgenic mice.**—Gijón, M. A., S. Zarini, and R. C. Murphy. **Biosynthesis of eicosanoids and transcellular metabolism of leukotrienes in murine bone marrow cells.** ***J. Lipid Res.*** **2007.** 48: **716–725.**

Supplementary key words murine neutrophils • leukotriene B_4 • leukotriene C_4 • MEG-01 cells • mass spectrometry

Leukotrienes (LTs) are derivatives of arachidonic acid (AA) that play a role in innate immune responses and are potent mediators of inflammation. They belong to a wider family of oxidized metabolites of AA collectively called eicosanoids, which also includes prostaglandins (PGs) and thromboxanes (TXs) (1). All of these lipids are rapidly synthesized in response to extracellular stimuli and act through specific receptors in a paracrine manner to signal a variety of responses. Effects of LTs include leukocyte chemotaxis, release of bactericidal compounds such as defensins and reactive oxygen species, release of cytokines, bronchoconstriction, and vasodilation. As a result, LTs are implicated in human clinical situations such as vascular disease and asthma (1, 2). Drugs that inhibit LT biosynthesis (zileuton) (3) or antagonize LT receptors (montelukast, zafirlukast) (4) have been or are currently being used for the treatment of asthma.

Arachidonate esterified at the *sn*-2 position of phospholipids is the source of activation-induced substrate for LTs and other eicosanoid biosynthesis. AA is released upon hydrolysis catalyzed by phospholipases A_2, most commonly group IVA cytosolic phospholipase A_2 ($cPLA_2\alpha$), which exhibits a preference for AA-containing phospholipids and is rapidly activated in response to extracellular stimuli (5). Upon activation, in a calcium-dependent manner, $cPLA_2\alpha$ translocates from the cytosol to intracellular membranes that include the Golgi apparatus, the endoplasmic reticulum, and the nuclear envelope; at this location, it is in close proximity to some of the enzymes that use AA for eicosanoid biosynthesis. Cyclooxygenases (COX-1 and COX-2) are microsomal membrane proteins that catalyze the oxidation of AA to the endoperoxide PGH_2, which can then be enzymatically converted to biologically active eicosanoids, including PGD_2 (by the action of PGD_2 synthase), PGE_2 (by PGE_2 synthase), prostacyclin (PGI_2; by PGI_2 synthase), and TXA_2 (by TXA_2 synthase) (1). Alternatively, AA can be converted to LTs by 5-lipoxygenase (5-LO), functionally coupled to 5-LO-activating protein, an integral membrane protein present on the nuclear envelope that presents AA to 5-LO. Like $cPLA_2\alpha$, 5-LO translocates to the nuclear envelope upon cell activation (6) and catalyzes the formation of 5-hydroperoxyeicosatetraenoic acid and subsequently LTA_4. 5-Hydroperoxyeicosatetra-

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Abbreviations: AA, arachidonic acid; COX, cyclooxygenase; $cPLA_2\alpha$, cytosolic phospholipase A_2; cys-LT, cysteinyl leukotriene; 5-HETE, 5-hydroxyeicosatetraenoic acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; 5-LO, 5-lipoxygenase; LT, leukotriene; mBMC, mouse bone marrow cell; PG, prostaglandin; PMN, polymorphonuclear leukocyte; TX, thromboxane.

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enoic acid can be reduced by a peroxidase to form 5-hydroxyeicosatetraenoic acid (5-HETE). LTA_4 is an unstable intermediate that can be enzymatically hydrolyzed by LTA_4 hydrolase to generate LTB_4 or conjugated with glutathione by LTC_4 synthase to generate LTC_4, which can be released and further metabolized extracellularly to LTD_4 and LTE_4. LTC_4, LTD_4, and LTE_4 are known collectively as cysteinyl leukotrienes (cys-LTs). LTA_4 in an aqueous environment is quickly and nonenzymatically degraded to Δ^6-*trans*-LTB_4s or 5,6-diHETEs (7). In some cells, LTA_4 is converted to LTC_4 not by LTC_4 synthase but by microsomal glutathione *S*-transferase II (8).

Neutrophils, or polymorphonuclear leukocytes (PMNs), are essential cells in innate immune and inflammatory responses. They are usually the first cells to migrate out of the blood stream and into sites of inflammation, and they help control infection by phagocytizing microbes, by releasing toxic substances such as reactive oxygen species, enzymes (such as lysozyme or serine proteases) (9), or peptides (such as defensins) (10), and by producing mediators that help recruit other cells to the site, such as LTB_4 or cytokines such as interleukin-8 (11) or macrophage-inflammatory proteins (12). Deficiencies in the number of circulating PMNs in the blood (neutropenia) are associated with increased risk of bacterial and fungal infection (13, 14). AA-containing phospholipids are abundant in intracellular membranes of PMNs (15), and PMNs are a major source of eicosanoids, particularly LTB_4, at sites of inflammation (16).

An interesting feature of the synthesis of LTs and other eicosanoids is the common occurrence of transcellular metabolism (17). Although many cells do not contain the complete enzyme cascade necessary to produce eicosanoids from phospholipids, it is possible for cells to produce and export intermediates that are taken up by neighboring cells for further metabolism. One example of this occurrence is the transfer of LTA_4 produced by PMNs into platelets, which are then able to produce LTC_4 (18).

The overwhelming majority of studies concerning the production of LTs by PMNs have been performed with human cells, undoubtedly because of the relevance to human pathophysiology but also because of the ability and relative ease of obtaining high numbers of cells from volunteers. However, the potential of using animal models in PMN research is indisputable. For instance, the mouse offers an enormous wealth of well-established models of human diseases and a constantly growing supply of transgenic animals that could provide unique genetic approaches to test specific biochemical pathways. Yet, the small yield of PMNs from mouse peripheral blood has slowed progress in the knowledge of mouse PMNs, their capacity to generate LTs, and fundamental studies of in vivo LT regulation using knockout mice. Recently, however, it has been reported that mouse bone marrow PMNs can be obtained in useful numbers that are functionally very similar to peripheral blood PMNs (19, 20). In this study, the synthesis of eicosanoids, both COX and 5-LO products, by mouse bone marrow PMNs from wild-type and knockout mice was examined in response to extracellular stimuli. Also, the ability to transfer LTA_4 from murine PMNs to a platelet-like cell line was tested. 5-LO knockout mice were used to establish, for the first time, the potential for PMN interaction with peritoneal macrophages to demonstrate transcellular biosynthesis between these two inflammatory cells.

EXPERIMENTAL PROCEDURES

Materials

Female 6–10 week old wild-type (C57BL/6J) and 5-LO-deficient (B6.129S2-Alox5^{tm1Fun}/J) mice were purchased from Jackson Laboratory (Bar Harbor, ME). Calcium ionophore A23187 and zymosan were purchased from Sigma-Aldrich (St. Louis, MO). Zymosan was prepared by boiling in phosphate-buffered saline, washing three times, and counting the particles with a phase-contrast microscope. Opsonized zymosan was prepared by incubating zymosan for 30 min at 37°C with fresh mouse serum. Percoll was obtained from Amersham Biosciences (Piscataway, NJ). Standard eicosanoids [d_4]LTB_4 (≥97 atom %D), [d_8]5-HETE (≥98 atom %D), [d_4]TXB_2 (≥98 atom %D), [d_4]PGE_2 (≥98 atom %D), LTF_4, and LTA_4 methyl ester were purchased from Cayman Chemical (Ann Arbor, MI). LTA_4 free fatty acid was prepared by hydrolysis of the methyl ester in acetone/NaOH as described previously (21).

Cell isolation, culture, and stimulation

Human PMNs (hPMNs) were isolated from peripheral blood of healthy volunteers as described previously (22). Mouse bone marrow cells (mBMCs) were obtained from the femurs and tibias of mice as described previously (19). Briefly, legs were dissected from the animals, bones were cleaned, and marrows were flushed with HBSS without calcium, magnesium, bicarbonate, or phenol red (Mediatech, Inc., Herndon, VA) using a 25 gauge needle. After gently dislodging cell aggregates with a plastic Pasteur pipette, cells were washed twice in HBSS, resuspended in 3 ml of HBSS, and layered on top of a three-step Percoll gradient (3 ml each of 52, 64, and 72% Percoll in HBSS). After a 30 min centrifugation at 1,000 *g*, three distinct fractions of cells were observed at the 0–52, 52–64, and 64–72% interfaces. Cells were harvested and washed in Ca/Mg-free HBSS before stimulation. The 64–72% interface cells have been characterized as being 94–95% mature neutrophils (19, 20). As an alternative to the Percoll gradient, mBMCs were subjected to hypotonic lysis of red blood cells by resuspending in 1 ml of 34 mM sodium chloride, followed by the addition of 1 ml of 0.3 M sodium chloride/0.01 M sucrose and washing in Ca/Mg-free HBSS.

Human megakaryoblast-like MEG-01 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and a penicillin/streptomycin solution.

Resident mouse peritoneal macrophages were obtained from peritoneal lavage as described previously (23), plated on tissue culture-treated 60 mm Petri dishes (2–4 × 10^6 cells/dish), and kept in a 37°C incubator for 2 h before the experiment. Macrophages were then washed three times in Ca/Mg-free HBSS to remove nonadherent cells and stimulated as described below.

PMNs, mBMCs, and MEG-01 cells were washed and resuspended in Ca/Mg-free HBSS at a concentration of 1 × 10^6 cells/ml and stimulated with calcium ionophore A23187 (0.1–2 μM, 10 min), fMLP (1 or 10 μM, 10 min), or zymosan (30 particles/cell, 60 min) at 37°C, after addition of $CaCl_2$ (2 mM) and $MgCl_2$ (0.5 mM), in a total volume of 1 ml.

Reactions were terminated by the addition of ice-cold methanol (1 ml) containing 2 ng each of internal standards [d_4]LTB_4 and [d_8]5-HETE and 5 ng each of [d_4]TXB_2, [d_4]PGE_2, and LTF_4. The synthetic leukotriene LTF_4, which has never been reported to be present in biological samples, was used as an internal standard for cys-LTs. Samples were diluted with water to a final methanol concentration of <15% and then extracted using a solid-phase extraction cartridge (Strata C18-E, 100 mg/ml; Phenomenex, Torrance, CA). The eluate (1 ml of methanol) was dried down and solubilized in 40 μl of HPLC solvent A (8.3 mM acetic acid buffered to pH 5.7 with ammonium hydroxide) plus 20 μl of HPLC solvent B (acetonitrile-methanol, 65:35, v/v).

Eicosanoid measurements by reverse-phase HPLC and electrospray mass spectrometry

An aliquot of each sample (25 μl) was injected into a C18 HPLC column (Columbus 150 × 1 mm, 5 μm; Phenomenex) and eluted at a flow rate of 50 μl/min with a linear gradient from 25% to 100% of HPLC solvent B, which was increased from 25% to 85% in 24 min, to 100% in 26 min, and held at 100% for a further 12 min. The HPLC system was directly interfaced into the electrospray source of a triple quadrupole mass spectrometer (Sciex API 3000; PE-Sciex, Thornhill, Ontario, Canada) for mass spectrometric analysis in the negative ion mode using multiple reaction monitoring of the specific transitions m/z 319 → 115 for 5-HETE, m/z 335 → 195 for LTB_4 and Δ^6-*trans*-LTB_4s, m/z 351 → 195 for 20-OH-LTB_4, m/z 365 → 195 for 20-COOH-LTB_4, m/z 335 → 115 for 5,6-diHETEs, m/z 624 → 272 for LTC_4, m/z 495 → 177 for LTD_4, m/z 438 → 333 for LTE_4, m/z 351 → 233 for PGD_2, m/z 351 → 271 for PGE_2, m/z 369 → 169 for TXB_2, m/z 327 → 116 for [d_8]5-HETE, m/z 339 → 197 for [d_4]LTB_4, m/z 567 → 171 for LTF_4, m/z 373 → 173 for [d_4]TXB_2, and m/z 355 → 275 for [d_4]PGE_2. Quantitation was performed using standard isotope dilution as described previously (24).

RESULTS

Eicosanoid production by mouse bone marrow PMNs and hPMNs

The ability of human peripheral blood PMNs to synthesize LTs in response to extracellular stimuli has been studied for more than two decades (25). However, very little is known about eicosanoid synthesis by mouse neutrophils. A well-known stimulus of the $cPLA_2\alpha$/5-LO pathway, the calcium ionophore A23187 was initially examined to induce LT production in mBMCs. Incubation of mBMCs with 2 μM A23187 for 10 min resulted in the formation of different COX- and 5-LO-derived eicosanoids, as determined by online liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques (**Fig. 1**). mBMCs were isolated from femurs and tibias and subjected to density gradient centrifugation. Previous studies had separated cells into bands based on Percoll concentration. The top band (band 1, 0–52% Percoll) contained mononuclear-like cells, the second band (band 2, 52–64% Percoll) was a mixture of mature and immature neutrophils, and the third band (band 3, 64–72% Percoll) was >95% mature neutrophils (19). Although the level of TXB_2 production was similar in these fractionated bone marrow cells, 5-LO products were abundantly synthesized by band 2 (Fig. 1B) and band 3 (Fig. 1C) cells. Δ^6-*Trans*-LTB_4 and 5,6-diHETE isomers (data not shown), which are nonenzymatic derivatives of LTA_4, were also present in band 2 and band 3. The unseparated mixture of mBMCs showed a very similar eicosanoid profile to the two denser fractions (Fig. 1D) and for this reason was used in all subsequent experiments. The production of eicosanoids from hPMNs stimulated with A23187 is shown for comparison (Fig. 1E).

The quantities of LTB_4 and 5-HETE produced by A23187-stimulated mBMCs were similar to those produced by hPMNs (**Table 1**). hPMNs synthesized much lower amounts of TXB_2 and PGE_2 than mBMCs. As reported previously (22), hPMNs did produce detectable levels of LTC_4, possibly because of contamination with eosinophils, and significant quantities of 20-OH-LTB_4 (Fig. 1E), an ω-oxidation derivative of LTB_4 mediated by CYP4F4 (26). This P450 metabolite of LTB_4 was completely absent in the stimulated mBMC incubation (Fig. 1A–D).

Quantitative comparison of the production of 5-LO-derived eicosanoids by hPMNs and mBMCs (Table 1), as determined by LC-MS/MS and the corresponding standard isotope dilution curves, revealed remarkable similarities in 5-LO pathway activation. Eicosanoid production by mBMCs could also be initiated by the formylated peptide fMLP and the phagocytic stimulus zymosan, coated (opsonized) or uncoated with complement fragments (Table 1). The concentration of fMLP necessary to trigger a robust synthesis of LTs was 10 μM, significantly higher than that observed for hPMNs (0.1 μM) (22), suggesting lower expression of the formylated peptide receptor in less mature PMNs. This was consistent with recent findings that a concentration as high as 10 μM fMLP is necessary for full superoxide production and primary granule release by mouse bone marrow PMNs (20).

LT production by mBMCs induced by A23187 was time- and dose-dependent (**Fig. 2**), with maximal effects observed at 0.5 μM and 10 min. These incubation conditions were used in all subsequent experiments with calcium ionophore.

Eicosanoid production and LTA_4 intake by MEG-01 cells

It is known that human platelets are able to capture LTA_4 produced by PMNs and use it to synthesize LTC_4, thus providing a source of this biologically active cys-LT (18). We tested mBMCs for the ability to transfer LTA_4 to other cells. As an acceptor cell, we tested the human megakaryoblastic cell line MEG-01. These cells have been shown to shed particles morphologically similar to platelets, express $cPLA_2\alpha$ and TXA_2 synthase, and release AA in response to A23187 (27, 28). On the other hand, the use of this cell line helps circumvent some of the problems associated with the use of primary platelets, mainly aggregation during isolation. Upon stimulation with 0.5 μM A23187 for 10 min (**Fig. 3A**), MEG-01 cells produced TXB_2 and PGE_2 but not 5-LO-derived eicosanoids. None of these eicosanoids was detected in nonstimulated, control cells (data not shown). When LTA_4 was added to the incubation medium of nonstimulated MEG-01 cells, LTB_4 and LTC_4 were produced (Fig. 3B), indicating that, like plate-

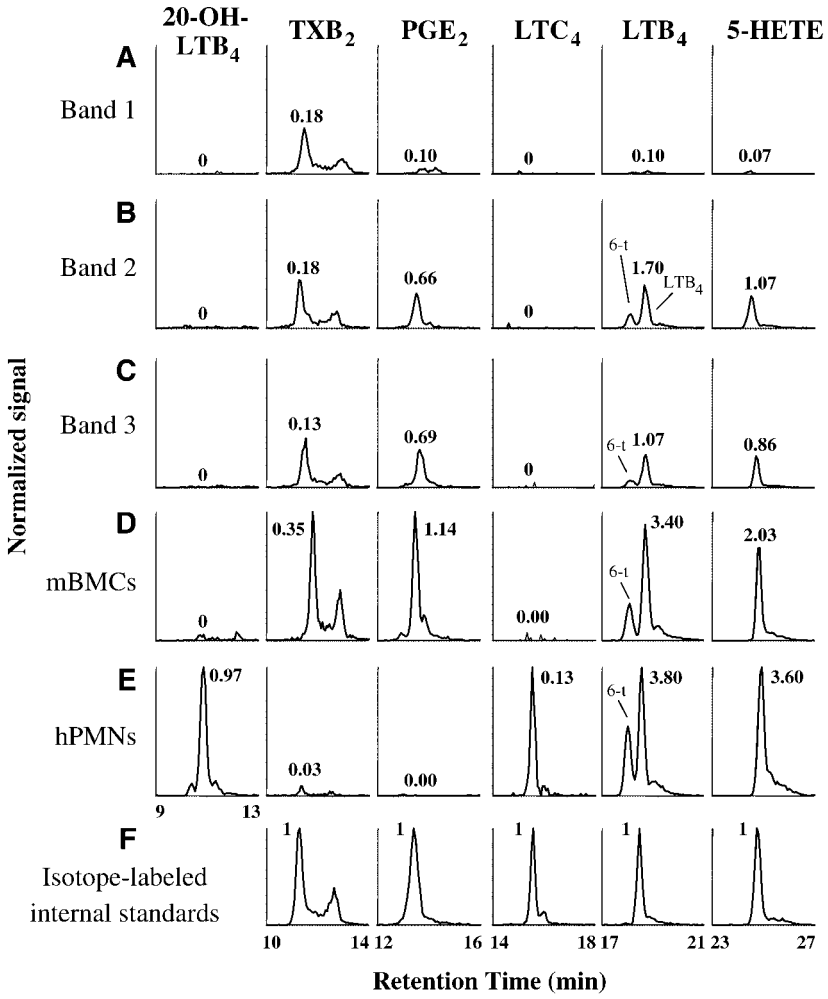

Fig. 1. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of eicosanoids produced by A23187-stimulated cells. Mouse bone marrow cells (mBMCs) separated according to their density on a Percoll gradient were stimulated for 10 min at 37°C with 2 μM A23187: band 1, 0–52% fraction (A); band 2, 52–64% fraction (B); band 3, 64–72% fraction (C). The complete mixtures of isolated mBMCs (D) and human peripheral blood polymorphonuclear leukocytes (hPMNs) (E) were stimulated under identical conditions. In all cases, 1×10^6 cells were used and eicosanoids were identified by their HPLC retention times and by specific collision-induced mass transitions in LC-MS/MS experiments, as detailed in Experimental Procedures. F: The ion intensity for each eicosanoid (column) is normalized to the deuterium-labeled internal standards as 1.0. Thromboxane B_2 (TXB_2) emerged from the reverse-phase HPLC column as two interconverting ring-opened and ring-closed forms. The two HPLC peaks in the leukotriene B_4 (LTB_4) column correspond to Δ^6-*trans*-LTB_4 isomers (6-t) and LTB_4. 5-HETE, 5-hydroxyeicosatetraenoic acid; PGE_2, prostaglandin E_2.

lets, these cells possess mechanisms to take up and stabilize exogenous LTA_4 for transcellular biosynthesis of cys-LTs, and they express LTC_4 synthase. Unlike platelets, however, MEG-01 cells also express LTA_4 hydrolase, because they are able to synthesize LTB_4 from exogenous LTA_4.

Transcellular synthesis of cys-LTs in mBMC/MEG-01 mixtures

When MEG-01 cells and mBMCs were incubated together in the presence of A23187 (**Fig. 4**), all eicosanoids produced by either cell alone were observed, as well as a new component corresponding to LTC_4 and a smaller peak corresponding to LTD_4 (based on mass spectrometric criteria). These cys-LTs were absent when both types of cells were incubated separately (Figs. 1, 3), providing evidence that LTA_4 generated in mBMCs, most likely mature PMNs, was transported into MEG-01 cells and used as a substrate for MEG-01 LTC_4 synthase. When the number of MEG-01 cells in the coincubation experiment was varied relative to number of mBMCs, the amount of LTC_4 produced correlated with the number of MEG-01 cells (**Fig. 5**), suggesting that the amount of LTC_4 synthase in the acceptor cell was the limiting factor for transcellular LTC_4 synthesis under these conditions.

The transfer of LTA_4 between mBMCs and MEG-01 cells, as determined by the synthesis of LTC_4, also occurred when opsonized zymosan was used as a stimulus (**Fig. 6**), demonstrating that the phenomenon of transcel-

TABLE 1. Quantitation of LTB_4, LTC_4, and 5-HETE produced by stimulated mBMCs or hPMNs

Sample	LTB_4	5-HETE	LTC_4
		ng/10^6 cells	
mBMCs			
Unstimulated	0.00 ± 0.00	0.01 ± 0.00	n.d.
A23187 (2 μM)	1.01 ± 0.16	2.71 ± 0.33	0.01 ± 0.00
hPMNs			
A23187 (2 μM)	1.04 ± 0.24	4.03 ± 0.54	0.44 ± 0.16
mBMCs			
Nonopsonized zymosan	0.14 ± 0.05	0.25 ± 0.09	n.d.
Opsonized zymosan	0.19 ± 0.05	0.42 ± 0.08	n.d.
fMLP (10^{-6} M)	0.02 ± 0.01	0.07 ± 0.02	n.d.
fMLP (10^{-5} M)	0.20 ± 0.05	0.52 ± 0.08	n.d.

5-HETE, 5-hydroxyeicosatetraenoic acid; hPMN, human peripheral blood polymorphonuclear leukocyte; LT, leukotriene; mBMC, mouse bone marrow cell; n.d., not detected. mBMCs and hPMNs (1×10^6 cells) were stimulated with 2 μM A23187 (10 min), 10^{-5} or 10^{-6} M fMLP (10 min), or 30 particles of nonopsonized or opsonized zymosan per cell (60 min). LTB_4, LTC_4, and 5-HETE were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and quantitated by standard isotope dilution. Values shown are averages ± SEM (n = 3).

lular metabolism may also play a role during physiological, phagocytic stimulation of mouse PMNs in the presence of other cells. Under these experimental conditions, LTD_4 was detected at higher levels compared with LTC_4 (**Table 2**). This may be attributable simply to the longer incubation time required for zymosan stimulation (1 h). An unexpected observation was the marked decrease in 5-HETE production in mBMC/MEG-01 cell mixtures compared with mBMCs alone (data not shown).

Absence of transcellular cys-LT production in 5-LO-null mBMC/MEG-01 cell mixtures

Mice with a targeted disruption of the gene encoding 5-LO were generated in 1994 (29), providing a very useful model to study the roles of LTs in models of disease. We tested mBMCs from these transgenic mice as a negative control in the transfer of LTA_4 to MEG-01 cells. When mBMCs from these animals were stimulated with A23187 (**Fig. 7A**), there was increased production of the COX-derived eicosanoids TXB_2 and PGE_2, like their wild-type counterparts, indicating an intact ability to stimulate $cPLA_2\alpha$ and release free arachidonate. However, as expected, none of the products of 5-LO were detected. When cells were coincubated with MEG-01 cells and stimulated with A23187 (Fig. 7B), no LTC_4 or LTD_4 was detected, verifying the complete absence of 5-LO in these mice and also supporting the hypothesis that mBMCs were the source of LTA_4 in this mixed cell system.

Transcellular production of LTC_4 in mixtures of mBMCs and mouse peritoneal macrophages

Interactions between PMNs and macrophages are understood to be an important part of the inflammatory response. Both types of cells are known sources of LTs: LTB_4 in the case of PMNs and LTC_4 in the case of resident peritoneal macrophages. Because of the ability of these cells to synthesize the final products of their LT synthesis pathways, transcellular exchange of LTA_4 has not been reported. We tested this possibility by using peritoneal macrophages from 5-LO-deficient mice as potential acceptor cells. Wild-type mouse peritoneal macrophages produced TXB_2 and PGE_2 as well as LTC_4, 5-HETE, and low levels of LTB_4 when stimulated with 2 μM A23187 (data not shown). As expected, macrophages from 5-LO-deficient mice showed no detectable 5-LO products but retained the ability to synthesize TXB_2 and PGE_2 (**Fig. 8A**). In fact, these prostanoids were produced at higher levels than in wild-type macrophages (data not shown), suggesting that the absence of 5-LO provided more AA to the COX pathway. When mBMCs from wild-type mice were coincubated with 5-LO-deficient peritoneal macrophages in the presence of A23187 (Fig. 8B), LTB_4 and LTC_4 became abundant 5-LO products, providing evidence that macrophages are able to synthesize LTC_4 by using LTA_4 from surrounding PMNs, a situation that conceivably could occur at sites of inflammation.

DISCUSSION

In spite of extensive interest in neutrophil biochemistry, very few studies have been performed on PMNs from sources other than human peripheral blood, particularly from the standpoint of arachidonate metabolism. Because PMNs originate and mature in the bone marrow before being released into the vasculature, bone marrow is a good source of these cells. It has been reported that human bone marrow PMNs are morphologically similar to peripheral blood PMNs, that they are able to phagocytize opsonized Oil Red O lipopolysaccharide particles, and that they respond to the bacterial peptide fMLP or the protein kinase C activator phorbol

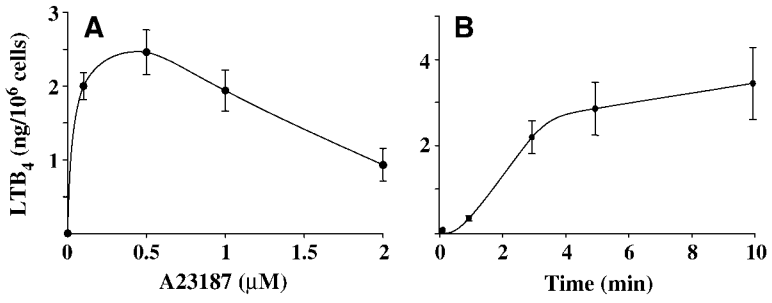

Fig. 2. Dose response and time course of A23187-induced LTB_4 synthesis by mBMCs. A: mBMCs (1×10^6 cells) were stimulated for 10 min at 37°C with the indicated concentrations of A23187. B: Cells were incubated with 0.5 μM A23187 for the times indicated. After incubation, LTB_4 was analyzed and quantified by LC-MS/MS and standard isotope dilution. Results shown are averages ± SEM (n = 3).

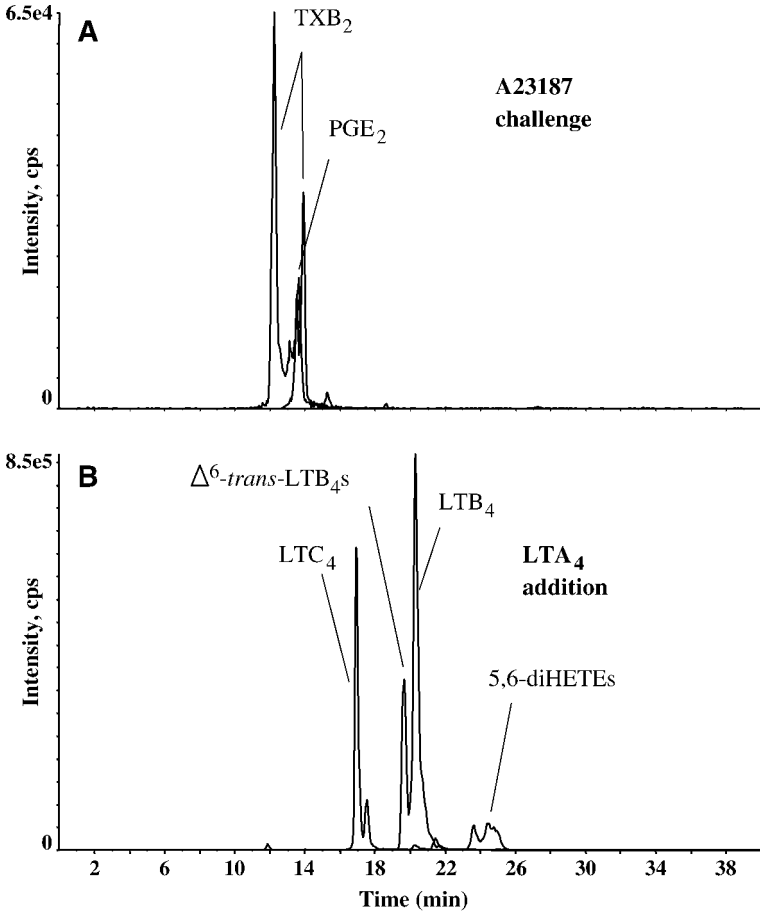

Fig. 3. Analysis of eicosanoids produced by MEG-01 cells stimulated with A23187 or incubated with exogenous LTA_4. MEG-01 cells (1×10^6) were incubated for 10 min at 37°C with 0.5 μM A23187 (A) or with 1 μM LTA_4 (B). Eicosanoids were analyzed by LC-MS/MS. Ion intensity is expressed as counts per second (cps). Responses for deuterium-labeled internal standards were identical (not shown).

myristate acetate by producing superoxide, although to a lesser extent than peripheral blood PMNs (30). A recent study with mouse bone marrow PMNs reported superoxide production in response to phorbol myristate acetate to a similar extent than in mouse peripheral blood PMNs, but significantly less than in mouse thioglycolate-elicited peritoneal PMNs (19). Both superoxide production and degranulation have been observed in mouse bone marrow PMNs treated with fMLP and the immunostimulatory peptide WKYMVm-NH_2 (20, 31). These data demonstrate that bone marrow PMNs exhibit at least some of the functional responses of circulating PMNs

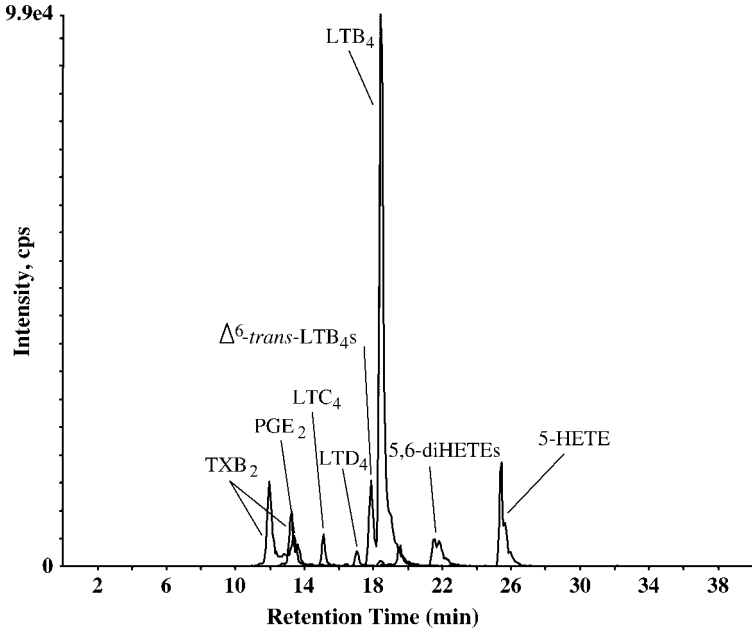

Fig. 4. Transcellular synthesis of LTC_4 in mixtures of mBMCs and MEG-01 cells stimulated with A23187. Mixtures of mBMCs (1×10^6) and MEG-01 cells (1×10^6) were incubated for 10 min at 37°C with 0.5 μM A23187. Eicosanoids were analyzed by LC-MS/MS. Ion intensity is expressed as counts per second (cps). Responses for deuterium-labeled internal standards for each eicosanoid are not shown.

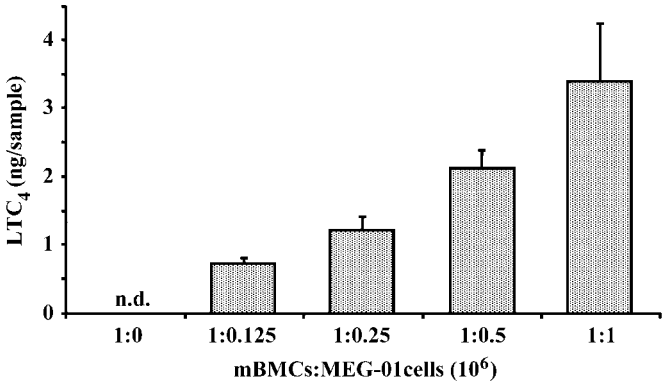

Fig. 5. Effects of the number of MEG-01 cells on the transcellular synthesis of LTC_4 in mixtures with mBMCs stimulated with A23187. mBMCs (1×10^6) were incubated with the indicated numbers of MEG-01 cells for 10 min at 37°C with 0.5 μM A23187. Eicosanoids were analyzed by LC-MS/MS, and LTB_4 was quantitated by standard isotope dilution. Results shown are averages ± SEM (n = 3). n.d., not detected.

TABLE 2. Quantitation of LTC_4 and LTD_4 produced by mBMCs and MEG-01 cells stimulated with A23187 or zymosan

Sample	LTC_4	LTD_4
	ng/sample	
A23187	3.39 ± 0.85	0.53 ± 0.12
Opsonized zymosan	0.10 ± 0.03	0.21 ± 0.05

MEG-01 cells (1×10^6) were incubated with mBMCs (1×10^6) and stimulated with 0.5 μM A23187 (10 min) or 30 particles of opsonized zymosan per mBMC (60 min). LTC_4 and LTD_4 were analyzed by LC-MS/MS and quantitated by standard isotope dilution. Values shown are averages ± SEM (n = 3).

and that PMNs display quantitatively different properties according to their maturation state.

Our experimental results provide evidence that mouse bone marrow PMNs are able to synthesize TXB_2, PGE_2, and LTB_4 when exposed to calcium ionophore A23187 or to physiological stimuli such as fMLP or the phagocytic stimulus zymosan. To our knowledge, this is the first report of eicosanoid production induced in bone marrow PMNs, and it is consistent with published results showing that other murine hematopoietic cells, such as the mouse thioglycolate-induced peritoneal PMNs, release PGE_2 and LTB_4 upon incubation with rickettsiae, the bacterial cause of typhus in humans (32). In both purified bone marrow PMNs and mBMCs, LTB_4 and 5-HETE were found to be synthesized at very similar levels as that in hPMNs, but PGE_2 and TXB_2 were produced in relatively higher amounts, indicating possible higher expression of COX or contamination of these cells with other COX-expressing cells. In contrast, although hPMN preparations showed reproducible synthesis of LTC_4, most likely as a result of contamination with eosinophils, this cys-LT was completely below detection levels in bone marrow PMNs under these experimental conditions.

The absence of LTC_4 background in mBMCs makes this an excellent cell preparation for studies of the transcellular transfer of LTA_4 to LTC_4 synthase-expressing cells. Transcellular metabolism of eicosanoids is a common mechanism for LT biosynthesis, and PMNs have been shown to synthesize and transfer LTA_4 to cells that are able to synthesize other LTs, such as red blood cells (LTB_4), endothelial cells (LTC_4), and platelets (LTC_4) (17). An elegant study involving bone marrow chimeras of 5-LO- and LTA_4 hydrolase-deficient mice has provided unequivocal evidence that transcellular synthesis of LTB_4 occurs in vivo during zymosan-induced peritonitis and AA-induced acute skin inflammation, thus illustrating

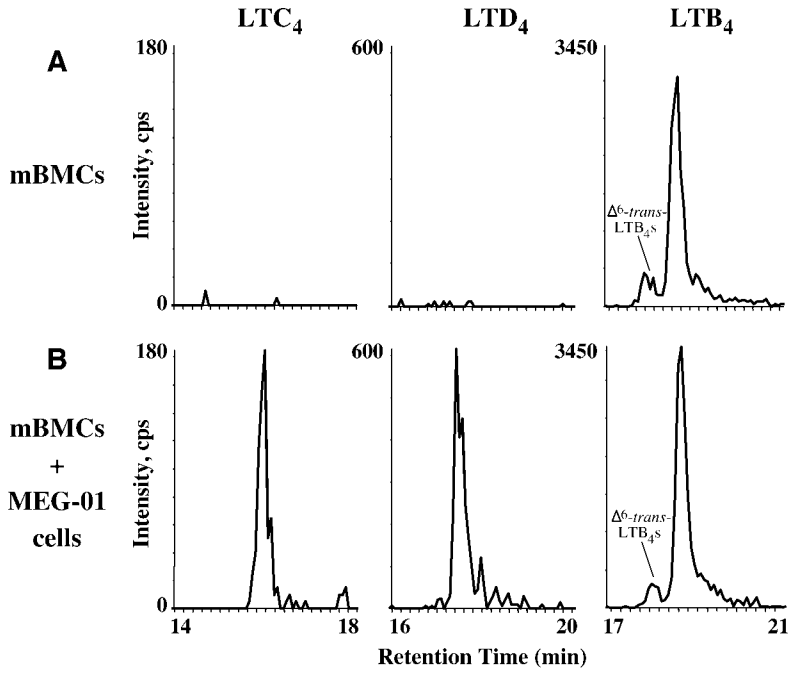

Fig. 6. Transcellular synthesis of LTC_4 in mixtures of mBMCs and MEG-01 cells stimulated with opsonized zymosan. mBMCs (1×10^6) were incubated without (A) or with (B) 1×10^6 MEG-01 cells and 3×10^7 particles of opsonized zymosan for 1 h at 37°C. Eicosanoids were analyzed by LC-MS/MS. Ion intensity is expressed as counts per second (cps). Responses for deuterium-labeled internal standards are not shown.

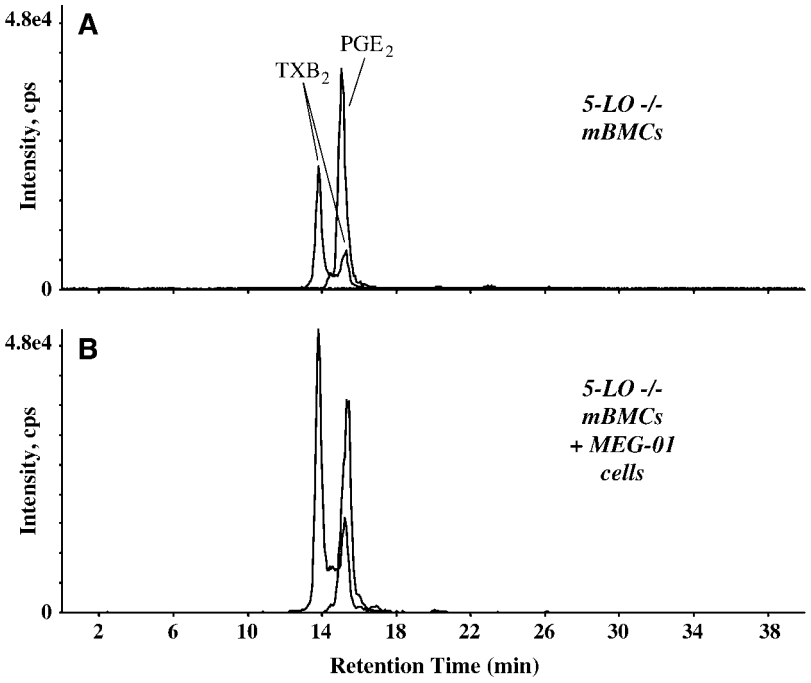

Fig. 7. Effects of A23187 on eicosanoid synthesis by mBMCs from 5-lipoxygenase (5-LO)-deficient mice. mBMCs (1×10^6) from 5-LO-deficient mice were incubated for 10 min at 37°C with 0.5 μM A23187 in the absence (A) or presence (B) of 1×10^6 MEG-01 cells. Eicosanoids were analyzed by LC-MS/MS. Ion intensity is expressed as counts per second (cps). Responses for deuterium-labeled internal standards are not shown.

the biological relevance of this phenomenon (33). When mBMCs were incubated with platelet-like MEG-01 cells and then challenged with A23187, transcellular synthesis of LTC_4 was detected, and even with the relatively low amounts of LTA_4 produced by zymosan-treated mBMCs, transcellular LTC_4 could be observed. LTC_4 production correlated with the number of MEG-01 cells present, as has been observed in experiments with human PMNs and platelets stimulated with A23187 (18) or opsonized zymosan (34).

The 5-LO-deficient mouse model has been studied extensively and has revealed interesting aspects of the biology of LTs. In these transgenic mice, no LT production was observed in in vivo models of inflammation or in primary cells, such as alveolar and peritoneal macrophages and bone marrow-derived mast cells (35). As expected, our

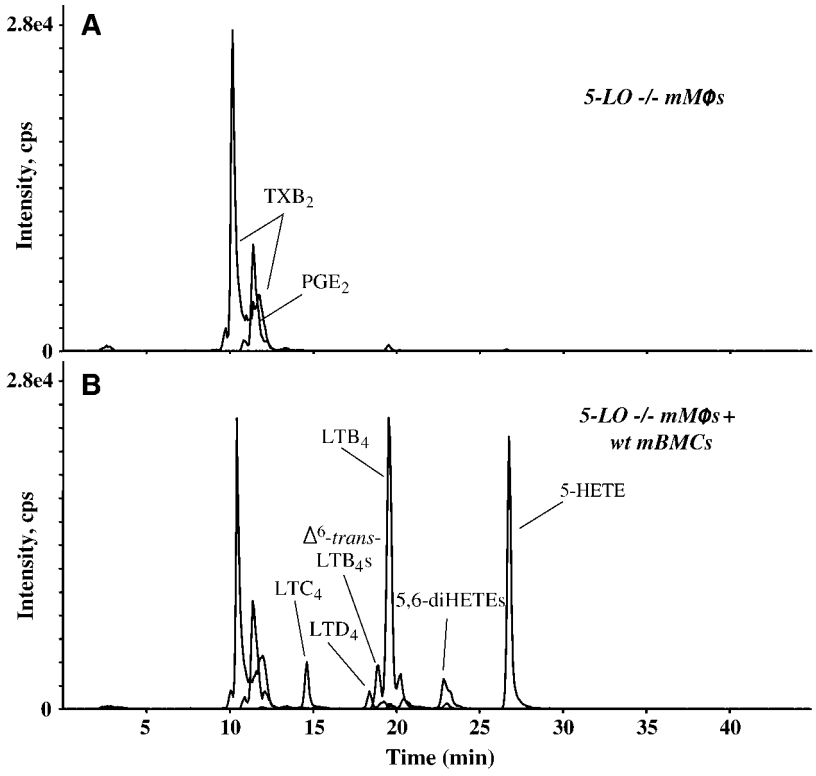

Fig. 8. Transcellular synthesis of LTC_4 in mixtures of mBMCs and peritoneal macrophages. Mouse peritoneal macrophages (mMΦs; 2×10^6) from 5-LO-deficient mice were incubated in the absence (A) or presence (B) of mBMCs (1×10^6) from wild-type mice for 10 min at 37°C with 2 μM A23187. Eicosanoids were analyzed by LC-MS/MS. Ion intensity is expressed as counts per second (cps). Responses for deuterium-labeled internal standards are not shown.

experiments confirmed the absence of LT production in 5-LO-deficient mBMCs and in peritoneal macrophages stimulated with A23187, whereas the ability to synthesize TXB_2 and PGE_2 was not impaired. Because of the central role of PMNs in inflammation, this lack of LT production helps explain the reduced signs of inflammation that 5-LO-deficient mice present under certain experimental conditions (36). It is interesting that although LTC_4 synthesis was impaired by the lack of 5-LO in peritoneal macrophages, these cells retained expression of LTC_4 synthase, as demonstrated by their ability to use PMN-produced LTA_4 to generate LTC_4. This observation extends the examples of the transcellular metabolism of LTs by including two of the main cellular components of inflammatory responses. The fact that this particular transcellular exchange would have been much more difficult to detect with human primary cells also highlights the usefulness of mBMCs in PMN research, especially regarding LT synthesis.

In summary, these results describe and characterize for the first time a useful model for the study of LT biosynthesis in mouse PMNs. This will now make it possible to use the increasing number of transgenic animals involving genes relevant to the synthesis and metabolism of LTs and other eicosanoids, as well as animal models of inflammation and disease. Such studies have not been feasible previously because of the enormous difficulty of obtaining peripheral blood PMNs in sufficient numbers from mice. Additionally, these data also validate the use of MEG-01 cells as acceptor cells in the transcellular biosynthesis of LTs, avoiding the difficulties associated with the use of platelets from human donors.

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