Maresin conjugates in tissue regeneration biosynthesis enzymes in human macrophages

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Macrophages are central in coordinating immune responses, tissue repair, and regeneration, with different subtypes being associated with inflammation-initiating and proresolving actions. We recently identified a family of macrophage-derived proresolving and tissue regenerative molecules coined maresin conjugates in tissue regeneration (MCTR). Herein, using lipid mediator profiling we identified MCTR in human serum, lymph nodes, and plasma and investigated MCTR biosynthetic pathways in human macrophages. With human recombinant enzymes, primary cells, and enantiomerically pure compounds we found that the synthetic maresin epoxide intermediate 13S,14S-eMaR (13S,14S-epoxy- 4Z,7Z,9E,11E,16Z,19Z-docosahexaenoic acid) was converted to MCTR1 (13R-glutathionyl, 14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z,19Z-docosahexaenoic acid) by LTC_4S and GSTM4. Incubation of human macrophages with LTC_4S inhibitors blocked LTC_4 and increased resolvins and lipoxins. The conversion of MCTR1 to MCTR2 (13R-cysteinylglycinyl, 14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z,19Z-docosahexaenoic acid) was catalyzed by γ-glutamyl transferase (GGT) in human macrophages. Biosynthesis of MCTR3 was mediated by dipeptidases that cleaved the cysteinyl-glycinyl bond of MCTR2 to give 13R-cysteinyl, 14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z,19Z-docosahexaenoic acid. Of note, both GSTM4 and GGT enzymes displayed higher affinity to 13S,14S-eMaR and MCTR1 compared with their classic substrates in the cysteinyl leukotriene metabolome. Together these results establish the MCTR biosynthetic pathway and provide mechanisms in tissue repair and regeneration.

proresolving mediators | inflammation | omega-3 fatty acids | regeneration | eicosanoids

Resolution of acute inflammation is an orchestrated host response to injury and/or infection that leads to the clearance of bacteria and tissue debris as well as tissue repair and regeneration (1–3). Central to the regulation of resolution responses is a novel genus of endogenous mediators termed specialized proresolving mediators (SPM) (2). They actively counterregulate production of inflammation-initiating signals including cytokines, chemokines, and lipid mediators and regulate leukocyte trafficking and phenotype as well as promote tissue repair and regeneration (1, 2, 4–6). At the site of inflammation leukocytes are key in the production of both inflammation-initiating (7, 8) and proresolving mediators (2, 4) because they carry the necessary enzymatic machinery for the stereoselective conversion of precursor essential fatty acids to the bioactive mediators.

Macrophages are central players in the acute inflammatory response governing both initiation and resolution phases (3, 4, 9–12). Distinct macrophage subtypes are involved in the regulation of these different phases of acute inflammatory responses, with macrophages from the resolution phase expressing higher levels of SPM biosynthetic enzymes (12). Recent evidence also demonstrates that lipid mediator profiles change with macrophage phenotype. Classic macrophages express higher levels of inflammation-initiating eicosanoids, whereas alternatively activated cells display higher levels of proresolving mediators (6, 13). Recently, we reported that macrophages produce a family of bioactive peptide-conjugated mediators coined maresin conjugates in tissue regeneration (MCTR) (4) and the complete stereochemistries of MCTR1 (13R-glutathionyl, 14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z,19Z-docosahexaenoic acid) [International Union of Pure and Applied Chemistry nomenclature: (4Z,7Z,9E,11E,13R,14S,16Z,19Z)-13-(((R)-2-amino-3-((carboxymethyl)amino)-3-oxopropyl)thio)-14-hydroxydocosa-4,7,9,11,16,19-hexaenoic acid], MCTR2 (13R-cysteinylglycinyl, 14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z,19Z-docosahexaenoic acid), and MCTR3 (13R-cysteinyl, 14S-hydroxy-4Z,7Z,9E,11E,13R,14S,16Z,19Z-docosahexaenoic acid) were established (14). Each displays potent bioactions in stimulating human phagocyte functions, promotes the resolution of bacterial infections, counterregulates the production of proinflammatory mediators, and promotes tissue repair and regeneration (14).

In the proposed MCTR biosynthetic pathway (4), human macrophage 12-lipoxygenase is the initiating enzyme, converting docosahexaenoic acid to 14S-hydro(peroxy)-4Z,7Z,10Z,12E,16Z,19Z-docosahexaenoic acid and then to 13S,14S-epoxy-4Z,7Z,9E,11E,16Z,19Z-docosahexaenoic acid (13S,14S-eMaR). The epoxide intermediate is then enzymatically converted to MCTRs.

Significance

We recently uncovered a family of macrophage-derived molecules, coined maresin conjugates in tissue regeneration, that regulate the system's ability to clear bacteria as well as repair and regenerate damaged tissues. In the present study, we identified enzymes involved in the formation of these potent molecules in human macrophages. These enzymes were shared with the classic cysteinyl leukotrienes, underscoring the presence of conserved biosynthetic motifs in these two functionally distinct lipid mediator families. Inhibition of these pathways upregulated the formation of several specialized proresolving mediator (SPM) families including D- and E-series resolvins. Thus, these illustrate the dynamic nature of the SPM biosynthetic pathways and provide new targets in the resolution of inflammation and regulation of tissue repair and regeneration.

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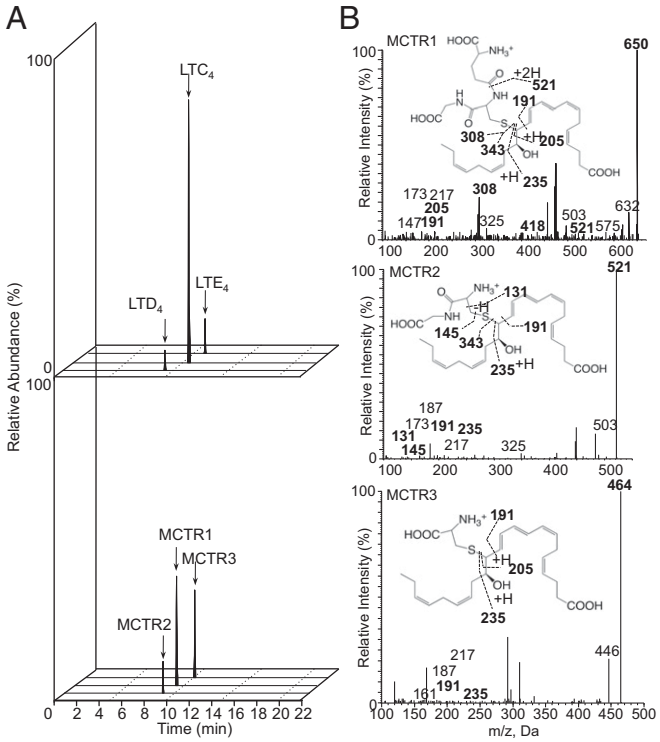

Fig. 1. MCTRs are produced in lymph nodes. Peptide conjugated lipid mediators obtained from human lymph nodes following C18 solid-phase extraction were identified using LM metabololipidomics (*Materials and Methods*). (*A*) Representative multiple reaction monitoring (MRM) chromatograms of peptide conjugated lipid mediators. (*B*) MS-MS spectra used for identification of (*Upper*) MCTR1, (*Middle*) MCTR2, and (*Lower*) MCTR3. Results represent $n = 7$ healthy donors.

For example, in planaria, a GST catalyzes the conversion of the epoxide to MCTR1, which in turn is the proposed precursor to MCTR2 and MCTR3 (4). The identity of the enzymes that catalyze the conversion of 13S,14S-eMaR to MCTR1 and those that catalyze the formation of MCTR2 and the bioactive MCTR3 in human macrophages are of interest. This is because in addition to establishing the biosynthetic route in specific cell types, determining the identity of these enzymes provides essential information on the intrinsic competition between substrates in distinct stages for the inflammation-resolution cascade. This in turn allows for development of more targeted therapeutic strategies because it sheds light on the contribution of each of these pathways in disease pathophysiology as well as provides potential novel therapeutic leads that can focus on selective regulation of resolution pathways. Given the potent biological actions of these molecules (4) and the differential expression of MCTRs in distinct macrophage subtypes (6), here we identified MCTRs in human lymph nodes, serum, and plasma and investigated the human macrophage enzymes involved in the biosynthesis of MCTRs. Both leukotriene C_4 synthase (LTC_4S) and GST-mu 4 (GSTM4) catalyze the formation of MCTR1 and contribute to its biosynthesis in human macrophages. Gamma-glutamyltransferase (GGT) converts MCTR1 to MCTR2, which is then further converted to MCTR3 by a dipeptidase. Together, these results establish the MCTR biosynthetic pathway and identify the enzymes that catalyze these reactions in human macrophages.

Results

MCTRs Are Produced in Human Tissues. To establish the production of MCTR in human systems we used liquid chromatography-tandem MS (LC-MS-MS)–based lipid mediator metabololipidomics to profile human lymph nodes, serum, and plasma. MCTR1 and MCTR3 were identified in all three human tissues, whereas MCTR2 was present in human lymph nodes and serum. Each of these molecules was identified in accordance with published criteria (4, 14), including matching retention times and MS-MS spectra (Fig. 1). We then assessed their amounts in relation to other peptide-conjugated lipid mediators, namely the cysteinyl leukotrienes (7) and protectin conjugates in tissue regeneration (PCTR) (6). MCTR levels in human serum and plasma demonstrated that serum contained significantly higher levels of MCTR1 and MCTR2 (Fig. 1, Fig. S1, and Table 1). In addition, levels for MCTR1, MCTR2, and MCTR3 in each of these tissues were comparable to those of the potent inflammation-initiating cysteinyl leukotrienes and the proresolving and tissue-regenerative PCTR (Table 1).

Role of Human Macrophage LTC_4S and GSTM4 in MCTR Formation. GST enzymes catalyze the formation of bioactive lipid mediators that are peptide-lipid conjugates (7, 8). The proposed homolog of human GSTM4 in planaria promotes formation of MCTR in planaria (4). Therefore, we investigated the expression of both GSTM4 and LTC_4S in human macrophages. Using flow cytometry and fluorescently conjugated antibodies, we found that human macrophages expressed both LTC_4S and GSTM4 (Fig. 2*A*). We next tested whether these enzymes were involved in MCTR biosynthesis. To this end, human macrophages were transfected with shRNA targeting LTC_4S or GSTM4 or a control sequence. In cells transfected with the shRNA to LTC_4S or GSTM4, we found >50% reduction in the expression of these enzymes compared with control scrambled (CS) shRNA (n= 4 independent experiments). We next investigated MCTR production in these cells, and using LC-MS-MS–based lipid mediator profiling found that transfection of cells with shRNA to GSTM4 led to a reduction in MCTR1 (~60%), MCTR2 (~60%), and MCTR3 (~55%; Fig. 2*D*) compared with CS-shRNA transfection. Of note, in these incubations we also observed a significant increase in both maresin (MaR) 1 and MaR2 (Fig. 2*E*). Similar results were obtained when macrophages were transfected with shRNA for LTC_4S (Fig. 2 *D* and *E*).

To further test the role of LTC_4S in MCTR biosynthesis and dynamic modulation of lipid mediator pathways in human macrophages we investigated the regulation of endogenous lipid mediator-SPM pathways by LTC_4S and LT biosynthesis inhibitors. Incubation of human macrophages with MK886 significantly reduced cysteinyl leukotrienes, with LTC_4 levels reduced by ~41%, LTD_4 by ~36%, and LTE_4 by ~29%, in line with published findings (15). MCTR levels were also reduced, with MCTR1 levels reduced from 3.0 ± 0.1 pg/4 $\times 10^6$ cells to 1.3 ± 0.4 pg/4 $\times 10^6$ cells, MCTR2 from 1.5 ± 0.5 pg/4 $\times 10^6$

Table 1. MCTR in human tissue: Relation to cysLT and PCTR

Mediator	Q1	Q3	Lymph node, pg/150 mg	Serum, pg/mL	Plasma, pg/mL
LTC_4	626	189	6.7 ± 3.1	5.9 ± 3.4	2.3 ± 1.0
LTD_4	497	189	0.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.0
LTE_4	440	189	0.5 ± 0.2	1.7 ± 0.4	0.3 ± 0.2
MCTR1	650	191	1.1 ± 0.3	1.9 ± 0.5	0.8 ± 0.1
MCTR2	521	191	0.3 ± 0.2	0.3 ± 0.1	—*
MCTR3	464	191	1.4 ± 0.3	1.1 ± 0.5	0.3 ± 0.0
PCTR1	650	231	2.9 ± 0.6	1.6 ± 0.5	0.6 ± 0.2
PCTR2	521	231	1.6 ± 0.9	0.8 ± 0.3	0.4 ± 0.1
PCTR3	464	231	1.6 ± 0.8	1.7 ± 0.8	0.7 ± 0.1

LMs were extracted using C18 SPE columns and products profiled using LC-MS-MS–based LM metabololipidomics. Products were identified from MS-MS spectra and quantified using MRM with calibration curves specific to each compound. Results are mean ± SEM, $n = 7$ for axillary human lymph nodes and serum and 10 for plasma (see Fig. S1).
*Below the limit of detection (~0.1 pg).

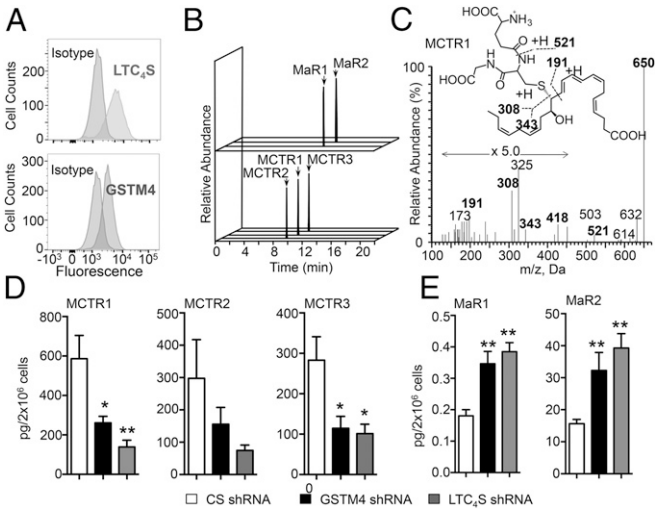

Fig. 2. LTC_4S and GSTM4 promote MCTR biosynthesis in human macrophages. (*A–E*) Human macrophages were prepared from peripheral blood mononuclear cells and the expression of LTC_4S and GSTM4 determined using flow cytometry and fluorescently conjugated antibodies. (*Upper*) Representative histogram plot for human macrophage incubated with anti-LTC_4S or isotype antibodies. (*Lower*) Representative histogram plot for human macrophage incubated with anti-GSTM4 or isotype antibodies. Human macrophages (5×10^6 cells/10 mL) were transfected with shRNA targeting human GSTM4, LTC_4S, or a CS-shRNA then were incubated with *Escherichia coli* (2.5×10^7 cfu/mL) and 14S-hydroperoxy-docosahexaenoic acid (100 nM; $PBS^{+/+}$, pH 7.45, 30 min, 37 °C). Incubations were stopped and products extracted and profiled using metabololipidomics (*Materials and Methods*). (*B*) Representative MRM chromatogram for each of the mediators identified and quantified. (*C*) MS-MS spectrum of MCTR1. (*D* and *E*) Specific bioactive mediators quantified using Q1: M-H (parent ion) and Q3: diagnostic ion in the MS-MS (daughter ion). Results in *A–C* are representative of $n = 4$ donors, *D* and *E* are mean ± SEM, $n = 4$ donors. $^*P < 0.05$, $^{**}P < 0.01$ vs. CS-shRNA.

cells to 0.8 ± 0.2 pg/4 × 10^6 cells, and MCTR3 from 2.4 ± 0.4 pg/4 × 10^6 cells to 0.9 ± 0.1 pg/4 × 10^6 cells (Fig. 3 and Table S1). In these incubations we also identified and quantified proresolving mediators from the arachidonic acid, eicosapentaenoic acid, and docosahexaenoic acid bioactive metabolomes, which were identified in accordance with published criteria including matching retention time and MS-MS fragmentation spectra (13). We also found, in these incubations, up-regulation of select proresolving mediators including resolving (Rv) D2, lipoxin (LX) A_4, and LXB_4. Incubation of human macrophage with another leukotriene and LTC_4S inhibitor, BAY-X-1005, also significantly inhibited both the cysteinyl leukotriene (~47%) and the MCTR (~31%) pathways (Table 1). In addition, we found that this inhibitor regulated several biosynthetic pathways up-regulating the levels of SPM from all three bioactive metabolomes including RvD1, RvD2, MaR1, RvD5, LXA_4, LXB_4, and RvE1 (Fig. 3 and Table S1). Of note, in these incubations we did not observe significant regulation of the inflammation-initiating eicosanoids including LTB_4 and PGE_2.

Given that LTC_4S catalyzes the reaction of glutathione with LTA_4 in the cysteinyl leukotriene biosynthetic pathway (7) we questioned whether this enzyme was responsible for catalyzing the reaction of 13S, 14S-eMaR with glutathione. Incubation of human recombinant (hr) LTC_4S with increasing concentrations of synthetic 13S, 14S-eMaR yielded MCTR1, a reaction that gave a maximum reaction rate (V_{max}) of 6.7 ± 1.6 mmol/min and a Michaelis Menten constant (K_m) of 70.5 ± 33.5 μM (Fig. 4*A*, *Left*) and a k_{cat}/K_m of 1.2 ± 0.4 $M^{-1}{\cdot}s^{-1}$. For direct comparison, conversion of LTA_4 to LTC_4 by hrLTC_4S gave a V_{max} of 8.7 ± 1.5 mmol/min and a K_m of 31.2 ± 13.8 μM (Fig. 4*A*, *Right*) with a calculated k_{cat}/K_m of 4.4 ± 1.4 $M^{-1}{\cdot}s^{-1}$. Incubation of hrGSTM4 with 13S, 14S-eMaR also yielded MCTR1 with a V_{max} of 5.1 ± 0.2 mmol/min and a K_m of 42.5 ± 3.8 μM (Fig. 4*B*, *Left*) and a k_{cat}/K_m of 1.4 ± 0.1 $M^{-1}{\cdot}s^{-1}$, whereas incubation of LTA_4 with GSTM4 gave V_{max} of 1.6 ± 0.6 mmol/min and K_m of 98.0 ± 6.6 μM (Fig. 4*B*, *Right*) and a k_{cat}/K_m of 2.1 ± 0.2 $M^{-1}{\cdot}s^{-1}$. Together these results demonstrate that LTC_4S and GSTM4 each convert 13S, 14S-eMaR to MCTR1 in human macrophages. In addition, GSTM4 gave higher affinity to 13S, 14S-eMaR, whereas LTC_4S has a higher affinity to LTA_4.

Allylic epoxides such as 13S, 14S-eMaR and LTA_4 can directly interact with biosynthetic enzymes, regulating their activity, as in the case of LTA_4 hydrolase that is inactivated by its substrate LTA_4 inhibiting the production of LTB_4 (7, 16). Thus, we next questioned whether these epoxides regulated the activity of either LTC_4S or GSTM4. Incubation of LTC_4S with 13S, 14S-eMaR did inhibit the conversion of 13S, 14S-eMaR to MCTR1, as evidenced by a doubling in MCTR1 levels in incubations with a second addition of the epoxide compared with incubations where only vehicle was added (Fig. 5 *A* and *B*). Addition of LTA_4 to hrLTC_4S also did not interfere with the conversion of LTA_4 to LTC_4. Similar results were also obtained with hrGSTM4 (Fig. 5*C*).

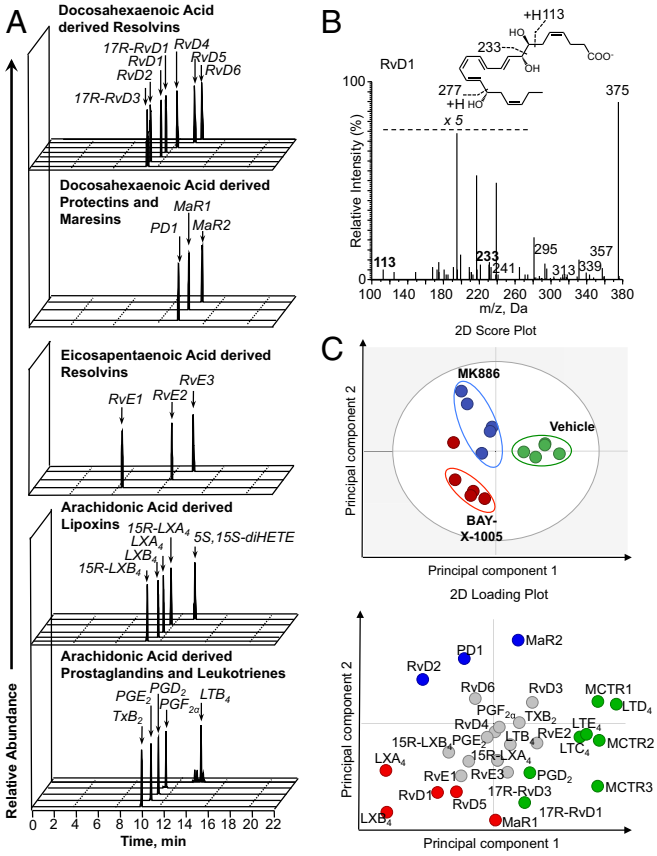

Fig. 3. Regulation of LM SPM profiles by LTC_4 inhibitors in human macrophages. Macrophages (2×10^7 cells) were incubated with vehicle (PBS containing 0.1% DMSO), MK886 (10 μM), or BAY-X-1005 (10 μM) for 20 min (room temperature, PBS containing 2% FCS, pH 7.45). Cells were then incubated with *E. coli* (2×10^8 cfu) and incubations were quenched with 2 volumes of ice-cold MeOH containing deuterium-labeled internal standards after 45 min. Lipid mediators were extracted, identified, and quantified using LM profiling. (*A*) MRM chromatograms for identified mediators. (*B*) Representative MS-MS spectrum used in the identification of RvD1. (*C*) PLS-DA for identified lipid mediator profiles. (*Upper*) 2D loading plot. (*Lower*) 2D score plot. Results are representative of $n = 5$ healthy volunteers.

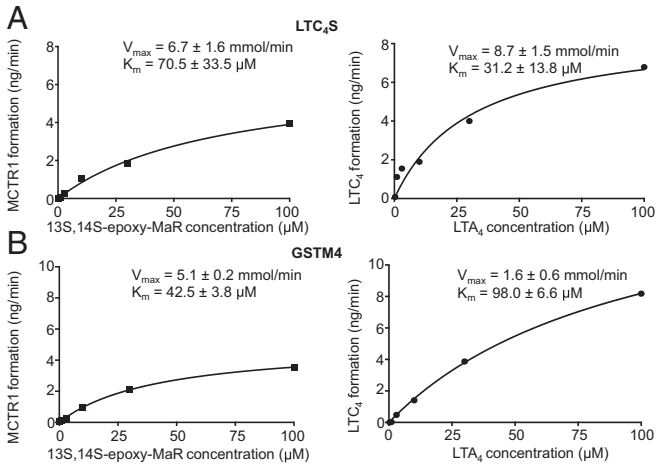

Fig. 4. Human LTC_4S and GSTM4 convert 13S,14S-eMaR to MCTR1. (*A*) Human recombinant LTC_4S (40 ng/20 μL; 0.12 μM) was incubated with the indicated concentrations of (*Left*) 13S,14S-eMaR or (*Right*) LTA_4 in 25 mM Tris·HCl, pH 7.8, 0.05% Triton X-100, and 5 mM glutathione at room temperature. (*B*) Human recombinant GSTM4 (61 ng/20 μL; 0.12μM) was incubated with the indicated concentrations of (*Left*) 13S,14S-eMaR or (*Right*) LTA_4 in 25 mM Tris·HCl, pH 7.8, 0.05% Triton X-100, and 5 mM glutathione at room temperature. MCTR1 and LTC_4 were each identified and quantified using LC-MS-MS metabololipidomics. Results are mean of three independent experiments.

Macrophage GGT Converts MCTR1 to MCTR2. MCTR1 is the proposed precursor to MCTR2 via the conversion of glutathione to cysteinyl-glycinyl (4). GGT enzymes are involved in the conversion of LTC_4 to LTD_4 by cleaving γ-glutamyl from the glutathione moiety in LTC_4 (7). Thus, we next tested whether MCTR1 was a precursor to MCTR2 and the role of GGT in catalyzing this step in human macrophages. Incubation of human macrophages with MCTR1 and either acivicin or serine borate, two GGT enzyme inhibitors, significantly reduced the MCTR2 and MCTR3 and significantly increased MCTR1. These results implicate GGT in macrophage production of MCTR2 (Fig. 6*A*).

To further test this, we incubated hrGGT with MCTR1 and assessed the kinetics of conversion to MCTR2. MCTR1 was rapidly converted to MCTR2 with 50% maximal kinetics similar to those observed for the conversion of LTC_4 to LTD_4 (Fig. 6*B*). Having found that hrGGT converts MCTR1 to MCTR2, we next assessed the catalytic efficiencies of hrGGT. MCTR1 gave a V_{max} of 8.1 ± 0.4 mmol/min a K_m of 4.6 ± 1.0 μM, and a k_{cat}/K_m of 6.0 ± 0.6 $M^{-1}{\cdot}s^{-1}$ for hrGGT. For direct comparison, LTC_4 gave a V_{max} of 8.9 ± 0.8 mmol/min, a K_m of 18.7 ± 5.0 μM, and a k_{cat}/K_m of 1.6 ± 0.4 $M^{-1}{\cdot}s^{-1}$ (Fig. 6*C*), suggesting that GGT has a higher affinity for MCTR1 than LTC_4. Together these results indicate that MCTR1 is a precursor to MCTR2 via enzymatic conversion by GGT as demonstrated in human macrophages and using recombinant human enzyme.

MCTR3 Is Produced by Dipeptidase Enzymes in Human Macrophages. We next assessed whether MCTR2 is a precursor to MCTR3, a step that would involve the cleavage of the cysteinyl-glycinyl bond. Given that dipeptidase enzymes are responsible for catalyzing this reaction in the cysteinyl leukotriene biosynthetic pathway (7) we next questioned whether this enzyme(s) is responsible for MCTR3 formation in human macrophages. For this purpose, human macrophages were incubated with MCTR2 in the presence or absence of cilastatin sodium, a dipeptidase enzyme inhibitor, and the formation of MCTR3 using lipid mediator (LM) metabololipidomics was assessed (Fig. 7). In cells incubated with cilastatin sodium we found significantly higher MCTR2 levels and significantly lower MCTR3 (Fig. 7*B*). These results suggest that macrophage dipeptidase catalyzes the conversion of MCTR2 to MCTR3.

To further evaluate the role of dipeptidases in MCTR biosynthetic pathway, human macrophages were incubated with MCTR1 in the presence or absence of cilastatin sodium, and the levels of MCTR1, MCTR2, and MCTR3 were assessed (Fig. 7*C*). In these incubations all three molecules were identified, with statistically significant increases in MCTR2 and decreases in MCTR3 in the presence of the dipeptidase inhibitors, indicating that dipeptidase contributes to the conversion of MCTR2 to MCTR3 in human macrophages. Together, these results support the proposed biosynthetic pathway, in which the epoxide 13S, 14S-eMAR is converted to MCTR1, which is then converted to MCTR2 and subsequently to MCTR3 (Fig. 8).

Discussion

In the present paper we establish the MCTR production in human tissues and biosynthetic pathway with human macrophages together with recombinant enzymes. Using material prepared by total organic synthesis, we found that 13S, 14S-eMaR is converted to MCTR1, a step that in human macrophages is catalyzed by both LTC_4S and GSTM4. Cleavage of the γ-glutamyl moiety of MCTR1 by GGT yields MCTR2. This mediator is then a precursor in the biosynthesis of MCTR3, where in human macrophages the cysteinyl-glycinyl bond is cleaved by a dipeptidase enzyme. Using LM metabololipidomics, we profiled human tissues identifying MCTR in human plasma, serum, and lymph nodes at concentrations (0.5–4.5 pM) commensurate with their known bioactive ranges (4, 14).

Tissue repair and regeneration are essential in the reestablishment of barrier function and return to homeostasis (1–3, 10, 17). Macrophages are central in orchestrating these responses, with cells of the alternative activated lineage being primarily linked with tissue repair and regeneration (2, 17, 18). In this context identification of MCTRs as macrophage-derived mediators with potent tissue protective and regenerative actions (4, 14) provides

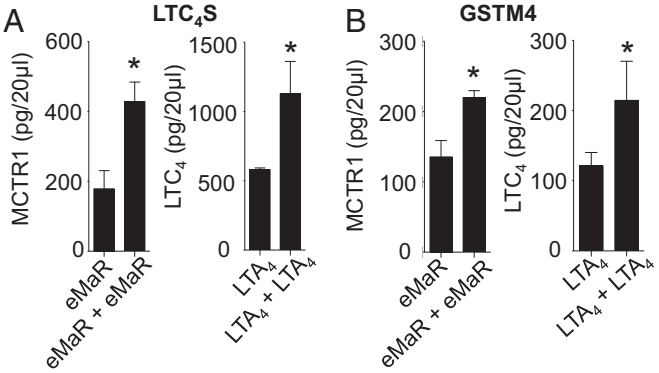

Fig. 5. Human LTC_4S and GSTM4 are not inactivated by LTA_4 or 13S,14S-epoxy-MaR. (*A* and *B*) LTC_4S (40 ng/20 μL; 25 mM Tris·HCl, 5 mM reduced glutathione, and 0.05% Triton X-100, pH 7.8) was incubated with 13S,14S-epoxy-MaR (5 μM, 2 min, 37 °C) then 13S,14S-epoxy-MaR (5 μM, 2 min, 37 °C) or vehicle. Incubations were then quenched using 2 volumes of ice-cold methanol and products profiled using LM metabololipidomics. (*A*) MCTR1 and LTC_4 produced by LTC_4S (40 ng/20 μL; 25 mM Tris·HCl, 5 mM reduced glutathione, and 0.05% Triton X-100, pH 7.8) incubated with eMaR followed by eMaR or LTA_4 (5 μM, 2 min, 37 °C) then LTA_4 (5 μM, 2 min, 37 °C) or vehicle. (*B*) GSTM4 (61 ng/20 μL; 0.12 μM; 25 mM Tris·HCl, 5 mM reduced glutathione, and 0.05% Triton X-100, pH 7.8) was incubated with 13S,14S-epoxy-MaR (5 μM, 2 min, 37 °C) then 13S,14S-epoxy-MaR (5 μM, 2 min, 37 °C) or vehicle. (*Left*) GSTM4 was incubated with LTA_4 (5 μM, 2 min, 37 °C) then LTA_4 (5 μM, 2 min, 37 °C) or vehicle. Incubations were then quenched and products profiled as above. Results are mean ± SEM, n = three independent incubations. *$P < 0.05$ vs. vehicle.

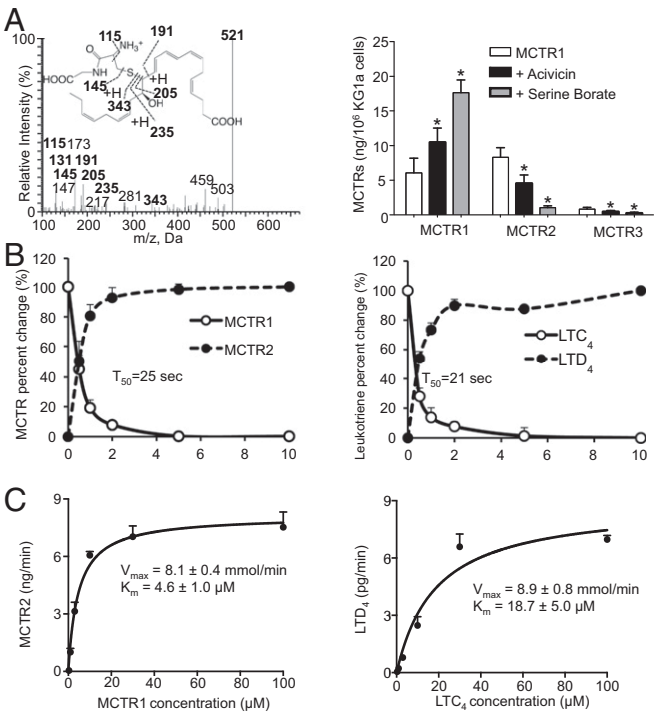

Fig. 6. Human macrophage GGT converts MCTR1 to MCTR2. KG1a cells (1×10^6 cells per mL) were incubated with acivicin (2.5 mM), serine borate (45 mM), or vehicle ($PBS^{+/+}$, pH 7.45, 15 min) then MCTR1 (0.33 μM) and serum-treated zymosan (0.1 mg, 37 °C, $PBS^{+/+}$, pH 7.45, 180 min). Incubations were stopped with ice-cold methanol and products profiled using lipid mediator metabololipidomics. (*A*, *Left*) Representative MS-MS spectrum of MCTR2 and (*Right*) MCTRs amounts in macrophage incubations. Results are mean ± SEM, $n = 4$ macrophage preparations. $^*P < 0.05$ vs. macrophages + MCTR1. (*B*) Time course: 4.4 nM of MCTR1 (*Left*) or LTC_4 (*Right*) were each incubated with human recombinant GGT (147 ng/20 μL, 185 mM Tris·HCl, pH 8.2, room temperature) for the indicated intervals. Results are mean ± SEM; $n = 4$ macrophage preparations. (*C*) Human recombinant GGT (147 ng/20 μL) was incubated with the indicated concentrations of (*Left*) MCTR1 or (*Right*) LTC_4 (185 mM Tris·HCl, pH 8.2, room temperature). All incubations were stopped with ice-cold methanol and extracted and products were profiled using LM metabololipidomics. Results are mean ± SEM; $n = 3$ independent incubations.

leads into pathways and mechanisms that control reestablishment of functions to damaged tissues. We also recently found that alternatively activated human macrophages produce higher levels of MCTRs than classically activated macrophages (6), underscoring the potential role of this pathway in tissue and organ repair in human tissue.

Bioactive mediators are produced via the stereoselective conversion of essential fatty acids that give rise to molecules with defined stereochemistries (2, 7). Hence, identifying the enzymes responsible for the formation of lipid mediators is of fundamental importance. This is because establishing the identity of these enzymes allows for a better appreciation of their biological roles during both health and disease. In the present study, we demonstrated that two enzymes from the GST family, GSTM4 and LTC_4S, catalyze the formation of MCTR1 from 13S,14S-eMAR (Figs. 2–5). Both of these enzymes also catalyze the conversion of LTA_4 to LTC_4, a lipid mediator that displays potent vasoactive and smooth muscle constricting actions (7). Of note, the two enzymes displayed different affinities to these substrates and whereas LTC_4S displayed a higher affinity to LTA_4, GSTM4 displayed a higher affinity toward 13S,14S-eMAR (Fig. 4). These findings suggest that in addition to substrate availability, the relative expression of the two enzymes in one cell type may determine the balance between the inflammation-, contraction-, and stress-initiating LTC_4 (7) vs.

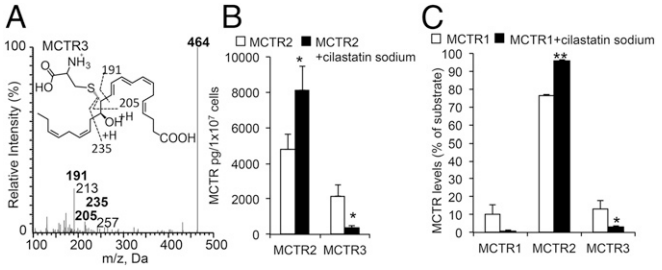

Fig. 7. MCTR3 is formed by human macrophage dipeptidase from MCTR2. (*A*–*C*) KG1a cells (1×10^6 cells/mL) were incubated with cilastatin sodium (2.3 mM), or vehicle ($PBS^{+/+}$, pH 7.45, 15 min) then MCTR2 (66.9 nM) or MCTR1 (83.4 nM) and serum-treated zymosan (0.1 mg, 37 °C, $PBS^{+/+}$, pH 7.45, 360 min). Incubations were stopped and extracted and products were profiled using LM metabololipidomics. (*A*) Representative MS-MS spectrum of MCTR3. (*B* and *C*) MCTR in macrophage incubations. Results are mean ± SEM; $n = 4$ independent experiments. (*B*) $^*P < 0.05$ vs. KG1a cells + MCTR2. (*C*) $^*P < 0.05$, $^{**}P < 0.01$ vs. KG1a cells + MCTR1.

the tissue-regenerative pathway of MCTRs. The second enzyme in the MCTR biosynthetic pathway that was identified in this report is GGT, which catalyzes the conversion of MCTR1 to MCTR2 (Fig. 6). This enzyme, and the third enzyme identified in the present study, the dipeptidase enzyme(s) that catalyzes the conversion of MCTR2 to MCTR3, are also shared with the cysteinyl leukotriene pathway (Figs. 6 and 7). Of note, substrate affinity for the GGT enzyme to MCTR1 was higher than to LTC_4 (Fig. 6), a finding that further underscores the role of these enzymes in determining the macrophage lipid mediator phenotype.

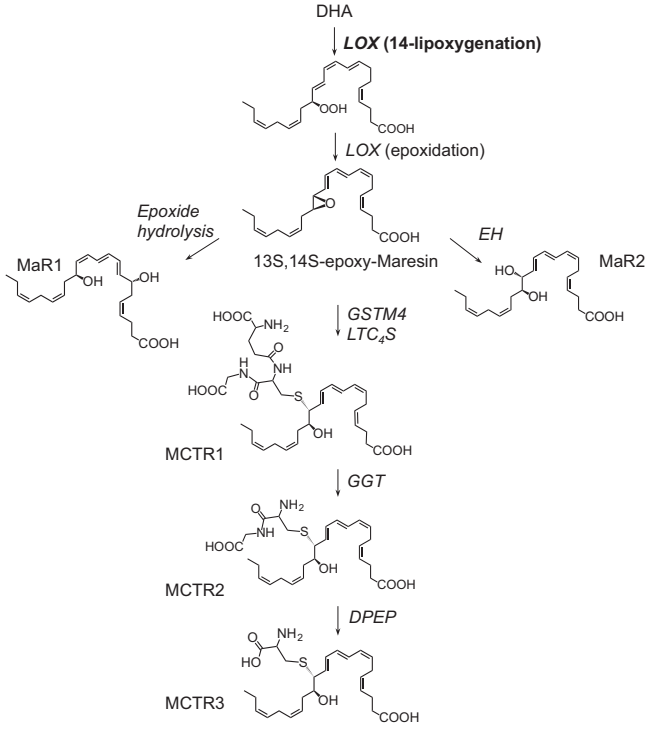

Fig. 8. MCTR biosynthetic pathway. Structures are illustrated in most likely conformations based on biosynthetic evidence (4, 14). Stereochemistry of MCTR1, MCTR2, MCTR3, MaR1, and the maresin-epoxide intermediate are established (14, 16). The lipoxygenase responsible for 14-lipoxygenation and epoxidation reactions in human macrophages is human 12-LOX (16, 21). DPEP, dipeptidase; EH, epoxide hydrolase; LOX, lipoxygenase.

In summation, in the present experiments using primary human macrophages, stereochemically defined materials prepared using total organic synthesis and human recombinant enzymes, we establish the MCTR biosynthetic pathway and precursor–product relationship(s) for MCTR1, MCTR2, and MCTR3. The identification of these potent proresolving and tissue-regenerative immunoresolvents in other human organs and tissues, including lymph nodes and serum, suggests that these pathways and mediators may be of interest in other human tissues. Given the differential affinity of enzymes identified herein to the cysteinyl leukotriene and MCTR pathways, their relative expression at sites of injury and/or inflammation may also assist in understanding disease processes. In addition, they also provide leads for targeted therapeutic strategies that may preferentially inhibit formation of inflammation-initiating cysteinyl leukotriene and up-regulate SPM formation.

Materials and Methods

Human Tissues and Cells. This study was conducted in accordance with Partners Human Research Committee Protocols 1999P001297 and 1999P001279 and a protocol approved by Barts and the London Research Ethics Committee [London (QMREC 2014:61)]. Informed consent was obtained from all participants.

LM Metololipidomics. Human lymph nodes (~150 mg) were defrosted on ice and carefully weighed, then 1 mL ice-cold methanol was added to each (see Table S2 for patient demographics and tissue source). Fresh serum and plasma (1 mL) were obtained from healthy donors and 4 mL of ice-cold methanol was added to each sample. Five hundred picograms of internal standards d_5-LTC_4, d_5-LTD_4, d_5-LTE_4, d_4-LTB_4, d_4-PGE_2, d_5-RvD2, and d_5-LXA_4 were added to each sample to facilitate identification and quantification. Samples were then kept at −20 °C for 1 h to allow for protein precipitation and products isolated as detailed in *SI Materials and Methods*.

Incubation Conditions: Enzymes. $hrLTC_4S$ (40 ng/20 μL; Origene) and hrGSTM4 (61 ng/20 μL; Creative Biomart) were suspended in 25 mM Tris·HCl containing 5 mM reduced glutathione and 0.05% Triton X-100 (pH 7.8) and incubated with 13S, 14S-eMaR or LTA_4 (0.3, 1, 3, 10, 30, and 100 μM) at room temperature for 2 min. The incubations were quenched using 2 volumes of MeOH and were profiled using lipid mediator metololipidomics. Synthetic epoxide eMaR was prepared as in ref. 16, and MCTR1, 2, and 3 were prepared as in ref. 19.

$hrLTC_4S$ (40 ng/20 μL; Origene) was suspended in 25 mM Tris·HCl containing 5 mM reduced glutathione and 0.05% Triton X-100 (pH 7.8). This was incubated with 13S,14S-eMaR (5 μM, 2 min, 37 °C) then with 13S,14S-eMaR (5 μM, 2 min, 37 °C) or vehicle. Incubations were then quenched using 2 volumes of ice-cold methanol and products profiled using lipid mediator metololipidomics. In separate experiments $hrLTC_4S$ (0.12 μM; 25 mM Tris·HCl, 5 mM reduced glutathione, and 0.05% Triton X-100, pH 7.8), was incubated with LTA_4 from Cayman Chemical (5 μM, 2 min, 37 °C) then with LTA_4 (5 μM, 2 min, 37 °C) or vehicle. Incubations were then quenched using 2 volumes of ice-cold methanol and products profiled using LM metololipidomics.

HrGSTM4 (61 ng/20 μL; Creative Biomart) was suspended in 25mM Tris·HCl containing 5 mM reduced glutathione and 0.05% Triton X-100 (pH 7.8). This was incubated with synthetic 13S,14S-epoxy-MaR (5 μM, 2 min, 37 °C) then with 13S,14S-epoxy-MaR (5 μM, 2 min, 37 °C) or vehicle. Incubations were then quenched and products profiled as above. Also, 61 ng/20 μL GSTM4 (25 mM Tris·HCl, 5 mM reduced glutathione, and 0.05% Triton X-100, pH 7.8) was incubated with LTA_4 (5 μM, 2 min, 37 °C) then with LTA_4 (5 μM, 2 min, 37 °C) or vehicle. Incubations were then quenched and products profiled as above.

MCTR1 (4.4 nM) and LTC_4 (4.4 nM) were suspended separately in Tris·HCl (185 mM, pH 8.2) and were incubated with GGT (147 ng/20 μL; Lee Biosolutions) for a total of 10 min. Aliquots were taken at predetermined intervals, placed in two volumes of ice-cold methanol, and mediator levels determined. GGT (147 ng/20 μL) was suspended in 185 mM Tris·HCl (pH 8.2) and incubated with 13S, MCTR1 or LTC_4 (0.3, 1, 3, 10, 30, and 100 μM) at room temperature for 2 min. All incubations were stopped using 2 volumes of MeOH, extracted, and profiled using LM metololipidomics. Human lymph nodes (deidentified) were purchased from Science Care and Ohio State University (OSU) Tissue Procurement Services.

Statistics. All results are expressed as means ± SEM. Differences between groups were compared using Student *t* test (two groups). The criterion for statistical significance was $P < 0.05$. Sample sizes for each experiment were determined on the variability observed in preliminary experiments and prior experience with the experimental systems. The criterion for statistical significance was $P < 0.05$. Partial least squares discriminant analysis (PLS-DA) was conducted as described in ref. 20 with mediators and macrophage lineage markers giving variable importance in projection scores greater than 1 taken as displaying significant correlation.

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