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Leukotriene B₄ Receptors BLT1 and BLT2: Expression and Function in Human and Murine Mast Cells

Katherine A. Lundeen, Binggang Sun, Lars Karlsson, and Anne M. Fourie¹*

Leukotriene B₄ (LTB₄) is a potent proinflammatory lipid mediator derived from arachidonic acid via the 5-lipoxygenase pathway (1–4). LTB₄ is known to be a chemotactic factor and activator for leukocytes, particularly granulocytes and T cells (5–8), and has been implicated in several allergic and inflammatory diseases. The action of LTB₄ is mediated by two cell surface receptors, BLT1, which is predominantly expressed in peripheral blood leukocytes, and BLT2, which is expressed more ubiquitously. Recently, BLT1 expression and LTB₄-dependent chemotaxis have been reported in immature mast cells (MCs). We now show the first evidence for BLT2 mRNA expression, in addition to BLT1, in murine bone marrow-derived MCs (mBMMCs) and in a human MC line (HMC-1). Protein expression of BLT1 was confirmed by mAb staining in HMC-1 cells and shown to be predominantly intracellular. Both HMC-1 cells and mBMMCs migrated to LTB₄ in a dose-dependent manner in chemotaxis assays. Migration to LTB₄ could be inhibited by either a BLT1- or BLT2-selective antagonist. Significant dose-dependent migration of mBMMCs also was observed to 12-(S)-hydroxyeicosatetraenoic acid, a BLT2-selective agonist, demonstrating functional BLT2 activity in these cells. Stimulation of mBMMCs with LTB₄ induced transient, dose-dependent, ERK phosphorylation and changes in Akt phosphorylation. Dose-dependent ERK phosphorylation also was observed in response to 12-(S)-hydroxyeicosatetraenoic acid, indicating signaling downstream of BLT2. Pretreatment of mBMMCs with stem cell factor significantly down-regulated expression of BLT1 and BLT2 mRNA and inhibited their migration to LTB₄. This study demonstrates expression of functional LTB₄ receptors, both BLT1 and BLT2, in murine and human MCs and a regulatory role for stem cell factor in their expression. These receptors may mediate recruitment and accumulation of MCs in response to LTB₄ production in areas of inflammation. The Journal of Immunology, 2006, 177: 3439–3447.

Leukotriene B₄ (LTB₄) is a potent proinflammatory lipid mediator derived from arachidonic acid via the 5-lipoxygenase pathway (1–4). LTB₄ is known to be a chemotactic factor and activator of leukocytes, particularly granulocytes and T cells (5–8), and has been implicated in several allergic and inflammatory diseases. The action of LTB₄ is mediated by two G protein-coupled receptors, BLT1 and BLT2, high- and low-affinity receptors, respectively (9–14). BLT1 is only activated by LTB₄, whereas BLT2 can be activated by LTB₄ and several hydroxyeicosotetraenoic acids (HETEs) (12). The tissue distribution of the two receptors is quite different. Although BLT1 expression in both mice and humans has been reported to be predominantly restricted to peripheral leukocytes, BLT2 expression in human seems to be fairly ubiquitous; with the highest level in the spleen, liver, and lymphocytes (10, 11, 13, 14).

Mast cells (MCs) have an established role in a wide range of both acute and chronic inflammatory conditions. In allergy, cross-linking of their surface receptors for IgE results in degranulation and release of inflammatory mediators. However, MCs also can be recruited and activated without degranulation in response to chemokines in a number of inflammatory conditions, including parasitic infections, allergic responses, and wound healing (15). Upon activation, MCs have been shown to secrete a wide range of cytokines and chemokines that activate and recruit neutrophils, eosinophils, and effector T cells (7, 8, 16). However, little is known about the mechanism of MC recruitment and migration to the site of inflammation. Several chemotactic factors for mouse bone marrow-derived MCs (BMMCs) in vitro have been described, including stem cell factor (SCF), IL-3, MCP-1, MIP-1α, and RANTES (17–20). For both human (h)HMC-1 and cord blood-derived MCs (CBMCs), SCF (18), IL-8 (20), C3a, and C5a (21) have been reported as chemotactic factors, and RANTES for CBMCs only (18). Kitaura et al. (22) reported mBMMC migration to LTB₄ in a chemotaxis assay but did not identify the receptors involved. Until recently, there have been no published data on LTB₄ receptor expression or function in MCs. Recently, Weller and colleagues (23) reported BLT1 expression and function in mouse and hMC progenitors, although the authors did not investigate potential expression or a functional role for BLT2. In the present study, we have established the expression of both LTB₄ receptors, BLT1 and BLT2, in hMCs and murine (m)MCs and have evidence to suggest that both receptors contribute to the migration of MCs to LTB₄. We show dose-dependent migration and ERK phosphorylation in response to 12-(S)-HETE, a BLT2-selective agonist, thus further demonstrating functional activity of BLT2 in MCs. We also demonstrate that SCF down-regulates expression of both receptors in mBMMCs, resulting in the loss of their chemotactic response to LTB₄.

Materials and Methods

Cell culture

The hMC line, HMC-1, was cultured in IMDM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin, and 1.2 mM α-thioglycerol (Sigma-Aldrich). mBMMCs were obtained by culturing primary femoral BM cells from BALB/c mice in RPMI 1640 medium supplemented with 10% FCS, 0.1 mM nonessential amino acids, 100 IU/ml penicillin, 50 μg/ml streptomycin, 10 ng/ml IL-3, and 20% WEHI-3 cell (line TIB-68; American Type Culture Collection) conditioned medium. Nonadherent cells were selected and medium replaced weekly, maintaining cell number at ~5 × 10⁵ cells/

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² Abbreviations used in this paper: LTB₄, leukotriene B₄; MC, mast cell; h, human; mBMMC, murine bone marrow-derived MC; CBMC, cord blood-derived MC; SCF, stem cell factor; HETE, hydroxyeicosatetraenoic acid; RT, reverse transcription.
ml. After 4 wk of culture, the cells typically consisted of >98% MCs as determined by c-kit receptor immunostaining and flow cytometry analysis. Cells were routinely tested for migration to 50 ng/ml mSCF and 100 nM LTβ as described below, before use in experiments.

Chemotaxis

For mBMMC migration assays, 24-well, 8-μm microchemotaxis chambers (Costar) were coated with 100 μl of 100 μg/ml bovine fibronectin (Sigma-Aldrich) in PBS and incubated for 30 min at room temperature. The fibronectin solution was aspirated from the wells, and a total of 2 × 106 MCs in 100 μl of chemotaxis medium (RPMI 1640 medium, 25 mM HEPES, 0.25% BSA, 100 IU/ml penicillin, 50 μg/ml streptomycin) was added to the upper chamber. The lower chamber contained 600 μl of chemotaxis medium with and without 50 ng/ml mSCF (Biosource) or different concentrations of LTβ2 (Sigma-Aldrich) or 12-(S)-hydroxyeicosatetraenoic acid (HETE) (Cayman Chemical). Either BLT1 antagonist U-75302 (Cayman Chemical) or BLT2 antagonist LY255283 (Cayman Chemical) was added at the appropriate concentrations to both the upper chamber and the lower compartment. Chemokinetic analysis was performed with LTβ2 in both the upper and lower chambers. After incubation for 3 h at 37°C, in 5% CO2, the Transwells were removed and discarded, and the cells in the lower chamber were quantitated in a 60-s suspension via FACs.

The hHMC-1 cell migration was measured using a 96-well, 8-μm Chemotaxis plate (Cytosystems). The filter was coated with 50 μg/ml human fibronectin (Sigma-Aldrich) in PBS for 1 h at room temperature, after which it was allowed to dry for 30 min. Wells were slightly overfilled (305 μl) with chemotaxis medium with or without hSCF (100 ng/ml; R&D Systems), LTβ2, or BLT2 antagonist. The microporous filter screen was attached, and 29 μl of cell suspension containing 1 × 104 cells in chemotaxis medium with and without antagonists was applied to drops placed to the top (as instructed by the manufacturer). Plates were incubated for 4 h at 37°C, 5% CO2. Without removing the filter screen, the plate was tipped at a 45° angle, and the nonmigrating cells on the surface of the screen were removed by rinsing with RPMI 1640 medium and then gently wiping with a damp paper towel. The screen was then removed, and the cells that had migrated into the filter screen were fixed by immersion in methanol for 1 min, stained in Wright-Giemsa-modified stain (WG-32; Sigma-Aldrich) on a rotary shaker for ~20 min, destained with water, and air dried. The number of cells migrating into the filter was counted microscopically and reported as the total number of cells observed in four randomly selected high-power fields.

RNA generation and genomic DNA removal

RNA was extracted from cells using RNeasy (Qiagen). Genomic DNA was removed using DNA-free, a DNase treatment and removal kit (Ambion). Briefly, 10 μl of DNAase buffer, and 5 μl of DNAase I were added to a total volume of 100 μl in diethyl pyrocarbonate-treated H2O. The reaction was incubated for 30 min at 37°C, then 10 μl of DNase-removal slurry was added and incubated for an additional 2 min at room temperature, centrifuged for 1 min, and RNA was removed and frozen at −80°C.

RT-PCR

RNA was reverse transcribed into cDNA and amplified using a OneStep RT-PCR kit (Qiagen) and a gene-specific antisense primer. Primers for mBLT1 were 5'-CCTGTGACCATCTGTGTTGAGAATAAGC-3' (sense) and 5'-CACCAAGGTTGAGACGACCGAGAACAA-3' (antisense). The primers for mBLT2 were 5'-ATCTGGCTGCGCTTTAGCCCGTC-3' (sense) and 5'-GACTGTTCCTTCGCCGCTCCTTCG-3' (antisense). The resulting PCR products for mBLT1 and mBLT2 were 444 and 387 bp, respectively. The primers for hBLT1 were 5'-TATGTCGCTTCGAGGATCAGCATGCAGTACC-3' (sense) and 5'-CCTGTGACCATCTGTGTTGAGAATAAGC-3' (antisense); and for hBLT2 were 5'-AGGCTGAGACAGGGCGCCTCCTGCG-3' (sense) and 5'-GCAGTACGGACCGGGTGCTCCTGAC-3' (antisense). The resulting PCR products for hBLT1 and hBLT2 were 345 and 320 bp, respectively. The hBLT1 and hBLT2 primer sequences were those published by Yokomizo et al. (24). The reaction mixture (50 μl) contained 100–500 ng of total RNA, 1 μl Qiagen OneStep RT-PCR buffer mix, 1× Q-solution, 400 μM each of dNTP, 0.2 μM each of gene-specific primer, 5 μl RNa inhibitor, and 2 μl of OneStep Enzyme mix (contains Omniscript and Sensiscript reverse transcriptase (RT) and HotStarTaq DNA polymerase). No-RT control contained HotStarTaq only. RT-PCR was performed on a Stratagene Robocycler, and the amplification profile was 50°C for 30 min (RT); 95°C 15 min (HotStarTaq activation); and 35–40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Cycling was completed with a 10-min extension at 72°C. The final PCR products were resolved on a 1.5% agarose gel containing ethidium bromide and visualized under UV light.

Stimulation of mBMMCs with SCF and LTβ4

The mBMMCs were stimulated for 16 h with 50 ng/ml mSCF. Cells were then washed four times and used for chemotaxis as described above, or RNA was extracted and used for TaqMan real-time PCR. Supernatant was collected and assayed by ELISA for LTβ4 (Assay Designs Correlate EIA) and mIL-6 (R&D Systems).

Quantitative real-time PCR (TaqMan) analysis

DNase-treated RNA was reverse transcribed using random hexamers with the Applied Biosystems High-capacity cDNA archive kit as per the manufacturer’s recommendations. The cDNA was quantitated using PicoGreen (Molecular Probes). Briefly, 5 μl of cDNA diluted in 100 μl of Tris-EDTA was added to 100 μl of pigcrogen working solution. After 5 min at room temperature, the assay was read in a fluorescent plate reader at 485-nm excitation, 580-nm emission. A standard curve was made using the cDNA standard that was the kit, and LTβ4 concentration of the unknown cDNA was determined by linear regression.

The experimental cDNA was diluted to a final concentration of 20 ng/μl. Five microliters of cDNA (100 ng) was amplified in the presence of 12.5 μl of universal master mix, 1.25 μl of gene-specific TaqMan probe, and 6.25 μl of H2O. Endogenous controls used gene-specific probes for β-actin. Probes used FAM as a reporter (hBLT1 Hs00609652.m1; hBLT2 Hs0025197342.m1; mBLT1 Mm00521839.m1; mBLT2 Mm00498941.s1; Applied Biosystems). Samples underwent the following amplification stages: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; and stage 3, 95°C for 15 s followed by 60°C for 1 min. Stage 3 was repeated 40 times. Gene-specific PCR products were measured by means of an Applied Biosystems 7500 Real-Time PCR instrument continuously during 40 cycles. Target gene expression was normalized between different samples based on the values of the endogenous control expression for each cDNA sample. The ΔΔCT and the comparative expression levels were calculated as described previously (25).

Flow cytometry analysis

For surface staining of BLT1, a total of 5 × 105 cells was blocked with 10% human serum in FACS buffer (PBS with 25 mM HEPES, 1 mM EDTA, 1% FBS) for 30 min at 4°C. Ten micrometers per milliliter of either FITC-conjugated anti-human BLT1 (BLT1 mAb (Abcam 781; or iso-type control was added and incubated for 30 min at 4°C and washed three times with FACS buffer, and BLT1 receptor expression was analyzed by flow cytometry. For intracellular staining, a total of 5 × 105 cells, after blocking, was resuspended in 250 μl of BD Cytoperm/Cytotox reagent (BD Pharmingen) for 20 min at 4°C, washed twice with BD Perm/Wash buffer, resuspended in 100 μl of Perm/Wash buffer with 10 μg/ml mAb for 30 min at 4°C, washed twice, and analyzed by flow cytometry. An appropriate irrelevant isotype control Ab was used to measure the extent of nonspecific binding.

LTβ4-mediated ERK and Akt phosphorylation

Activation of ERK and Akt by LTβ4 in mBMMCs was determined by immunoblotting using the phosphospecific anti-ERK p42/44 (Thr202/Tyr204) and anti-Akt (Ser473), respectively (Cell Signaling Technology). Briefly, a total of 3 × 105 cells was washed with serum-free RPMI 1640 medium and starved with medium containing 0.5% serum for 4 h. Cells were then resuspended at 107/ml with RPMI 1640 medium and stimulated with 10 and 100 nM LTβ4, respectively. Aliquots of cells (200 μl) were taken at 0, 2, 5, 10, 30, and 60 min and chilled in 1 ml of ice-cold PBS. For dose-dependent ERK activation, cells were treated with various concentrations of LTβ4 (1–100 nM) or 12-SHETE (0.1 to 10 μM) for 2 min only. Cells were then lysed in buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 50 mM β-glycerol phosphate, 5 mM EDTA, 1 mM Na2VO3, 10 mM sodium pyrophosphate, 1% Triton X-100, and protease inhibitor mixture (Roche). Approximately 20 μg of total proteins was separated on 10% SDS-PAGE and subjected to Western blot analysis using standard protocols.

Results

mBMMCs and HMC-1 express mRNA for BLT1 and BLT2

We investigated the possibility of MC expression of the LTβ4 receptors, BLT1 and BLT2, by RT-PCR analysis with specific primers in mBMMCs and in an hMC line, HMC-1. As shown in Fig. 1A, RT-PCR with primers specific for mBLT1 and mBLT2...
yielded PCR products of the expected sizes (444 and 387 bp, respectively). Controls without RT confirmed that the source of the products was indeed from mRNA, and not from contaminating genomic DNA. The same primers also were used to amplify BLT1 and BLT2 from mouse T cell RNA (data not shown), and the products were sequenced to confirm that they corresponded to the expected segments of mBLT1 and mBLT2 (NM.008519 and NM.019839). No-RT controls to test for genomic DNA contamination yielded no PCR products. Plasmids containing cDNA for each receptor were used as positive controls and yielded PCR products of the expected sizes in all cases (data not shown). Thus, mBMMC and hHMC-1 cells express mRNA for both BLT1 and BLT2.

Expression of intracellular BLT1 protein in HMC-1 cells

To demonstrate BLT1 receptor protein expression, flow cytometry was performed after staining the Jurkat T cell leukemia line and the HMC-1 MC line with a specific anti-hBLT1 mAb. Staining of Jurkat cells showed a rightward shifted peak relative to the isotype control staining, indicating that BLT1 receptor was expressed on the cell surface (Fig. 2A). In contrast in HMC-1 cells, there was a slight shoulder of positive surface staining indicating, at most, a very small population of BLT1 receptors on the surface (Fig. 2B). However, staining of fixed and permeabilized HMC-1 cells (Fig. 2C) showed a BLT1-positive cell population, indicating that the primary site of BLT1 protein expression in HMC-1 cells was intracellular. Incubations of HMC-1 cells with LTB₄ concentrations of 100 nM and 1 μM for different times varying from 15 min to overnight did not stimulate translocation of BLT1 to the surface (data not shown). There are currently no suitable flow cytometry Abs available for staining either hBLT2 or mBLT1/BLT2 proteins, thus precluding studies of their expression.

mBMMC chemotaxis

Because LTB₄ has been shown to be a chemotactic factor for leukocytes expressing LTB₄ receptors, we investigated whether LTB₄ could mediate chemotaxis of mBMMCs. Chemotaxis to SCF was used as a positive control for comparison. Fig. 3A shows the relative levels of migration of mBMMCs to LTB₄ compared with SCF-induced migration mediated through the c-kit receptor. Although the chemotactic response was not as pronounced as that to SCF, mBMMCs clearly demonstrated LTB₄-dependent migration (p = 0.0001). The migration of mBMMCs to LTB₄ also was tested over a range of concentrations. The mean results of three separate experiments in Fig. 3B indicate that mBMMCs migrated to LTB₄ in a dose-dependent manner, with an EC₅₀ of 5 nM, and reached maximum chemotaxis at 100 nM LTB₄, which was the highest concentration tested. To establish whether the LTB₄-dependent migration was due to chemotaxis and/or chemokinesis, migration was compared with only the lower well containing 100 nM LTB₄ vs 100 nM LTB₄ in both upper and lower wells. The results in Fig. 3C indicate that the level of chemotaxis (LTB₄ in lower wells only) was clearly higher than chemokinesis (LTB₄ in both upper and lower wells). At 100 nM LTB₄, there was no statistical difference between the control well and the chemokinesis well, whereas there was a significant difference between the chemotaxis and chemokinesis wells (p = 0.01).

To investigate the existence of functional BLT2 chemotactic activity in mBMMC, chemotaxis assays were performed using a range of concentrations (0.1–10 μM) of 12-(S)-HETE, a BLT2-selective agonist (12). In three separate experiments, significant, dose-dependent chemotaxis to 12-(S)-HETE was observed, reaching similar or greater magnitude, compared with that observed for 100 nM LTB₄, as shown in Fig. 3D. Although the most significant chemotactic activity was observed between 5 and 10 μM, statistically significant chemotaxis vs background was observed at 12-(S)-HETE concentrations as low as 1 μM (p < 0.05; Experiment 1); and 2.5 μM (p < 0.05; Experiment 3). In each experiment, a
steep dose-response to 12-(S)-HETE was observed, reaching maximal levels at 10 μM, the highest concentration tested.

Chemotaxis assays also were performed in the presence of selective BLT1 and BLT2 receptor antagonists to investigate the extent of involvement of BLT1 and/or BLT2 in the LTB₄-dependent migration. Dose-reponses were performed with both antagonists in mBMMCs. The results in Fig. 4A show that there was statistically significant inhibition of chemotaxis by U-75302 (a BLT1-specific antagonist), as low as 10 μM, and by LY255283 (a BLT2-specific antagonist), as low as 2.5 μM compound. Chemotaxis to 25 nM LTB₄ was significantly inhibited by 10 μM U-75302 or by 10 μM LY255283 (Fig. 4B). There was no additive effect when these concentrations of the two compounds were combined. To further investigate the specificity of the BLT antagonists, we tested their effect on chemotaxis to SCF. Fig. 4C shows that, at concentrations of 20 μM compound, there was no inhibition by either antagonist of chemotaxis to SCF, and there was, in fact, a significant enhancement effect. Furthermore, no evidence for cytotoxicity was observed after overnight incubation of mBMMCs with either antagonist at 20 mM (data not shown).

**LTB₄-dependent signaling in mBMMC**

To further support the functional expression of BLT1 and BLT2 in mBMMCs, we examined LTB₄-mediated signaling events. It has been shown that ligation of LTB₄ to BLT1 and/or BLT2 activates PI-3 and MAPK in a variety of experimental systems, including cell lines, transfected cell lines, and primary cells (26–31). We investigated ERK1/2, Akt, and p38 phosphorylation in mBMMCs in response to 10 nM and 100 nM LTB₄ or LPS. Background ERK1/2, Akt, and p38 phosphorylation was observed in the absence of stimulants, and even starvation of mBMMC in 0.5% serum for up to 24 h failed to eliminate the background (data not shown). After 10-min incubation with LPS, a small, transient increase over background in ERK1/2 and Akt phosphorylation was observed, which significantly decreased by 45 min (Fig. 5, last two lanes). LTB₄, at 10 or 100 nM, induced transient activation of ERK1/2, peaking at around 2 min and progressively declining thereafter to a much lower level by 30–60 min. Although the kinetics for ERK activation by the two different concentrations of LTB₄ were similar, 100 nM LTB₄ seemed to result in higher and more sustained activation than 10 nM. In response to LTB₄, Akt phosphorylation initially decreased from the relatively high background, followed by transient phosphorylation, peaking at around 10 min and declining thereafter. In contrast with ERK1/2 and Akt, p38 MAPK phosphorylation in mBMMC was relatively unchanged in the presence of LTB₄ or LPS. Similar results were obtained in three separate experiments. Thus, transient LTB₄-mediated ERK1/2 phosphorylation and changes in Akt phosphorylation were observed in mBMMC at both 10 and 100 nM, and the relative magnitude of the responses was similar to the relative chemotactic responses observed at 10 vs 100 nM LTB₄.

In a separate experiment, ERK activation in mBMMCs was examined in response to a 2-min stimulation with LTB₄ (1–100 nM)
or 12-(S)-HETE (0.1–10 μM). Dose-dependent ERK phosphorylation in response to LTB4 was clearly observed, reaching maximal levels between 10 and 100 nM. Treatment of mBMMCs with 12-(S)-HETE also induced ERK phosphorylation. Marginal phosphorylation was observed at 2 μM, but a clear increase in phospho-ERK could be observed at 5 μM and even more so at 10 μM (see Fig. 5B), similar to the concentration range where the most significant chemotaxis to 12-(S)-HETE was observed (Fig. 3D).

hHMC-1 chemotaxis

Chemotaxis assays were performed using HMC-1 cells and, as for mBMMCs, LTB4-dependent migration was observed (Fig. 6A). Migration due to chemokinesis was measured as described above and was higher than spontaneous migration of untreated control cells (p = 0.01) but significantly lower than the LTB4-dependent chemotactic response (p = 0.0008). Migration of HMC-1 cells was tested over a range of concentrations of LTB4. The mean results of three separate experiments (Fig. 6B) showed dose-dependent HMC-1 chemotaxis to LTB4, with an EC50 of ~25 nM LTB4. Chemotaxis of HMC-1 cells to 100 nM LTB4 was completely inhibited by both the BLT1-specific receptor antagonist, U-75302, and the BLT2-specific antagonist, LY255283, at a concentration of 500 nM (Fig. 6C). No difference was observed when combining the two compounds. Neither antagonist showed any evidence of cytotoxicity at 20 μM, after overnight incubation with HMC-1 cells (data not shown).

SCF regulation of BLT receptors in mBMMCs

SCF is an important growth and differentiation factor for MCs and stimulates both chemotaxis and the production of chemokines and cytokines, including IL-6, in mBMMCs. We found that incubation of mBMMCs with SCF for 16 h resulted in dose-dependent LTB4 production, with an EC50 of 60 ng/ml SCF (Fig. 7A). Similarly, SCF induced a dose-dependent production of IL-6 with an EC50 of 75 ng/ml (Fig. 7B), and also induced a small amount of MCP-1 (data not shown). However, similar incubations with LTB4 did not induce IL-6 or MCP-1 (data not shown). Because SCF stimulated LTB4 production in mBMMCs, we also explored the effects of SCF on BLT1 and BLT2 mRNA expression. Cells were treated for 16 h with 50 ng/ml SCF, after which RNA was extracted for RT and real-time PCR. The ΔΔCt method for relative quantitation of gene expression in the TaqMan system was used. The calculated fold differences in Tables I and II show that SCF treatment of mBMMCs down-regulated BLT1 and BLT2 receptor expression, respectively. This result is shown graphically in Fig. 8A.

SCF regulation of MC chemotaxis to LTB4 and SCF

Because we observed down-regulation of BLT1 and BLT2 expression in SCF-treated mBMMC cells, we tested chemotaxis to LTB4 and SCF in this same cell population. After a 16-h incubation with 50 ng/ml SCF, mBMMCs were washed four times to remove any
residual SCF and tested in chemotaxis assays to 100 nM LTB4 or 50 ng/ml SCF. Pretreatment with SCF completely eliminated the LTB4-dependent migration of mBMMCs (Fig. 8B), which correlated with the reduction observed in mRNA expression for both BLT1 and BLT2 receptors (Fig. 8A). Conversely, the ability to migrate to SCF was enhanced ~2-fold (Fig. 8C).

Discussion

LTB4 is a potent leukocyte activator and chemoattractant, which mediates its biological action via the LTB4 receptors, BLT1 and BLT2. Recently, BLT1 expression and LTB4-dependent chemotaxis have been reported in immature MCs (23). In the present study, we have shown the first evidence for LTB4 expression in mBMMCs and in HMC-1 cells. Both HMC-1 cells and mBMMCs migrated to LTB4 in a dose-dependent manner, and this migration was inhibited by either a BLT1- or BLT2-selective antagonist (Fig. 8A). Conversely, the ability to migrate to SCF was enhanced ~2-fold (Fig. 8C).

FIGURE 6. HMC-1 chemotaxis: Human HMC-1 migration was measured as described in Materials and Methods. A, HMC-1 chemotaxis to 100 nM LTB4, compared with chemokinesis with 100 nM LTB4 in both top and bottom wells (p = 0.0008). B, Dose-dependent migration of HMC-1 cells in response to LTB4 (EC50 = 25 nM; mean of three separate experiments). C, Inhibition of HMC-1 chemotaxis to 100 nM LTB4 by 500 nM BLT1 antagonist (U75302), 500 nM BLT2 antagonist (LY255283), or both antagonists combined, each at 500 nM (*, p < 0.05; **, p < 0.01; ***, p < 0.005).

FIGURE 7. SCF-stimulated LTB4 and IL-6 production in mBMMCs: Cells were washed and stimulated for 16 h with 50 ng/ml mSCF in chemotaxis buffer. Cell supernatant was collected, and LTB4 and IL-6 were measured by ELISA. A, Dose-dependent SCF stimulation of LTB4 production (EC50 = 60 ng/ml SCF). B, Dose-dependent IL-6 production in response to SCF (EC50 = 75 ng/ml SCF).

down-regulated expression of both BLT1 and BLT2 mRNA and inhibited their ability to migrate to LTB4. Our study thus demonstrates the expression of functional LTB4 receptors, both BLT1 and BLT2, in mMCs and hMCs, and a regulatory role for SCF in their expression.

Mature MCs are normally found interspersed throughout the tissue. Mast cell hyperplasia is a hallmark of many inflammatory conditions such as allergic rhinitis (32), asthma (33), scleroderma (34), psoriasis (35), tissue transplant rejection (36), rheumatoid arthritis (37), and mastocytosis (38). The factors regulating MC recruitment during inflammation are still not fully understood. This relocalization of tissue MCs has been hypothesized to be regulated by chemotaxins such as complement proteins, C3a and C5a (21), growth factors, and chemokines such as CXCL2, platelet-activating factor, CCL5/RANTES, TGF-β, SCF, IL-3, and/or MCP-1 (39–43). Our study and that of Weller et al. (23) show that LTB4 also may play a significant role in regulating MC migration in vivo through both BLT1 and BLT2 receptors.

We also have shown LTB4-dependent signaling events in mBMMCs. Although LTB4 clearly induced transient phosphorylation of ERK, the predominant effect of LTB4 on the Akt pathway (Fig. 5) appeared to be an initial dephosphorylation of Akt, followed by a transient rephosphorylation, which may represent LTB4-stimulated phosphorylation, or a decline in LTB4-induced inhibition of phosphorylation. Chemotactant-induced inhibition of Akt phosphorylation has previously been observed: Heit et al. (44) demonstrated that IMLP inhibited the Akt phosphorylation induced in neutrophils by IL-8, suggesting that IMLP had a direct
inhibitory effect on the PI3K/Akt pathway. The initial dephosphorylation of Akt that we observed at both 10 and 100 nM LTB4 suggests that LTB4 may have similar effects on this pathway.

Until very recently, there had been no reports of BLT2 expression in any murine cells or tissues. Tager and Luster (45) indicated by Northern blot analysis that mouse BLT2 expression was not detected in neutrophils, macrophages, T cells, lymph node, spleen, or lung. However, Iizuka et al. (30) recently detected mouse BLT2 expression predominantly in small intestine, followed by skin, and a small amount in spleen and colon. In addition to our data in this study showing expression of both BLT1 and BLT2 in mMCs, we also have evidence from RT-PCR analysis that murine T cells and T cell lines express mRNA for both receptors (K. Lundein and A. Fourie, unpublished observations).

In the Jurkat human T cell leukemia line, we found that BLT1 receptor protein was expressed on the cell surface, whereas our staining of HMC-1 cells showed predominantly intracellular expression of BLT1. We observed chemotaxis to LTB4 in HMC-1 cells, despite the apparent intracellular location of BLT1. It is difficult to attribute chemotaxis to a completely intracellular pool of receptors. It is possible that BLT1 would externalize under certain conditions, but we were unable to detect BLT1 translocation to the surface in response to LTB4 at 100 nM or 1 μM in a time course ranging from 15 min to overnight. There is precedence for the intracellular localization of chemotactic factor receptors. fMLP is a neutrophil chemotactic factor for which the receptor is located in the secretory vesicles of neutrophils. Upon stimulation with fMLP, they are rapidly translocated to the membrane creating a dramatic up-regulation of fMLP receptors on the neutrophil surface (46). Lippert et al. (20) also have reported intracellular expression of the IL-8 receptor CXCR2 in HMC-1 cells, while CXCR1 was expressed on the cell surface. These authors also showed that IL-8 stimulation did not induce translocation of CXCR2 to the cell surface, similar to the apparent lack of translocation of BLT1 receptor to the surface we observed upon stimulation with exogenous LTB4 of HMC-1 MNCs (data not shown). It is possible that a very small population of BLT1 on the surface may mediate chemotaxis or that there are additional surface BLT1 receptors not recognized by the particular mAb used for BLT1 staining.

Another possibility is that BLT2 plays a predominant role in mediating LTB4-dependent chemotaxis of MCs. BLT2 is clearly capable of mediating chemotaxis when transfected into cells lacking LTB4 receptors (24). We have evidence to suggest that, in mouse MCs and hMCs, both BLT1 and BLT2 receptors are functional. Chemotactic activity could be blocked by either a BLT1- or BLT2-selective LTB4 receptor antagonist. U-75302 is a BLT1 antagonist with IC50 values of 1 μM and >10 mM for BLT1- and BLT2-transfected Chinese hamster ovary cells, respectively. The IC50 values for LY255283 are 10 μM for BLT1 and ~1 μM for BLT2 (12, 47). In our studies, both antagonists resulted in inhibition of migration at concentrations where they would be selective for BLT1 or BLT2, respectively. We were unable to demonstrate an additive effect of the two antagonists. A similar observation was made by Kitaura et al. (22) where there was very little, if any, additive inhibitory effect when these two antagonists were combined, at 1 μM each, on BMMC migration to IgE-sensitized cells and Ag. One possible explanation for our data is that the two receptors do not function independently. For example, it is known that BLT1 forms homodimers, and there is a growing body of evidence showing that many G protein-coupled receptors form heterodimers, which can alter their signaling and their intracellular transport. Thus, it is possible that BLT1 and BLT2 may function as heterodimers. This could explain why both selective antagonists could maximally inhibit chemotaxis, even at concentrations where they should inhibit LTB4 binding to only BLT1 or BLT2, respectively.

Further support for functional BLT2 receptors in mBMMCs was the evidence for dose-dependent chemotaxis to concentrations between 1 and 10 μM 12-(S)-HETE, previously shown by Yokomizo et al. (12) to be a selective agonist for BLT2. Although a minimal amount of chemotaxis for BLT1-transfected cells was observed in their study at 10 μM 12-(S)-HETE, the relative amount, compared with that observed for LTB4 at 100 nM, was essentially negligible (12). In contrast, we have shown significant chemotaxis to 12-(S)-HETE at concentrations as low as 1 μM, reaching similar magnitude at 10 μM 12-(S)-HETE to that observed at 100 nM LTB4.

### Table I. Quantitation of mRNA levels for BLT1 in SCF-treated vs untreated mBMMCs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average CΤ BLT1</th>
<th>Average CΤ β–Actin Control</th>
<th>ΔCΤ BLT1–β–Actin</th>
<th>ΔΔCΤ SCF Treated–Untreated</th>
<th>Fold Difference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>24.91 ± 0.09</td>
<td>13.86 ± 0.13</td>
<td>11.05 ± 0.16</td>
<td>0.00 ± 0.6</td>
<td>1 (0.9–1.1)</td>
</tr>
<tr>
<td>SCF</td>
<td>25.58 ± 0.1</td>
<td>13.21 ± 0.1</td>
<td>12.37 ± 0.14</td>
<td>1.32 ± 0.14</td>
<td>0.4 (0.36–0.44)</td>
</tr>
</tbody>
</table>

Fold difference of BLT1 mRNA expression in nontreated cells vs cells stimulated for 16 h with 50 ng/ml mSCF. DNase-treated RNA was reverse transcribed using the Applied Biosystems cDNA archive kit, and mRNA levels were measured in the TaqMan PCR system using predesigned gene-specific probes.

* Fold difference was calculated using the formula 2−ΔΔCΤ with ΔΔCΤ = s and ΔΔCΤ = S, where s is the standard deviation of ΔCΤ triplicates.

### Table II. Quantitation of mRNA levels for BLT2 in SCF-treated vs untreated mBMMCs

<table>
<thead>
<tr>
<th>Sample</th>
<th>Average CΤ BLT2</th>
<th>Average CΤ β–Actin Control</th>
<th>ΔCΤ BLT2–β–Actin</th>
<th>ΔΔCΤ SCF Treated–Untreated</th>
<th>Fold Difference*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>20.79 ± 0.01</td>
<td>13.86 ± 0.13</td>
<td>6.93 ± 0.13</td>
<td>0.00 ± 0.13</td>
<td>1 (0.99–1.1)</td>
</tr>
<tr>
<td>SCF</td>
<td>22.2 ± 0.07</td>
<td>13.21 ± 0.1</td>
<td>8.99 ± 0.12</td>
<td>2.06 ± 0.12</td>
<td>0.24 (0.22–0.26)</td>
</tr>
</tbody>
</table>

Fold difference of BLT2 mRNA expression in nontreated cells vs cells stimulated for 16 h with 50 ng/ml mSCF. DNase-treated RNA was reverse transcribed using the Applied Biosystems cDNA archive kit, and mRNA levels were measured in the TaqMan PCR system using predesigned gene-specific probes.

* Fold difference was calculated using the formula 2−ΔΔCΤ with ΔΔCΤ = s and ΔΔCΤ = S, where s is the standard deviation of ΔCΤ triplicates.
LTB₄ RECEPTORS, BLT1 AND BLT2, MEDIATE MC CHEMOTAXIS

These results are very similar to those reported for BLT₂-transfected cells (12) and support the existence of functional BLT₂ receptors in mBMMCs.

SCF is known to be an important growth and differentiation factor that stimulates the production of numerous cytokines and chemokines in mBMMCs (48). Our observation that SCF-stimulated mBMMCs secreted LTB₄ in a dose-dependent manner, similarly to IL-6 and MCP-1 production, may thus have interesting implications in MC biology. Another consequence of SCF stimulation of mBMMCs was a reduction in BLT₁ and BLT₂ mRNA levels at 16 h and then tested for LTB₄- and SCF-dependent chemotaxis, compared with untreated cells. A Graphical representation of mRNA expression data from Table I (BLT₁) and Table II (BLT₂) reported as fold change with untreated cells.

FIGURE 8. Effect of SCF preincubation on mRNA levels for BLT₁ and BLT₂ and chemotaxis to LTB₄ and SCF. Cells were treated with SCF (50 ng/ml) for 16 h and then tested for LTB₄- and SCF-dependent chemotaxis, compared with untreated cells. A. Graphical representation of mRNA expression data from Table I (BLT₁) and Table II (BLT₂) reported as fold change with untreated cells. B. Prestimulated vs nonstimulated mBMMC chemotaxis to 100 nM LTB₄. C. Prestimulated vs nonstimulated mBMMC chemotaxis to 50 ng/ml mSCF.

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