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Leukotriene B_4, an activation product of mast cells, is a chemoattractant for their progenitors

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Mast cells are tissue-resident cells with important functions in allergy and inflammation. Pluripotential hematopoietic stem cells in the bone marrow give rise to committed mast cell progenitors that transit via the blood to tissues throughout the body, where they mature. Knowledge is limited about the factors that release mast cell progenitors from the bone marrow or recruit them to remote tissues. Mouse femoral bone marrow cells were cultured with IL-3 for 2 wk and a range of chemotactic agents were tested on the c-kit$^+$ population. Cells were remarkably refractory and no chemotaxis was induced by any chemokines tested. However, supernatants from activated mature mast cells induced pronounced chemotaxis, with the active principle identified as leukotriene (LT) B_4. Other activation products were inactive. LTB_4 was highly chemotactic for 2-wk-old cells, but not mature cells, correlating with a loss of mRNA for the LTB_4 receptor, BLT1. Immature cells also accumulated in vivo in response to intradermally injected LTB_4. Furthermore, LTB_4 was highly potent in attracting mast cell progenitors from freshly isolated bone marrow cell suspensions. Finally, LTB_4 was a potent chemoattractant for human cord blood–derived immature, but not mature, mast cells. These results suggest an autocrine role for LTB_4 in regulating tissue mast cell numbers.

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Abbreviations used: 5-LO, 5-lipoxygenase; ACN, acetonitrile; BMMC, BM-derived mast cell; CBMC, cord blood–derived mast cell; CMFDA, 5-chloromethylfluorescein diacetate; HSA, human serum albumin; i.d., intradermal; LT, leukotriene; mMCP, murine mast cell protease; SCF, stem cell factor.

Mast cells are long-lived cells that reside in tissues, where they play important roles in inflammation, angiogenesis, and wound healing. They are principally recognized for their effector functions in allergic reactions and in host defense to helminth parasites, but they also have roles as sentinel cells in responses to microbial infections (1). Mast cells have FcεR1 receptors that bind IgE with high affinity, and recognition of polyvalent antigen triggers receptor cross-linking. This results in the release of degranulation products and the de novo synthesis of mediators with potent inflammatory activity (e.g., smooth muscle spasmogens), vasopermeability agents, and chemoattractants, as well as cytokines with a range of activities.

Mast cells are derived from pluripotential hematopoietic stem cells in the bone marrow (2). Under the influence of growth factors, these cells give rise to committed mast cell progenitors. The progenitors are released from the bone marrow into the blood from where they localize to different tissues throughout the body. Once in the tissues, mast cell maturation proceeds, with local factors determining the mature phenotype appropriate for the particular location. Two major subtypes of mast cells have been identified: connective tissue type, particularly localized in skin, around blood vessels, and in the peritoneal cavity; and mucosal type, which is associated with mucosal surfaces such as those in the gut or airways. These subtypes have a characteristic expression of particular serine proteases (3–5). Studies in mice have revealed important information on the nature of mast cell progenitors and their transit between compartments of the body, but specific details of the mechanisms involved in their release from the bone marrow and recruitment to the tissues remain to be established. The importance of mucosal mast cells in certain host defense reactions to parasites

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and in allergic reactions is demonstrated by the localized mast cell hyperplasia that occurs in the affected tissues (6, 7). Animals lacking stem cell factor (SCF), such as the $WCB6_{F1}$-Sl/Sl^d mouse (8), or its receptor, c-kit, such as in the $WBB6_{F1}$-W/W^v mouse (2), have few tissue mast cells constitutively and fail to develop mast cell hyperplasia. Thus, SCF and its receptor are essential for mast cell maturation and/or localization.

Studies of mast cell progenitors in tissues are difficult because of their very low numbers in situ. A minor population of circulating c-kit$^+$ committed mast cell progenitors has been reported in mouse fetal blood (9). Recently, sequential immunomagnetic isolation of adult mouse bone marrow has revealed a 0.02% population of undifferentiated mast cells characterized as CD34$^+$, CD13$^+$, c-kit$^+$, and FcεR1$^-$ (10). Another approach, using limiting dilution assays, has been used to determine the numbers of mast cell progenitors in different tissues, including the small and large intestine, lung, spleen, and bone marrow (11). It has also been demonstrated that the $\alpha_4\beta_7$ integrin is essential for mast cell progenitor homing to the small intestine (11). A c-kit$^+\alpha_4^{hi}\beta_7^+$ mast cell progenitor has been reported in mouse bone marrow 5 d after infecting the small intestine with *Trichinella spiralis* (12). Loss of these cells from the bone marrow was followed by their appearance in the blood, with mature mast cells becoming detectable in the gut after 3 d (12).

Analogy with the recruitment of mature leukocytes would suggest that soluble chemoattractants, acting in concert with adhesion molecules, may regulate the population of tissues with mast cell progenitors. Such chemotactic factors may also be involved in the release of progenitors from the bone marrow, as demonstrated previously for mature leukocytes (13–15) and their precursors (16). Thus, we have investigated the chemotactic responses of immature c-kit$^+$ mast cells cultured from mouse femoral bone marrow in the presence of IL-3. Such cells have been shown to be capable of populating tissues in vivo after intravenous injection in mice (17). These cells were found to be remarkably selective

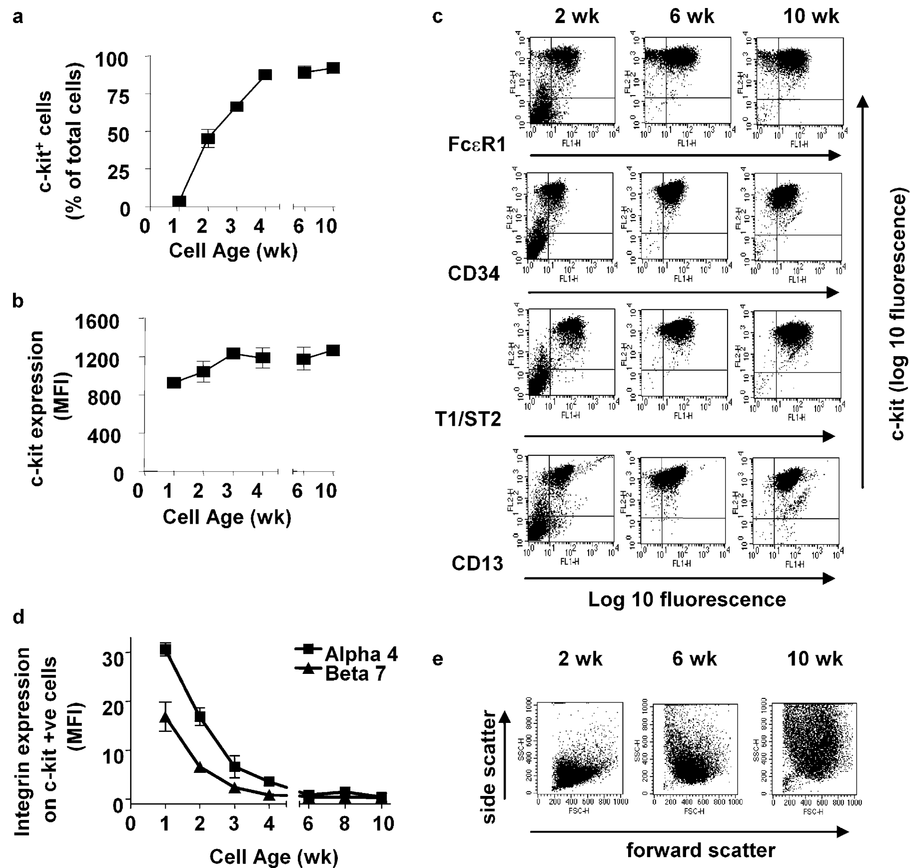

Figure 1. Characterization of BMMC cultures. The percentage of c-kit$^+$ cells in the total BMMC culture (a) and the mean fluorescence intensity of c-kit expression on the c-kit$^+$ BMMCs (b) were measured by flow cytometry. Data shown are ±SEM ($n = 9$). (c) Representative dot plots showing double staining of 2-wk- (left), 6-wk- (middle), and 10-wk-old (right) BMMCs with anti–c-kit–PE (c-kit) and either IgE followed by anti-IgE–FITC (top, FcεR1), anti-CD34–FITC (top middle, CD34), anti-T1/ST2–FITC (bottom middle, T1/ST2), or anti-CD13–FITC (bottom, CD13). Data shown are ±SEM ($n = 6$). (d) The mean fluorescence intensity of α_4 and β_7 integrins on c-kit$^+$ BMMCs decreases from 1 to 10 wk. (e) Representative dot plots showing forward and side scatter of 2-wk- (left), 6-wk- (middle), and 10-wk-old (right) BMMCs. Data shown are ±SEM ($n = 4$).

in their chemotactic responses (e.g., no responses were elicited by any of the chemokines tested). We investigated whether mature mast cells release factors during activation that are chemotactic for immature mast cells. Our evidence demonstrates that leukotriene (LT) B_4 is such a factor that potently attracts mouse immature mast cells. Interestingly, this effect is transient and is lost on cell maturation, correlating with the loss of the LTB_4 receptor, BLT1. Human mast cells exhibit a similar transient responsiveness. Mouse and human immature mast cells were unresponsive, or relatively poorly responsive, to SCF; this suggests that its receptor, c-kit, is not efficiently linked to the locomotor machinery at an early stage in mast cell development.

LTB_4 was originally discovered as an arachidonate metabolite that stimulates neutrophils (18) and has recently been shown to be a chemoattractant for effector $CD4^+$ and $CD8^+$ T-lymphocytes (19–21), acting via the high affinity receptor BLT1. The results presented here suggest that LTB_4 released from activated mature mast cells may also have an important autocrine role in regulating the release of mast cell progenitors from the bone marrow and/or their recruitment into tissues before maturation.

RESULTS

Effects of chemotactic agents on BM-derived mast cells (BMMCs)

Mast cells cultured in IL-3 were analyzed in terms of purity and maturation state for up to 10 wk, with the aim of studying chemotactic responses in cells of minimal maturity at a time when numbers were such that experiments were practicable. Cells were analyzed weekly by flow cytometry; results are shown in some cases for 2-, 6-, and 10-wk-old BMMCs (Fig. 1, c and e). After 1 wk, ~1% of cells exhibited high c-kit expression, but this rose to ~50% by 2 wk in culture (Fig. 1 a). At 4 wk, >85% of cells were c-kit$^+$, and this was maintained up to 10 wk (Fig. 1 a). The intensity of c-kit expression was high at 1 wk; this increased until 3 wk and was then maintained (Fig. 1 b). Identification of c-kit$^+$ cells as mast cells was confirmed by double labeling with antibodies to other mast cell markers, including FcεR1, CD34, T1/ST2 (22), and CD13 (Fig. 1 c). Expression of FcεR1 and TI/ST2 did not change detectably from 2 to 10 wk in culture, whereas CD34 and CD13 did not reach maximum levels until 3–4 wk. Expression of the integrins α_4 and β_7 was observed on 1-wk-old c-kit$^+$ BMMCs, which was consistent with the progenitor mast cells previously described as c-kit$^+\alpha_4^{hi}\beta_7^+$ (12). This expression decreased from 1 to 4 wk and remained at low levels up to 10 wk in culture (Fig. 1 d). At 2 wk in culture, the granularity of the cell population measured as side scatter by flow cytometry was low. By 6 wk, some cells showed an increase in side scatter; at 10 wk, most of the cell population exhibited substantial granularity, which was consistent with mature mast cells (Fig. 1 e).

Chemotactic responses of 2-wk-old BMMCs were measured using 96-well microchemotaxis plates. Migrated cells were recovered from the lower chambers after 3 h at 37°C and double stained with monoclonal antibodies to c-kit (mast cells) and Gr-1 (a marker for the major contaminating population). The c-kit$^+$ cells migrated in response to SCF when present in the lower chambers. With SCF used as the positive control in further experiments, none of the following were found to be chemotactic for c-kit$^+$ cells under the conditions of our experiments: (a) cytokines/growth factors (NGF, TGF-β_1, activin-A, and VEGF); (b) CC chemokines (JE [CCL2], MIP-1α [CCL3], RANTES [CCL5], Eotaxin-1 [CCL11], MIP-3β [CCL19], and MEC [CCL28]); (c) CXC chemokines (KC and MIP-2 [orthologues of the human GRO family], MIG [CXCL9], IP-10 [CXCL10], I-TAC [CXCL11], and SDF-1α [CXCL12]); and (d) peptides (fMLP, bradykinin, and substance P). In the same assays, Gr-1$^+$ cells present in the upper chambers migrated in response to KC, MIP-2, MIP-1α, JE, RANTES, and fMLP (e.g., KC at 0.1, 1, and 10 nM induced the migration of 49 ± 29, 118 ± 35, and 219 ± 34 Gr-1$^+$ cells, respectively, compared with 9 ± 4 cells for the control; $n = 4$ experiments).

Migration of 2-wk-old BMMCs in response to mast cell activation products

We investigated the possibility that mature mast cells, on activation, release a chemoattractant for immature cells. 10-

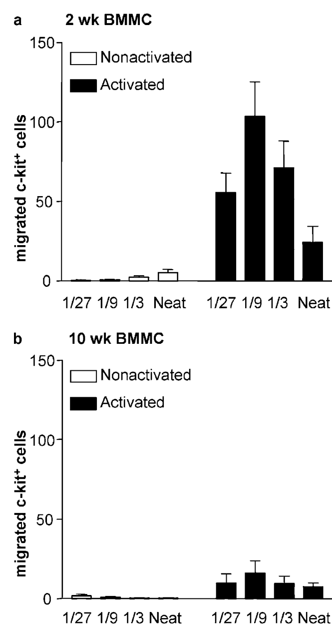

Figure 2. Activated mast cell supernatants induce migration of 2-wk-old BMMCs. 10-wk-old BMMCs were either nonactivated (open bars) or activated through FcεR1 cross-linking for 2 h (closed bars), and cell-free supernatants (undiluted [NEAT] or diluted at 1:3, 1:9, and 1:27) were added to the lower wells of a 96-well chemotaxis plate. BMMCs cultured for 2 (a) or 10 wk (b) were added to the top wells. After a 3-h incubation, migrated cells were removed from the wells, double stained with anti–c-kit–PE and anti–Gr-1–FITC, and c-kit$^+$ BMMCs were counted by flow cytometry. No cells migrated to assay buffer alone. Data are mean ± SEM ($n = 3$) for 10-wk-old cultures to generate the supernatants.

wk-old BMMCs were passively sensitized with IgE and activated by the specific antigen, DNP, conjugated to human serum albumin (HSA). Supernatants were then tested in chemotaxis assays on 2-wk-old immature and 10-wk-old mature BMMCs. Supernatants from nonactivated cells induced negligible chemotaxis; however, supernatants from activated cells induced pronounced migration of 2-wk-old cells with a bell-shaped concentration–response curve and a maximal response at a 1:9 dilution (Fig. 2 a). In comparison, only very weak responses were obtained using 10-wk-old cells in the chemotaxis assay (Fig. 2 b).

Identification of chemoattractant activity in supernatants from activated mast cells

Supernatants from activated and nonactivated 10-wk-old BMMCs were applied to reverse phase HPLC and fractions were collected for chemotaxis assay on 2-wk-old BMMCs. Virtually all the chemotactic activity from activated mast cell supernatants was present in fraction 20 (41% acetonitrile [ACN]), with a small amount in fraction 21 (Fig. 3). No chemotactic activity was detectable in the other fractions or in any of the fractions from the nonactivated cell supernatants (Fig. 3). Known products of mast cell activation were subsequently run through the same column as authentic standards and their retention times were recorded by absorbance at 280 nm (for LTs) or 200 nm (for non-LT standards). LTB_4 eluted with a retention time indistinguishable from that of the activated mast cell–derived activity (fraction 20), in contrast to the peptido-LTs, LTC_4 (fraction 17/18), LTD_4 (fraction 21), and LTE_4 (fraction 21/22). As shown in Fig. 3, other mast cell activation products—PGD_2, histamine, and 5-hydroxytryptamine—eluted much earlier in the gradient than the mast cell–derived chemotactic activity, as did the 20-hydroxy metabolite of LTB_4. Thus, the chemotactic activity in the supernatants of activated mast cells coeluted with authentic LTB_4 and not with any other known product of acute activation. Furthermore, ELISA of the HPLC fractions of activated mast cell supernatants showed that the majority of LTB_4 immunoreactivity was in fraction 20 (10.6 nM) with a smaller amount (4.6 nM) in fraction 21.

The generation of chemotactic activity for 2-wk-old BMMCs was abolished by pretreating mature mast cells with MK-886, an inhibitor of 5-lipoxygenase (5-LO) activating protein before activation (Fig. 4 a). Supernatants were assayed on 2-wk-old BMMCs as before. MK-886 at 100 nM re-

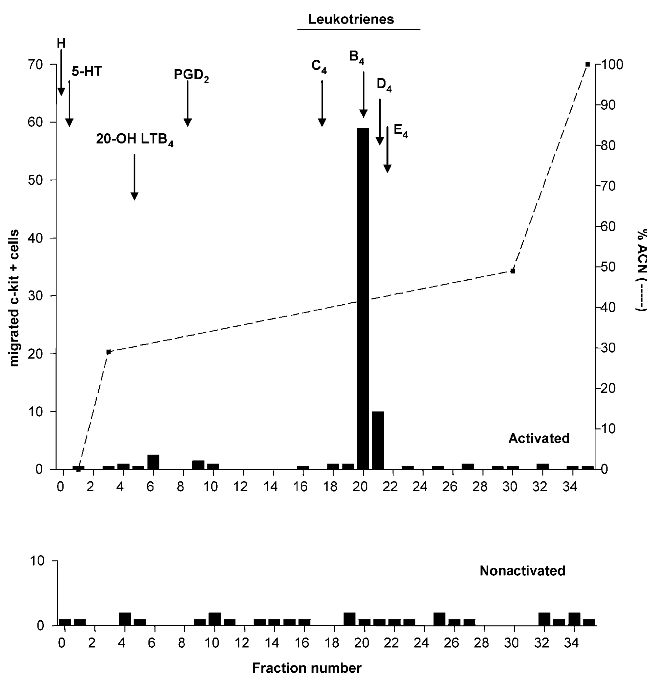

Figure 3. HPLC of the mast cell progenitor chemoattractant activity released by mature mast cells. 10-wk-old BMMCs were either activated via FcεR1 cross-linking for 2 h (top), or nonactivated (bottom), and the supernatants applied to reversed phase HPLC. Fractions were tested for chemotaxis of c-kit$^+$ 2-wk-old BMMCs. This result is representative of two independent experiments using supernatants from different 10-wk-old BMMC cultures tested on different 2-wk-old cultures. Arrows indicate the elution of LT (B_4, C_4, D_4, E_4, and 20-hydroxy-LTB_4) and non-LT (histamine [H], 5-hydroxy tryptamine [5-HT], and PGD_2) standards.

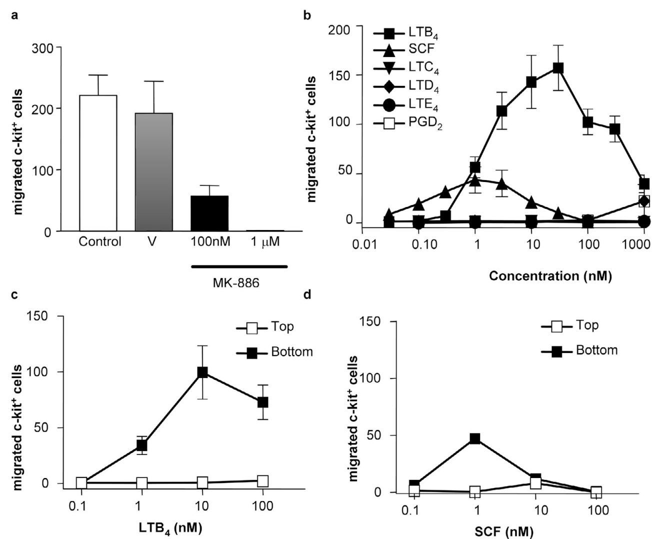

Figure 4. LTB_4 is chemotactic for 2-wk-old BMMCs. (a) 10-wk-old BMMCs were activated through FcεR1 cross-linking for 2 h after pretreatment for 10 min at 37°C with a 5-LO inhibitor (black bars, MK-886), vehicle alone (gray bar, V), or buffer (open bar, control). Cell supernatants were added to the lower wells of chemotaxis plates at a 1:9 dilution and 2-wk-old BMMCs were added to the upper wells. After 3 h, migrated cells were removed from the wells and c-kit$^+$ BMMC migration was counted. MK-886 inhibited migration of 2-wk-old BMMCs at 100 nM and 1 μM ($P < 0.05$). Data are ±SEM ($n = 3$). (b) SCF (0.1–100 nM), LTB_4, LTC_4, LTD_4, LTE_4, and PGD_2 (0.1–1,000 nM) were tested for their ability to induce migration of 2-wk-old BMMCs. LTB_4 and SCF induced migration of 2-wk-old BMMCs, but LTC_4, LTD_4, LTE_4, and PGD_2 induced no migration. Data are mean ± SEM for LTB_4 and SCF ($n = 8$), LTC_4 ($n = 4$), and LTD_4, LTE_4, and PGD_2 ($n = 3$). LTB_4 (c) or SCF (d) were added to either the top or the bottom wells of a 96-well chemotaxis plate and 2-wk-old BMMCs were added to the top wells. Migrated cells were removed and c-kit$^+$ BMMCs counted. Data are mean ± SEM ($n = 3$). No cells migrated to assay buffer alone in any experiment.

duced chemotactic activity substantially, whereas 1 μM gave complete inhibition. Supernatants from untreated activated mast cells contained 46 nM LTB_4 (3.1 ng/10^6 cells), measured by ELISA, which was reduced to 4 nM and <0.06 nM with 100 nm and 1 μM MK-886, respectively.

Comparison of the chemotactic activities of lipid mediators on 2-wk-old BMMCs

The chemotactic activity of lipid mediators tested on 2-wk-old BMMCs was compared with that of SCF (Fig. 4 b). LTB_4 had a high potency and efficacy. SCF had a much lower efficacy and the peptido-LTs C_4, D_4, and E_4 were inactive. In addition, PGD_2 (Fig. 4 b), 5-hydroxytryptamine, histamine, and platelet activating factor (unpublished data) were also inactive as chemotactic agents on these cells. 20-hydroxy-LTB_4, a metabolite of LTB_4, exhibited chemotactic activity, but was 100-fold less potent than its parent (unpublished data). The c-kit$^+$ cells migrated in response to both LTB_4 and SCF when the agents were present in the lower chambers, but not when added with the cells above the filter, characteristic of a chemotactic response requiring a gradient, rather than a chemokinetic response, representing increased random movement (Fig. 4, c and d). As well as being active as a chemoattractant for c-kit$^+$ cells cultured for 2 wk in IL-3, LTB_4 was also active on cells cultured for 2 wk in a combination of IL-3 and SCF (unpublished data).

Comparison of the chemotactic effect of LTB_4 on 2- and 10-wk-old BMMCs

The chemotactic effect of LTB_4 was examined on BMMCs at different stages of maturity. LTB_4 was highly potent and efficacious on immature mast cells cultured for 2 wk. By 6 wk, chemotactic responses were very weak and remained so at 10 wk (Fig. 5 a). In contrast, no notable decrease in responsiveness to SCF was observed as the cells matured (Fig. 5 b).

The loss of responsiveness to LTB_4 with mast cell maturation was associated with a reduction in mRNA expression of BLT1. Cells cultured for 2, 6, and 10 wk were purified by positive selection using CD117 (c-kit) immunomagnetic beads. Fig. 6 a shows, using RT-PCR, the decrease in mRNA as the mast cells matured. Using real-time PCR analysis, 2-wk-old BMMCs were shown to express fourfold more BLT1 mRNA than 6-wk-old cells, and 10-fold more than 10-wk-old cells (Fig. 6 b).

Chemotactic effect of LTB_4 on freshly isolated cell suspensions from mouse femoral bone marrow

Fresh cell suspensions of mouse femoral bone marrow were tested in 96-well chemotaxis assays using LTB_4 and SCF at concentrations effective on cultured BMMCs (Fig. 4 b). After 3 h of incubation, migrated cells were recovered from the lower chambers and cultured for 2 wk in IL-3, IL-9, SCF, and TGF-β1, a medium favoring rapid maturation toward a mucosal mast cell phenotype (23, 24) to enable quantitation of mast cell numbers by their content of mast cell–specific

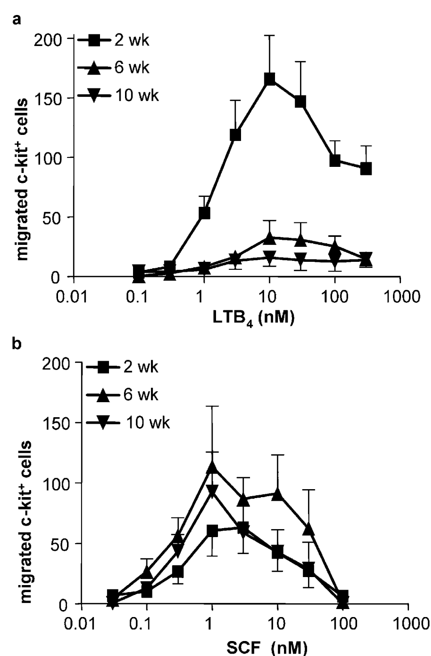

Figure 5. LTB_4 is a more efficacious chemoattractant for 2-wk-old than for 6- or 10-wk-old BMMCs. LTB_4 (a) or SCF (b) were added to the bottom wells of a 96-well chemotaxis plate and 2-, 6-, and 10-wk-old BMMCs were added to the top wells. After 3 h, migrated cells were removed, stained, and c-kit$^+$ BMMCs were counted. No cells migrated to assay buffer alone. Data are mean ± SEM for 2- and 10-wk-old BMMCs ($n = 6$), and 6-wk-old BMMCs ($n = 4$).

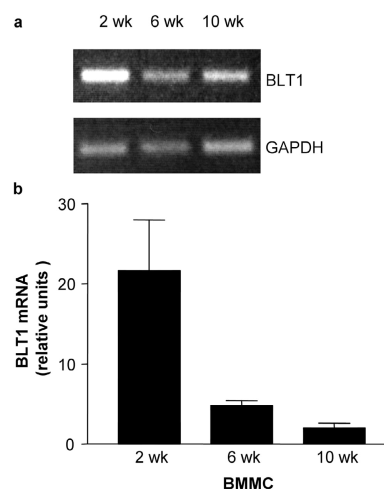

Figure 6. Expression of BLT1 mRNA in c-kit$^+$ 2-wk-old BMMCs. (a) BLT1 mRNA expression, analyzed by RT-PCR and compared with the housekeeping gene GAPDH, was higher in 2-wk-old than in either 6- or 10-wk-old BMMCs. The result is representative of three independent experiments using RNA isolated from separate cell cultures. (b) Real-time PCR analysis of BLT1 mRNA expression in 2-, 6-, and 10-wk-old BMMCs. Fold increases in BLT1 mRNA in BMMCs were normalized to GAPDH, with an internal 18S control to which both GAPDH and BLT1 were normalized. 2-wk-old BMMCs expressed more BLT1 mRNA than 6- or 10-wk-old BMMCs ($P < 0.05$). Data are mean ± SEM ($n = 3$) using RNA isolated from separate cell cultures.

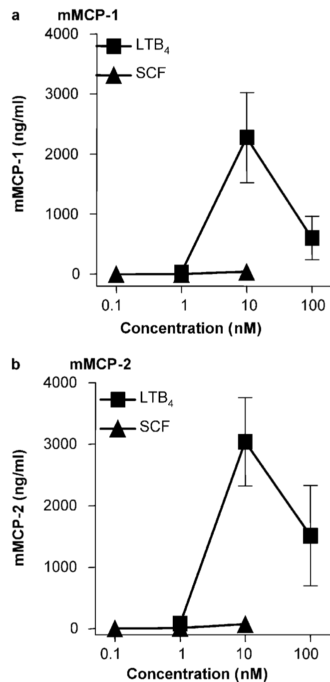

Figure 7. Freshly isolated bone marrow cells that migrate in response to LTB_4 develop into mMCP-1– and -2–expressing mature mast cells. LTB_4 and SCF were added to the bottom wells of a 96-well chemotaxis plate, and freshly isolated bone marrow cells were added to the top wells. After a 3-h incubation, migrated cells were cultured for 14 d in the presence of TGF-β1, SCF, IL-3, and IL-9. On day 14, cells were frozen, lysed, and assayed for levels of mMCP-1 (a) and -2 (b). LTB_4 -induced migration of cells that developed into mMCP-1– and -2–expressing cells (mast cells), but SCF did not induce migration of this cell population. Migration to buffer alone gave mMCP-1 and mMCP-2 values below the detection limits of the assays (>0.04 ng/ml). Data are mean ± SEM (n = 3) for different bone marrow cell preparations.

proteases. Cells were then lysed by freeze-thawing and the released murine mast cell proteases (mMCP)-1 and -2 were quantitated using ELISA. Although the number of mast cell progenitors in the bone marrow was low (25) and, thus, the migrating cells were few in number, this system provided amplification to enable a measurement in proportion to the number of migrated mast cell progenitors. LTB_4 induced a concentration-related increase in both mMCP-1 and -2, with a maximal effect, seen at 10 nM (Fig. 7, a and b). Interestingly, SCF failed to induce a detectable response; no proteases were detected nor were any cells observed microscopically in the cultures before the lytic step (Fig. 7, a and b). Chemokines acting via CCR3, Eotaxin-1, and CXCR2, KC and MIP-2, also failed to demonstrate a chemotactic effect in this system (unpublished data).

Localization of prelabeled BMMCs in vivo after intradermal (i.d.) injection of LTB_4

2-wk-old 5-chloromethylfluorescein diacetate (CMFDA)–labeled BMMCs were either negatively selected by immunomagnetic bead separation to remove the contaminating $Gr\text{-}1^+$ cells, or c-kit$^+$ BMMCs were positively selected. The isolated cells were injected intravenously into mice, followed by i.d. injections of 150 pmol LTB_4 or vehicle control at separate sites in the dorsal skin of each mouse. Animals were killed 1 h after i.d. injections, and the skin was removed and processed for microscopy. Negatively selected CMFDA-labeled BMMCs accumulated at the LTB_4 injection sites, compared with the few cells present in the vehicle control injection sites (Fig. 8, a–c; $P < 0.05$). The same was seen for positively selected CMFDA-labeled BMMCs (Fig. 8 d), with 23.2 ± 4.7 cells/50 mm^2 accumulating at the LTB_4 site compared with 9.4 ± 1.9 cells/50 mm^2 accumulating at the vehicle control site (n = 5, $P < 0.05$). The majority of CMFDA-labeled BMMCs observed were extravascular. Fig. 8 (b–d) shows representative images of CMFDA-labeled BMMCs detected in the skin by confocal microscopy.

Chemotactic effect of LTB_4 on immature mast cells derived from human umbilical cord blood–derived mast cells (CBMCs)

Mast cells were cultured from human umbilical cord blood using an established method (26). A uniform population of c-kit$^+$ cells was evident at ~4 wk that exhibited the charac-

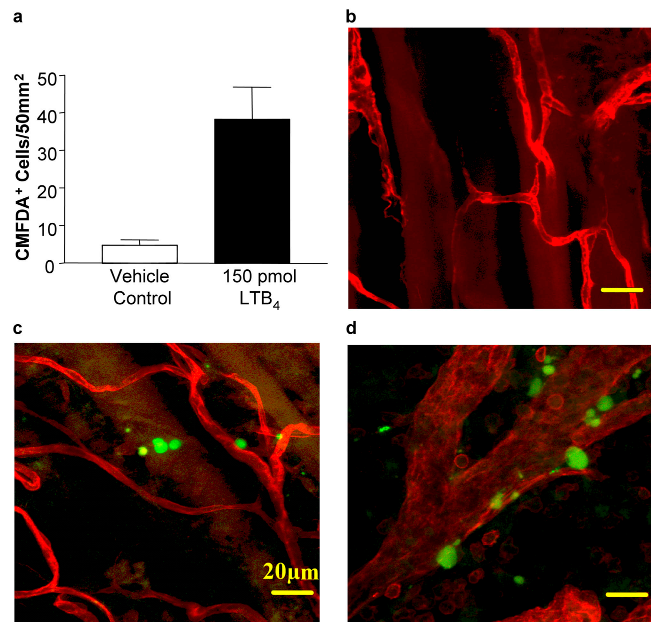

Figure 8. Localization of prelabeled BMMCs in mouse skin in response to i.d. LTB_4. (a) Increased numbers of CMFDA-labeled BMMCs (negative selection) in skin injected with 150 pmol LTB_4 compared with vehicle control sites. Data are mean ± SEM (n = 4; $P < 0.05$). (b–d) Representative confocal micrographs of whole-mount mouse skin. Prelabeled CMFDA-labeled BMMCs are green in skin samples counter-stained with GSL-1 Isolectin B4, staining endothelium, and neutrophils red. (b) Vehicle control (negative BMMC selection). (c) LTB_4 injection site showing extravascular CMFDA-labeled BMMCs and capillaries of 4–6-μm diameter (negative BMMC selection). (d) LTB_4 injection site showing CMFDA-labeled BMMCs migrating from venules of 18–25 μm (positive BMMC selection). Bars, 20 μm.

teristics of immature mast cells, such as detectable but low FcεR1 expression, low side scatter, and lack of specific markers for other cell types, including CD14 (monocytes), CD16 (neutrophils), and CD123 (basophils). In subsequent weeks of culture, a population of mast cells with a more mature phenotype was also observed, with higher expression of both c-kit and FcεR1, but no expression of CD14, CD16, or CD123, and increased side scatter, indicating increased granularity. By 8–9 wk, only the more mature c-kit^{high} FcεR1^{high} mast cell population was present, also in agreement with Ochi et al. (26). The two populations are illustrated in Fig. 9 (a–e) in a representative 7-wk-old culture. Chemotaxis of the cultured cells was investigated between 5 and 7 wk when both the c-kit$^+$ and c-kit^{high} mast cell populations were present. The proportions of the two populations varied between cord blood preparations and over the 5–7-wk period, from 32 to 80% (56.2 ± 8.3%, n = 5) c-kit$^+$ and 20 to 68% (43.8 ± 8.3%, n=5) c-kit^{high} cells. Chemotaxis experiments were performed in 96-well plates using the mixed cultured cells, and the numbers of migrated cells from each of the two populations were determined according to intensity of c-kit expression by flow cytometry. LTB_4 was a highly potent and efficacious chemoattractant for the immature c-kit$^+$ mast cell population (Fig. 9 f), but the mature c-kit^{high} mast cells were unresponsive (Fig. 9 g). In contrast, the mature cells responded to SCF, albeit with low efficacy (Fig. 9 g), whereas the immature cells showed no response (Fig. 9 f).

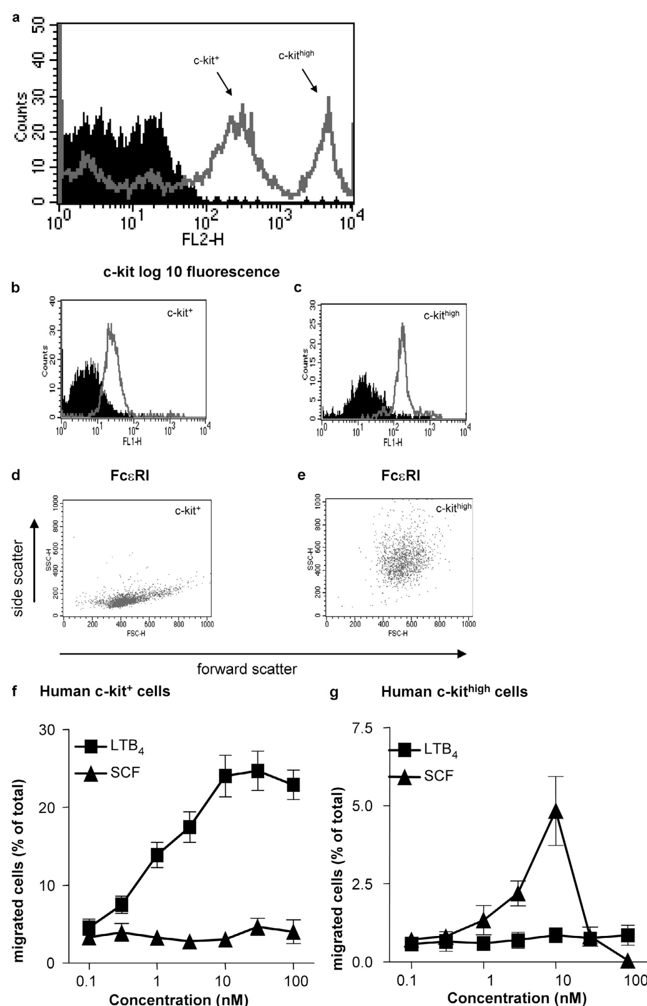

Figure 9. LTB_4 induces migration of immature, but not mature, human CBMCs. (a) Representative histogram of CBMCs cultured for 7 wk and stained with anti–human c-kit–PE (open histogram) or isotype control (shaded histogram). Two populations of c-kit–expressing mast cells can be seen (c-kit$^+$ and c-kit^{high}). (b and c) Histograms showing that FcεR1 expression (open histogram) is detectable above the control antibody (shaded histogram) on both c-kit$^+$ and c-kit^{high} cells. (d and e) Representative dot plots showing forward and side scatter of the c-kit$^+$ and c-kit^{high} cells. Migration of 5–7 wk c-kit$^+$ (f) and c-kit^{high} (g) mast cells to LTB_4 and SCF. Migrated cells were stained with anti–human c-kit–PE and anti–human CD14–FITC and counted. Migration to assay buffer alone was 4.5 ± 1.4% c-kit$^+$ cells and 0.7 ± 0.15% c-kit^{high} cells. Data are mean ± SEM (n = 5) using cells cultured from separate donors.

DISCUSSION

Cell migration is of critical importance at several stages during the life history of the mast cell. Committed mast cell progenitors, derived from pluripotential stem cells in the bone marrow stroma, must first transit toward and through the sinus endothelium to gain access to the general circulation. The progenitors then attach to and migrate through the microvascular endothelium of peripheral tissues. There, under the influence of local factors, the progenitors begin to mature and can migrate away from blood vessels toward their final destination, where they exhibit a phenotype characteristic of their location. We are particularly interested in mucosal mast cells whose numbers increase markedly in apposition to epithelial surfaces in response to allergic stimuli, a process associated with the migration of cells from the lamina propria and thought to include recruitment from microvessels in this region.

To investigate potential chemoattractant molecules involved in progenitor recruitment from the blood, we cultured mouse bone marrow cells with IL-3 and measured their responses in chemotaxis chambers using cells as immature as possible at a time when c-kit$^+$ cells were of sufficient numbers for study. Under the conditions of our experiments, these immature c-kit$^+$ cells were remarkably unmoved by a range of known chemotactic agents. The cells responded chemotactically to SCF but not to other factors tested, such as chemokines acting through CXCR2 or CCR3, receptors previously described on mast cells (26–28). We were surprised at this lack of responsiveness, which contrasts with many reports of chemotactic responses of mast cells to a range of mediators, including chemokines (27, 29–33). We believe that this difference is largely attributable to the stage of maturity of the cells as we concentrated on very immature mast cells, but differences in methodology may also play a part.

In light of the mast cell hyperplasia observed in sensitized individuals exposed to allergen, we investigated whether activation of mature mast cells by cross-linking their FcεR1 results in the production of a chemoattractant for immature mast cells. We demonstrate that immature mast cells cultured for 2 wk from mouse bone marrow are highly responsive in vitro to the chemotactic effect of LTB_4 released on activation of mature mast cells. In addition, immature cells were able to accumulate in response to intradermally injected LTB_4 in vivo. The results shown in Fig. 7 indicate that mast cell progenitors in fresh bone marrow also respond highly to LTB_4 in vitro, although it is not possible from this study to determine precisely when the receptor is first expressed. The responsiveness to LTB_4 is transient and is lost as the cells mature, correlating with the loss of mRNA for BLT1. These results are consistent with those in a recent study showing a weak chemotactic effect of LTB_4 on BMMCs (chemotactic index of ∼2) as cells of 4–6 wk in culture were used (34). Interestingly, results obtained with human CBMCs were very similar to those obtained with mouse BMMCs. Human immature mast cells were highly responsive to LTB_4, an effect lost on maturation.

The lack of responses of mature mast cells to LTB_4 may explain why this mediator has previously been identified only as a product of this cell rather than as its chemoattractant. Human and mouse mast cells have been reported to produce between 1 and 4 ng $LTB_4/10^6$ cells after FcεR1 cross-linking (35–37). Accordingly, we found that our activated BMMC supernatants contained 3.1 ng $LTB_4/10^6$ cells, and nonactivated mast cells produced no detectable LTB_4. BLT1 expression has been investigated previously on mast cells in tissues during allergic reactions, but was reported to be very low (38). In contrast, cysteinyl LT receptors are expressed on mast cells in tissues during allergic disease and by human CBMCs (38–40). The observation that immature mast cells respond strongly to a major activation product of mature cells may be more than a coincidence. The evanescent responsiveness of mast cells to LTB_4, correlating with transient BLT1 mRNA expression, strongly implies an important function at a critical stage in their life history. We suggest that LTB_4 produced by activated mast cells in tissues is able to recruit circulating mast cell progenitors and contribute to mast cell hyperplasia at sites of allergic reactions (41, 42). Furthermore, LTB_4 may contribute to the movement of immature mast cells from the lamina propria to the epithelium of affected tissues. Once at their final location, the mature cells then become insensitive to LTB_4 because of the loss of BLT1. The reason for this may be to ensure that mature mast cells do not congregate exclusively at areas of focal allergen stimulation, leaving adjacent areas of epithelium depleted.

Recruitment of mast cell progenitors would also be expected to result from LTB_4 production by cells other than mast cells, e.g., neutrophils. Irrespective of the cellular source of LTB_4, progenitor recruitment would only result in population with mature cells if the appropriate maturation factors were present. The general mechanisms we describe may also operate in response to foreign organisms; e.g., helminths and microorganisms. It will be of interest to investigate the importance of these mechanisms in disease models in which mast cells have a critical role. One example is a mouse autoimmune arthritis model in which mast cells are obligatory (43). LTB_4 may drive both the recruitment of mast cell progenitors and neutrophils in this model. Similarly, a mouse model of experimental allergic encephalomyelitis is described as mast cell dependent (44–46), and an LTB_4 receptor antagonist has been shown to suppress disease pathology in this model (47).

Chemoattractants generated at sites of inflammation serve an important function in recruiting inflammatory cells. These same mediators, having gained access to the general circulation, can act remotely and release leukocytes from the bone marrow by establishing a chemotactic gradient across the sinus endothelium (15), as exemplified by eotaxin-1, that can release both mature eosinophils and their progenitors (14, 16). The experiments described here using freshly isolated bone marrow cell suspensions demonstrate that mast cell progenitors in the marrow can respond to low concentrations of LTB_4. Thus, circulating LTB_4 may induce progenitor release in vivo. Although c-kit, the receptor for SCF, is a classical marker for mast cells, it may be important that fresh mouse mast cell progenitors and the immature human CBMCs were shown to be unresponsive to SCF as a chemoattractant. SCF clearly has a vital role in mast cell development, but we suggest that this factor may not have a role in releasing mast cell progenitors from the bone marrow or their recruitment into tissues. Indeed, SCF gradients established in the bone marrow as the result of local production in the stroma would be in the wrong direction to mediate movement of cells into sinuses by chemotaxis, although theoretically such gradients could be reversed by elevation of circulating SCF as a consequence of production in other tissues. The observation that the mouse bone marrow cells cultured for 2 wk with IL-3 did respond chemotactically to SCF (albeit with relatively low efficacy) suggests that these cells are a little more advanced in maturity than the bone marrow progenitor mast cells; these 2-wk-old cells retain responsiveness to LTB_4, but now have the c-kit receptor coupled to the locomotor machinery. Both mouse and human mature mast cell did respond chemotactically to SCF, so it is possible that this mediator is involved with relocating mature or maturing mast cells within tissues once the progenitors have been recruited. This would be an additional function to the critical role of SCF in mast cell proliferation and maturation.

Thus, LTB_4 may serve the function of an autocrine mediator produced by activated mast cells, mediating the supply of their progenitors to the tissue. This may be important in the mast cell hyperplasia that is overt in allergic reactions and responses to helminth parasite infections. If this is the case, it would follow that interventions resulting in an increase in mast cell activation would increase tissue mast cell numbers. Such an increase in numbers has been observed in a recent

study after deletion of RabGEF1, which is a component of a negative feedback pathway that makes mast cells more readily activated (48). *Rabgef1*$^{-/-}$ mice showed spontaneous inflammation in the skin associated with mast cell degranulation and increased mast cell numbers (48). Alternatively, interventions aimed at suppressing the production or actions of LTB_4 e.g., using 5-LO inhibitors or BLT1 antagonists, may suppress mast cell hyperplasia and, thus, be of benefit in allergic diseases, such as allergic rhinitis and asthma.

MATERIALS AND METHODS

Reagents. BSA, PBS, 2-ME, Histopaque 1077, human TGF-β1, FMLP, bradykinin, Substance P, anti–DNP IgE (clone SPE-7), HSA-DNP, and 0.22-μm spin filters were obtained from Sigma-Aldrich. TFA (peptide synthesis grade) was obtained from Rathburn, and ACN (far UV grade for HPLC) was obtained from Merck. RPMI 1640, DMEM, Hepes, penicillin, streptomycin, L-glutamine, FBS, fungizone, sodium pyruvate, and nonessential amino acids were purchased from Invitrogen. PE–rat anti–mouse CD117 (2B8), FITC–rat anti–mouse IgE (R35-72), FITC–rat anti–mouse CD34 (RAM 34), FITC–rat anti–mouse Gr-1 (RB6-8C5), FITC–rat anti–mouse CD13 (R3-242), rat anti–mouse CD16/32 (2.4G2), PE–mouse anti–human CD117 (YB5.B8), FITC–mouse anti–human CD14 (M5E2), FITC–mouse anti–human CD16 (3G8), Cy5–mouse anti–human CD123 (9F5), biotin–rat anti–mouse α4 (R1-2), FITC–rat anti–mouse β7 (M293), the appropriate isotype controls, and FACSFlow were obtained from BD Biosciences. FITC–rat anti–mouse T1/ST2 (2.4G2) was purchased from Morwell Diagnostics GmbH, and polyclonal rabbit anti–human FcεR1 was obtained from Upstate Ltd. Alexa 568–conjugated GSL-1 isolectin B4, AlexaFluor 488 goat anti–rabbit IgG (A-11008), TO-PRO-3, and Cell Tracker green CMFDA were purchased from Molecular Probes. All eicosanoids were obtained from Cayman Chemical. Murine IL-3, SCF, VEGF, human SCF, IL-6 and IL-10, and all chemokines were purchased from PeproTech. Murine IL-9 and human Activin A were obtained from R&D Systems. Murine NGF was purchased from Promega. All microbeads were obtained from Miltenyi Biotec. RNeasy mini kit, HotStar Taq DNA Polymerase, and Omniscript Reverse Transcriptase were obtained from QIAGEN. TaqMan universal PCR mastermix, BLT1, GAPDH, and 18S-specific primers (Assays-on-Demand), and the sequence detection system were (model ABI Prism 7700) purchased from Applied Biosystems.

Mouse BMMC culture. Murine BMMCs were obtained by culture of BALB/c femoral BM cells at 5 × 10^5 cells/ml in RPMI 1640 with 10% FBS, 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 ng/ml murine IL-3. BMMC cultures were maintained for up to 10 wk at 37°C in 5% CO_2. The flask was replaced weekly for up to 3 wk to remove adherent cells, with weekly replacements of half the medium throughout. An aliquot of cells was taken at weekly intervals for immunofluorescence staining. In some experiments, BM cells were cultured with 15 ng/ml SCF in addition to IL-3 (28).

Human CBMC culture. Human mast cells were derived from heparin-treated umbilical cord blood obtained after routine deliveries, according to the method of Ochi et al. (26), with minor modifications. Whole blood was diluted 1:3 with RPMI 1640 and centrifuged through Histopaque 1077 to isolate the mononuclear cells. Residual erythrocytes were removed by hypotonic lysis, and the mononuclear cells were resuspended in RPMI 1640 with 10% FBS, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were seeded at 10^6 cells/ml and cultured in the presence of 100 ng/ml SCF, 50 ng/ml IL-6, and 10 ng/ml IL-10. Cells were cultured for up to 8 wk, and the culture medium and flask were replaced weekly. An aliquot of cells was taken at weekly intervals for immunofluorescence staining.

Cell surface immunofluorescence and flow cytometry. BMMCs or CBMCs were resuspended at 2–5 × 10^6 cells/ml in ice-cold staining buffer (PBS, 0.1% sodium azide, and 0.5% BSA) containing either anti–mouse CD16/32 or goat IgG, respectively, to block Fc receptors. After a 10-min incubation on ice, specific mAbs were added at saturating concentrations and incubated for a further 20–30 min in the dark. Cells were then washed, resuspended in FACSFlow containing TOPRO-3 (1:10,000, to identify dead cells), and analyzed on a flow cytometer (model FACSCalibur; BD Biosciences). For FcεR1 staining, after Fc receptor blocking, BMMCs were incubated with anti-DNP IgE and then FITC-anti–mouse IgE. CBMCs were incubated with polyclonal rabbit anti-FcεR1, followed by AlexaFluor 488 goat anti–rabbit IgG (1:1,000).

Mouse BMMC chemotaxis. Chemotactic responses of BMMCs were examined using 96-well chemotaxis plates (ChemoTx) with 5-μm pore size polycarbonate filters. 20 μl of BMMCs were added to the top wells at 2 × 10^6 cells/ml in RPMI 1640 with 25 mM Hepes and 0.1% BSA (assay buffer), and 30 μl of agonist or buffer was added to the lower wells. After a 3-h incubation at 37°C, migrated cells were removed, the wells washed with 25 μl of assay buffer, and stained with PE-anti–mouse c-kit (1:130) and FITC-anti–mouse Gr-1 (1:160) for 10 min at 22°C to distinguish the mast cells from nonmast cells. 100 μl FACSFlow containing 1:10,000 TO-PRO-3 was added to the cells before counting by flow cytometry. In some assays, the activation products from mature BMMCs were added to the lower wells. To investigate chemokinesis, BMMCs were mixed with agonist before addition to the upper assay chamber and migration to the buffer was determined. Assays were performed in duplicate, and results were expressed as the absolute number of cells migrating.

Mouse freshly isolated BM cell chemotaxis. Chemotaxis of freshly isolated BM cells was measured in a similar way to the cultured BMMCs, except that 20 μl BM cells at 10^7 cells/ml were added to the upper wells of chemotaxis plates immediately after isolation from femurs and aspiration through a 19-gauge needle. Migrated cells were removed and cultured for up to 14 d in DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml fungizone, 1 mM sodium pyruvate, 1 ng/ml TGF-β1, 1 ng/ml murine IL-3, 5 ng/ml mIL-9, and 50 ng/ml mSCF (24). Assays were performed in quadruplicate and, after cell lysis, cells were enumerated by specific ELISAs for mMCP-1 and -2 (49, 50).

In vivo mast cell tracking. 2-wk-old BMMCs (2 × 10^7 cells/ml) were labeled with 25 μM Cell Tracker Green CMFDA for 40 min at 37°C. Cells were purified by immunomagnetic separation by either negative selection using Gr-1 FITC followed by anti-FITC microbeads, to remove Gr-1$^+$ contaminating cells, or by positive selection using anti-CD117 (c-kit) microbeads. The isolated cells (10^6 cells/BALB/c mouse) were administered by tail vein injection 5 min before i.d. injections into dorsal skin of 50 μl LTB_4 in Tyrode's solution (150 pmol/site) and vehicle control in each mouse. Mice were killed 1 h after i.d. injection. The skin was removed and fixed in 4% paraformaldehyde for 24 h. A quarter segment from an 8-mm-diameter biopsy (Schuco International) was whole mounted in a hard+set mounting medium (VECTASHIELD; Vector Laboratories) for examination by standard epifluorescence microscopy. The number of CMFDA$^+$ cells in each one fourth skin biopsy was counted. A second quarter segment of each skin biopsy was counterstained with 10 μg/ml Alexa 568–conjugated GSL-1 isolectin B4, which is a marker for mouse endothelial cells and neutrophils (unpublished data). This tissue was then whole mounted and examined by confocal microscopy using a TCS NT system (Leica). Home Office guidelines for animal welfare based on the Animals (Scientific Procedures) Act 1986 were strictly observed.

Human CBMC chemotaxis. CBMCs cultured for 5–7 wk were used to measure chemotaxis of human mast cells in the same way as BMMCs, except that 20 μl of cells at 10^6 cells/ml were used. The mast cells were identified by staining with PE-anti–human c-kit (1:100) and FITC-anti–human CD14 (1:100) for 10 min at 22°C before diluting with FACSFlow containing TOPRO-3 and counted. Assays were performed in duplicate, and the

migration of c-kit$^+$ or c-kit^{high} mast cells was expressed as a percentage of the total c-kit$^+$ or c-kit^{high} cells added to the wells, respectively.

Mast cell activation. Mature (10 wk) BMMCs (5×10^6 cells/ml) were incubated with 10 μg/ml anti-DNP IgE for 1 h at 37°C. Cells were washed in RPMI 1640 with 0.1% BSA, resuspended in HSA-DNP at 30 ng/ml, and incubated for 2 h at 37°C. Some cells were left untreated, or treated with either anti-DNP IgE or HSA-DNP alone to control for basal secretion of chemoattractant mediators. The supernatants were collected by centrifugation through a 0.22-μm filters. Supernatants were either used undiluted (NEAT) or diluted at 1:3, 1:9, or 1:27 into RPMI 1640 with 0.1% BSA. In some assays, cells were preincubated with MK-886 (5-LO inhibitor; Alexis Biochemicals) for 10 min at 37°C before activation, along with DMSO-only controls (vehicle) and cells that were treated with neither MK-886 nor DMSO.

Reverse phase HPLC. 400 μl of activated or nonactivated 10-wk-old mast cell supernatants, adjusted to pH 2.0 with 20% TFA and further diluted in 0.08% TFA, pH 2.0, or 500 pmol of eicosanoid standards, 400 nmol histamine, and 40 nmol 5-hydroxytryptamine in 0.08% TFA were applied to a μRPC C2/C18 PC 3.2/3 column (Pharmacia Smart System; GE Healthcare). The flow rate was one column volume (240 μl)/min in 0.08% TFA, and the gradient was started 15 min after sample injection: 0–30% ACN at 5% ACN/min, 30–50% ACN at 1% ACN/min, and 50–100% ACN at 5% ACN/min. Fractions were collected from the start of the gradient (2-min fractions for 0–10 min, 0.5-min fractions for 10–22 min, and 2-min fractions for the remainder of the gradient into carrier BSA), freeze-dried, and dissolved in 400 μl RPMI 1640 with a final concentration of 0.1% BSA for bioassay and ELISA.

ELISA. LTB_4 was quantified using a specific ELISA kit (R&D Systems) with a detection limit of 60 pM.

RT-PCR. 2-, 6-, and 10-wk-old BMMCs were isolated with anti–mouse CD117 (c-kit) immunomagnetic beads and total RNA was extracted using an RNeasy mini kit. First strand cDNA was generated from 0.5 μg of total RNA using Omniscript Reverse Transcriptase with oligodeoxythymidine$_{12-18}$ primers. PCR was performed using primers for mouse BLT1 (available from GenBank/EMBL/DDBJ under accession no. AF044030) 5′-TAAAGTCTTCCATCTGCTCTTCGAA-3′ and 5′-ACTTCGAAGACTCAGGAATGGTGGA-3′ or a using GAPDH control primer set. PCR products were generated using HotStarTaq DNA Polymerase and resolved on a 1% agarose gel containing 0.025 μg/ml ethidium bromide.

Real-time PCR analysis. Total RNA was extracted from purified BMMCs as described in the RT-PCR section and cDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen) with both oligodeoxythymidine and random hexamer primers. 25-μl PCR procedures were performed in triplicate using the sequence detection system (model ABI Prism 7700), with TaqMan universal PCR mastermix and BLT1, GAPDH, and 18S specific primers. Samples were standardized to 18S, and BLT1 mRNA was quantified using the comparative threshold for detection method (http://appliedbiosystems.com).

Statistics. The data are expressed as mean ± SEM. Statistical differences between groups of three or more was estimated using a Kruskal Wallis test with pairs of groups compared with Dunn's multiple comparison test. For groups of two, the Mann-Whitney test was used. $P < 0.05$ was considered significant.

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