Inflammatory Chemoreceptor Cross-Talk Suppresses Leukotriene B4 Receptor 1-Mediated Neutrophil Calcium Mobilization and Chemotaxis After Trauma

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Inflammatory Chemoreceptor Cross-Talk Suppresses Leukotriene B₄ Receptor 1-Mediated Neutrophil Calcium Mobilization and Chemotaxis After Trauma

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G protein-coupled chemoattractants recruit neutrophils (PMN) to sites of injury and infection. The leukotrienes (LT) and CXC chemokines (CXC) and their receptors (BLT1/BLT2 and CXCR1/CXCR2) are all known to play roles in these responses. Each system has been studied separately in vitro, but in vivo they act concurrently, and the clinical interactions between the two systems are unstudied. We prospectively studied calcium mobilization and chemotactic responses to LTB₄ in PMN from major trauma patients. The responses of the high affinity BLT1 receptor were suppressed at the 3-day postinjury time point, but recovered by 1 wk. Trauma patients had transient elevations of plasma LT and CXC levels. Functional deficits identical with those in trauma PMN were reproduced in vitro by exposing healthy PMN to CXC at the elevated plasma concentrations found. Functional responses to LTB₄ were suppressed by cross-talk with CXC and BLT2 receptors that desensitize BLT1. Since the suppression of intracellular calcium mobilization was prominent, we also studied the role of suppressed cell calcium mobilization in the defective chemotactic responses to LTB₄. We noted that PMN chemotaxis to LTB₄ showed far more dependence on store-operated calcium entry than on the release of cellular calcium stores, and that store-operated calcium responses to BLT1 activation were markedly inhibited during the same time period as was chemotaxis. The intermittent release of inflammatory mediators after injury can blunt PMN responses to LTs by suppressing BLT1 as well as downstream calcium entry. Diminished LT receptor activity due to cross-talk with CXC receptors can inhibit PMN recruitment to infective sites. This may predispose injured patients to septic complications.

Trauma patients are highly susceptible to infections like pneumonia, and neutrophil (PMN) recruitment to the lung is critical for the control of pulmonary infection. Both infections and injury lead to the production of inflammatory chemoattractants that act on G protein-coupled (GPC) receptors to recruit PMN to sites of inflammation. After receptor binding, PMN become polarized and migrate toward areas of increasing chemoattractant concentration (1). Leukotrienes and chemokines are two key classes of GPC receptor agonists that have been implicated in these responses. Matsukawa et al. (2) suggested that cross-talk between these two classes of agonist may exist, in that leukotriene (LT) production may be chemokine dependent and crucial for normal host responses to peritonitis. No studies exist, however, that examine cross-talk between LTs and other inflammatory PMN chemoattractants in clinical human disease.

The LT are inflammatory lipid mediators produced by the serial actions of phospholipase A2 and lipoxygenases on membrane lipids. Leukotriene B₄ (LTB₄) is a potent chemoattractant that can cause PMN aggregation, degranulation, adhesion to endothelial cells, and respiratory burst (3–5). Moreover, Marleau (6) has shown in animal models that LTB₄ synthesis plays a role in PMN responses to other chemoattractants, and that high circulating LTB₄ levels may modulate PMN aggregation. LTB₄ has recently been shown to bind to two cell surface GPC receptor subtypes currently termed BLT1 and BLT2. BLT1 is a high affinity LT receptor found predominantly on PMN. The more recently discovered BLT2 is a lower affinity LT receptor that is more widely distributed (4, 7). LTB₄ binding releases intracellular calcium ([Ca²⁺]ᵢ) via the phospholipase C-inositol triphosphate pathway (8). The depletion of PMN microsomal calcium stores then leads to calcium influx via a complex of PMN store-operated calcium (SOC) entry pathways (9). Such stimulated calcium influx pathways provide the prolonged elevations of [Ca²⁺]ᵢ that are essential for many inflammatory processes (10, 11) and can be pathologically altered after major injury (12). IL-8 (IL-8, CXCL8) is a CXC chemokine that plays an important role the pathogenesis of both pneumonia and acute respiratory distress syndrome (13–15). IL-8 is released into the circulation after both mechanical trauma and infections, and in several studies elevated plasma IL-8 levels have been predictive of organ failure and mortality (16, 17). Like the LTs, CXC chemokines act via two related GPC receptors, CXCR1 and CXCR2. CXCR1 and CXCR2 are expressed predominantly on PMN, and both mobilize intracellular calcium as a second messenger. CXCR1 is a somewhat lower affinity receptor and predominantly binds IL-8, CXCR2 is a promiscuous receptor that binds a wide variety of CXC chemokines. PMN stimulation with IL-8 desensitizes CXCR2 and, to a lesser degree, CXCR1 by receptor internalization (18–23). CXCR receptor desensitization has been shown clinically in sepsis (24), and we have noted CXCR2 desensitization after mechanical trauma (25). We hypothesized that...
BLT1/BLT2 and CXCR1/CXCR2 provide parallel and redundant mechanisms for PMN recruitment to the lung, and that such inflammatory receptor systems might interact to down-regulate PMN responses to LTs after injury.

We therefore prospectively assessed PMN responses to LTB4 in major trauma patients, studying PMN calcium mobilization and chemotactic responses to LTB4 at the concentrations found both chronically and acutely in trauma plasma. We then sought to define the role of possible cross-talk with CXC chemokine receptors in the modulation of PMN responses to LTB4 at each LT receptor type. Last, since [Ca²⁺], mobilization is a key function of the LT receptors and may be abnormal after trauma, we studied the role of cell calcium mobilization in chemotactic responses to LTB4.

Materials and Methods

All studies were performed in compliance with the institutional review board of University of Medicine and Dentistry of New Jersey-New Jersey Medical School. Informed consent for blood sampling from trauma patients was obtained from their next of kin. Informed consent for blood sampling was also obtained from volunteer donors.

Trauma patients

Neutrophil samples were obtained prospectively from 36 major trauma patients admitted to the New Jersey State Trauma Center with an injury severity score > 17. Potential study patients were excluded if significant injuries were limited to severe head trauma or if patients were thought unlikely to survive 48 h due to uncontrollable hemorrhage. The mean injury severity score (26) of the final study group was 27.5 (95% confidence interval, 23.5–31.5). Twenty-nine patients were men, and seven were women. Patients ranged from 18 to 68 years of age, with an average age of 35.6 years (95% confidence interval, 30.2–40.0). Twenty-eight patients had a blunt mechanism of injury; eight had suffered penetrating trauma. All patients survived. Samples were obtained on the first day of admission (mean ± SE, 14 ± 1 h after injury), on day 3, and on day 7. The responses of PMN samples to a range of chemoattractants at different doses were tested. The clinical data in this report focus on a subset of patients whose PMN were assayed using high (3 nM) and low (100 pM) concentrations of LTB4.

Volunteers

Age (±5 years), sex-, and ethnicity-matched healthy volunteer controls were identified for each of the trauma patients prospectively studied. Matched volunteer PMN were isolated and studied contemporaneously with and identically to the patient samples.

Neutrophil isolation

Our methods were described in detail previously (27). Briefly, heparinized whole blood samples (25 U/ml) were obtained via indwelling catheters or direct venipuncture and were centrifuged at 150 × g for 10 min. Plasma was reserved, centrifuged at 300 × g, aliquoted, and stored at −80 °C. The entire buffy coat and RBC were then layered onto Polymorphomorph centrifugation medium (Robbins Scientific Corp., Sunnyvale, CA) and centrifuged at 300 × g for 30 min. The supernatant and PBMC layer were discarded. The PMN layer was removed and mixed with an equal volume of 0.45% NaCl solution to restore osmolality. After 5 min of rest, the cells were washed with RPMI solution (10/1) and centrifuged for 10 min at 150 × g. The PMN pellet was then resuspended in 2 ml of HEPES buffer solution: 1.040 mol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl₂, 10 mmol/L glucose, 20 mmol/L HEPES, and 0.1% fatty acid-free BSA, pH adjusted to 7.4). PMN were counted and assessed for purity using flow cytometry. These methods routinely yield PMN of 96–99% purity and > 98% viability by trypan blue.

Calcium dye loading

After adjusting the PMN suspension calcium concentration to 1 mM with CaCl₂ and buffered to pH 7.4 at 37 °C using Tris, PMN were resuspended in 1 ml of HEPES buffer (pH 7.4) with 2 µg/ml fura-2-acetoxymethyl ester (fura-2/AM; Molecular Probes, Eugene, OR). PMN were then divided into aliquots (2 × 10⁶ PMN) and placed on ice in the dark. Just before study, PMN were centrifuged for 5 s at 4500 rpm in a programmable microcentrifuge. The supernatants were removed, and the cells were resuspended in 200 µl of HEPES buffer with or without 1 mM CaCl₂, and then injected into cuvettes containing 2.8 ml of the same buffer for spectrofluorometric study.

Spectrofluorometry

The intracellular free calcium concentration ([Ca²⁺]i) was determined at 37 °C with constant stirring by measuring Fura fluorescence at 505 nm using 340/380-nm dual wavelength excitation in a spectrofluorometer (Fluoromax-2; SPEX, Edison, NJ) using our adaptations of the methods of Grynkiewicz (28, 29). Calibration is achieved by permeabilizing PMN at the end of each experiment with 100 µM digitonin and measuring the Fura fluorescence in 1 mM CaCl₂ solution (Rmax) then adding 15 mM EGTA for a zero calcium solution (Rmin). The fluorescence of cell suspensions treated with 100 µM digitonin and 2 mM MnCl₂ was subtracted from the total fluorescence. The order of study of PMN isolates were alternated to avoid bias related to duration of dye loading or time of cell study.

PMN [Ca²⁺]i responses to these agonists were assessed as the peak transient [Ca²⁺]i concentration change in nanomoles per liter. For experiments performed in nominally calcium-free environments, EGTA was added to calcium-free HEPES buffer to a final concentration of 0.3 mM. This maintains the Ca²⁺ concentration of the medium at or near normal cytosolic calcium levels (~50 nM), thus removing the gradient that normally drives calcium influx. In such nominally calcium-free studies, CaCl₂ can be readded to 1 mM after the [Ca²⁺]i release transient has returned to baseline. This maneuver is used to isolate and assay SOC entry. For experiments performed in zero calcium environments, EGTA was added to calcium-free HEPES buffer to a final concentration of 5 mM.

Plasma IL-8 and LTB4 determinations

Plasma aliquots from trauma patients and their matched volunteer controls were thawed and placed on ice. The plasma concentration of IL-8 was determined by ELISA using a kit (BD Pharmingen, San Diego, CA) according to the manufacturer’s instructions. Samples were diluted appropriately, and the OD₅₇₀ was read with an Automatic Microplate Reader (MR500; Dynatech, Guernsey, Channel Islands). The plasma LTB4 concentration was measured in a similar manner with an LTB4 ELISA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions.

Calcium mobilization by LTB4

After study of basal [Ca²⁺]i, for 30 s, volunteer or patient PMN isolated on days 1, 3, and 7 were initially stimulated with low doses of LTB4 (50–300 pM) that approximated the LTB4 concentration in plasma (see below) and higher doses of LTB4 (3 nM; HD-LTB4) that approximated the concentrations of LTB4 found at inflammatory sites (see below). Volunteer PMN were found to respond to 100 pM LTB4 with [Ca²⁺]i transients between 50–100 nM (Fig. 1A), whereas responses to 3 nM LTB4 produced transients with a 150–250 nM deflection (Fig. 1B). We preliminarily noted that responses to lower doses diminished after injury, but responses to higher doses did not. To evaluate whether this simply reflected a dose-response phenomenon, further studies were performed in the presence of U75302 (Cayman Chemical). U75302 has been reported to be a specific inhibitor of human BLT1 (41). In assays to confirm its specificity, we found that concentrations of U75302 between 50–500 nM totally ablated PMN calcium flux response to 100 pM LTB4 with minimal effects on PMN [Ca²⁺]i mobilization by 3 nM LTB4 (data not shown). U75302 also had no direct effect on IL-8 signaling (Fig. 1C).

After preliminary dose-response studies in the presence of U75302, we determined that 100 pM LTB4 (low dose LTB4; LD-LTB4) acted as a specific agonist for BLT1 and that 3 nM LTB4 (HD-LTB4) acted to mobilize calcium overwhelmingly through BLT2. Clearly, 3 nM LTB4 can elicit 20% of the total response without any effect on BLT2. Where blockade of BLT1 receptor was used specifically (e.g., in cross-talk and chemotaxis experiments; see below), PMN were preincubated with 500 nM U75302 for 5 min at 37°C.

Chemotaxis assays

PMN chemotaxis was determined using Matrigel-coated Transwell systems (Corning, Corning, NY). Transwell systems with polycarbonate membranes and 3-µm pores were coated with Biomatix (Biomedical Technologies, Newton, MA) to create a modified Boyden chamber, where the gel on the filter approximates the components found in vascular basement membrane (30). Chemoattractants were placed in the lower wells, and PMN in the upper wells. For all chemotaxis studies, PMN were isolated and suspended in 2 ml of HEPES buffer with 1 mM Ca²⁺, exactly as for Fura loading. The cells were then incubated in 5 µl of calcium-ace- toxymethyl ester (calcine-AM; Molecular Probes) for 30 min in a 37°C water bath in the dark. The PMN concentration was then adjusted to 10⁵
cells/ml. Approximately 2 × 10^6 cells were set aside for use in the standard curve.

For each experiment under each condition, two wells were set up: in one the lower chamber contained LTB₄ diluted in buffer, and the other blank contained buffer (with vehicle where appropriate) only. The blanks were used to determine the random migration (chemokinesis) of an identically treated aliquot of PMN in the absence of chemoattractant in the lower well. Where EGTA or gadolinium (Gd³⁺, an inhibitor of PMN SOC (9)) was used, the same concentration was always present in both the upper and lower chambers. In cases where PMN were preincubated with IL-8 (1.25 nM for 15 min at 37°C), the PMN suspension was centrifuged for 10 s at 4500 rpm to remove residual IL-8. The PMN were then resuspended in HEPES buffer at a concentration of 10⁷ PMN/ml before being placed in the chemotactic wells.

After each upper chamber was loaded with 100 μl of PMN (10⁶ cells/well), the system was incubated for 90 min at 37°C in the dark. The insert wells were then removed without any attempt made to dislodge any adherent PMN. Adherent PMN in the lower chamber were resuspended by vigorous pipetting. PMN aliquots from each lower chamber were then transferred, in duplicate, to a 96-well, U-bottom plate. The cells that had been set aside for a standard curve were diluted in ascending concentrations of PMN to be used as a standard curve. The plate was read for calcein fluorescence using a FL500 Microplate Reader (Bio-Tek Instruments, Winooski, VT) at an excitation of 410/25 and an emission of 530/40. Fluorescent intensities were converted directly to the number of cells using the standard curve. Finally, the number of PMN in the corresponding experimental blanks was subtracted from the total number of neutrophils migrating under experiment conditions to assess specific chemotaxis.

**Statistical analysis**

All [Ca²⁺]ᵢ transient results reported are measured as the mean (±SEM) change from basal [Ca²⁺]ᵢ in nanomoles per liter. SOC influx was assessed as the area under the [Ca²⁺]ᵢ influx curve for 100 s after recalciﬁcation of the medium and was calculated as nanomoles × seconds per liter (nM·s) from the [Ca²⁺]ᵢ influx trace by a computer algorithm (GRAMS/32; Galactic Industries, Salem, NH). All study data were assessed for statistical significance using one-way ANOVA testing with Tukey’s post hoc test or unpaired t tests where appropriate. All data are reported as the mean ± SEM, and statistical signiﬁcance was accepted at p ≤ 0.05.

**Results**

**Plasma LTB₄ and IL-8 levels after clinical trauma**

To simulate the clinical environment of trauma and thus study how PMN respond to inflammatory mediators in vivo, we serially measured the concentrations of LTB₄ and IL-8 present in the plasma of a subset of trauma patients (n = 13) on the day of admission (day 1) as well as days 3 and 7 postinjury. Control plasma was obtained from age-, sex-, and ethnicity-matched volunteers. All samples were obtained between 0800–1000 h. Plasma IL-8 and LTB₄ concentrations were also assessed in a subset of trauma patients (n = 8) who had open-packed abdomens subsequent to damage control abdominal exploration, with those plasma samples obtained at the time of re-expansion for removal of the abdominal packing.

**LTB₄.** The mean random plasma LTB₄ concentration in the volunteers was 83 ± 24 pM. In trauma plasma, mean LTB₄ was 92 ± 24 pM on day 1, 65 ± 12 pM on day 3, and 71 ± 15 pM on day 7. Thus, we found no differences in random plasma LTB₄ levels in any of these groups (Fig. 2A). In distinction, plasma LTB₄ levels increased acutely to 685 ± 160 pM at the time of laparotomy for removal of abdominal packing (p < 0.01). LTB₄ levels in the simultaneously sampled abdominal fluids were ~10-fold higher than the plasma levels. Plasma LTB₄ levels were elevated for a subset of trauma patients (p < 0.01) and in those patients who returned to the OR for removal of packing (p < 0.01). ND, levels below the limit of detection for the assay (8 pM).

**FIGURE 1.** A, Volunteer PMN responses to LD-LTB₄ (100 pM). The trace represents the combined [Ca²⁺]ᵢ traces of PMN from eight paired-matched volunteer controls. The hatch marks are the SE of the mean [Ca²⁺]ᵢ response. B, Volunteer PMN responses to HD-LTB₄ (3 nM). The trace represents the combined [Ca²⁺]ᵢ traces of the same eight patients studied in A. C, The BLT1 receptor antagonist U75302 at 50–500 nM causes complete inhibition of signaling by 100 pM LTB₄, but has no measurable effect on signaling at BLT2 by 3 nM LTB₄ or on IL-8 signaling.

**FIGURE 2.** A and B, Plasma LTB₄ and IL-8 concentrations in a subset of 13 trauma patients on the day of admission (D1) and on days 3 and 7 postinjury. Plasma was also obtained from a subset of eight trauma patients at the time of reoperative abdominal exploration for the removal of packing (OR). There were no significant differences between plasma LTB₄ levels at any time point, except during operative manipulation of the abdominal cavity (p < 0.01). Plasma IL-8 was significantly elevated on day 1 (p < 0.05 vs volunteers) and in those patients who returned to the OR for removal of packing (p < 0.01). ND, levels below the limit of detection for the assay (8 pM).
still (7.2 ± 2.2 nM) and were similarly elevated (7.9 ± 1.3 nM) in the pleural fluids in another subset of these patients (n = 7) sampled at the time of drainage of sympathetic effusions.

**IL-8.** IL-8 was undetectable (<8 pM) in the plasma of volunteers. The mean random morning plasma IL-8 in the patient group was elevated to 51 ± 23 pM on day 1 (p < 0.05 vs controls), fell to 13 ± 4 pM on day 3, and generally became undetectable again on day 7 (Fig. 2B). Thus, trauma patients have small, but significant, increases in the basal plasma IL-8 concentration on day 1 that decay over the first week after their injury. However, although these increases were statistically significant, the concentrations were too low to signal at either CXC receptor (29) and thus were unlikely to affect circulating PMN. Conversely, as with LTB4, a transient spike in plasma IL-8 levels was found at the time of abdominal unpacking. The plasma concentrations of IL-8 at those times (1.8 ± 0.4 nM; p < 0.01) are sufficient to activate both CXCR1 and CXCR2. Thus, intermittent mobilization of IL-8 and LTB4 occurs during the manipulation of inflammatory sites where such agonists are present in mid-nanomolar concentrations (31, 32), resulting in biologically active plasma concentrations.

**PMN LTB4 calcium signaling**

Freshly isolated PMN from 12 trauma patients were studied on days 1, 3, and 7 postinjury. Cells from age-, sex-, and ethnicity-matched volunteers were studied similarly. Cells were stimulated with LTB4 in nominally calcium-free buffer, and their peak calcium mobilization was recorded. SOC entry responses to LTB4 were then quantified after readdition of CaCl2 to 1 mM. Preliminary studies showed that PMN responses to 100 pM LTB4 were completely specific for BLT1 and that responses to 3 nM LTB4 were predominantly due to BLT2, with BLT1 contributing only 10–20% of the [Ca2+]i mobilization.

**BLT1 responses (to 100 pM, LD-LTB4).** The peak transient [Ca2+]i response to LD-LTB4 in volunteers was 57 ± 12 nM (Fig. 1A). The responses of trauma patients (Fig. 3A) were similar on day 1, were decreased on day 3, and returned to normal levels by day 7 (p < 0.05, by ANOVA/Tukey’s test). After injury, the LD-LTB4-dependent SOC was 4010 ± 570 nM/s on day 1, but dropped to 1892 ± 265 nM/s on day 3, returning toward control levels (2674 ± 312 nM/s) at 1 wk after injury (p < 0.05, by ANOVA/Tukey’s test). Thus, calcium mobilization from stores as well as SOC in response to stimulation with the high affinity BLT1 receptor were suppressed on day 3 after trauma.

**BLT2 responses (3 nM, HD-LTB4).** HD-LTB4 acts at BLT2 to produce far higher [Ca2+]i than does BLT1 (Fig. 1B). In distinction to the clear modulation of BLT1 responses after trauma, both peak [Ca2+]i, and SOC in trauma PMN by BLT2 were stable and indistinguishable from the responses of matched volunteer control PMN at all time points (Fig. 3B). Thus, the calcium mobilization function of the low affinity BLT2 receptor appeared unaffected by trauma.

**BLT2 cross-talk with CXCR1/R2.** Volunteer PMN (n = 3/condition) were exposed to 3 nM LTB4 in the presence of U75302H (Fig. 4C) or CXCR2 (Fig. 4A). Conversely, doses of LTB4 active at BLT1 showed no tendency to down-regulate either CXCR1 (Fig. 4C) or CXCR2 (Fig. 4A).

**BLT2 cross-talk with CXCR1/R2.** Volunteer PMN (n = 3/condition) were exposed to 3 nM LTB4 in the presence of U75302H (Fig. 4C) or CXCR2 (Fig. 4A). Conversely, doses of LTB4 active at BLT1 showed no tendency to down-regulate either CXCR1 (Fig. 4C) or CXCR2 (Fig. 4A).

**BLT1/BLT2 cross-talk.** Stimulation of PMN with the 100-pM LTB4 concentration active at BLT1 and present in plasma had little or no effect on BLT2 responses to 3 nM LTB4. Conversely, prior PMN stimulation at BLT2 using the 3-nM LTB4 concentration found in the plasma only during acute inflammatory events completely abolished BLT1-specific signaling (Fig. 6, A vs B).

**Chemotaxis to LTB4**

We next performed a series of experiments to evaluate PMN chemotaxis to LTB4 and to assess the role of LTB receptor cross-talk with chemokines on PMN function.

**Trauma suppresses PMN chemotaxis to LTB4**

PMN were isolated from nine trauma patients on day 3 postinjury. Chemotactic responses to LTB4 were assessed over a range of LTB4 doses from 0.1–100 nM and compared with vol-
unteer PMN responses \((n = 5)\). As shown in Fig. 7, chemotaxis to \(\text{LTB}_4\) is maximal at \(\sim 10 \text{ nM}\) in this system and is desensitized at higher doses. This bell-shaped curve is typical of PMN responses to other GPC chemoattractants (data not shown) and probably represents homologous receptor desensitization at very high agonist concentrations. PMN from trauma patients demonstrate suppressed chemotaxis to \(\text{LTB}_4\) over a broad range of concentrations. When U75302 was used to block BLT1, chemotaxis to \(\text{LTB}_4\) was markedly suppressed, with little PMN migration seen until nanomolar (BLT2 activating) levels of \(\text{LTB}_4\) were present. Thus, net PMN chemotaxis to \(\text{LTB}_4\) appears to be the sum of the responses to \(\text{LTB}_4\) at the two LT receptors. BLT1 seems to be responsible for PMN chemotaxis to picomolar \(\text{LTB}_4\) concentrations. The higher total chemotaxis seen at nanomolar \(\text{LTB}_4\) concentrations appears to represent the additive effect of \(\text{LTB}_4\) acting at both receptors.

FIGURE 4. A–D, Calcium mobilization at the BLT1 receptor is suppressed by stimulation of either CXCR2 (A vs B) or CXCR1 (C vs D) at clinical agonist concentrations. Conversely, prior stimulation of BLT1 by 100 pM \(\text{LTB}_4\) has no effect on subsequent CXCR1 or R2 function (B vs A and D vs C).

FIGURE 5. A–D, BLT2-specific receptor signals were evoked by 3 nM \(\text{LTB}_4\) in the presence of U75302. BLT2 is moderately suppressed by IL-8 (A vs B). BLT2 was not suppressed by GRO-\(\alpha\) (C vs D). These two findings infer that CXCR1, but not CXCR2, can act to desensitize BLT2. Prior BLT2 stimulation has minimal effects on either GRO-\(\alpha\) signaling at CXCR2 (D vs C) or IL-8 signaling at CXCR1/R2 (B vs A).
Effects of IL-8 on normal PMN chemotaxis to LTB₄

A series of experiments was next performed to evaluate the effects of IL-8 on PMN chemotaxis to LTB₄. PMN were obtained from healthy volunteers, and their chemotaxis to LTB₄ was assessed with and without prior incubation with 1.25 nM IL-8 (n/511005) (Fig. 8). Prior exposure to IL-8 at concentrations achieved in the plasma during abdominal unpacking suppressed chemotaxis to 100 pM LTB₄ by 53% (11006 vs 11006103 PMN/well; p < 0.01). PMN chemotaxis to 3 nM LTB₄ was also decreased by prior incubation with IL-8 (1104 vs 277 ± 4 × 103 PMN/well; p < 0.01). The absolute decreases in PMN migration caused by IL-8 under the two sets of conditions were essentially identical (53 vs 63 × 103 PMN/well). Thus, like the suppression of calcium flux, the suppression of chemotaxis by IL-8 appeared compatible with desensitization of the high affinity BLT1 receptor by about half, with no effect on BLT2 activity.

Dependence of LTB₄ chemotaxis upon calcium entry

Since both peak calcium release and SOC responses to LTB₄ were diminished in trauma, we studied the relative dependence of LTB₄ chemotaxis on the influx of extracellular calcium as opposed to the release of intracellular calcium stores. PMN were studied in the presence of graded amounts of extracellular calcium (n = 5 experiments/condition; Fig. 9). PMN chemotaxis to 3 nM LTB₄ (stimulating both BLT1 and BLT2) was studied in medium with 1 mM Ca²⁺ (physiologic Ca²⁺ conditions), with no added calcium plus 0.3 mM EGTA (nominally calcium free; Ca²⁺ concentration in medium, ~50 nM), and with no added calcium plus 5 mM EGTA (zero calcium). PMN in nominally calcium-free environments have normal store release transients, but no transmembrane Ca²⁺ gradient is present to drive SOC. Under these conditions PMN demonstrated significantly decreased chemotaxis compared with physiologic Ca²⁺ conditions (62 ± 11 vs 227 ± 4 × 10³ cells/well; p < 0.01; Fig. 9, center bar). In zero calcium environments PMN calcium stores become diminished, and cells show
Thus, chemotaxis to LTB₄ is dependent upon SOC, but the isolated inhibition of nonspecific cationic entry channels.

Since chemotaxis was highly dependent upon calcium influx, we sought to determine the route of that influx. At micromolar concentrations Gd³⁺ is a specific inhibitor of SOC, which is probably the major source of calcium entry into PMN (27, 33). Moreover, we have shown that at nanomolar concentrations, Gd³⁺ only blocks SOC through nonspecific divalent cation channels, whereas at low micromolar concentrations it blocks all SOC (9). We therefore studied PMN chemotaxis to a BLT1 active dose of LTB₄ (300 pM) in calcium-replete buffer in the presence of 500 nM or 10 μM Gd³⁺. Control was defined as PMN chemotaxis in the absence of Gd³⁺ (Fig. 10). Chemotaxis was unaffected by 500 nM Gd³⁺, but PMN treated with 10 μM Gd³⁺ showed suppression of chemotaxis to the levels seen in nominally calcium-free medium (p < 0.01). Thus, chemotaxis to LTB₄ is dependent upon SOC, but the isolated inhibition of nonspecific entry channels is insufficient to block chemotaxis.

Discussion

Pneumonia and sepsis are common after trauma, and are often major determinants of patient survival (34). PMN are recruited to sites of bacterial invasion by GPC chemoattractants. There they take part in immune surveillance and the control of infection (35). In vitro, LTs, chemokines, and other chemoattractants can act individually to recruit PMN (14, 15, 36–39). In vivo, however, PMN must respond to arrays of mediators in a multistep process to be recruited to inflammatory sites. Early responses by circulating PMN are likely to require high affinity receptors to respond to low concentrations of plasma agonists. The responses of BLT1 to the picomolar LTB₄ concentrations present in normal plasma probably exemplify this type of receptor-ligand interaction. Subsequent interactions probably entail progressively lower affinity receptors responding to higher agonist concentrations at the tissue level. Within inflammatory collections per se, only those receptors most resistant to desensitization are likely to persist. We hypothesized that such sequential responses to chemoattractants would become aberrant after major injury, potentially impairing PMN recruitment and predisposing to infection.

The present study examined the relationship between LTs and chemokines as a paradigm for such complex interactions. The data demonstrate that circulating PMN develop suppressed receptor signaling responses to LTB₄ after trauma. This suppression appears specific for the high affinity BLT1 receptor, develops in the middle of the first week after injury, is associated with chemotactic dysfunction, and tends to abate toward the end of the week. Both chemokines and LTs at the high concentrations found during inflammatory events can desensitize BLT1. Changes in postreceptor PMN calcium mobilization also appear to play a role in suppressing chemotaxis. It is likely that other inflammatory GPC chemoattractants can act similarly.

After trauma, IL-8 and LTB₄ were generated in large amounts at sites of tissue injury and inflammation and were released intermittently into the circulation in high titer. Even though such agonist release is intermittent, subsequent desensitization of GPC receptors can be prolonged (40). This desensitization results in the suppression of PMN function. These results shed important light on the natural history of PMN responses to inflammation after injury, but they also allowed us to replicate the precise clinical concentrations of LTB₄ and IL-8 encountered by circulating PMN for in vitro studies.

BLT1 has been reported to be activated at picomolar LTB₄ concentrations, and the reported K₅₀ of the BLT2 receptor is in the nanomolar range (4, 7). Our present experience suggests that BLT2 may have a lower 50% effective concentration in native PMN than the values reported in expression systems. Nonetheless, the present data clearly confirm that the two BLT receptors have entirely different roles in the response to inflammation. BLT1 is probably active in recruiting circulating PMN, since it is activated at ambient clinical plasma LTB₄ concentrations. BLT2 is probably more crucial for PMN activity at sites of inflammation, where LTB₄ is present at concentrations close to its EC₅₀. Moreover, BLT1 appears to undergo heterologous desensitization by chemokines such as GRO-α and IL-8 at clinical concentrations (Fig. 4) as well as heterologous desensitization by stimulation of BLT2 (Fig. 6). In distinction, BLT2 appears more resistant to desensitization, being resistant to GRO-α (Fig. 5, C and D), partially sensitive to IL-8 (Fig. 5, A and B), and unaffected by stimulation of BLT1 (Fig. 6). The suppression of chemotaxis to LTB₄ seen in clinical trauma PMN, in volunteer PMN treated with IL-8, and in PMN treated with U75302 are all compatible with the right-shifting of PMN dose responses to LTB₄ expected on the basis of BLT1 desensitization. Before such regulation, however, they may act in a combinatorial or additive fashion, as has been suggested for other PMN receptors (41).

In all conditions studied, the rate of PMN chemotaxis was directly related to the degree of calcium mobilization by LTB₄. GPC receptors mobilize a variety of second messengers, so mobilization of cell calcium stores per se may simply be a marker for other GPC signal processes. The marked dose dependence of LT-mediated chemotaxis on external calcium, however, suggests that this is not the case here. Partial blockade of SOC had little, if any, effect, but chemotaxis was markedly suppressed in the absence of SOC imposed by nominal calcium-free conditions or by micromolar Gd³⁺. No chemotaxis was seen in the absence of external calcium. Thus, prolonged entry of external calcium is a clear requirement for PMN chemotaxis to LTB₄, and decreased clinical PMN chemotaxis coincided with diminished SOC.

We conclude that after injury, chemokines and other inflammatory mediators are periodically released into the circulation in high
titer. In our patients this often occurred due to surgical manipulation of inflammatory sites 24–48 h postinjury, when the local content of inflammatory mediators is maximal (17, 32). These events appear to suppress PMN chemotaxis to LTB₄ via desensitization of BLT₁ as well as by a more global suppression of stimulated calcium entry. The data suggest that suppression of chemotaxis to LTB₄ will be manifest as diminished BLT₁-dependent recruitment of PMN from the circulation to tissue sites of inflammation rather than as diminished BLT₂-dependent activity at inflammatory sites. Suppressed PMN recruitment from the circulation to sites of bacterial inoculation should predispose to infections, and indeed, similar study populations have pneumonia rates of 30–50% (42). Further studies are ongoing to establish the relationship between BLT₁ desensitization and the establishment of infections after trauma.

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References