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Cutting Edge: Interleukin 4-Dependent Mast Cell Proliferation Requires Autocrine/Intracrine Cysteinyl Leukotriene-Induced Signaling

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Reactive mastocytosis (RM) in epithelial surfaces is a consistent Th2-associated feature of allergic disease. RM fails to develop in mice lacking leukotriene (LT) C₄ synthase (LTC₄S), which is required for cysteinyl leukotriene (cys-LT) production. We now report that IL-4, which induces LTC₄S expression by mast cells (MCs), requires cys-LTs, the cys-LT type 1 receptor (CysLT₁), and Gi proteins to promote MC proliferation. LTD₄ (10–1000 nM) enhanced proliferation of human MCs in a CysLT₁-dependent, pertussis toxin-sensitive manner. LTD₄-induced phosphorylation of ERK required transactivation of c-kit. IL-4-driven comitogenesis was likewise sensitive to pertussis toxin or a CysLT₁-selective antagonist and was attenuated by treatment with leukotriene synthesis inhibitors. Mouse MCs lacking LTC₄S or CysLT₁ showed substantially diminished IL-4-induced comitogenesis. Thus, IL-4 induces proliferation in part by inducing LTC₄S and cys-LT generation, which causes CysLT₁, to transactivate c-kit in RM. The Journal of Immunology, 2006, 177: 2755–2759.

M ast cells (MCs) are central to allergy and host mucosal defense (1). They develop in tissues from circulating committed progenitor cells (MCps) and require the c-kit receptor tyrosine kinase (RTK) and its ligand, stem cell factor (SCF) (2, 3). Constitutive stromal expression of SCF ensures that MCs thrive in normal connective tissues. In contrast, normal intestinal and respiratory epithelial surfaces contain few MCs, although MCs selectively accumulate in these mucosal surfaces with inflammation (reactive mastocytosis (RM)). RM is required for the clearance of intestinal helminths (4). The MCs arising in such circumstances generate large quantities of cysteinyl (cys) leukotrienes (LTs) (cys-LTs) (5), lipid mediators that induce vascular leak, smooth muscle constriction, and intracrine signaling (6). Asthma, rhinitis, and allergic esophagitis in humans all are associated with RM in the involved mucosal epithelia (reviewed in Ref. 1) and high levels of cys-LTs. The effector role of MCs in both mucosal defense and allergy makes the mechanisms controlling RM and its relationship to cys-LT generation of considerable importance.

RM requires both c-kit (7) and T cells (8). Abs against IL-3 and IL-4 in vivo block RM but not constitutive MC development (9). Each T cell-derived cytokine implicated in RM amplifies SCF-dependent proliferation of MC and/or MCp in vitro (comitogenesis) (10, 11). IL-4 is comitogenic with SCF for human MCs (hMCs) isolated from the human intestine (12) or derived in vitro from peripheral blood (13). IL-4 induces the expression of LTC₄S synthase (LTC₄S), an enzyme obligatory for the generation of cys-LTs, by cord blood-derived hMCs (14) and enhances calcium flux and ERK phosphorylation (15, 16) by hMCs through the type 1 cys-LT receptor (CysLT₁), one of two cys-LT-specific G protein-coupled receptors (17). By regulating both cyst-LT synthesis and CysLT₁ function, IL-4 may induce unique cys-LT-dependent autocrine/intracrine signaling events in MCs.

In a MC-dependent model of allergic airway disease, mice lacking LTC₄S showed diminished Th2 cytokine generation and airway inflammation as compared with identically treated wild-type controls (18). The numbers of MCs in the tracheal epithelia of allergen-challenged Ltc₄s⁻/⁻ mice were decreased by >90% compared with wild-type control mice but were normal in the adjacent submucosa. These findings implied a previously unrecognized requirement for cys-LTs in T cell-dependent RM, a process that involves in situ proliferation of MCs and/or MCp. In this study, we demonstrate that cys-LTs are autocrine/intracrine regulators of IL-4-driven comitogenesis. RM, a pathogenetically relevant feature of all allergic mucosal diseases, may involve a novel IL-4-mediated signaling mechanism requiring the induction of LTC₄S and the transactivation of c-Kit by CysLT₁.

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3 Abbreviations used in this paper: MC, mast cell; hMC, human MC; MCp, mast cell progenitor; mBMMC, mouse bone marrow-derived MC; LT, leukotriene; cys-LT, cysteinyl LT; CysLT₁, type 1 receptor for cys-LTs; LTC₄S, LTC₄ synthase; PTX, pertussis toxin; RM, reactive mastocytosis; RTK, receptor tyrosine kinase; SCF, stem cell factor.

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Materials and Methods

Reagents

LTs, MK886 (an inhibitor of 5-lipoxygenase-activating protein and LTC₄S), and the CysLT₁-selective antagonist MK571 were purchased from Cayman Chemical. Pertussis toxin (PTX) (Sigma-Aldrich), the c-kit inhibitor STI571 (Novartis Pharmaceuticals), the 5-lipoxygenase inhibitor zileuton (Critical Therapeutics), and UO126 (Promega), an inhibitor of MEK, were purchased from the indicated vendors. Recombinant cytokines were purchased from R&D Systems.

Derivation of MCs

hMCs were derived from cord blood mononuclear cells using SCF (100 ng/ml), IL-6 (50 ng/ml), and IL-10 (10 ng/ml) as described (19) and harvested when ≥95% stained with toluidine blue (typically 6 wk). In some experiments, various concentrations of cys-LTs were added weekly to the cells from the start of the culture. hMCs were also derived from peripheral blood CD34⁺ cells from healthy adult volunteers using magnetic beads (Dynal Invitrogen) according to manufacturer’s protocol. Cells were seeded at 1.5 × 10⁴/ml in StemPro-34 medium (Invitrogen Life Technologies) containing 20% charcoal-filtered FCS, SCF (100 ng/ml), GM-CSF (10 pg/ml), and 4% conditioned medium from an immortalized B cell line. After 2 wk, cells were maintained in new flasks at a density of 1 × 10⁵/ml, and the medium was replaced weekly. hMCs grown under these conditions were 100% toluidine blue-positive and highly c-Kit-positive (19) at 4 wk. Mouse bone marrow-derived MCs (mBMMCs) were derived from marrow in the presence of IL-3 (10 U/ml) and were studied at 4–6 wks when virtually all stained with toluidine blue. Mitogenic assays were performed in triplicate on cells suspended in fresh medium at a concentration of 0.5 × 10⁶/ml in the presence of SCF (1–100 ng/ml) with or without LTD₄ (0.01–1 μM), IL-4 (10 ng/ml), or both. LTB₄ was used as a control stimulus.

FIGURE 1. Comitogenic effects of cys-LTs. A, Dose-response to LTD₄ for 3 days (top panel). Some samples were treated with MK571 for 30 min before the addition of LTD₄. Results are the mean ± SD of triplicate samples in a single experiment representative of five performed. Bottom panel, response to LTD₄ at the indicated concentrations as compared with the response to LTD₄ (1 μM). Results are mean ± 0.5 range from two experiments. B, Numbers of cells (expressed as a percentage of the numbers in cultures maintained without cys-LTs) arising from 4-wk (top panels) and 6-wk (bottom panels) cultures maintained with SCF, IL-6, and IL-10 along with the weekly additions of LTC₄, LTD₄, and LTE₄ at the indicated concentrations. Data are mean ± SEM from three or four experiments. Toluidine blue staining (right panels) of cells from representative LTE₄-treated cultures are shown. C, Annexin V staining of 6-wk-old cells maintained in SCF-, IL-6-, and IL-10-supplemented medium with or without the indicated cys-LT at 1 μM. Results in a second experiment were similar.

FIGURE 2. Signaling events for cys-LT-mediated comitogenesis. A, Proliferation of hMCs cultured for 3 days with SCF (10 ng/ml) without or with LTD₄ (1 μM). Some of the LTD₄-treated cells were preincubated overnight with PTX (100 ng/ml) or for 30 min with the MEK inhibitor UO126 (5 ng/ml) before the addition of LTD₄. Data are mean ± SD from triplicate samples in a single experiment representative of five (for PTX) and two (for UO126) performed. B, Phosphorylation of c-kit and ERK in hMCs stimulated for 5 min with SCF (100 ng/ml), medium alone, or LTD₄ (500 nM). Some cells were treated with STI571 for 30 min before stimulation. Data are from a single experiment representative of three performed. Quantitative densitometry showing the effect of STI571 on c-kit (C) and ERK (D) phosphorylation. Data in C and D are expressed as density relative to the positive control (SCF) and are mean ± SEM from three experiments. *, p = 0.03 relative to cells not treated with STI571.
In some experiments, PTX (100 ng/ml), MK571 (1 μM), MK886 (1 μM), U0126 (5 μg/ml), or zileuton (1 μM) was added at the same time as the mitogens. At 72 h, the cells were pulsed overnight with thymidine, and counts were analyzed by β counting. Apoptosis was assessed by cytofluorographic binding of annexin V (BD Pharmingen). Cys-LTs were measured in supernatants of IL-4-stimulated hMCs by ELISA (Cayman Chemical).

Results and Discussion

All nonlymphoid leukocytes of the respiratory mucosa in asthma or rhinitis express CysLT1 (23, 24). Unlike granulocytes, MCs proliferate in the peripheral tissues, which, along with MCp recruitment, results in RM. The association between RM and high levels of cys-LT generation (5) suggested a potential link between them. IL-4 is the only Th2 cytokine implicated in RM in vivo that is comitogenic for mature tissue-derived MCs and MCps from both rodents and humans in vitro (10, 12, 13, 25). The induction of LTC₄S and CysLT₁ function by IL-4 and the lack of RM in the airway epithelium of the Ltc₄s⁻/⁻ mouse (18) led us to suspect a role for endogenous cys-LTs as amplifiers of MC proliferation in the context of mucosal inflammation.

Of the cys-LTs, LTD₄ has the highest affinity for CysLT₁ (17). We therefore tested whether LTD₄ could directly induce proliferation of cord blood-derived hMCs maintained with SCF. LTD₄ (1 μM) increased the proliferation of hMCs at every SCF concentration tested, most markedly at doses of SCF sufficient to sustain cell viability (1–10 ng/ml) but below the EC₅₀ for proliferation. Even in the absence of SCF, LTD₄ caused hMC proliferation in a dose-dependent manner (n = 2; data not shown). The 2–2.5-fold amplification of SCF-dependent proliferation induced by LTD₄ was ~70% blocked by MK571 (as shown for one experiment; Fig. 1A; n = 5, p = 0.01; data not shown), implicating CysLT₁, LTβ₁, an LT that is chemotactic for MCp in vitro through the BLT₁ receptor for each experiment where indicated. Differences between treatment groups were determined with Student’s t test.

Statistics

Data are expressed as mean ± SEM from at least three experiments except where otherwise indicated. Data were converted to a percentage of control for figures.

FIGURE 3. Requirement for cys-LTs, CysLT₁, and CysLT₂, and Gi proteins in IL-4-induced comitogenesis of hMCs. A, Cord blood hMCs (left panel) and peripheral blood hMCs (right panel) were maintained in the presence of SCF (10 ng/ml) with or without IL-4 (10 ng/ml) for 3 days in the absence or presence of MK571 (1 μM) or PTX (100 ng/ml). Data are mean ± SEM of n = 6 and 4, respectively, for cord and peripheral blood hMCs. B, Dose-dependent effect of MK571 (1 μM) on IL-4-mediated comitogenesis of cord blood hMCs. Results in a second experiment were similar. C, Cord blood hMCs were treated with SCF with or without IL-4 in the presence or absence of MK886 (1 μM). LTD₄ (1 μM) was added to the indicated samples. Data are the mean ± SEM from four separate experiments. *p = 0.04 relative to IL-4-stimulated cells without MK886.
(26), failed to induce proliferation of hMCs (n = 2; Fig. 1A) although it stimulated robust calcium flux (n = 3, not shown); thus, the mitogenic effect was specific to cys-LTs. The abrogation of the LTD₄-mediated comitogenic signal by treatment of the cells with either PTX or the MEK inhibitor U0126 (Fig. 2A) and the loss of LTD₄-mediated ERK phosphorylation in cell samples treated with PTX (not shown) imply that CysLT₁ used Gi protein-dependent MEK/ERK signaling to cause proliferation.

When LTC₄, LTD₄, or LTE₄ were added at weekly intervals from the start of culture, each increased the numbers of hMCs even with the maximally effective dose of SCF (100 ng/ml) and the comitogenic cytokines IL-6 and IL-10. Cys-LTs did not alter the percentages of mature hMCs as determined by toluidine blue staining (Fig. 1B) and expression of c-Kit (data not shown). Although LTE₄ (the weakest CysLT₁ agonist) (17) induced the most significant increase in the number of MCs (p = 0.02 and 0.04 at 4 and 6 wk relative to untreated controls; n = 4), it was similar to LTD₄ in stimulating thymidine incorporation (115 ± 19, 132 ± 6, and 202 ± 3% of the SCF control at 0.1, 0.5, and 1 µM, respectively; n = 2), and its effect was blocked by MK571. LTE₄ could activate a second (uncharacterized) MK571-sensitive receptor, or it may induce a qualitatively different signal than LTD₄ through CysLT₁ to account for differences in cell numbers. Because none of the cys-LTs protected hMCs from apoptosis (Fig. 1C), the near doubling of hMCs arising over the 4–6 wk of culture likely reflects proliferation, largely through CysLT₁ and the Gi/MEK/ERK signal sequence.

Whereas some G protein-coupled receptors stimulate Ras/MEK/ERK signaling directly through G proteins, others do so through transactivation of RTKs (reviewed in Ref. 27), promoting cell proliferation in circumstances where protein growth factor concentrations are low. Transactivation is implicated in fibrosis and malignancies. LTD₄-induced c-Kit phosphorylation (Fig. 2B) and LTD₄-dependent ERK phosphorylation were abrogated by STI571 (n = 3, Fig. 2C; as shown for one experiment, Fig. 2B). SCF, but not LTD₄, strongly induced phosphorylation of c-Jun terminal kinase (n = 2, not shown), and p38 was weakly and constitutively phosphorylated under each of these conditions (not shown). Thus, CysLT₁ requires transactivation of c-Kit to integrate the cys-LT-induced Gi/MEK/ERK cascade. These findings carry potential implications for mechanisms controlling proliferation of MCs and other c-Kit-dependent cell lineages as well as cys-LT-dependent proliferation of other cell types that express RTKs. The observation that LTD₄ cannot completely substitute for SCF reflects the ability of the latter to induce additional signaling events (i.e., JNK activation) and cytoprotection.

Like LTD₄, IL-4 amplifies SCF-dependent MC proliferation through MEK/ERK (25). Because IL-4 also induces LTC₄S expression, we suspected that IL-4-dependent comitogenesis might involve autocrine actions of endogenous cys-LTs. We tested this hypothesis with hMCs both derived from cord blood and from adult peripheral blood to ensure that the pathway was operative regardless of progenitor origin or culture methodology. IL-4 increased proliferation of the hMCs grown with both methods (Fig. 3A). Both PTX and MK571 decreased IL-4-induced comitogenesis in both hMC types (Fig. 3A). MK886, which inhibits cys-LT production by blocking both 5-lipoxygenase-activating protein and LTC₄S, decreases IL-4-mediated proliferation of cord blood-derived hMCs in a dose-dependent manner (Fig. 3B) but not SCF-induced basal proliferation or LTD₄-mediated proliferation (Fig. 3C). Exogenous LTD₄ restored the response of hMCs to IL-4 in the presence of MK886. Thus, endogenous cys-LTs contribute 40–60% of the IL-4-mediated comitogenic signal in hMCs, likely reflecting the capacity of this cytokine to both up-regulate LTC₄S and enhance CysLT₁-mediated ERK signaling. Extracellular cys-LTs were below limits of detection in these experiments, suggesting likely synaptic functions of low-level endogenous cys-LTs at membrane or nuclear receptors (6).

Restimulated lymphocytes from Ltc4s⁻/⁻ mice with OVA-induced airway disease were deficient in the generation of Th2 cytokines (18). Their complete lack of RM could therefore reflect a loss of cys-LT-dependent autocrine/intracrine signaling combined with diminished Th2 cytokine production. We studied IL-4-dependent comitogenesis of mBMMCs from both enzyme- and receptor-null mice. IL-4 induced LTC₄S protein expression in wild-type mBMMCs within 72 h (Fig. 4A). Although IL-4 was comitogenic...
for mBMMCs from both genotypes, the net response of the LTC₄S⁻/⁻ mBMMCs to IL-4 was only ~40% of the increment in the wild-type mBMMCs (n = 3; Fig. 4B). Moreover, CysH₂r⁻/⁻ but not CysH₁r⁻/⁻ mBMMCs showed markedly impaired comitogenic responses to IL-4 (Fig. 4C). MK571, MK886, zileuton (data not shown), and PTX attenuated the IL-4-driven comitogenic response only in the wild-type and CysH₂r⁻/⁻ mBMMCs. Thus the LTC₄S/CysLT/CysLT₁/Gi sequence is a major autocrine/intracrine signal amplification pathway contributing to IL-4-driven comitogenesis, a central event in mucosal RM. The specialized functions of LTB₄ (McP recruitment) and cysteines (comitogenesis) may synergize to facilitate RM in allergic disease. CysLT₁ antagonists and 5-lipoxygenase inhibitors could interrupt these pathways as part of their therapeutic mechanism in asthma.

Disclosures
The authors have no financial conflict of interest.

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