T Helper Cell Type 2 Cytokines Coordinately Regulate Immunoglobulin E–dependent Cysteinyl Leukotriene Production by Human Cord Blood–derived Mast Cells: Profound Induction of Leukotriene C_4 Synthase Expression by Interleukin 4

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Abstract

Human mast cells (hMCs) derived in vitro from cord blood mononuclear cells exhibit stem cell factor (SCF)-dependent comitogenic responses to T helper cell type 2 (Th2) cytokines. As cysteinyl leukotriene (cys-LT) biosynthesis is a characteristic of immunoglobulin (Ig)E-activated mucosal hMCs, we speculated that Th2 cytokines might regulate eicosanoid generation by hMCs. After passive sensitization for 5 d with IgE in the presence of SCF, anti-IgE–stimulated hMCs elaborated minimal cys-LT (0.1 ± 0.1 ng/10^6 hMCs) and abundant prostaglandin (PG)D_2 (16.2 ± 10.3 ng/10^6 hMCs). Priming of hMCs by interleukin (IL)-4 with SCF during passive sensitization enhanced their anti-IgE–dependent histamine exocytosis and increased their generation of both cys-LT (by 27-fold) and PGD_2 (by 2.5-fold). Although priming with IL-3 or IL-5 alone for 5 d with SCF minimally enhanced anti-IgE–mediated cys-LT generation, these cytokines induced further six- and fourfold increases, respectively, in IgE-dependent cys-LT generation when provided with IL-4 and SCF; this occurred without changes in PGD_2 generation or histamine exocytosis relative to hMCs primed with IL-4 alone. None of these cytokines, either alone or in combination, substantially altered the levels of cytosolic phospholipase A_2 ($cPLA_2$), 5-lipoxygenase (5-LO), or 5-LO activating protein (FLAP) protein expression by hMCs. In contrast, IL-4 priming dramatically induced the steady-state expression of leukotriene C_4 synthase (LTC_4S) mRNA within 6 h, and increased the expression of LTC_4S protein and functional activity in a dose- and time-dependent manner, with plateaus at 10 ng/ml and 5 d, respectively. Priming by either IL-3 or IL-5, with or without IL-4, supported the localization of 5-LO to the nucleus of hMCs. Thus, different Th2-derived cytokines target distinct steps in the 5-LO/LTC_4S biosynthetic pathway (induction of LTC_4S expression and nuclear import of 5-LO, respectively), each of which is necessary for a full integrated functional response to IgE-dependent activation, thus modulating the effector phenotype of mature hMCs.

Key words: eicosanoids • asthma • allergy • prostaglandin D_2 • FcεRI

Introduction

Mast cells (MCs)[1] are stem cell factor (SCF)-dependent hematopoietic cells that home to tissues as committed progenitors and then mature and differentiate into heterogeneous phenotypes (1–3). When stimulated by their high-affinity Fc receptor for IgE (FcεRI), MCs generate a range of bioactive products implicated in allergic and asthmatic inflammation. Among these products are the eicosanoid metabo-

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[1]*Abbreviations used in this paper:* BMMC, bone marrow–derived MC; $cPLA_2$, cytosolic phospholipase A_2; cys-LT, cysteinyl leukotriene; FcεRI, high-affinity Fc receptor for IgE; FLAP, 5-LO activating protein; hMC, human MC; 5-LO, 5-lipoxygenase; LT, leukotriene; LTC_4S, LTC_4 synthase; MC, mast cell; PGD_2S, PGD_2 synthase; PGHS, prostaglandin endoperoxide H synthase; RP, reverse phase; SCF, stem cell factor.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/2001/01/123/11 $5.00
Volume 193, Number 1, January 1, 2001 123–133
http://www.jem.org/cgi/content/full/193/1/123

lites of cell membrane–derived arachidonic acid: PGD_2 (4), a product of the prostaglandin endoperoxide H synthase (PGHS)/PGD_2 synthase (PGD_2S) pathway, and leukotriene (LT)C_4, a product of the 5-lipoxygenase (5-LO)/LTC_4 synthase (LTC_4S) pathway (5). Cell activation by FcεRI initiates both pathway sequences with liberation of membrane stores of arachidonic acid by a calcium-dependent cytosolic phospholipase A_2 ($cPLA_2$; reference 6). Constitutive PGHS-1 and inducible PGHS-2, integral proteins of the perinuclear membrane and endoplasmic reticulum, provide substrate, PGH_2, to cytosolic, glutathione-dependent PGD_2S (7–9). Human MCs (hMCs) release PGD_2 during the early bronchoconstrictor response to inhaled allergen challenge (10). PGD_2 may contribute to bronchoconstriction in aspirin-sensitive asthma (11) and to the development of allergic airway inflammation through its interaction with its receptor on bronchial epithelial cells (12).

In response to cell activation, 5-LO reversibly translocates from either the nucleoplasm or cytoplasm, depending on the cell type, to the perinuclear region (13), and acts in concert with 5-LO activating protein (FLAP [14]), an integral perinuclear protein, to convert arachidonic acid sequentially to the unstable intermediates 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and then to LTA_4 (15). LTA_4 either is converted by LTA_4 hydrolase (16) to LTB_4 (as occurs in neutrophils and monocytes) or is conjugated to reduced glutathione by LTC_4S (17–19), an integral perinuclear membrane protein with homology to FLAP that is expressed by eosinophils, basophils, MCs, and monocytes. LTC_4 is released by a distinct cellular export mechanism (20) and converted sequentially to the receptor-active cysteinyl LTs (cys-LTs), LTD_4 and LTE_4, by extracellular γ-glutamyl transferase and dipeptidase, respectively (21, 22). LTC_4, LTD_4, and LTE_4 then act at specific receptors, including the CysLT1 receptor (23) and CysLT2 receptor (24), to mediate a variety of cellular effects germane to asthma, including bronchoconstriction, alterations in venular permeability, leukocyte extravasation, and mucus hypersecretion (25–28). The role for the cys-LT in asthma is now substantiated by the clinical efficacy of pharmacologic agents that interfere with the actions of 5-LO or that block the CysLT1 receptor (29, 30).

hMCs differ in their profiles of eicosanoid biosynthesis in response to FcεRI-dependent activation after isolation from various dispersed tissue sources. Both total cys-LT generation (from 3.5 ng/10^6 hMCs from skin to 45 ng/10^6 hMCs from uterus) and the cys-LT/PGD_2 ratio (1:12 for skin hMCs; 1:3, 1:2, and 1:1 for lung, uterine, and intestinal hMCs, respectively) are marked by tissue-related differences that are both quantitative and relative (31–34). Because MCs in all tissues derive from a single lineage of circulating committed progenitors (2, 35, 36) under the influence of constitutively expressed SCF, we postulated that their heterogeneous profiles of eicosanoid generation would be determined by the absence or presence of additional local factors, particularly the cytokines derived from the Th2 lymphocytes that associate with mucosal surfaces in allergic diseases.

We recently reported the derivation in vitro of hMCs from umbilical cord blood mononuclear cells cultured in the presence of recombinant human SCF, IL-6, and IL-10 (37). These cells were characterized by uniformly high levels of *c-kit* expression, expression of CD13 and low-level FcεRIα, and uniform toluidine blue metachromasia and immunoreactivity for both tryptase and chymase. The receptors for IL-3 and IL-5 were detected on these hMCs by flow cytometry, and the corresponding recombinant ligands induced a comitogenic response when provided with SCF. We now demonstrate that IL-4, in addition to its recognized inductive effect on FcεRI expression (38), strongly and selectively upregulates the expression of LTC_4S mRNA, protein, and biosynthetic function. However, the inclusion of either IL-3 or IL-5 with IL-4 during priming selectively further increases IgE-dependent cys-LT production by hMCs, without altering PGD_2 production, by favoring nuclear import of 5-LO. Thus, the distinct effects of Th2 cytokines control the integrated function of the 5-LO/LTC_4S pathway in SCF-dependent hMCs.

Materials and Methods

Cytokines. Recombinant human SCF was a generous gift from Amgen. The cytokines IL-3, IL-4, and IL-5 (PeproTech), IL-6 (R&D Systems), and IL-10 (Endogen) were purchased as noted.

Cell Culture. Cord blood was obtained from human placentas after routine Caesarian section in accordance with established institutional guidelines. hMCs were derived by the culture of the mononuclear cell fraction as described previously (37). In brief, heparin-treated cord blood was sedimented with 4.5% dextran solution to remove erythrocytes. The buffy coats were layered onto 1.77 g/liter Ficoll-Hypaque (Amersham Pharmacia Biotech), and mononuclear cell interfaces were obtained after centrifugation. Residual erythrocytes were removed by hypotonic lysis, and the remaining mononuclear cells were suspended in RPMI 1640 (GIBCO BRL) containing 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 μg/ml gentamycin (all from Sigma-Aldrich), and 0.2 μM 2-mercaptoethanol (GIBCO BRL). Cells were seeded at a concentration of 10^6 cells/ml and were cultured in the presence of 100 ng/ml SCF, 50 ng/ml IL-6, and 10 ng/ml IL-10. The nonadherent cells were transferred every week for up to 9 wk into culture medium containing fresh cytokines. Cytospin preparations were examined weekly from samples of 2×10^4 cells using a cytocentrifuge (Shandon) and were stained with toluidine blue to assess metachromasia. Once cells reached maturity, defined by >95% toluidine blue positivity and positive immunostaining for both tryptase and chymase (37), no other immunocytochemical or functional differences were noted between 6- and 9-wk cells. Therefore, cells were used for this study when they reached >95% toluidine blue positivity rather than a specific number of weeks in culture. No quantitative or qualitative changes in cell responsiveness to cytokine treatments were observed that were age dependent.

Analysis of Cys-LT and PGD_2 Production and Histamine Release by hMCs after Passive IgE Sensitization and Anti-IgE Activation. hMCs were washed twice in medium alone and were resuspended in medium containing SCF (100 ng/ml) and semipurified human myeloma IgE (10 μg/ml; Chemicon). Cells were incubated with combinations of additional cytokines, including IL-3 (5 ng/ml), IL-4 (10 ng/ml), and IL-5 (5 ng/ml). The 10 ng/ml concentration of IL-4 and the 5-d period of priming were each chosen so as to optimize the expression and function of FcεRI (38). The concentration of IL-3 was selected based on preliminary dose–response experiments for cys-LT generation with 10 ng/ml IL-4 and SCF. The concentration-response for 1, 5, and 10 ng/ml IL-3 on IL-4–primed hMCs was 3.4, 16.0, and 25.5 ng cys-LT/10^6 hMCs, respectively. The dose of 5 ng/ml IL-3 was chosen as it was also the optimal concentration for comitogenesis (37). The dose of IL-5 was chosen for similar reasons. The hMCs were stimulated with a rabbit anti–human IgE Ab (ICN Biomedicals) at a concentration of 1 μg/ml for 30 min at 37°C. Cell supernatants were harvested and stored at −70°C before assay. Cell pellet fractions were resuspended in medium and lysed by three cycles of rapid freezing and thawing. Histamine in the supernatant and cellular pellet fractions was measured by histamine ELISA (ICN Biomedicals). Percentage of histamine release was quantitated by the equation: histamine in supernatant/(histamine in supernatant + histamine in pellet) × 100. Cys-LT generation in the supernatant was measured with an ELISA for $LTC_4/D_4/E_4$ (Amersham Pharmacia Biotech). For this ELISA, the cross-reactivity with LTC_4 is 100%, LTD_4 is 100%, LTE_4 is 70%, and LTB_4 is 0.3%. PGD_2 generation in the supernatant was measured with an ELISA for PGD_2 (Cayman Chemical). For the PGD_2 ELISA, the cross-reactivity with TXB_2, PGF_{2a}, or PGE_2 is <0.01%. The ratios of cys-LT to PG-D_2 were calculated as the mean ± SD of the respective ratios determined for each condition in each individual experiment.

For measurement of cys-LT generation by reverse phase (RP)-HPLC, hMCs were primed and activated as above. Cell supernatants were collected and three volumes of cold methanol containing 400 ng/ml of PGB_2 were added. After centrifugation in a microcentrifuge (Eppendorf) for 5 min at maximum speed at room temperature, the clarified methanolic extracts were removed and applied to a 5-μm 4.6 × 250 mm C18 Ultrasphere RP-HPLC column (Beckman Coulter) equilibrated with 100% methanol/acetonitrile/water/acetic acid (10:15:100:0.2, vol/vol, pH 6.0; solvent A). RP-HPLC was performed with a model 126 dual pump system and a model 167 scanning UV detector (Beckman Instruments) with Beckman System Gold software. After injection of the sample, the column was eluted at a flow rate of 1 ml/min with a programmed concave gradient (System Gold curve 6) to 55% of the equilibrated solvent A and 45% methanol (solvent B) over 2.5 min. After 5 min, solvent B was increased linearly to 75% over the next 15 min and was maintained at this level for an additional 15 min. UV absorbance at 235 and 280 nm was recorded. The retention times for PGB_2, LTB_4, LTC_4, LTD_4, LTE_4, and 5-HETE were 20.7, 24.0, 21.6, 23.6, 26.0, and 30.2 min, respectively. The resolved products were quantitated by calculating the ratio of the peak areas to the area of the internal standard PGB_2 (18). When synthetic LTC_4 or mixtures of synthetic LTD_4 and LTE_4 (Cayman Chemical) were analyzed using both RP-HPLC and ELISA, the total product measured by ELISA was consistently greater than that measured by RP-HPLC by a maximum of 20%.

SDS-PAGE Immunoblot Analysis. Whole cell extracts were prepared by washing cells in cold PBS and then boiling them in Tris-glycine/bromophenol blue lysis buffer (Novex) containing 0.5% 2-ME at a concentration of 10^7 cells/ml of lysis buffer for 10 min. After SDS-PAGE with 14% Tris-glycine gels (Novex), the proteins from 10^5 cells/lane were electrophoretically transferred to 0.45-μm nitrocellulose membranes (Bio-Rad Laboratories). Nonspecific binding was blocked with 3% wt/vol nonfat milk (Bio-Rad Laboratories) in Tris-buffered saline (TBS) containing 0.1% wt/vol Tween 20 and 0.5% normal goat serum (Caltag). Detection of LTC_4S with primary Abs used a 1:500 dilution of affinity-purified rabbit polyclonal antipeptide Ab (0.192 mg/ml) directed against the carboxyl-terminal 15 amino acids of human LTC_4S (protein sequence RAALLGRLRTLLWPA). This Ab does not cross-react with human FLAP, human m-glutathione-S-transferase II, or mouse LTC_4S on immunoblot, and recognizes only human LTC_4S in multiple tissue and cellular lysates. Additional primary Abs used in this study included rabbit polyclonal anti–5-LO Ab (J. Evans, Merck-Frosst Centre, Quebec, Canada [15]) at 1:5,000 dilution, rabbit polyclonal anti-FLAP Ab (15) at 1:5,000 dilution, and rabbit polyclonal anti-$cPLA_2$ Ab (Santa Cruz Biotechnology, Inc.) at 1:1,000 dilution. After the membranes were washed, the proteins were detected with a secondary goat anti–rabbit IgG labeled with horseradish peroxidase (Bio-Rad Laboratories) followed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech). SDS-PAGE immunoblots were quantitated using a ChemiImager 4400 densitometer with AlphaEase v5.0 software (Alpha Innotech Corporation).

RNA Blot Analysis. Total RNA was extracted from 10^7 cells with Tri-reagent (Molecular Research Center [39]). After extraction with chloroform followed by overnight precipitation in isopropanol, total RNA was washed with 70% ethanol and purity was assessed by spectrophotometry (DU 640; Beckman Coulter). Then, 14 μg of total RNA was loaded into a 1.2% agarose gel with 1× MOPS (3-[*N*-morpholino] propan-sulfonic acid) buffer containing 20% formaldehyde. After electrophoresis, RNA was transferred to nylon membranes (Micron Separations) by capillary action overnight. RNA was fixed to the membrane by baking at 80°C for 1 h. The blot was prehybridized in 5× standard sodium phosphate with EDTA (SSPE; GIBCO BRL) containing 2× Denhardt's solution, 0.25% SDS, 50% formamide, and 100 μg/ml denatured salmon sperm DNA (GIBCO BRL) overnight at 43°C. The blot was then probed with human LTC_4S cDNA (18) or human 18S ribosomal RNA (CLONTECH Laboratories, Inc.) that had been labeled with [^{32}P]dCTP incorporated by random priming with RediPrime II (Amersham Pharmacia Biotech). Blots were washed at high stringency with 0.2× SSPE at 55°C, and hybridization signals were detected by autoradiography with Kodak XAR film (Eastman Kodak Co.).

Analysis of LTC_4S Activity in Lysed Cells. LTC_4S activity was determined as described (18, 39). 2 × 10^5 hMCs were washed into 250 μl of 50 mmol/liter of Hepes, pH 7.6, with 10 mmol/liter $MgCl_2$. The cells were sonicated on ice with a Branson sonicator three times for 5 s each. Reduced glutathione and LTA_4 methyl ester were added to the cell lysates at room temperature at final concentrations of 10 mmol/liter and 20 μmol/liter, respectively. After 10 min, the reaction was terminated by adding 2 volumes of cold methanol containing 400 ng/ml of PGB_2. After centrifugation in a microcentrifuge (Eppendorf) for 5 min at maximum speed, the supernatants were removed and applied to a 5-μm 4.6 × 250 mm C18 Ultrasphere RP-HPLC column (Beckman Coulter) equilibrated with 100% methanol/acetonitrile/water/acetic acid (10:15:100:0.2, vol/vol, pH 6.0; solvent A). After injection of the sample, the column was eluted at 1 ml/min with a programmed concave gradient (System Gold curve 6) to 30% of the equilibrated solvent A and 70% methanol (solvent B) over 0.2 min. After 2.8 min, solvent B was increased linearly to 90% over 2 min and was maintained at this level for an additional 10 min. UV absorbance at 280 nm and the UV spectra were recorded. The retention times for PGB_2 and LTC_4 methyl ester were 8.5 and 10.1 min, respectively. LTC_4 methyl ester was quantitated by calculating the ratio of the peak area to the area of the internal standard PGB_2.

Immunofluorescence and Immunocytochemistry. hMCs were fixed in suspension with 2% paraformaldehyde in PBS for 10 min at 4°C, washed once in HBSS without calcium or magnesium containing 0.1% BSA (HBA), permeabilized with 100% methanol for 20 min at −20°C, and spun for 5 min at 500 rpm in a cytocentrifuge onto glass coverslips. The slides were then blocked in HBA containing 5% normal horse serum (Jackson ImmunoResearch Laboratories) for 1 h. Primary Abs rabbit polyclonal anti–5-LO Ab (J. Evans, Merck-Frosst Centre [15]) or normal rabbit IgG (Chemicon) were added at a 1:800 dilution in HBA, and the cells were incubated for 1 h at room temperature. The cells were then washed in HBA and treated for 1 h at room temperature with the nuclear dye bis-benzimide (Hoechst no. 33258; Sigma-Aldrich) at a 1:1,000 dilution and/or goat anti–rabbit IgG conjugated to FITC (Jackson ImmunoResearch Laboratories) at a 1:100 dilution. After washing in HBA, cells were mounted in a 33% glycerol solution in PBS containing 15% w/v vinol 205 (Air Products and Chemicals) and 0.1% sodium azide. Cells were visualized with 40× or 100× oil objective lenses with a FXA microscope (Nikon). At least 100 cells per cover slip were counted in each experiment. The presence or absence of nuclear staining was determined by superimposition of the FITC-stained images with the Hoechst-stained images of the same cells. Each cover slip was scored for nuclear staining of 5-LO by two independent investigators blinded to the experimental conditions. The results reported are the average of the percentage of positive cells reported by the two investigators for each experiment.

For immunocytochemistry, slides with 2×10^4 hMCs were prepared by cytocentrifugation, air dried, and fixed in Carnoy's fluid (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 10 min at room temperature. After being washed with PBS three to four times, the slides were blocked with 2% chicken egg albumin (Sigma-Aldrich) for 30 min at room temperature, and were incubated with an appropriate dilution of antitryptase (Chemicon) or with an equivalent dilution of the corresponding isotype-matched negative control (BD PharMingen). After application of the appropriate secondary Ab, alkaline phosphatase was used as a chromogenic reporter (37).

Statistical Analysis. Unless otherwise indicated, the results are reported as the mean ± SEM from at least three independent experiments with the cells from different donors. As consistent trends for cytokine effects on cys-LT generation were observed despite wide variability among subjects in absolute quantities produced, the data for eicosanoid generation were calculated both as absolute quantities and as the mean ratio of cys-LT/PGD_2 for each experiment. Statistical differences in immunostaining and eicosanoid generation were determined with the independent group Student's *t* test.

Results

Effect of Th2-type Cytokines on Cys-LT and PGD_2 Generation by Anti-IgE–activated hMCs. hMCs developed from human umbilical cord blood mononuclear cells cultured in the presence of SCF, IL-6, and IL-10 (37) were washed and maintained with SCF (100 ng/ml) alone or with SCF plus IL-4 (10 ng/ml) for 5 d of passive sensitization with human IgE. When activated by the addition of anti-IgE, hMCs primed by IL-4 released fivefold more of their secretory granule–associated histamine than hMCs stimulated after maintenance with SCF alone ($P = 0.0006$, $n = 4$; Fig. 1), compatible with previous reports (38, 40). hMCs treated with SCF alone during passive sensitization generated only 0.1 ± 0.1 ng cys-LT/10^6 cells, whereas those primed with IL-4 in the presence of SCF responded to IgE-dependent activation with a 27-fold increase in cys-LT production (2.7 ± 1.0 ng cys-LT/10^6 cells, $P = 0.03$, $n = 4$; Fig. 1). PGD_2 generation by hMCs in SCF alone increased from a substantial amount (16.2 ± 10.3 ng/10^6 hMCs) to 39.1 ± 18.5 ng/10^6 hMCs ($P = 0.17$, $n = 4$; Fig. 1) when the cells were primed with IL-4. IL-4 priming produced an increment in both cys-LT and PGD_2 production in every experiment.

The modest cys-LT generation even after IL-4–induced priming for IgE-dependent activation prompted a search for additional priming factors, with attention to the Th2 cytokines that had previously shown maximal comitogenic activities for hMCs, IL-3, and IL-5. Priming with IL-3 alone produced a small increment in IgE-dependent cys-LT

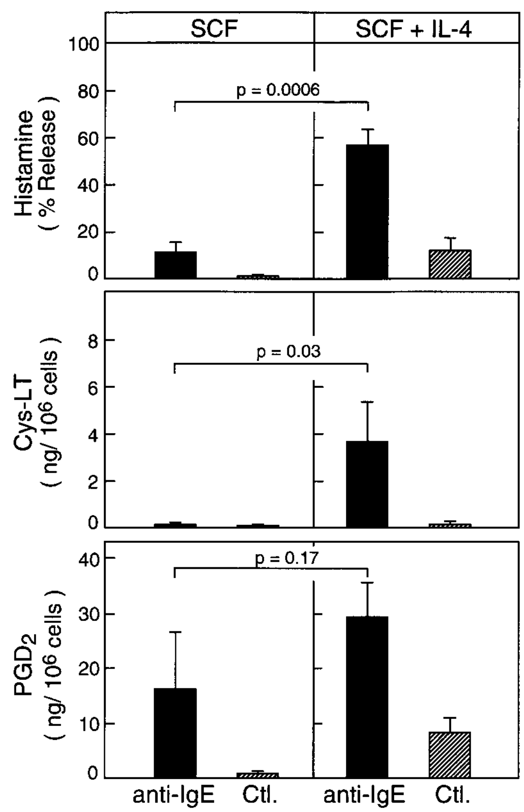

Figure 1. Effect of IL-4 priming of hMCs on IgE-mediated histamine release and eicosanoid generation as measured by ELISA. hMCs were maintained in SCF and IgE for 5 d with and without 10 ng/ml of IL-4. Results depict percent histamine release, cys-LT generation, and PGD_2 production after 5 d of priming and passive sensitization followed by activation with anti-IgE (black bars) or treatment with buffer alone (hatched bars). Results are the mean ± SEM of four experiments and the IL-4 effect was significant for histamine ($P = 0.0006$) and cys-LT ($P = 0.03$). Ctl., control.

generation above cells maintained in SCF alone that was evident by 24 h and maximal by 3 d (2.7 ± 2.0 ng vs. 0.9 ± 1.8 ng, $n = 3$). Priming with IL-5 was comparable ($n = 1$). When added with SCF and IL-4 for 5 d of priming, neither IL-3 (5 ng/ml) nor IL-5 (5 ng/ml) increased the exocytosis of histamine by passively sensitized hMCs challenged with anti-IgE (55 ± 9% and 53 ± 9% release with the added priming of IL-3 and IL-5, respectively, vs. 73 ± 8% release with IL-4 priming alone, $n = 4$; Fig. 2). In contrast, in each of these experiments, the inclusion of IL-3 during priming with IL-4 increased IgE-dependent cys-LT generation (from 5.7 ± 3.1 to 33.0 ± 13.1 ng/10^6 hMCs, $P = 0.05$, $n = 5$). The effect of IL-5 priming was similar and present in every experiment (22.7 ± 16 ng/10^6 hMCs, $P = 0.17$, $n = 3$; Fig. 2). Priming by IL-3 plus IL-4 also modestly increased PGD_2 generation relative to priming with IL-4 alone (from 27.2 ± 5.4 to 40.3 ± 12.3 ng/10^6 hMCs, $n = 4$), whereas IL-5 did not add to the effect of IL-4 priming alone for PGD_2 generation (28.3 ± 2.0 ng/10^6 hMCs, $n = 3$; Fig. 2). Because the effects of IL-3 and IL-5 were each relatively selective for cys-LT generation, their addition significantly altered the ratio of cys-LT to PGD_2 generated (from 1:14.6 for hMCs primed with IL-4 to 1:2.9 for hMCs primed with IL-3 plus IL-4 [$P = 0.05$] and 1:3.7 for hMCs primed with IL-5 plus IL-4 [$P = 0.04$]).

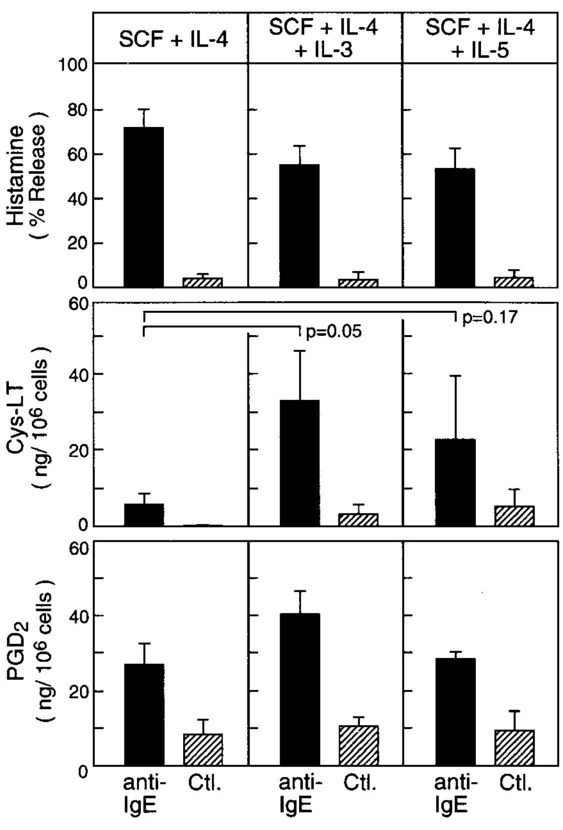

Figure 2. Effect of IL-3 or IL-5 treatment during IL-4 priming of hMCs for IgE-mediated release of histamine and generation of cys-LTs and PGD_2, as measured by ELISA. hMCs were activated with anti-IgE (black bars) or buffer (hatched bars) after 5 d of passive sensitization and priming with IL-4 in the presence of SCF with or without the addition of IL-3 or IL-5. Results are mean ± SEM for five (for SCF plus IL-4 and SCF plus IL-4 plus IL-3) or three (SCF plus IL-4 plus IL-5) experiments. Ctl., control.

The effects of each cytokine on integrated cellular 5-LO function can be quantitatively assessed only by RP-HPLC analysis, permitting the simultaneous measurement of cys-LT, LTB_4, and the proximal metabolites 5-HETE and 6-trans-LTB_4 (derived from nonenzymatic breakdown of 5-HPETE and LTA_4, respectively). With this analysis, hMCs maintained with SCF alone generated 0.1 ± 0.2 pmoles cys-LT/10^6 hMCs with IgE-dependent activation, whereas hMCs primed with IL-3 in the absence of IL-4 produced 2.7 ± 1.3 pmoles cys-LT/10^6 hMCs ($n = 3$ for both conditions). Comparable quantities were produced by IL-5–primed hMCs. hMCs primed by IL-4 with SCF generated 5.0 ± 2.4 pmoles cys-LT/10^6 hMC ($n = 5$) after activation, which was increased threefold by the inclusion of IL-3 (14.8 ± 4.2 pmoles/10^6 hMCs, $P = 0.05$, $n = 4$), and to a lesser extent by the inclusion of IL-5 (8.6 ± 2.9 ng/10^6 hMCs, $P = 0.19$, $n = 3$). No 5-HETE or 6-trans LTB_4 was detected under any experimental conditions. Peaks corresponding to LTC_4, LTD_4, and LTE_4 were detected, with most of the product being converted to LTE_4.

Effect of Th2 Cytokines on LTC_4S Expression and Function. Compared with maintenance in SCF alone, priming by IL-4 (10 ng/ml) for 5 d resulted in a marked increase in LTC_4S protein, with a slight increase in $cPLA_2$ and no apparent change in either FLAP or 5-LO (Fig. 3 a). As quantitated by densitometry, the mean increase in LTC_4S protein signal after IL-4 treatment was fivefold (Fig. 3 b; $n = 11$, $P = 0.0005$). IL-13 (10 ng/ml) did not affect the expression of LTC_4S or any of the other pathway proteins, as shown for LTC_4S (Fig. 4 a). With the concentration of SCF held constant at 100 ng/ml, treatment of hMCs for 5 d with increasing concentrations of IL-4 induced a dose-dependent increase in immunodetectable LTC_4S protein in every

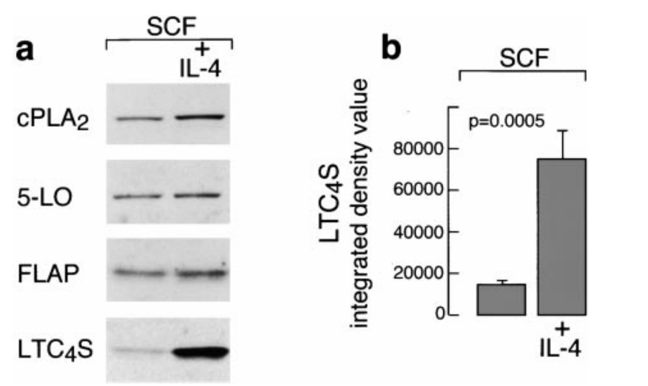

Figure 3. Effect of IL-4 priming on 5-LO/LTC_4S pathway protein expression by hMCs. (a) SDS-PAGE immunoblot was performed with lysates from hMCs (10^5/lane), treated for 5 d with SCF with or without IL-4, with polyclonal Abs specific for $cPLA_2$, 5-LO, FLAP, and LTC_4S, as specified in Materials and Methods. The displayed blot is a single experiment representative of three experiments. (b) Quantitative densitometry revealed a fivefold increase in LTC_4S signal after IL-4 treatment for 5 d ($n = 11$, $P = 0.0005$).

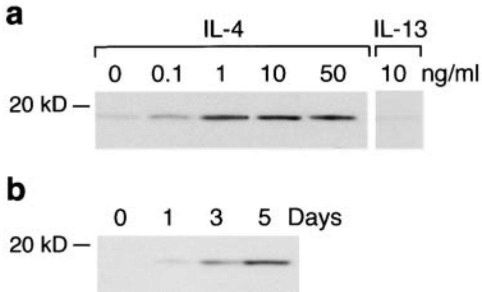

Figure 4. Dose dependence and kinetics for the IL-4–mediated upregulation of LTC_4S protein expression. (a) Dose-dependent effects at 5 d of IL-4, from 0.1 to 50 ng/ml, on LTC_4S protein with a constant concentration of SCF (100 ng/ml), as demonstrated by SDS-PAGE immunoblot. Single lanes containing lysates from 10^5 hMCs treated for 5 d with SCF alone, and with SCF plus IL-13 (10 ng/ml), are included. (b) Kinetic effects of IL-4 (10 ng/ml) on LTC_4S protein in hMCs incubated for 0 to 5 d with SCF (100 ng/ml) to maintain viability. The displayed blots are representative of three experiments each.

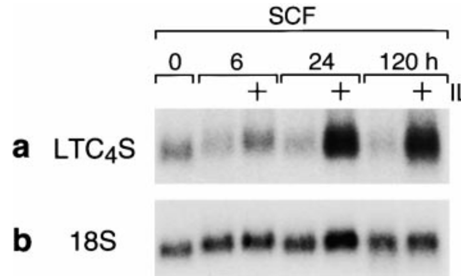

Figure 6. IL-4–mediated upregulation of steady-state LTC_4S mRNA. (a) RNA blot analysis depicting hybridization signals for LTC_4S mRNA in samples of total RNA (14 μg/lane) extracted from hMCs that were harvested at the indicated times after being transferred to fresh SCF-containing medium with (+) or without IL-4. (b) Ribosomal 18S RNA signal in the corresponding lanes. Results are representative of two experiments performed.

experiment (n = 3), apparent at the lowest concentration tested (0.1 ng/ml), and maximal at 10 ng/ml (Fig. 4 a). The effect of 10 ng/ml IL-4 on LTC_4S protein was apparent by 1 d and maximal at 5 d (Fig. 4 b). LTC_4S activity, as measured by the conversion of LTA_4 methyl ester to LTC_4 methyl ester, remained unchanged in lysates of hMCs treated with SCF alone for 1 and 5 d as compared with hMCs harvested from the original developmental triad of SCF, IL-6, and IL-10. In contrast, lysates of IL-4–primed hMCs revealed a sevenfold increase in LTC_4S activity over this time frame (138.5 pmol to 980.5 pmol $LTC_4/10^6$ hMCs, $n = 4$, $P = 0.02$; Fig. 5). Compared with maintenance in SCF alone, the addition of IL-4 (10 ng/ml) enhanced steady-state levels of LTC_4S mRNA expression by 6 h, with a plateau at 24 h that continued unchanged for 5 d ($n = 2$, as shown for a representative experiment; Fig. 6).

Effect of IL-3 and IL-5 on the Cellular Localization of 5-LO. Compared with hMCs primed with SCF alone, neither IL-3 nor IL-5 priming altered the immunodetectable quantities of 5-LO or FLAP, and they modestly increased the baseline expression of $cPLA_2$. IL-5, but not IL-3, also slightly increased immunodetectable LTC_4S protein (Fig. 7). When added in combination with IL-4 in the presence of SCF, neither IL-3 nor IL-5 produced further increases in LTC_4S protein or altered the quantities of 5-LO or FLAP proteins, and each induced slight increases in the expression of $cPLA_2$ above that induced by IL-4 alone (n = 3, data not shown).

hMCs maintained for 5 d in SCF alone displayed weak 5-LO immunoreactivity predominantly in a diffuse, cytoplasmic distribution (as shown for one experiment; Fig. 8 d). Under these conditions, 11 ± 2% of the hMCs exhibited some staining of the nucleus after 5 d, which was enhanced only slightly by the addition of IL-4 (19 ± 6%, P =

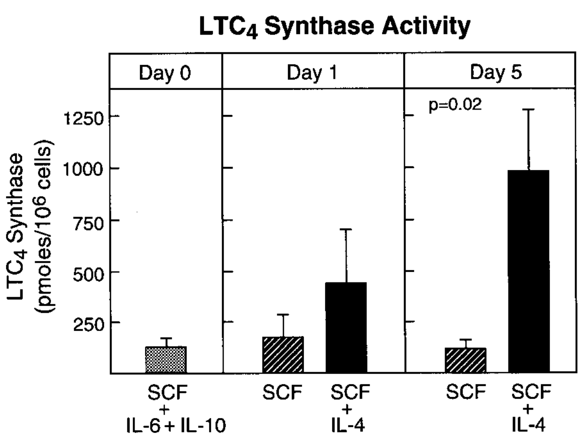

Figure 5. IL-4–mediated upregulation of LTC_4S biosynthetic activity. LTA_4 methyl ester and reduced glutathione were provided as substrates to the sonicated lysates from 2×10^5 hMCs for each condition. Sonicates were prepared from hMCs harvested from their original medium containing SCF, IL-6, and IL-10 at the start of the experiment (day 0), and again after these hMCs were treated with SCF alone (100 ng/ml) or with SCF and IL-4 (10 ng/ml) for the indicated times. Products were resolved by RP-HPLC and quantitated based on the internal standard PGB_2 as indicated in Materials and Methods. Results are mean ± SEM for four experiments.

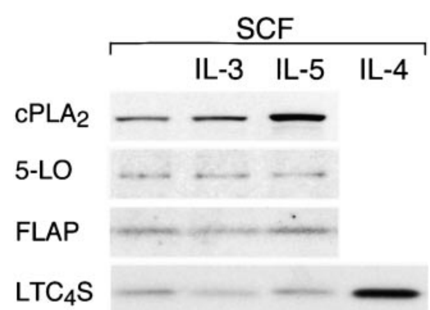

Figure 7. Effect of IL-3 or IL-5 priming on 5-LO/LTC_4S pathway protein expression by hMCs. SDS-PAGE immunoblot was performed with lysates from hMCs (10^5/lane), treated for 5 d with SCF (100 ng/ml) with or without IL-3 or IL-5 (5 ng/ml each), using polyclonal Abs specific for $cPLA_2$, 5-LO, FLAP, and LTC_4S as specified in Materials and Methods. The LTC_4S signal from hMCs primed for 5 d with IL-4 is included for comparison. The displayed blot is a single experiment representative of three experiments.

0.12). When compared with maintenance in SCF alone or with SCF plus IL-4, treatment with either IL-3 or with IL-5 increased the proportion of hMCs exhibiting nuclear staining for 5-LO by as early as 3 d. The differences were significant by 5 d (46 ± 12% with nuclear staining for hMCs maintained with IL-3 and IL-4 plus SCF, $P = 0.05$; and 38 ± 7% for hMCs maintained with IL-5 and IL-4 plus SCF, $P = 0.03$). IL-3 and IL-5 each also increased the intensity of the nuclear stain ($n = 3$, as shown for a representative experiment; Fig. 8, h and j, respectively). The ef-

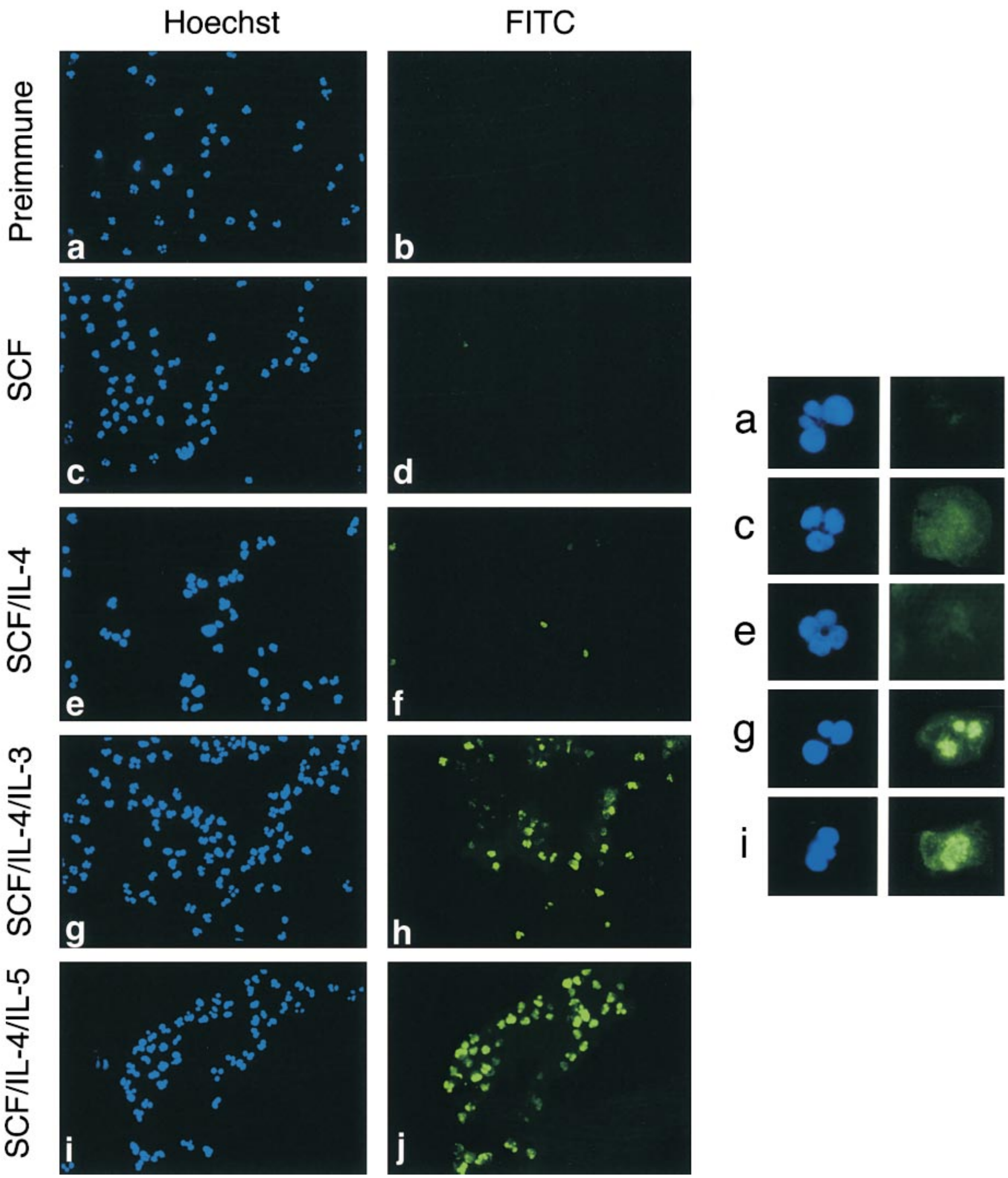

Figure 8. Effects of IL-3 and IL-5 on immunolocalization of 5-LO in hMCs. hMCs were maintained for 5 d in SCF alone (a–d), with 10 ng/ml of IL-4 (e and f), with IL-4 plus 5 ng/ml of IL-3 (g and h), or IL-4 plus 5 ng/ml of IL-5 (i and j). Images on the left are photographed through a 40× objective, whereas those on the right are photographed at 100× for nuclear detail. Identical fields of hMCs are photographed under blue fluorescence (a, c, e, g, and i) to show location of nuclei as identified by Hoechst staining, and under green fluorescence (FITC) to demonstrate localization of 5-LO immunoreactivity in the same cells (d, f, h, and j). FITC staining with a preimmune rabbit IgG (b) is included as a specificity control. The images are taken from a single experiment representative of three performed, for which the mean data are presented in the text.

fects of IL-3 and IL-5 on 5-LO localization were similar in experiments where IL-4 was omitted (55 ± 19% and 34 ± 14% positive, respectively, mean ± 1/2 range for two of the experiments presented above). Immunofluorescence with control rabbit IgG gave almost no background staining (Fig. 8 b).

Discussion

The effector molecules implicated in the pathogenesis of bronchial asthma include the lipid mediators provided by hMCs and eosinophils (10, 25, 41) and the cytokines provided by Th2 cells. hMCs derived from cord blood with the triad of SCF, IL-6, and IL-10 express the receptors for IL-3 and IL-5 (37), and both of these Th2 cytokines mediate comitogenic responses from hMCs in the presence of SCF. hMCs respond to a third Th2 cytokine, IL-4, with augmented FcϵRI expression and IgE-dependent activation responses (38, 40). IL-3 and IL-5 promote the development in vitro of cord blood–derived eosinophils that express all proteins of the 5-LO/LTC_4S pathway (39) and that generate cys-LT after stimulation with calcium ionophore. Unlike eosinophils, which do not produce PGD_2, hMCs generate both PGD_2 and cys-LT, the latter of which exhibits wide variability among hMCs obtained from various dispersed tissues (31–34). Thus, both arms of the eicosanoid-generating pathways of hMCs were assessed for the regulatory effects of Th2 cytokines. We found that although cord blood–derived hMCs maintained in SCF alone generated abundant PGD_2 after IgE-dependent activation even without Th2 cytokine priming, their optimal cys-LT generation required the coordinate actions of IL-4 with either IL-3 or IL-5, which mediate separate and distinct steps in priming the 5-LO/LTC_4S pathway for an integrated functional response.

For all priming conditions, SCF was included to ensure maximal hMC viability. A marked (27-fold) increase in cys-LT generation (Fig. 1) was observed when hMCs were primed with IL-4 before activation. This was attributable to two events. First, IL-4 priming augmented IgE-dependent exocytosis of histamine by fourfold (Fig. 1), an effect attributable to the previously recognized upregulation of FcϵRI by IL-4 (38, 40). Second, and unexpectedly, IL-4 induced a dramatic upregulation of LTC_4S transcript (Fig. 6), protein (Figs. 3 and 4), and function (Fig. 5). This marked induction by IL-4 was relatively selective for LTC_4S among the 5-LO/LTC_4S pathway proteins (Fig. 3). IL-13 at a concentration of 10 ng/ml did not upregulate LTC_4S protein (Fig. 4). In previous studies, IL-13 failed to augment FcϵRI expression by cord blood hMCs (38), and was not comitogenic with SCF (37). The fact that hMCs respond markedly to IL-4 but not to IL-13 suggests that they may express the $\alpha\gamma$ IL-4 receptor heterodimer associated with T cells rather than the IL-4 receptor type II consisting of the IL-4Rα and IL-13Rα1 subunits found mainly in B cells and nonhematopoietic cells (42). The parallel IL-4–mediated regulation of both FcϵRI and LTC_4S, the biosynthetic enzyme responsible for cys-LT leukotriene generation, would fit the pivotal role of Th2 cells in bronchial asthma and supports a direct regulatory role for lymphocytes in the control of hMC function.

Although IL-4 priming of hMCs did induce their IgE-dependent cys-LT generation, the modest quantities of cys-LTs produced, relative to the abundant generation of PGD_2, led us to explore possible additional priming events. IL-3 and IL-5 were maximally comitogenic for hMCs from among a panel of Th2 cytokines tested in our earlier studies (37). When added without IL-4, IL-3 or IL-5 provided an increment in cys-LT production comparable to IL-4 alone by hMCs after IgE-dependent activation. In contrast, the inclusion of IL-3 or IL-5 with IL-4 resulted in a six- or fourfold increase, respectively, in IgE-dependent cys-LT production, without a dramatic change in either PGD_2 generation or percentage of histamine release (Fig. 2). These findings indicate a selective action of IL-3 and IL-5 on the function of the 5-LO/LTC_4S pathway. The priming effect of IL-3 and IL-5 involved the redistribution of 5-LO to the hMC nucleus (Fig. 8), which did not require the presence of IL-4, and was temporally concomitant with a functional effect evident by incremental cys-LT generation. Our studies thus reveal two Th2-dependent requirements for the integrated function of the 5-LO/LTC_4S pathway in cultured hMCs. IL-4 is necessary to upregulate FcϵRI expression and to induce LTC_4S expression, whereas IL-3 or IL-5 positions 5-LO for its subsequent utilization of arachidonic acid in the presence of FLAP at the nuclear envelope after IgE-dependent activation. Neither event alone is sufficient for the full expression of cys-LT production, but each acts synergistically with the other to promote a marked phenotypic change in hMCs.

The human LTC_4S gene is localized to chromosome 5q35 (43), close to a locus identified by linkage analysis to have gene candidates for asthma and atopy (44, 45). Regulatory cis-acting elements and transcription factors for the proximal core promoter of the human LTC_4S gene in THP-1 cells include a non–cell-specific basal promoter and a cell-specific upstream enhancer region (46). The effect of IL-4 on LTC_4S expression may reflect increased LTC_4S transcription or could result from increased mRNA stability. A monocyte-like cell line, THP-1, responds to TGF-β with increased steady-state LTC_4S mRNA expression by a transcription-dependent mechanism, without an effect on LTC_4S transcript stability (47). IL-4 could also influence trafficking of nascent LTC_4S protein to the perinuclear membrane and endoplasmic reticulum. The latter may account for the lag between plateau mRNA expression (24 h) and protein expression (5 d) in this study. Maximal expression of LTC_4S steady-state mRNA also preceded the plateau for expression of immunodetectable LTC_4S protein by several days in culture-derived eosinophils (39). The findings in earlier studies that IL-3– and IL-5–driven eosinophil differentiation from cord blood progenitors in vitro was accompanied by marked induction of LTC_4 transcript and protein (39), and the findings in this study that maximal expression of LTC_4S by hMCs requires induction by IL-4, indicate that cell-specific regulation of LTC_4S expression differs among effector cell types. Such cell-specific regulation may explain the profound upregulation of LTC_4S in eosinophils, but not hMCs, in lung tissue biopsy specimens from patients with aspirin-sensitive asthma (48).

Although mouse bone marrow–derived MCs (BMMCs) can develop in vitro in response either to SCF with IL-6 and IL-10 (36) or to IL-3 alone (49), normal MC development in mice in vivo is SCF-dependent (50–52). IL-3–derived BMMCs express 5-LO in their nucleus at baseline (13) and preferentially generate cys-LTs over PGD_2 when activated via FcϵRI. However, priming of these IL-3–driven BMMCs in vitro with SCF preferentially increases their capacity for PGD_2 generation by augmenting their expression of $cPLA_2$, PGHS-1, and PGD_2S (53). The apparently innate capacity for all tissue subpopulations of hMCs to generate PGD_2 may therefore reflect their SCF dependency in vivo. Our data confirm that SCF alone is sufficient to support a PGD_2-producing hMC population in vitro that is similar in eicosanoid product profile to hMCs from dispersed human skin (32). The previously reported capacity for IL-3 to upregulate cys-LT generation by SCF-driven mouse BMMCs (54) was associated with progressive increases in 5-LO and FLAP expression over 2 wk, followed at 4 to 5 wk by augmented LTC_4S expression concomitantly with a 12-fold increase in total BMMC numbers. In contrast, hMCs in our study did not increase in number during 5 d of cytokine priming, indicating that their cytokine-induced cys-LT biosynthetic capacity represented a phenotypic change, with a transition from a PGD_2-dominant profile of arachidonic acid metabolism to a nearly equivalent PGD_2/cys-LT profile reminiscent of dispersed lung or intestinal hMCs (33, 34).

Under resting conditions 5-LO localizes to the cytosol of neutrophils, but is in the nuclear euchromatin of alveolar macrophages, RBL cells, and mouse BMMCs cultured in WEHI medium (which contains murine IL-3 [55–58]). In each of these cells, 5-LO translocates to the nuclear envelope during activation-dependent LT generation. Nuclear import of 5-LO from the cytoplasm of neutrophils is associated with priming for subsequent enhanced calcium ionophore A23187-induced LT generation (58), possibly reflecting enhanced proximity to the other enzymes involved in LT biosynthesis. In our study, only a small minority of hMCs incubated with SCF alone or SCF plus IL-4 for 5 d showed nuclear staining for 5-LO. Priming with IL-3 or IL-5 increased the proportion of hMCs exhibiting nuclear staining for 5-LO at 5 d (as shown in a representative experiment; Fig. 8), an effect which did not require the presence of IL-4. Our study thus suggests that the priming effects of IL-3 and IL-5 for cys-LT production by hMCs includes the nuclear import of 5-LO, and may involve other mechanisms such as a slight upregulation of $cPLA_2$ (Fig. 7). Translocation of 5-LO from the cytosol to the nucleus has been proposed to explain IL-5–mediated priming of ionophore-stimulated LTC_4 production by human peripheral blood eosinophils (59).

An array of Th2 cytokines, including both IL-5 and IL-4, is strongly expressed through the influx of Th2 cells in bronchial biopsy specimens from patients with asthma relative to individuals without asthma (60). IL-3 protein is localized to the bronchial epithelium in individuals with and without asthma (61). The increased numbers of hMCs in the bronchial mucosa of patients with newly diagnosed asthma (62) may reflect the comitogenic actions of Th2 cytokines on this SCF-dependent lineage. The fact that endobronchial allergen challenge elicits markedly increased quantities of cys-LTs in the bronchoalveolar lavage fluids of patients with asthma relative to control individuals with allergic rhinitis alone (41) may reflect disease-related phenotypic modifications of local hMCs induced by these same cytokines. Our study demonstrates that Th2 cytokines alter the profile of eicosanoids generated by mature hMCs, and it is the first to suggest a mechanistic basis for this phenotypic change. Furthermore, our findings suggest that hMCs, which depend on SCF for their normal development and survival, have a constitutive arachidonic acid phenotype that is characterized by PGD_2 generation predominating markedly over cys-LT generation. This arachidonic acid profile of $PGD_2 >>$ cys-LT is substantially modified, with a profound increment in cys-LTs, by priming the hMCs with IL-4 to induce LTC_4S and with IL-3 or IL-5 to maintain 5-LO at the nucleus before FcϵRI-mediated activation.

This work was supported by National Institutes of Health grants AI01305, AI31599, AI22531, and HL36110, and by a grant from the Hyde and Watson Foundation. Dr. Hsieh is the recipient of grants from Glaxo-Wellcome Pharmaceuticals.

Submitted: 25 May 2000
Revised: 20 November 2000
Accepted: 28 November 2000

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