

Differential expression of leukocyte immunoglobulin-like receptors on cord blood-derived human mast cell progenitors and mature mast cells

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Abstract: The leukocyte Ig-like receptors (LILRs) comprise a family of cell-surface immunoregulatory receptors with activating and inhibitory members. The inhibitory LILRs possess cytoplasmic ITIMs that down-regulate signaling by nonreceptor tyrosine kinase cascades. The activating members have a truncated cytoplasmic domain and signal through the FcR γ chain. We examined the expression of LILRs on human mast cells during their development in vitro. Progenitor mast cells expressed cell surface inhibitory LILRB1, -B2, -B3, and -B4 and activating LILRA1. However, although mature cord blood-derived mast cells (*hMCs*) had detectable mRNA encoding multiple LILRs, none were expressed on the cell surface. Culture of progenitor mast cells or *hMCs* with various cytokine combinations failed to retain or induce cell surface expression of the LILRs. It is interesting that *hMCs* expressed LILRB5 in cytoplasmic granules and upon cross-linking of the high-affinity IgE receptor, released LILRB5 into the culture medium. Our results demonstrate that LILRs are developmentally regulated in human mast cells and that LILRB5 is expressed in mast cell granules and the release of soluble LILRB5 following IgE FcR-dependent stimulation, which has potential for amplification of mast cell-dependent, inflammatory responses. *J. Leukoc. Biol.* 83: 000–000; 2008.

Key Words: immunoreceptor tyrosine-based inhibitory motifs · immunoreceptor tyrosine-based activating motifs · immune regulation · mast cell activation

INTRODUCTION

Mast cells are derived from hematopoietic progenitor cells that home to tissue as committed progenitors [1–3]. Maturation and differentiation of mast cells occur in tissue in response to local production of stem cell factor (SCF) [4]. Mast cells can also undergo significant changes in numbers or in phenotype during allergic or nonallergic inflammation [1, 5–9]. These cells play a crucial role in IgE-dependent immune responses that medi-

ate immediate hypersensitivity reactions associated with allergic phenomena and host resistance to parasites [5, 10]. They also participate in innate immunity to bacterial infection [11] and play a direct role in the pathogenesis of inflammatory arthritis [8, 9, 12–14]. Upon activation, mast cells produce a number of preformed and newly synthesized mediators, such as cytokines and proteases [14], that could be harmful to the host; hence, it is likely that their activation in vivo is tightly regulated by a network of inhibitory and activating signals similar to many other effector cells [15–17]. However, the nature and mechanisms of these signals on human mast cells remain largely unknown.

The leukocyte Ig-like receptors (LIRs or LILRs), also termed Ig-like transcripts and given the CD designation CD85a–h [18], are Ig superfamily proteins expressed by leukocytes [19, 20]. The inhibitory LILRs (LILRB1–5) have long cytoplasmic domains with two to four ITIMs [19–21]. These receptors mediate inhibition of cell activation by recruiting the *src* homologue 2 domain containing phosphatase 1 to the phosphorylated ITIM to inhibit or terminate signaling through nonreceptor tyrosine kinase cascades [21]. The activating LILRs (LILRA1, -2, -4, -5, and -6) have a truncated cytoplasmic domain and possess a charged arginine residue in their transmembrane domain, through which they associate with the ITAM-containing FcR γ chain [22]. LILRA3 is expressed exclusively as a soluble molecule with no transmembrane domain [19, 23]. Soluble forms of LILRB2 [24] and LILRA5 have also been described [25].

The expression of LILRs is well documented on a variety of leukocytes such as monocytes, dendritic cells (DCs), T cells, B cells, NK cells, eosinophils, basophils, and neutrophils [17, 19, 26–28], as well as other cells such as endothelial cells [29]. The immunoregulatory function of inhibitory and activating LILRs in vitro has been studied in some of leukocyte subsets [18, 21, 22, 28, 30–32]. Recent studies have demonstrated that expression of LILRs and related molecules might be develop-

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mentally regulated [31, 33–36]. A number of LILRs are differentially expressed on the surface of immature and mature DCs [31], and their expression pattern varies in different subsets of primary DCs [34]. Furthermore, activation of DCs with various inflammatory stimuli can up- or down-regulate expression of inhibitory LILRB1 and LILRB4 [37]. Conversely, LILRB1 expression changes during the course of B cell differentiation: It is absent in the pre-B stage, and expression increases as these cells mature [36]. It is interesting that a family of LILR-related molecules, known as leukocyte-associated Ig-like receptor-1 (LAIR-1), is also expressed by hematopoietic progenitors and might be implicated in regulating hematopoiesis [33, 38]. B cells are also known to express LAIRs, but their expression decreases as these cells differentiate to plasma cells [35]. Expression of LAIRs is also down-regulated during maturation of promyeloid cells to neutrophils [39].

We now show that human cord blood-derived progenitor mast cells (*hPrMCs*) express LILRB2, LILRB3, and LILRA2 on their surface. However, despite the presence of mRNA for multiple LILRs in mature cord blood-derived mast cells (*hMCs*), we were unable to detect any of the LILRs on the surface of these cells. Nevertheless, intracellular staining for various LILRs showed selective expression of LILRB5 that was colocalized with mast cell tryptases within the granules. In one out of three experiments, activation of mast cells, using anti-IgE to cross-link IgE bound to its high-affinity FcR (FcεRI), caused partial mobilization of LILRB5 to the cell surface. However, Western blot analysis using a mAb to LILRB5 consistently revealed an immunoreactive component of ~65 kDa, similar to the size of LILRB5, without post-translational modification and a protein of ~55 kDa in supernatants of cells activated with IgE and anti-IgE, whereas supernatants from cells treated with IgE alone contained only the ~65-kDa protein.

Our findings demonstrate that LILRs are developmentally regulated in mast cells, suggesting a role in their maturation and differentiation. This is the first study to demonstrate the intracellular expression of a LILR in mast cell granules and its mobilization in response to cross-linking of the high-affinity FcR for IgE. To date, LILRB5 mRNA transcripts were restricted to NK cells [27]. We show LILRB5 mRNA and protein in in vitro-derived cord blood mast cells. LILRB5 may be a novel, counter-regulatory receptor released during mast cell activation.

MATERIALS AND METHODS

Cytokines

Recombinant human (rh)SCF was a generous gift from Amgen (Thousand Oaks, CA, USA). The cytokines IL-6, TNF-α, IFN-γ, IL-4, IL-5, and TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA). rhIL-10 was purchased from Pierce Endogen (Woburn, MA, USA).

Antibodies

Mouse IgG₁ mAb against LILRA1 and -A2 and LILRB1–5, which are designated as m401, m471/473, m421, m431, m451, m467, and m481, respectively, from Amgen, were generated in BALB/c mice by immunization with LIR-Fc

fusion proteins containing the LIR extracellular domains fused to the Fc region of human IgG1 as described [27]. The antibodies were screened for binding specificity by ELISA against a panel of LIR-Fc fusion proteins and by FACS analysis using COS-1 cells transfected with full-length LIR cDNAs [27]. Irrelevant mouse IgG1-negative control was purchased from Biosource International (Camarillo, CA, USA). mAb to the following cell surface markers were used to characterize cultured mast cells: FcεRIα (clone 22E7, IgG₁ mAb that recognizes the α chain of FcεRI, provided by Dr. R. Chizzonite, Hoffmann-LaRoche, Nutley, NJ, USA) and *c-kit* (clone SR-1, IgG2a, provided by Dr. Virginia Broudy, University of Washington, Seattle, WA, USA, and clone K45, IgG2a, purchased from Biosource International). PharMingen (Mountain View, CA, USA) supplied the following mouse IgG₁ mAb: anti-CD13-FITC, anti-CD16-FITC (FcγRIII), anti-CD32-FITC (FcγRII), anti-CD64-FITC (FcγRI), and anti-CD123 (IL-3Rα chain). Beckman Coulter (Fullerton, CA, USA) supplied the following mouse mAb pairs: anti-CD34-FITC/anti-CD33-PE, anti-CD45-FITC/anti-CD14-PE, anti-CD3-FITC/anti-CD56-PE, and control IgG1-FITC/IgG2a-PE. Normal goat serum and FITC or Cy3-conjugated goat F(ab')₂ anti-mouse IgG [F(ab')₂-specific] with minimum cross-reactivity to human, rat, and bovine serum were supplied by Jackson ImmunoResearch (West Grove, PA, USA). Alexa 488-conjugated F(ab')₂ goat anti-mouse IgG [F(ab')₂-specific] was purchased from Molecular Probes Inc. (Eugene, OR, USA). Mouse IgG₁ mAb that detect human α and β tryptase (clone AA1) and human chymase were purchased from Dako (Glostrup, Denmark) and Chemicon International (Temecula, CA, USA), respectively. Zenon mouse IgG₁ labeling kit was used to directly conjugate anti-LILRB5 with Alexa 488 and antitryptase with Alexa 568, according to the manufacturer's instructions (Molecular Probes Inc.).

Culture of *hPrMCs* and *hMCs*

Cord blood from human placentas after routine Caesarian section was obtained from the Australian Cord Blood Bank at the Royal Women's Hospital (Sydney, New South Wales, Australia) or the Brigham and Women's Hospital (Boston, MA, USA). Cord blood was collected in accordance with established institutional guidelines, and the institutional ethics committees of the respective institutions approved this study. *hPrMCs* and *hMCs* were derived by the culture of the mononuclear cell fraction as described [40]. In brief, heparin-treated cord blood was sedimented with 4.5% dextran to remove erythrocytes. The buffy coat was layered onto Ficoll-Hypaque (Amersham Pharmacia Biotech, UK) and centrifuged at 300 *g* for 25 min at room temperature. The mononuclear cells were collected from the interface, washed with PBS containing 5 mM EDTA, suspended at 2×10^6 /ml in high-glucose RPMI 1640 (Gibco-BRL, Grand Island, NY, USA) containing 10% FBS, 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 Chemical Co., St. Louis, MO, USA), and 0.2 μM 2-ME (Gibco-BRL), and cultured in 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 10 weeks into culture medium containing fresh cytokines. From the 4th week onwards, *PrMCs* and *hMCs* were assessed weekly by flow cytometry using a panel of mouse mAb against human surface antigens. Every week, 2×10^4 cells were spun onto glass slides in a cytocentrifuge (Cytospin® 2, Shandon, UK) and were stained with toluidine blue or used for immunohistochemical staining. In some experiments, IL-4 or IFN-γ was included in the culture between 2 and 4 weeks during mast cell maturation.

Immunohistochemistry

Cytospin slides were air-dried and fixed with acetone or Carnoy's solution (60% ethanol, 30% chloroform, and 10% glacial acetic acid) for 10 min. After two washes in PBS, slides were re-equilibrated in TBS and blocked with 20% goat serum for 20 min at room temperature. Slides were then incubated with TBS/2% BSA containing antitryptase (5 μg/ml), antichymase (10 μg/ml), or irrelevant control (10 μg/ml) antibodies overnight at 4°C. After four washes with TBS, sections were incubated with biotinylated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. After four washes with TBS, sections were incubated with streptavidin-alkaline phosphatase conjugate (Vector Laboratories) for 45 min at room temperature. Immunoreactivity was detected using colorimetric alkaline phosphatase substrate (Vector Red, Vector Laboratories) and brief counter-staining with hematoxylin [41].

Flow cytometric studies

Flow cytometric studies were performed as described [28]. In brief, cord blood-derived mast cells were washed with cold PBS containing 0.05% NaN₃ and 1% BSA (PAB buffer) and suspended in the same buffer at 2×10^6 cells per ml. Human serum (final concentration of 10%) was added to the cell suspension. Cells were then incubated for 30 min at room temperature with mAb to LILRs (5 µg/ml), c-kit, FcεR1, or CD123 (all 10 µg/ml), control mouse IgG1, saturating amounts of directly conjugated mAb to CD13, CD16, CD32, CD64, or the following pairs of molecules: CD45/CD14, CD34/CD33, CD3/CD56, and isotype-matched control antibody pairs. After two washes with cold PAB buffer, 500 µl 1% paraformaldehyde in PBS was added to cells stained with directly conjugated antibodies and stored at 4°C in the dark. Meanwhile, cells stained with unconjugated antibodies were washed in PAB buffer and incubated on ice for 45 min with 10 µl (10 µg/ml) FITC or Cy3-conjugated F(ab')₂ goat anti-mouse IgG [F(ab')₂-specific], which was preabsorbed in an equal volume of normal goat serum. Cells were washed twice with PAB buffer, fixed with 1% paraformaldehyde in PBS, and analyzed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). PBMCs from healthy subjects were used as controls for LILR staining. Monocytes, gated on the basis of positive CD14 staining, expressed all LILRs (positive control), and CD3-positive T cells only weakly expressed LILRB1 and thus, were used as a negative control. A right shift in fluorescence intensity of cells stained with specific antibodies compared with cells stained with the isotype-matched, negative control antibodies was considered positive.

RNA extraction and RT-PCR

hMCs of high purity (99% toluidine blue-positive) were isolated from 8- to 10-week-old cultures by FACS sorting based on their granularity (high side-scatter) and high level of surface expression of c-kit. Total RNA was extracted from the purified *hMCs*, and cells were collected from 3-, 5-, and 7-week-old *PrMCs* using RNeasyTM total RNA extraction kit (Promega, Sydney, Australia). Total RNA from PBMCs was extracted and used as a positive control. RT was performed on 1 µg RNA using AdvantageTM RT-for-PCR kit in a total volume of 20 µl (BD Clontech, Palo Alto, CA, USA). Aliquots (1 µl) of cDNA were then used for PCR using primers designed to identify single LILRs and control GAPDH specifically (Table 1). The specificity of the LILR primer pairs was confirmed by the size and sequence of the cDNA amplified by each pair [42]. PCR Optimized Buffer KitTM (Invitrogen Corp., Carlsbad, CA, USA) was used to select the optimal MgCl₂ concentration and pH for each primer set. PCR reaction mixtures contained 1 µl cDNA, 50 pmol each primer, 0.25 mM dNTPs, 15 mM ammonium sulfate, and 1.25 units Taq polymerase (Perkin Elmer Biosystems, Foster City, CA, USA) in 60 mM Tris-HCl. mRNA encoding LILRB1, LILRB2, LILR3, LILRA3, LILRB4, LILRA2, LILRB5, and GAPDH was amplified in 2.5 mM Mg²⁺ at pH 8.5. mRNA encoding LILRA2 was amplified in 2.5 mM Mg²⁺ at pH 9.5. In all cases, a 5-min hot start at 94°C was performed to denature the double-stranded cDNA, followed by 35 cycles of PCR (each cycle: 94°C, 45 s; 60°C, 45 s; 72°C, 45 s), and the reactions were terminated with a 7-min extension at 72°C, except for LILRB4 and GAPDH, for which the annealing temperatures were 58°C and 62°C, respectively. Cycle number was predetermined so that the products formed fell within the linear portion of the amplification curve. A 100-bp ladder (New England BioLabs, Ipswich, MA, USA) was run in adjacent lanes.

Modulation of LILR expression on *hMCs* by cytokines

Seven- to 9-week-old *hMCs* were washed with media and treated in 24-well plates (Costar, Corning, NY, USA) with 25 ng/ml TNF-α, 25 ng/ml rhIL-1β, 20 ng/ml TGF-β1, 20 ng/ml IL-4, or 25 ng/ml IFN-γ for 24–72 h, with or without 100 ng/ml SCF. The surface expression of all LILRs was then determined by flow cytometry.

Modulation of LILR expression on *PrMCs* by cytokines

In an attempt to retain the expression of LILRs, 4-week-old cord blood cells were cultured at 1×10^6 /ml with one of the following combinations of cytokines: 100 ng/ml SCF, 50 ng/ml IL-6, and 10 ng/ml IL-10; 100 ng/ml SCF and 50 ng/ml IL-6; 100 ng/ml SCF and 10 ng/ml IL-10; 100 ng/ml SCF and 25 ng/ml TNF-α; 100 ng/ml SCF and 25 ng/ml rhIL-1β; 100 ng/ml SCF and 20 ng/ml TGF-β1; 100 ng/ml SCF and 25 ng/ml IFN-γ; 100 ng/ml SCF and 20 ng/ml IL-4; or 100 ng/ml SCF alone. A sample of cells was harvested weekly, and fresh media and cytokines were added; the expression of LILRs was determined weekly for up to 9 weeks.

Activation of cord blood-derived *hMCs* by IgE cross-linking

Activation of cord blood-derived mast cells was induced after passive IgE sensitization and anti-IgE stimulation as described [40]. In brief, *hMCs* were washed twice with medium alone and were resuspended at 2×10^6 cells/ml in RPMI containing SCF (100 ng/ml). Cells were then primed with semipurified human myeloma IgE (10 µg/ml, Chemicon, El Segundo, CA, USA) and primed with IL-4 (10 ng/ml) for 5 days. This was followed by a single wash of the cells with serum-free RPMI, adjustment of cells to 2×10^6 /ml in serum-free RPMI, and activation of cells with 1 µg/ml rabbit anti-human IgE (ICN Biomedicals, Aurora, OH, USA) for 30 min at 37°C in 5% CO₂. Cell supernatants were harvested and stored at –70°C. Cell pellets were resuspended in serum-free medium and lysed by three cycles of rapid freezing and thawing. Histamine in the supernatant and cell pellets was measured by ELISA (ICN Biomedicals). The percentage of histamine released was quantified by the equation: histamine in supernatant/(histamine in supernatant+histamine in pellet) × 100.

Immunofluorescence staining for intracellular protein expression

Eight- to 10-week-old-cultured mast cells were washed with cold PAB buffer and prefixed with 4% paraformaldehyde in PBS at 5×10^6 cells/ml for 10 min at room temperature. Cells were washed with 10 vol cold PAB buffer and resuspended at 2×10^6 cells/ml in 0.5% saponin in PBS containing 1% BSA (permeabilization medium) for 45 min at room temperature with constant shaking. Aliquots (50 µl) of the cell suspension in permeabilization medium were then incubated with unconjugated mAb to LILRs (5 µg/ml) or human trypsin (5 µg/ml), with PE-conjugated anti-c-kit (10 µg/ml) or control mouse IgG1 (10 µg/ml) for 30 min at room temperature. After a single wash with the permeabilization medium, cells stained with unconjugated antibodies were incubated with FITC-conjugated F(ab')₂ goat anti-mouse IgG [F(ab')₂-spe-

TABLE 1. Primers Designed for Specific Amplification of cDNAs for LILRs

Primers	Forward	Reverse	Product (bp)
LILRB1	5' ggC AgC TgA TgA CCC ATg g 3'	5' gAg gTg ggg CCT gTT gTC g 3'	222
LILRB2	5' ggg gGA ACg TgA CCT TCg 3'	5' ggg gGC Tgg AAC CCA Tgg Ag 3'	506
LILRB3	5' gAA CAC CAg CTC CCC Cgg AC 3'	5' gGA CAg gGC Cct gCA ggg 3'	226
LILRB4	5' CCC ATC CAT gAC AgA ggA CT 3'	5' AgA Agg AAA gTg TCC ATT ggg CTC C 3'	210
LILRB5	5' ATC CAC ggT gTA TgA CAg 3'	5' gTC CTg ggg AgC TTC TgT T 3'	237
LILRA1	5' CAC AAA ACA AgA CTg CCT CA 3'	5' gAA AAT gTC TCT gCA ATC CAA CAC 3'	313
LILRA2	5' AgC ACC AAg CTC AgC AgA 3'	5' ggg TgT Tgg Cct Agg gAT 3'	166
LILRA3	5' CgC TgC gTc TAA AAT CAA AgC g 3'	5' CAC CCA gCT CCT CTT gAC A 3'	287
GAPDH	5' CCA CCC ATg gCA AAT TCC ATg GCA 3'	5' TCT AgA Cgg CAg gTC Agg TCC ACC 3'	500

cific], preabsorbed with normal goat serum, washed with PAB buffer, postfixed in 1% paraformaldehyde in PBS, and analyzed by flow cytometry.

Initial immunofluorescent staining using nonconjugated anti-LILRB5 antibody and fluorochrome-conjugated secondary antibody suggested LILRB5 localization within mature mast cell granules. To confirm this, anti-LILRB5 and antitrypsin (AA1) antibodies (both IgG₁ isotype) were freshly conjugated with Alexa 488 and Alexa 568, respectively, using Zenon IgG₁ labeling kits (Molecular Probes), and double immunofluorescent staining was performed. In brief, cytopsin slides were air-dried and fixed with 4% paraformaldehyde in PBS for 10 min. Slides were then washed twice in PBS and permeabilized and blocked simultaneously with 0.5% saponin in PBS containing 1% BSA and 20% goat serum for 20 min at room temperature. After flicking off the solution, cells were first incubated with 50 μ l Alexa 488-conjugated anti-LILRB5 antibody (5 μ g/ml) for 40 min at room temperature, washed twice with permeabilization/blocking buffer, and then incubated with Alexa 568-conjugated antitrypsin antibody (1.25 μ g/ml) for 1 h at room temperature. Slides washed four times with TBS were counterstained with 100 nmol 4'-diamidino-2-phenylindole (DAPI; Molecular Probes), washed three times with PBS, mounted with Vector Shield antifade media, and visualized by fluorescence microscopy (Olympus BX51). Alexa 488- and Alexa 568-conjugated, irrelevant IgG₁ antibodies were used as negative controls.

Western blot analysis of LILRB5 in culture supernatants

Supernatants (250 μ l) from *hMC* treated with IgE or medium alone, followed by anti-IgE or medium alone, were concentrated tenfold using 10 kDa cutoff 1.5 ml centricon concentrators (Millipore, Australia). Equal volumes of 2 $\times$ SDS-PAGE loading dye with 20 mM DTT were added, and the concentrates were boiled for 5 min and loaded onto 10% polyacrylamide-reducing gels. Cell lysates from 10⁶ *hMCs* or fresh PBMCs were prepared in Western lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris HCl, pH 7.5, 20 mM iodoacetamide, 5 mM EDTA, and a cocktail of protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). Lysates (25 μ g) in SDS-PAGE loading dye with 20 mM DTT were separated. Proteins were transferred to PolyScreenTM polyvinylidene fluoride membranes (Perkin Elmer Life Sciences, Boston, MA, USA), which were rinsed briefly with TBS and blocked at 4°C overnight in 5% powdered skim milk in TBS. Membranes were then incubated with 5 μ g/ml anti-LILRB5 antibody in TBS/2% BSA for 2 h at room temperature, washed extensively in TBS containing 0.1% Triton X-100 (TBST; 3 $\times$ 5 min), and incubated with a 1:10,000 dilution of HRP-conjugated goat anti-mouse IgG in TBST/5% skim milk (BioRad, Australia) for 1 h at room temperature. Membranes were washed (3 $\times$ 5 min) in TBST and imaged by chemiluminescence (Western LightningTM, Perkin Elmer Life Sciences) and an automated LAS 3000 image analyzer that acquired digital images (Fuji, Japan). A prestained molecular weight protein ladder (BioRad) was run in adjacent lanes.

RESULTS

Phenotypic characterization of *hMCs* and *hPrMCs*

To validate the identity of the culture-derived mast cells, we performed flow cytometric analysis of cell surface markers and immunohistochemical staining for granule-associated serine proteases [40]. Immunohistochemical staining of cytopsin from 8- to 10-week-old *hMCs* showed abundant expression of tryptase (Fig. 1A, a) and chymase (Fig. 1A, b). They also displayed dense, granular cytoplasm and intense metachromatic staining with acidic toluidine blue (Fig. 1A, inset). Cytopsin from 2- to 4-week-old *hPrMCs* showed minimal immunostaining to tryptase or chymase and were less granular with poor metachromatic staining (data not shown).

hMCs and *hPrMCs* from the same culture at a given time were gated on the bases of their granularity (side-scatter) and

size (forward-scatter), as described previously [40]. *hMCs* were highly granular (high side-scatter) and larger (high forward-scatter; Fig. 1B, R1) compared with *hPrMCs* (Fig. 1B, R2). Mature mast cells showed uniformly high surface expression of c-kit and CD13, were uniformly positive for Fc ϵ R1, and were negative for IL-3R α (Fig. 1C, upper row). By contrast, *hPrMCs* were c-kit^{low}, did not express Fc ϵ R1, were positive for IL-3R α , and showed heterogeneous expression of CD13 (Fig. 1C, lower row). Unlike the pluripotent stem cells of the bone marrow, *hPrMCs* were CD34-negative but were strongly positive for CD33, a marker of more committed myeloid progenitors [43] (Fig. 1D, lower row). *hPrMCs* (lower row) and *hMCs* (upper row) were uniformly negative for CD16 (Fc γ RIII) and positive for CD32 (Fc γ RII), but only *hPrMCs* were positive for CD64 (Fc γ RI; Fig. 1D). As described previously [40], *hPrMCs* and *hMCs* express the pan leukocyte marker CD45 and were negative for markers for NK cells (CD56) and T cells (CD3; data not shown). A subset of *PrMCs* expressed CD14, which was expressed at a low level on *hMC*. Thus, *PrMC* and *hMC* had the cell-surface characteristics, protease phenotype, and staining pattern described previously for these cord blood-derived cells. Specifically, the low side-scatter population of cells exhibited low-level expression of c-kit with high-level expression of CD13 and IL-3R α , as described previously for human progenitor mast cells [40], whereas the high side-scatter population of cells exhibited high-level expression of c-kit and CD13 with uniformly positive staining for tryptase and chymase and metachromatic mast cell granules, typical of mature human mast cells [40]. Using this established culture technique, we characterized expression of LILRs during differentiation of human mast cells in vitro.

Surface expression of LILRs

The surface expression of LILRs on 3- to 10-week-old-cultured mast cells was studied using *hMCs* and *hPrMCs* from the same culture at early time-points in which the two populations could be gated based on their granularity and size (Fig. 1B). *hPrMCs* showed variable and biphasic surface expression of several inhibitory LILRs (B1–4) and uniformly high expression of the activating LILRA2 but did not express LILRA1 or LILRB5 (Fig. 2, middle row). By contrast, *hMCs* were negative for surface expression of all LILRs, with the exception of marginal expression of LILRB2 and -B4 in some cultures (Fig. 2, top row). Neither of the activating LILRs, A1 and A2, was expressed on the surface of *hMCs* (Fig. 2, top row).

LILR mRNA transcript in *hMCs*

RNA extracted from cord blood cultures at Weeks 3, 5, and 7, FACS-sorted *hMCs*, and PBMCs were used for RT-PCR using specific primers to LILRB1–B5, LILRA1, LILRA2, and LILRA3. Transcripts for LILRB1, -B2, -B3, -A3, and -A2 (Fig. 3) were readily detected in *hMCs* harvested at Week 3 (Lanes 1), Week 5 (Lanes 2), Week 7 (Lanes 3), and in FACS-sorted *hMCs* (Lanes 4). Transcripts for LILRB4 were amplified less readily in *hMC* and PBMCs but were detectable at all stages of mast cell development. LILRB5 mRNA was only found in 7-week mast cell cultures (Lanes 3) and FACS-sorted (Lanes 4) *hMCs* (Fig. 3). Except for early mast cell culture (Week 3,

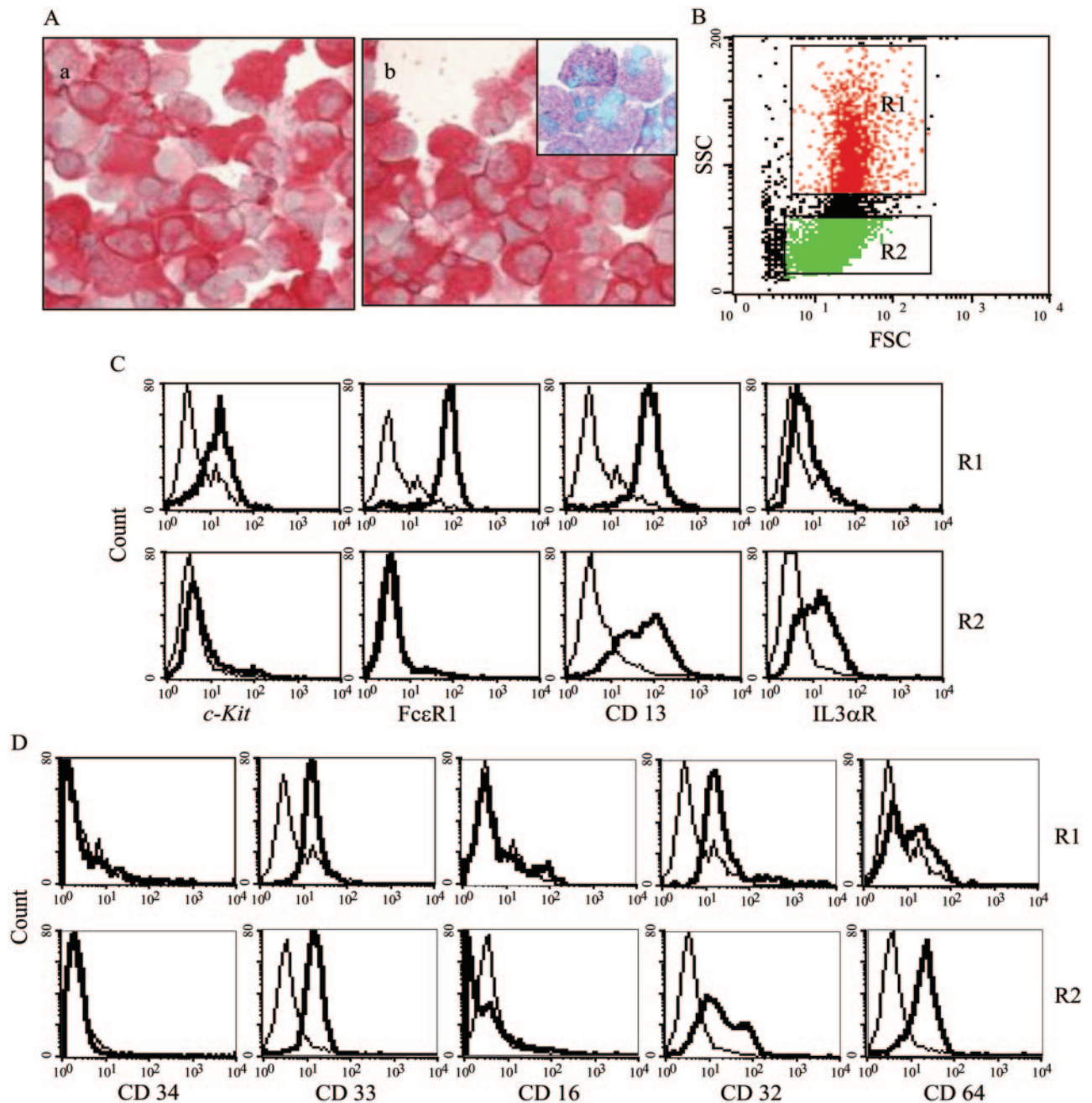


Fig. 1. Characterization of cord blood-derived *hMCs* and *PrMCs*. (A) *hMCs* were histochemically stained with antibodies to mast cell tryptase (a) and chymase (b) showing >99% tryptase- and chymase-positive cells. Metachromatic staining of these cells with toluidine blue shows a high degree of granularity in all cells (inset). Mast cells from six independent cultures were stained with a number of cell surface markers starting from the 4th week until the 10th week of culture and analyzed by flow cytometry. (B–C) Representative data from a 5-week culture that contained 65% *hMCs* (R1) and 35% *PrMCs* (R2) showing the scatter profile and the differential expression of a number of cell surface receptors by *hMCs* (C and D, upper rows) and *PrMCs* (C and D, lower rows). The nonbold histogram in each plot shows staining of cells with isotype-matched, negative control antibody. SSC, Side-scatter; FSC, forward-scatter.

Lanes 1), we did not detect LILRA1 transcripts in any other samples (Fig. 3). All LILR transcripts were present in PBMCs, used as a positive control (Lanes 5).

Modulation of LILR expression by cytokines

Although we demonstrated several LILRs on the surface of *PrMCs* and despite the presence of mRNA for some activating and inhibitory LILRs in *hMCs* (Fig. 3), no significant surface expression of any LILR was found on these cells. We previ-

ously demonstrated LILRA2 protein in tryptase-positive cells in rheumatoid synovium by immunohistochemistry [41], suggesting that LILR expression may be retained or re-expressed by mast cells in an appropriate cytokine microenvironment. We therefore examined surface LILR expression on *hMCs* cultured in cytokines that are known to be abundantly present in rheumatoid synovium or those involved in allergic inflammation. However, short-term treatment (24–72 h) of *hMCs* (7- to 9-weeks old) with various doses of TNF-α, IL-1β, IFN-γ,

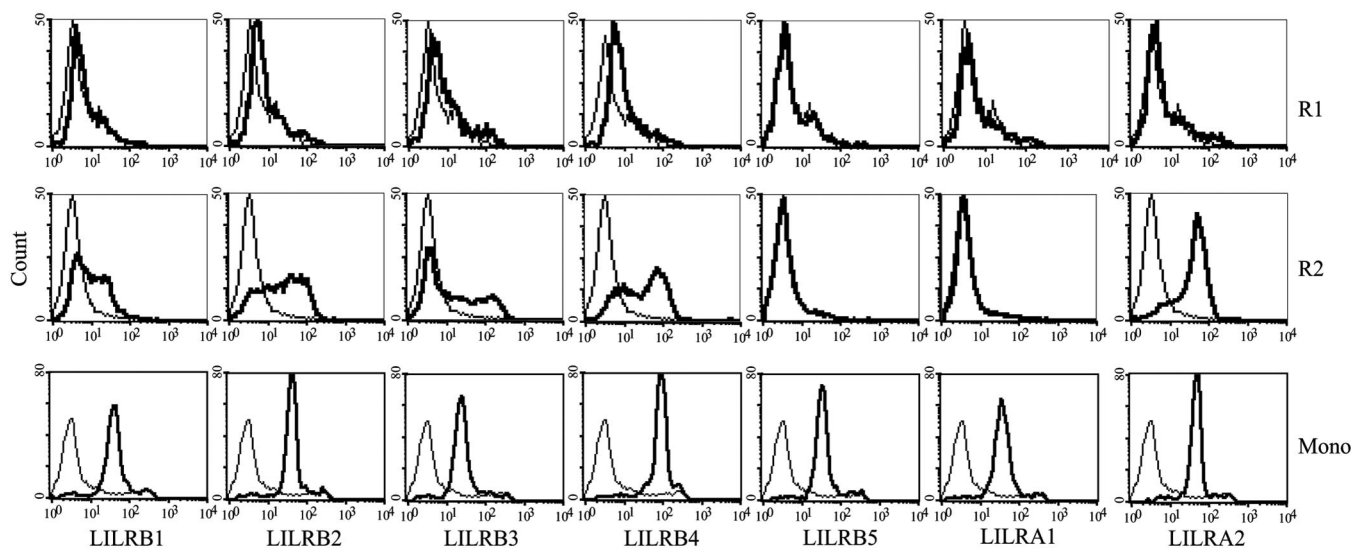


Fig. 2. The expression of inhibitory and activating LILRs on the surface of *hMCs* and *PrMCs*. The surface expression of the inhibitory LILRB1, -B2, -B3, -B4, and -B5 and the activating LILRA1 and -A2 was studied on 5-week-old-cultured mast cells by flow cytometry. *hMCs* (top row, R1) and *PrMCs* (middle row, R2) were gated on the basis of their size and granularity. There was no surface expression of LILRs on *hMCs*, but there was variable expressions of a number of LILRs on *PrMCs* (middle row). In contrast to mast cells, monocytes (Mono) from healthy subjects expressed all LILRs on their surface (bottom row). Similar data were obtained in more than 10 independent experiments. The nonbold histogram in each plot shows staining of cells with isotype-matched, negative control antibody.

IL-4, IL-5, or TGF- β 1 did not promote de novo expression of LILRs on the surface of *hMCs* (data not shown). Similarly, withdrawal of IL-6 or IL-10 from the culture media did not elicit re-expression of LILRs on *hMCs* (data not shown). Furthermore, when 2- to 4-week-old *PrMCs* were cultured with one of the above cytokines in addition to SCF, IL-6, and IL-10, they consistently failed to retain significant expression of LILRs during maturation (data not shown).

Intracellular expression of LILRs

To examine the possible presence of intracellular LILRs in *hMCs*, 10-week-old, mature mast cells were prefixed with paraformaldehyde and permeabilized with 0.5% saponin prior to staining. Flow cytometric analysis of permeabilized *hMCs* showed high intracellular expression of LILRB5 (**Fig. 4**). None of the other inhibitory or activating LILRs was detected (**Fig. 4A**). Intracellular LILB5 expression was consistent in three additional independent experiments (**Fig. 4B**). The validity and reproducibility of the intracellular staining procedure were confirmed using a mAb (AA1, Dako) directed against intracellular mast cell tryptase (**Fig. 4**). Isotype-matched mouse IgG₁ primary mAb was used as an irrelevant negative control (**Fig. 4**). Intracellular localization of LILRB5 was confirmed by immunofluorescence staining of mature mast cells, which showed extensive LILRB5 expression in a granular pattern (**Fig. 5E**). Double-immunofluorescence staining confirmed colocalization of LILB5 with tryptase within mast cell granules (**Fig. 5, A–C**). Immunofluorescence staining with isotype-matched control antibodies was negative (**Fig. 5F**).

Mobilization of LILRB5 from mast cell granules

Upon sensitization with IgE and activation with anti-IgE, *hMCs* released substantial quantities of the secretory granule mediator histamine (40–50% of total histamine content; **Fig. 6A**). Flow cytometric analysis of *hMCs* after cross-linking of cell-surface IgE showed mobilization of LILRB5 to the cell surface (**Fig. 6B, b**), which was not observed in cells treated with IgE alone (**Fig. 6B, a**). Although there was a substantial amount of intracellular LILRB5 (**Fig. 6B, c and d**), the mobilization of LILRB5 to the surface of *hMC* after IgE-dependent activation was observed in only one of three experiments. In contrast, Western blotting using anti-

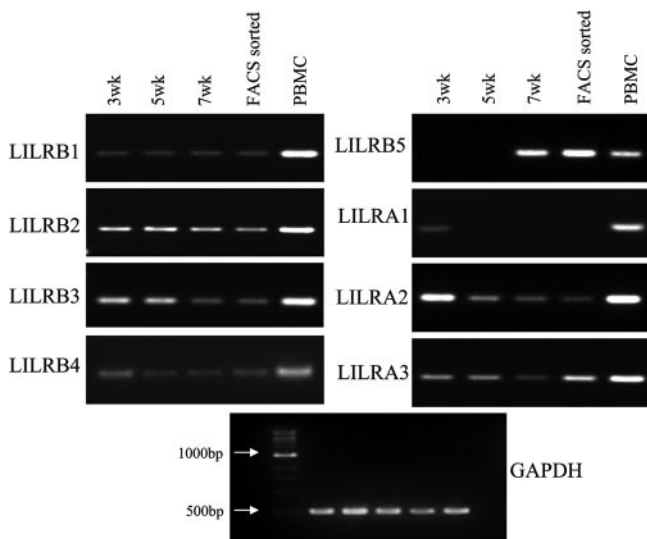


Fig. 3. RT-PCR for LILRs in mast cells determined by agarose gel electrophoresis stained with ethidium bromide. RNA was extracted from 3-week (Lanes 1), 5-week (Lanes 2), and 7-week (Lanes 3) mast cell cultures and 8-week-old mast cells that were sorted by FACS to 99% purity (Lanes 4). RNA was extracted from PBMCs as a positive control (Lanes 5). Transcripts for LILRs and GAPDH were detected by RT-PCR using specific primers.

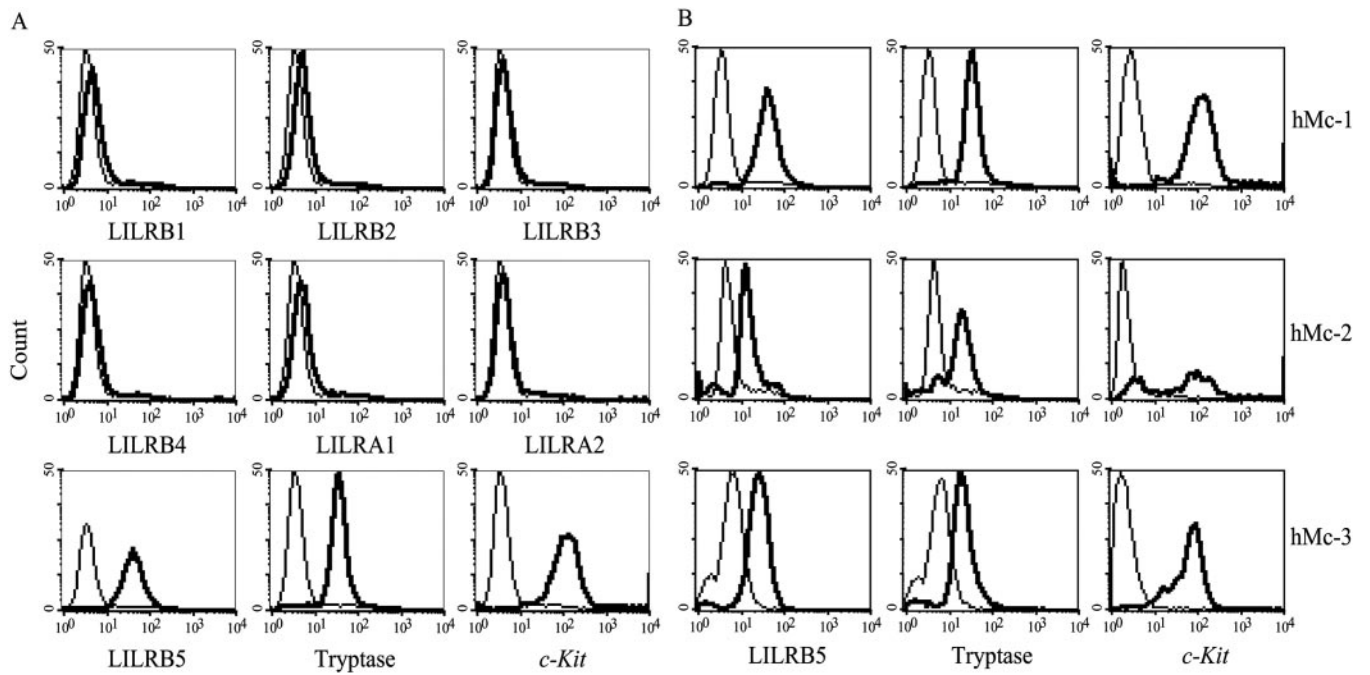


Fig. 4. Intracellular localization of LILRs on *hMCs*. (A) Mature mast cells (>95% tryptase, chymase, toluidine blue, and *c-kit*-positive) were permeabilized with saponin and stained with mAb to LILRs, showing only LILRB5 was positive in these cells. Antibodies against tryptase and *c-kit* were used as positive controls, and isotype-matched, control antibody was used as a negative control (nonbold histograms). (B) Expression of LILRB5, tryptase, and *c-kit* in permeabilized *hMCs* from another three independent donors.

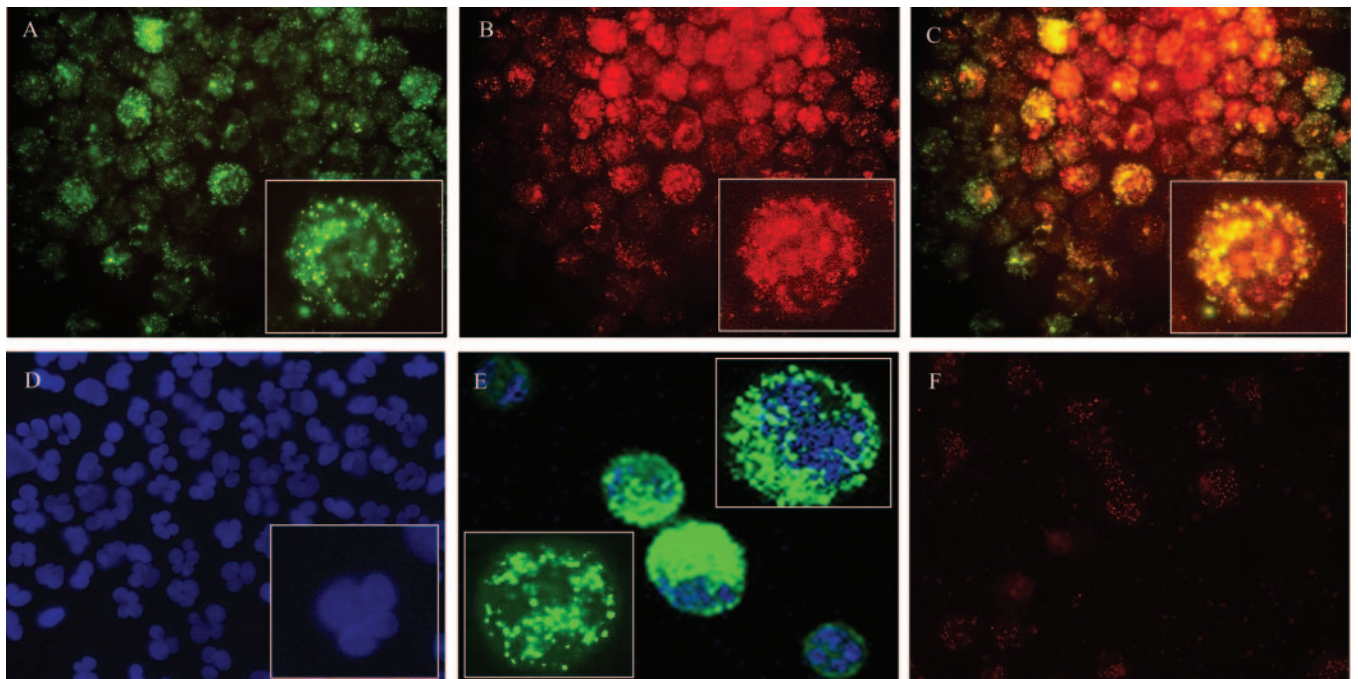


Fig. 5. Colocalization of LILRB5 and tryptase in *hMC* granules. Double-immunofluorescence staining was performed using Alexa 488-conjugated anti-LILRB5 mAb (A) and Alexa 568-conjugated antitryptase mAb (B) on cytopins prepared from *hMCs*, which were prefixed with paraformaldehyde and permeabilized with 0.5% saponin. (C) An overlay of A and B showing colocalization of LILRB5 and tryptase in mast cell granules; (D) nuclear counter-staining with DAPI. (E) 400 $\times$ original magnification of cells stained with anti-LILRB5 antibody followed by Alexa 488-conjugated goat anti-mouse secondary antibody and nuclear counter-staining with DAPI. The insets show a single cell at 1000 $\times$ original magnification, with and without DAPI counterstaining, demonstrating an exclusively cytoplasmic localization of LILRB5. (F) Staining of cells with isotype-matched IgG₁ control followed by Alexa 568-conjugated goat anti-mouse secondary antibody. Results are representative of three independent experiments.

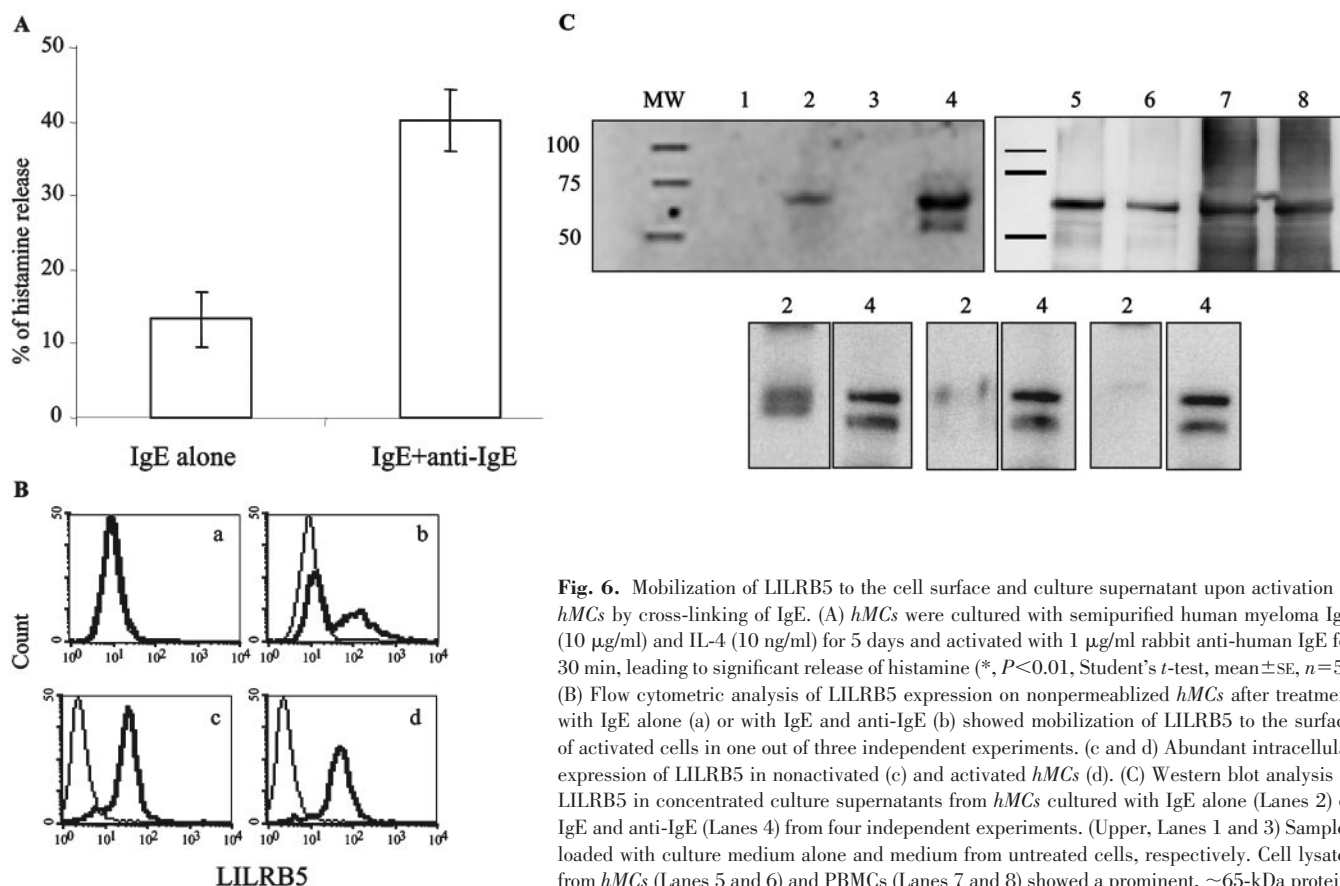


Fig. 6. Mobilization of LILRB5 to the cell surface and culture supernatant upon activation of *hMCs* by cross-linking of IgE. (A) *hMCs* were cultured with semipurified human myeloma IgE (10 μ g/ml) and IL-4 (10 ng/ml) for 5 days and activated with 1 μ g/ml rabbit anti-human IgE for 30 min, leading to significant release of histamine (*, $P < 0.01$, Student's *t*-test, mean $\pm$ SE, $n = 5$). (B) Flow cytometric analysis of LILRB5 expression on nonpermeabilized *hMCs* after treatment with IgE alone (a) or with IgE and anti-IgE (b) showed mobilization of LILRB5 to the surface of activated cells in one out of three independent experiments. (c and d) Abundant intracellular expression of LILRB5 in nonactivated (c) and activated *hMCs* (d). (C) Western blot analysis of LILRB5 in concentrated culture supernatants from *hMCs* cultured with IgE alone (Lanes 2) or IgE and anti-IgE (Lanes 4) from four independent experiments. (Upper, Lanes 1 and 3) Samples loaded with culture medium alone and medium from untreated cells, respectively. Cell lysates from *hMCs* (Lanes 5 and 6) and PBMCs (Lanes 7 and 8) showed a prominent, ~ 65 -kDa protein.

LILRB5 antibody reproducibly (four of four experiments) showed two immunoreactive bands at ~ 55 kDa and ~ 65 kDa in supernatants from cells treated with IgE and anti-IgE (Fig. 6C, Lanes 4) and a weaker, ~ 65 -kDa band in supernatants from cells treated with IgE alone (Fig. 6C, Lanes 2). No immunoreactive bands were present in culture medium alone (Fig. 6C, Lane 1) or medium from untreated cells (Fig. 6C, Lane 3). Western blotting of cell lysates from two mature mast cell cultures (Fig. 6C, Lanes 5 and 6) and two fresh PBMC isolates (Fig. 6C, Lanes 7 and 8) showed a prominent, ~ 65 -kDa product. No immunoreactive proteins were detected with control IgG₁ (data not shown).

DISCUSSION

The potential importance of the LILRs in regulating immune responses is illustrated by in vitro studies of the human receptors and in vivo studies of mice, in which expression of gp49B (mouse LILRB4) and paired Ig-like receptor B (mouse LILRB3) have been disrupted [44]. Mouse mast cells express paired Ig receptors and gp49 [44], and we have previously identified LILRA2 expression on human mast cells in rheumatoid synovium [41]. Mast cells are critical effector cells in allergic disease and innate immunity and link innate and adaptive immune responses [7, 10–12]. We therefore evaluated LILR expression on human mast cells with a view to examining their function. To do this, we used an established

culture system, in which mature, functional human mast cells are derived from culture of cord blood mononuclear cells.

As described previously, culture of cord blood mononuclear cells in SCF, IL-6, and IL-10 generated two populations of mast cells. The poorly granulated population of smaller cells (gate R2 in Fig. 1) differentiated to *hMC* over a period of 7 weeks [40]. Prior to maturation, these cells were *c-kit*^{lo}, Fc ϵ RI^{neg}, CD13^{pos}, and CD123 (IL3 α R)^{pos} (Fig. 1C). They also expressed CD33, two Fc γ Rs, CD32 and CD64 (Fig. 1D), and LILRB1, -B2, -B3, -B4, and -A2. The lack of LILRA1 expression distinguishes these cells from cells of the monocyte lineage. Like cells of the granulocyte lineage [17, 28], they express LILRB1, -B2, -B3, and -A2, but unlike granulocytes, they also express LILRB4. Thus, the *PrMC* population has a unique pattern of LILR expression that distinguishes these cells from granulocytes [17, 28], monocytes (Fig. 2, bottom row), T cells, B cells, and NK cells [26]. However, upon differentiation into mature, functional mast cells (gate R1 in Fig. 1), which are *c-kit*^{hi}, Fc ϵ RI^{pos}, CD13^{pos}, and CD123^{neg} [41] (Fig. 1C), they lost surface expression of CD64 (Fig. 1D) and all LILRs (Fig. 2, top row), suggesting that they are developmentally regulated. In this respect, this is similar to the findings of regulated expression of a related molecule, LAIR-1, expression of which is lost during differentiation of T cells [45] and neutrophil maturation [39]. We also observed decreased expression of LILRA2 and -A5 mRNA and protein in in vitro-differentiated, primary macrophages (unpublished data). Loss in expression of other receptors such as the chemokine

receptors during maturation from *hPrMCs* to *hMCs* has also been described [40]. This gives rise to the hypothesis that LILR expression may regulate mast cell development. However, in preliminary studies, culture of cord blood mononuclear cells on plates coated with antibodies to LILRs did not affect mast cell maturation in vitro (data not shown). Further examination of this hypothesis requires the identification of natural ligands for LILRs, which are at present limited to recognition of MHC class I by LILRB1, -B2, and -A1.

Highly restricted expression of LILRB5 mRNA has been described in NK cells [19] and in microarray of tissue from patients with lepromatous leprosy [46]; however, LILRB5 protein has never been reported. Here, we demonstrated surface expression of this molecule on monocytes and showed intracellular localization of LILRB5 protein within hMC granules (Fig. 6B, c). Moreover, this is the first description of constitutive, intracellular expression of a member of the LILR family in mast cell granules. The significance of granule-associated LILRB5 expression is speculative. Although an inhibitory receptor, LILRB4 can be internalized efficiently upon cross-linking and is believed to deliver its ligand to an intracellular compartment, where it is processed and presented to T cells [31].

LILRB5 in mast cells was mobilized to the surface and released in response to Fc ϵ RI cross-linking. However, surface expression of LILRB5 following Fc ϵ RI cross-linking was inconsistent. Interestingly, we showed surface expression of LILRB5 on blood monocytes in only one of five apparently healthy donors [28], despite finding the protein in PBMC lysates of all subjects (unpublished data). The reasons for selective expression of LILRB5 on the surface of monocytes and mast cells from a subset of donors require further investigation. In contrast, the release of LILRB5 into an extracellular milieu after activation of these cells was highly reproducible. Several questions regarding this secreted protein remain unanswered. First, we do not know the nature of the LILRB5 molecules present in or released from the mast cell granules. Although the predicted mass of unprocessed LILB5 is 64 kDa, similar to the size found in immunoblots (Fig. 6C), this protein has several potential glycosylation sites that may affect its size and like other members of the LILR family, is likely to be highly glycosylated. We attempted to characterize the secreted protein by mass spectrometric analysis of trypsin-derived peptides of anti-LILRB5-immunoprecipitated supernatants without success, primarily as a result of poor yield. We speculate that the protein is not post-translationally modified or may represent an alternately spliced form lacking the transmembrane domain, similar to human LILRA3 [23], human LILRA5 [25], and mouse LILRB4 [47]. Alternatively, the full-length protein might be proteolytically cleaved to yield soluble products after mobilization to the cell surface. It is also possible that LILRB5 is released in association with fragments of cell membranes with the potential to be taken up and expressed by neighboring cells through the process of trogocytosis, although this is thought to be a cell contact-dependent process. The observation that the mass of secreted LILRB5 is the same as that in lysates of PBMCs favors the latter hypothesis. One would speculate that it is a decoy receptor. Pursuit of this hypothesis requires the identification of the natural LILRB5 ligand.

In summary, we provide data for the developmental regulation of LILRs on human mast cells. We speculate that they may play a role in mast cell maturation and/or may serve as markers for developing mast cells. We provide the first description for the intracellular granule-associated expression of a LILR. The functional consequences of LILRB5 release upon mast cell activation are unknown. We speculate that LILRB5 released in response to mast cell activation may act as a decoy receptor to down-regulate the inhibitory function of LILRB5, expressed on neighboring cells, leading to amplification of mast cell responses.

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REFERENCES

1. Nakano, T., Sonoda, T., Hayashi, C., Yamatodani, A., Kanayama, Y., Yamamura, T., Asai, H., Yonezawa, T., Kitamura, Y., Galli, S. J. (1985) Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal and intravenous transfer into genetically mast cell-deficient *W/W^v* mice. Evidence that cultured mast cells can give rise to both connective tissue type and mucosal mast cells. *J. Exp. Med.* **162**, 1025–1043.
2. Metcalfe, D. D., Baram, D., Mekori, Y. A. (1997) Mast cells. *Physiol. Rev.* **77**, 1033–1079.
3. Williams, C. M., Galli, S. J. (2000) The diverse potential effector and immunoregulatory roles of mast cells in allergic diseases. *J. Allergy Clin. Immunol.* **105**, 847–859.
4. Galli, S. J., Zsebo, K. M., Geissler, E. N. (1994) The kit ligand, stem cell factor. *Adv. Immunol.* **55**, 1–16.
5. Madden, K. B., Urban Jr., J. F., Ziltener, H. J., Schrader, J. W., Finkelman, F. D., Katona, I. M. (1991) Antibodies to IL-3 and IL-4 suppress Helminth-induced intestinal mastocytosis. *J. Immunol.* **147**, 1387–1391.
6. Friend, D. S., Ghildyal, N., Austen, K. F., Gurish, M. F., Matsumoto, R., Stevens, R. L. (1996) Mast cells that reside at different locations in the jejunum of mice infected with *Trichinella spiralis* exhibit sequential changes in their granule ultrastructure and chymase phenotype. *J. Cell Biol.* **135**, 279–290.
7. Galli, S. J. (2000) Mast cells and basophils. *Curr. Opin. Hematol.* **7**, 32–39.
8. Malone, D. G., Wilder, R. L., Saavedra-Delgado, A. M., Metcalfe, D. D. (1987) Mast cell numbers in rheumatoid synovial tissues. Correlations with quantitative measures of lymphocytic infiltration and modulation by anti-inflammatory therapy. *Arthritis Rheum.* **30**, 130–137.
9. Gotis-Graham, I., McNeil, H. P. (1997) Mast cell responses in rheumatoid synovium. Association of the MCTC subset with matrix turnover and clinical progression. *Arthritis Rheum.* **40**, 479–489.
10. Abraham, S. N., Malaviya, R. (1997) Mast cells in infection and immunity. *Infect. Immun.* **65**, 3501–3508.
11. Echtenacher, B., Mannel, D. N., Hultner, L. (1996) Critical protective role of mast cells in a model of acute septic peritonitis. *Nature* **381**, 75–77.
12. Lee, D. M., Friend, D. S., Gurish, M. F., Benolst, C., Mathis, D., Brenner, M. B. (2002) Mast cells: a cellular link between autoantibodies and inflammatory arthritis. *Science* **297**, 1689–1692.
13. Woolley, D. E., Tetlow, L. C. (2000) Mast cell activation and its relation to proinflammatory cytokine production in the rheumatoid lesion. *Arthritis Res.* **2**, 65–74.
14. Tetlow, L. C., Woolley, D. E. (1995) Mast cells, cytokines and metalloproteinases at the rheumatoid lesion: dual immunolocalization studies. *Ann. Rheum. Dis.* **54**, 896–903.

15. Ravetch, J. V., Lanier, L. L. (2000) Immune inhibitory receptors. *Science* **290**, 84–89.
16. Daeron, M., Malbec, O., Espinosa, E., Pina, P., Pasmans, S., Fridman, W. H. (1995) The same tyrosine-based inhibition motif in the intracytoplasmic domain of Fc ϵ RIIB regulates negatively BCR, TCR and FcR-dependent cell activation. *Immunity* **3**, 635–646.
17. Sloane, D., Tedla, N., Awoniyi, M., Macglashan Jr., D. W., Borges, L., Austen, K. F., Arm, J. P. (2004) Leukocyte immunoglobulin-like receptors: novel innate receptors for human basophil activation and inhibition. *Blood* **104**, 2832–2839.
18. Banham, A. H., Colonna, M., Cella, M., Micklem, K. J., Pulford, K., Willis, A. C., Mason, D. Y. (1999) Identification of the CD85 antigen as ILT2, an inhibitory MHC class I receptor of the immunoglobulin superfamily. *J. Leukoc. Biol.* **65**, 841–845.
19. Borges, L., Cosman, D. (2000) LIRs/ILTs/MIRs, inhibitory and stimulatory Ig-superfamily receptors expressed in myeloid and lymphoid cells. *Cytokine Growth Factor Rev.* **11**, 209–217.
20. Samaridis, J., Colonna, M. (1997) Cloning of novel immunoglobulin superfamily receptors expressed on human myeloid and lymphoid cells: structural evidence for new stimulatory and inhibitory pathways. *Eur. J. Immunol.* **27**, 660–665.
21. Saverino, D., Fabbii, M., Ghiotto, F., Merlo, A., Bruno, S., Zarcone, D., Tenca, C., Tiso, M., Santoro, G., Anastasi, G., Cosman, D., Grossi, C. E., Ciccone, E. (2000) The CD85/LIR-1/ILT2 inhibitory receptor is expressed by all human T lymphocytes and down-regulates their function. *J. Immunol.* **165**, 3742–3755.
22. Nakajima, H., Samaridis, J., Angman, L., Colonna, M. (1999) Human myeloid cells express an activating ILT receptor (ILT1) that associates with Fc receptor γ -chain. *J. Immunol.* **162**, 5–8.
23. Arm, J. P., Nwankwo, C., Austen, K. F. (1997) Molecular identification of a novel family of human Ig superfamily members that possess immunoreceptor tyrosine-based inhibition motifs and homology to the mouse gp49B1 inhibitory receptor. *J. Immunol.* **159**, 2342–2349.
24. Chang, C. C., Ciubotariu, R., Manavalan, J. S., Yuan, J., Colovai, A. I., Piazza, F., Lederman, S., Colonna, M., Cortesini, R., Dalla-Favera, R., Suciu-Foca, N. (2002) Tolerization of dendritic cells by T(S) cells: the crucial role of inhibitory receptors ILT3 and ILT4. *Nat. Immunol.* **3**, 237–243.
25. Borges, L., Kubin, M., Kuhlman, T. (2003) LIR9, an Ig-superfamily, activating receptor is expressed as transmembrane and a secreted molecule. *Blood* **101**, 1484–1486.
26. Colonna, M., Nakajima, H., Cella, M. (2000) A family of inhibitory and activating Ig-like receptors that modulate function of lymphoid and myeloid cells. *Semin. Immunol.* **12**, 121–127.
27. Borges, L., Hsu, M. L., Fanger, N., Kubin, M., Cosman, D. (1997) A family of human lymphoid and myeloid Ig-like receptors, some of which bind to MHC class I molecule. *J. Immunol.* **159**, 5192–5196.
28. Tedla, N., Bandeira-Melo, C., Tassinari, P., Sloane, D. E., Samplaski, M., Cosman, D., Borges, L., Weller, P. F., Arm, J. P. (2003) The activation of human eosinophils by leukocyte immunoglobulin-like receptor-7 (ILT-1). *Proc. Natl. Acad. Sci. USA* **100**, 1174–1179.
29. Manavalan, J. S., Kim-Schulze, S., Scotto, L., Naiyer, A. J., Vlad, G., Colombo, P. C., Marboe, C., Mancini, D., Cortesini, R., Suciu-Foca, N. (2004) Alloantigen specific CD8+CD28–FOXP3+ T suppressor cells induce ILT3+ ILT4+ tolerogenic endothelial cells, inhibiting alloreactivity. *Int. Immunol.* **16**, 1055–1068.
30. Colonna, M., Samaridis, J., Cella, M., Angman, L., Allen, R. L., O'Callaghan, C. A., Dunbar, R., Ogg, G. S., Cerundolo, V., Rolink, A. (1998) Human myelomonocytic cells express an inhibitory receptor for classical and nonclassical MHC class I molecules. *J. Immunol.* **160**, 3096–3100.
31. Colonna, M., Navarro, F., Bellon, T., Llano, M., Garcia, P., Samaridis, J., Angman, L., Cella, M., Lopez-Botet, M. (1997) A common inhibitory receptor for major histocompatibility complex class I molecule on human lymphoid and myelomonocytic cells. *J. Exp. Med.* **186**, 1809–1818.
32. Kim-Schulze, S., Scotto, L., Vlad, G., Piazza, F., Lin, H., Liu, Z., Cortesini, R., Suciu-Foca, N. (2006) Recombinant Ig-like transcript 3-Fc modulates T cell responses via induction of Th anergy and differentiation of CD8+ T suppressor cells. *J. Immunol.* **176**, 2790–2798.
33. Nakajima, H., Asai, A., Okada, A., Ping, L., Hamajima, F., Sata, T., Isobe, K. (2003) Transcriptional regulation of ILT family receptors. *J. Immunol.* **171**, 6611–6620.
34. Cella, M., Nakajima, H., Facchetti, F., Hofmann, T., Colonna, M. (2000) ILT receptors at the interface between lymphoid and myeloid cells. *Curr. Top. Microbiol. Immunol.* **251**, 161–166.
35. van der Vuurst de Vries, A. R., Clevers, H., Logtenberg, T., Meyaard, L. (1999) Leukocyte-associated immunoglobulin-like receptor-1 (LAIR-1) is differentially expressed during human B cell differentiation and inhibits B cell receptor-mediated signaling. *Eur. J. Immunol.* **29**, 3160–3167.
36. Colonna, M., Nakajima, H., Navarro, F., Lopez-Botet, M. (1999) A novel family of Ig-like receptors for HLA class I molecules that modulate function of lymphoid and myeloid cells. *J. Leukoc. Biol.* **66**, 375–381.
37. Ju, X. S., Hacker, C., Scherer, B., Redecke, V., Berger, T., Schuler, G., Wagner, H., Lipford, G. B., Zenke, M. (2004) Immunoglobulin-like transcripts ILT2, ILT3 and ILT7 are expressed by human dendritic cells and down-regulated following activation. *Gene* **331**, 159–164.
38. Xu, M., Zhao, R., Zhao, Z. J. (2000) Identification and characterization of leukocyte-associated Ig-like receptor-1 as a major anchor protein of tyrosine phosphatase SHP-1 in hematopoietic cells. *J. Biol. Chem.* **275**, 17440–17446.
39. Verbrugge, A., de Ruiter, T., Geest, C., Coffey, P. J., Meyaard, L. (2006) Differential expression of leukocyte-associated Ig-like receptor-1 during neutrophil differentiation and activation. *J. Leukoc. Biol.* **79**, 828–836.
40. Ochi, H., Hirani, W. M., Yuan, Q., Friend, D. S., Austen, K. F., Boyce, J. A. (1999) T helper cell type 2 cytokine-mediated comitogenic responses and CCR3 expression during differentiation of human mast cells in vitro. *J. Exp. Med.* **190**, 267–280.
41. Tedla, N., Gibson, K. A., McNeil, H. P., Cosman, D., Borges, L., Arm, J. P. (2002) The co-expression of activating and inhibitory leukocyte immunoglobulin-like receptors in rheumatoid synovium. *Am. J. Pathol.* **160**, 425–431.
42. Liu, W. R., Kim, J., Nwankwo, C., Ashworth, L. K., Arm, J. P. (2000) Genomic organization of the human leukocyte immunoglobulin-like receptors within the leukocyte receptor complex on chromosome 19q13.4. *Immunogenetics* **51**, 659–669.
43. Griffin, J. D., Linch, D., Sabbath, K., Larcom, P., Schlossman, S. F. (1984) A monoclonal antibody reactive with normal and leukemic human myeloid progenitor cells. *Leuk. Res.* **8**, 521–534.
44. Katz, H. R. (2006) Inhibition of inflammatory responses by leukocyte Ig-like receptors. *Adv. Immunol.* **91**, 251–272.
45. Jansen, C. A., Crujisen, C. W., de Ruiter, T., Nanlohy, N., Willems, N., Janssens-Korpela, P. L., Meyaard, L. (2007) Regulated expression of the inhibitory receptor LAIR-1 on human peripheral T cells during T cell activation and differentiation. *Eur. J. Immunol.* **37**, 914–924.
46. Bleharski, J. R., Li, H., Meinken, C., Graeber, T., Ochoa, M. T., Yamamura, M., Burdick, A., Sarno, E. N., Wagner, M., Rollinghoff, M., Rea, T. H., Colonna, M., Stenger, S., Bloom, B. R., Eisenberg, D., Modlin, R. L. (2003) Use of genetic profiling in leprosy to discriminate clinical forms of the disease. *Science* **301**, 1527–1530.
47. Castells, M. C., Wu, X., Arm, J. P., Austen, K. F., Katz, H. R. (1994) Cloning of the gp49B gene of the immunoglobulin superfamily and demonstration that one of its two products is an early-expressed mast cell surface protein originally described as gp49. *J. Biol. Chem.* **269**, 8393–8401.