

Human mast cells produce IL-13 by high-affinity IgE receptor cross-linking: Enhanced IL-13 production by IL-4-primed human mast cells

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Background: Mast cells play a central role not only in the early phase of the allergic reaction, but also participate in the late phase of the allergic reaction through the allergen and IgE-dependent release of multifunctional cytokines.

Objective: Using the recently established culture system for human mast cells, we examined the expression of a variety of cytokines in cord blood-derived human cultured mast cells (HCMCs) in response to different stimuli.

Methods: HCMCs were grown from cord blood mononuclear cells in the presence of stem cell factor and IL-6 for 10 weeks. Cytokine mRNA expression in HCMCs by the different stimuli was examined by RT-PCR. Then taking 2 important cytokines, IL-13 and IL-4, that share several functional properties and play important roles in allergic diseases, we examined protein as well as mRNA expression of both cytokines in HCMCs.

Results: HCMCs did not express either IL-13 or IL-4 spontaneously. Stimulation with PMA + A23187 induced the expression of IL-4 protein, as well as IL-13 protein, in their cytoplasm, although IL-4 secreted in the supernatant was below detectable levels in contrast to a significant amount of IL-13. Stimulation of HCMCs by cross-linking of the high-affinity IgE receptor (FcεRI) induced the expression of IL-13 mRNA and protein, but not IL-4. Although we previously found that IL-4 upregulates FcεRI expression on HCMCs, when HCMCs were first cultured in the presence of IL-4 and then activated through FcεRI cross-linking, remarkable increase was found in IL-13 production. Furthermore, although IL-4 was still undetectable at protein level, IL-4 mRNA expression was induced in the IL-4-primed HCMCs stimulating FcεRI cross-linking. In addition, we examined the effects of these cytokines on the surface molecule expression in HCMCs. Although IL-4 remarkably upregulated lymphocyte function-associated antigen-1, intercellular adhesion molecule-1, and FcεRI expression and downregulated c-kit expression in HCMCs, IL-13 did not. **Conclusions:** Our observation that HCMCs produce IL-13 on cross-linking of FcεRI, which was enhanced by IL-4 priming, supports an important role of mast cells in amplification of allergic reaction and further suggests one of the mechanisms enhancing

mast cell function in the microenvironment. (*J Allergy Clin Immunol* 1998;102:491-502.)

Key words: IL-4, IL-13, FcεRI α chain, SCF, LFA-1, ICAM-1, HMC-1, KU812F, allergy, IgE

Recent studies indicate that in addition to the release of chemical mediators that initiate the early phase of allergic reactions, such as histamine and leukotrienes, mast cells are a source of a variety of cytokines. Murine peritoneal mast cells or bone marrow-derived cultured mast cells were reported to produce IL-1, IL-3, IL-4, IL-6, IL-13, and TNF-α.¹⁻⁶ In the human system IL-4, IL-5, IL-6, IL-8, TNF-α, and IL-13 expression by mast cells of the human airway and skin have been documented.⁷⁻¹² These mast cell-derived cytokines may contribute to the late phase of allergic reaction through the recruitment of inflammatory cells, such as lymphocytes, eosinophils, and basophils, into the site of allergic reaction. However, little is known about the cytokine profile of human mast cells because of difficulties in purification of human mast cells, small numbers of these cells, and the lack of appropriate cell lines that retain the functions of normal mast cells. Analysis of the cytokine production by human mast cell (HMC)-1 (an immature mast cell line)¹³ was performed by stimulation with phorbol myristate acetate (PMA) + A23187, but not by means of the high-affinity IgE receptors (FcεRI) because this cell line fails to express FcεRI.^{13,14} Thus the cytokine profile of human mast cells stimulated by means of FcεRI has not yet been completely elucidated.

The recently established culture system of human cultured mast cells (HCMCs) derived from cord blood mononuclear cells grown in suspension culture in the presence of stem cell factor (SCF) and IL-6 for 10 weeks¹⁵ serves as an adequate model to study the cytokine profile of human mast cells and their response to different stimuli. Over 99% of the cultured cells express the c-kit receptor as detected by flow cytometric analysis, and 99% are tryptase positive as detected by immunohistochemistry.^{15,16} These HCMCs contain $5.62 \pm 1.88 \mu\text{g}$ histamine per 10^6 cells. A few FcεRI could be detected on the surface of HCMCs by flow cytometric analysis by using mAbs to human FcεRI α-chain.

IL-13 and IL-4 are cytokines that play important roles in the allergic reaction. Although it was recently reported

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Abbreviations used

HCMC:	Human cultured mast cell
HMC:	Human mast cell
ICAM:	Intercellular adhesion molecule
LFA:	Lymphocyte function-associated antigen
PMA:	Phorbol myristate acetate
SCF:	Stem cell factor

that activated HMC-1 can express IL-13 on the basis of the studies on HMC-1 cells that were stimulated with PMA + A23187,⁶ there is yet little evidence of the expression of IL-13 in human mast cells activated by means of FcεRI. Furthermore, there is much controversy about the expression of IL-4 in mast cells.^{1,2,17} We therefore asked whether activated cultured mast cells could express IL-13 and IL-4 by means of FcεRI both at the mRNA and protein levels. We report here that HCMCs activated by means of FcεRI express IL-13, but not IL-4. Because we previously found that IL-4 upregulates FcεRI expression in HCMCs,¹⁸ HCMCs were first cultured in the presence of IL-4 and then activated by FcεRI cross-linking. As a result, a remarkable increase was found in IL-13 production in the IL-4-primed HCMCs. Furthermore, although IL-4 was still undetectable at the protein level, IL-4 mRNA expression was induced in these IL-4-primed HCMCs stimulated by FcεRI cross-linking. Previously, we have reported that IL-4 has a significant effect on the surface molecule expression in HCMCs, upregulating intercellular adhesion molecule (ICAM)-1, lymphocyte function-associated antigen (LFA)-1, and FcεRI and downregulating c-kit.^{18,19} Then we examined whether IL-13 had IL-4-like effects on HCMCs and found that IL-13 did not have such effects on HCMCs. This showed the differences between the effects of the 2 cytokines on HCMCs.

METHODS**Cells**

HCMCs were obtained as previously described,¹⁵ with some modification. Briefly, cord blood mononuclear cells were grown in flasks (Becton Dickinson & Company, NJ) in α-MEM (GIBCO BRL, Life Technologies Inc, Gaithersburg, Md) and supplemented with 20% FCS (Hyclone Sterile Systems, Inc., Logan, Utah) in the presence of 100 ng/mL SCF (AMGEN, Thousand Oaks, Calif) and 80 ng/mL IL-6 (Ajinomoto Co, Ltd, Tokyo, Japan). The medium was changed weekly. After 10 weeks of culture, more than 99% of the cultured cells were tryptase positive as detected by immunohistochemistry with the anti-human tryptase mAb (Chemicon International Inc, St Elsegundo, Calif) and were c-kit positive (anti-human c-kit mAb; Nichirei Co, Ltd, Tokyo, Japan) as determined by flow cytometric analysis.

The human basophilic leukemia cell line (KU812F)²⁰ was obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and grown in RPMI 1640 supplemented with 10% FCS. HMC-1 was a generous gift from Dr Joseph H. Butterfield (Mayo Clinic, Canada) and was grown in Iscove's modified Dulbecco's medium as previously described.¹³

TABLE I. Sequences of specific primers and probes for cytokines

IL-4	5' ATG GGT CTC ACC TCC CAA CTG CT 3' 5' CGA ACA CTT TGA ATA TTT CTC TCT CAT 3' probe: oligoprobe cocktail (R&D Systems Europe Ltd, UK)
IL-13	5' ACG GTC ATT GCT CTC ACT TGC C 3' 5' CTT CCC GCC TAC CCA AGA CAT T 3' probe: cDNA (a generous gift from Dr René de Waal Malefyt, DNAX)
IL-5	5' G CTT CTG CAT TTG AGT TTG CTA GCT 3' 5' TG GCC GTC AAT GTA TTT CTT TAT TAA G 3' probe: 5' CTG AGG ATT CCT GTT CCT G 3'
IL-6	5' GCG CCT TCG GTC CAG TTG 5' ATG TTA CTC TTG TTA CAT GT probe: 5' CTC CTT TCT CAG GGC TGA G 3'
IL-10	5' AAG CTG AGA ACC AAG ACC CAG ACA TCA AGG CG 3' 5' AGC TAT CCC AGA GCC CCA GAT CCG ATT TTG G 3' probe: 5' GGC CGT GGA GCA GGT GAA G 3'
IFN-γ	5' GCA TCG TTT TGG GTT CTC TTG GCT GTT ACT GC 3' 3' CTC CTT TTT CGC TTC CCT GTT TTA GCT GCT GG 3' probe: oligoprobe cocktail (R&D Systems Europe Ltd, UK)
IL-12p35	5' GAG ACA CAG AAG GAG ACA G 3' 5' TAC TAC TAA GGC ACA GGG C 3' probe: 5' GCT GAG GGC CGT CAG CAA CAT G 3'
IL-12p40	5' CAG CAG TTG GTC ATC TCT TGG 3' 5' CTT GAG CTT GTG AAC GGC ATC 3' probe: 5' GGA CCA GAG CAG TGA GGT CTT A 3'
β-Actin	5' GTG GGG CGC CCC AGG CAC CA 3' 5' CTC CTT AAT GTC ACG CAC GAT TTC 3' probe: oligoprobe cocktail (R&D Systems Europe Ltd, UK)

mAbs

The mAbs specific for human IL-4 (25D2) and IL-13 (JES-5A2) were from DNAX (Palo Alto, Calif). For flow cytometric analysis, the mAbs specific for ICAM-1 (RR1/1),²¹ α-chain of LFA-1 (TS1/22),²¹ β-chain of LFA-1 (TS1/18),²¹ FcεRI α-chain (CRA-1),^{18,22,23} and c-kit (Nichirei Co, Ltd, Tokyo, Japan) were used. Mouse IgG1 or IgG2b (Coulter Immunology, Hialeah, Fla) was used as isotype-matched control antibodies.

Immunohistochemical staining for tryptase

HCMCs cultured in the presence of SCF and IL-6 for 10 weeks were cytocentrifuged onto each glass slide and fixed with fixing solution (Muto Pure Chemicals Ltd, Tokyo, Japan), consisting of formaldehyde (8.75%) and acetone (45%), for 1 minute. They were then washed 3 times with Tris-buffered saline. The samples were then blocked with rabbit serum for 10 minutes followed by incubation with mouse anti-human tryptase monoclonal antibody (Chemicon International, Inc, Calif) (10 μg/mL) or control mouse IgG1 at room temperature for 1 hour. After washing 3 times with Tris-buffered saline, the samples were reacted with rabbit anti-mouse IgG (MBL, Nagoya, Japan) (1:50) at room temperature for 1 hour and then washed 3 times. The samples were further reacted with soluble complexes of alkaline phosphatase and mouse monoclonal anti-alkaline phosphatase (DAKO Co Ltd, Glodstrup, Denmark) (1:50) at room temperature for 1 hour and then washed 3 times.

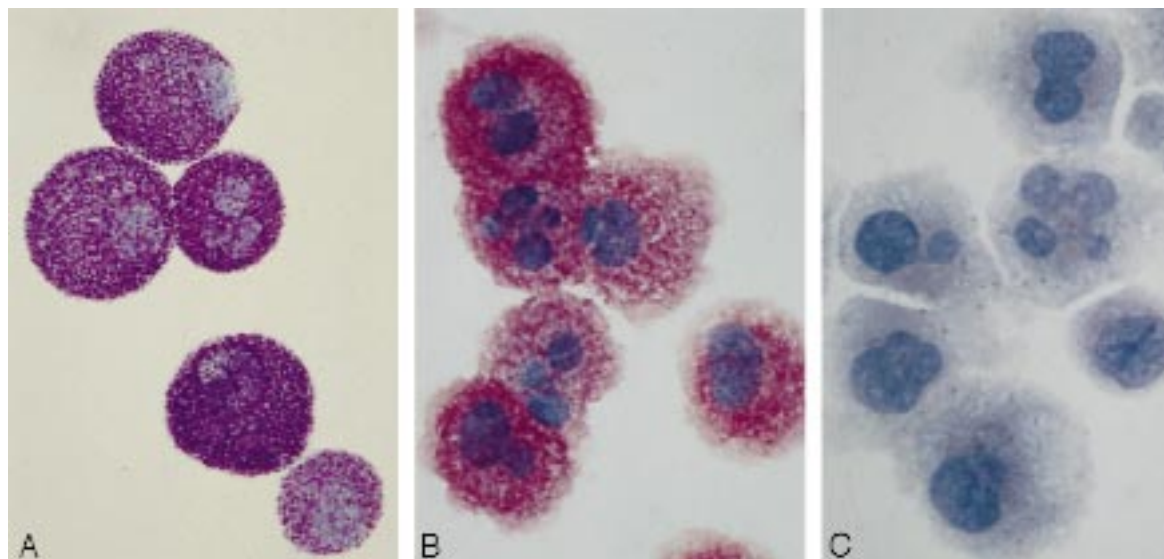


FIG 1 HCMCs derived from cord blood mononuclear cells grown in suspension culture in presence of SCF and IL-6 for 10 weeks. **A**, Cells have many granules in their cytoplasm (Wright stain). **B**, Although half of the cells have a polylobed nucleus, which is different from the round to oval shaped nucleus of *in vivo* mast cells, more than 99% of the cultured cells were tryptase positive as detected by immunohistochemistry with the anti-human tryptase mAb. **C**, Controls were prepared by substituting the primary antibodies with mouse IgG1.

Finally, they were developed with chromogenic substrate at 37° C for 30 minutes.

Analysis of the mRNA expression of cytokines in HCMCs by RT-PCR and Southern blotting

The mRNA expression of several cytokines in HCMCs was studied by RT-PCR and Southern hybridization using primers and probes specific for each cytokine. Briefly, cultured mast cells (5×10^6) were stimulated with PMA (20 ng/mL) + A23187 (0.5 μ mol/L) (Sigma Chemical Co, St. Louis, Mo) for 3 hours. Alternatively, the cells were incubated with human myeloma IgE (2 μ g/mL) (Chemicon International Inc, St Elsegundo, Calif) at 4° C for 1 hour and then cross-linked with anti-human IgE (1 μ g/mL) (Cappel Laboratories, Malvern, Pa) for 3 hours. KU812F cells and HMC-1 cells were stimulated with PMA + A23187 for 3 hours. Cells were homogenized in phenol-guanidium isothiocyanate (TRIzol reagent; GIBCO BRL, Life Technologies Inc, Gaithersburg, Md), and total RNA was extracted according to the method of Chomczynski and Sacchi.²⁴ cDNA was synthesized from 1 μ g of RNA by using random primers (hexadeoxynucleotide mixture: pd[N]₆; Takara Co, Ltd, Kyoto, Japan) and moloney murine leukemia virus RT (M-MLV RT) (GIBCO BRL, Life Technologies Inc) in the presence of ribonuclease inhibitor (Promega Corporation) in a total volume of 25 μ L, which was expanded up to 250 μ L with H₂O after the reaction was finished. cDNA (5 μ L) was amplified with specific primers for 35 cycles in a total volume of 10 μ L with Taq DNA polymerase (Perkin-Elmer Corp, Foster City, Calif). Each PCR cycle was composed of denaturation at 94° C for 45 seconds, annealing at 55° C for 45 seconds, and extension at 72° C for 2 minutes. The sequence of primers and probe used for each cytokine are as listed in Table I. The PCR products were analyzed on 1.8% agarose gels containing ethidium bromide. For Southern blot analysis, the PCR products were transferred onto nylon membranes (Hybond-N+; Amersham International, Buckinghamshire, UK), and the PCR products were confirmed with specific digoxigenin-labeled cDNA probes (IL-13 cDNA, a

generous gift from Dr René de Waal Malefyt, DNAX, Palo Alto, Calif) or oligoprobe listed on Table I and detected by means of a DIG DNA labeling kit (DIG DNA labeling kit for cDNA probes and a DIG oligonucleotide tailing kit for oligoprobes; Boehringer Mannheim, Mannheim, Germany). After prehybridization at 42° C for 1 hour, hybridization with DIG-labeled specific probe was done at 42° C overnight. The hybridized probe was then detected by using an anti-DIG antibody conjugated with alkaline phosphatase, which showed a chemiluminescent reaction with the substrate Lumigen PPD.

Immunohistochemical staining for the analysis of IL-13 and IL-4 in HCMCs

HCMCs stimulated with IgE and anti-IgE or PMA + A23187 for 2, 3, 4, 6, 8, 12, and 24 hours were fixed with periodate poly-L lysine paraformaldehyde at room temperature for 15 minutes and washed in PBS with 15% sucrose. The cells were cytocentrifuged (1×10^6 /mL) onto silane-coated glass slides (DAKO Japan Co, Ltd, Kyoto, Japan). Before staining, the cells were permeabilized with 0.1% saponin and treated with 10% normal rabbit serum for 20 minutes. Subsequently, the cells were incubated overnight in optimal concentrations of the relevant anti-cytokine mAb. The cells were stained by the streptavidin biotin method (ABC kit, Vector) as previously described.¹¹ The reaction was developed with the amino ethyl carbazole substrate and counterstained with Mayer's hematoxylin. Negative controls were prepared by substituting the primary antibodies with isotype-matched antibodies. Time-course analysis was performed to estimate the cytokine-positive cells under a microscope.

Priming HCMCs with IL-4

The 10-week cultured HCMCs were divided into 2 aliquots, and 1 aliquot was cultured in α -MEM supplemented with 20% FCS in the presence of IL-4 (10 ng/mL; a generous gift from DNAX), SCF (100 ng/mL), and IL-6 (80 ng/mL), and the other aliquot was cultured in α -MEM with 20% FCS in the presence of only SCF and IL-6. Each aliquot was cultured for 5 days. The cells in each aliquot were washed with PBS for 3 times and stimulated in microtiter plates (1×10^5 cells/200 μ L/well) with anti-IgE (1 μ g/mL) after sensitiza-

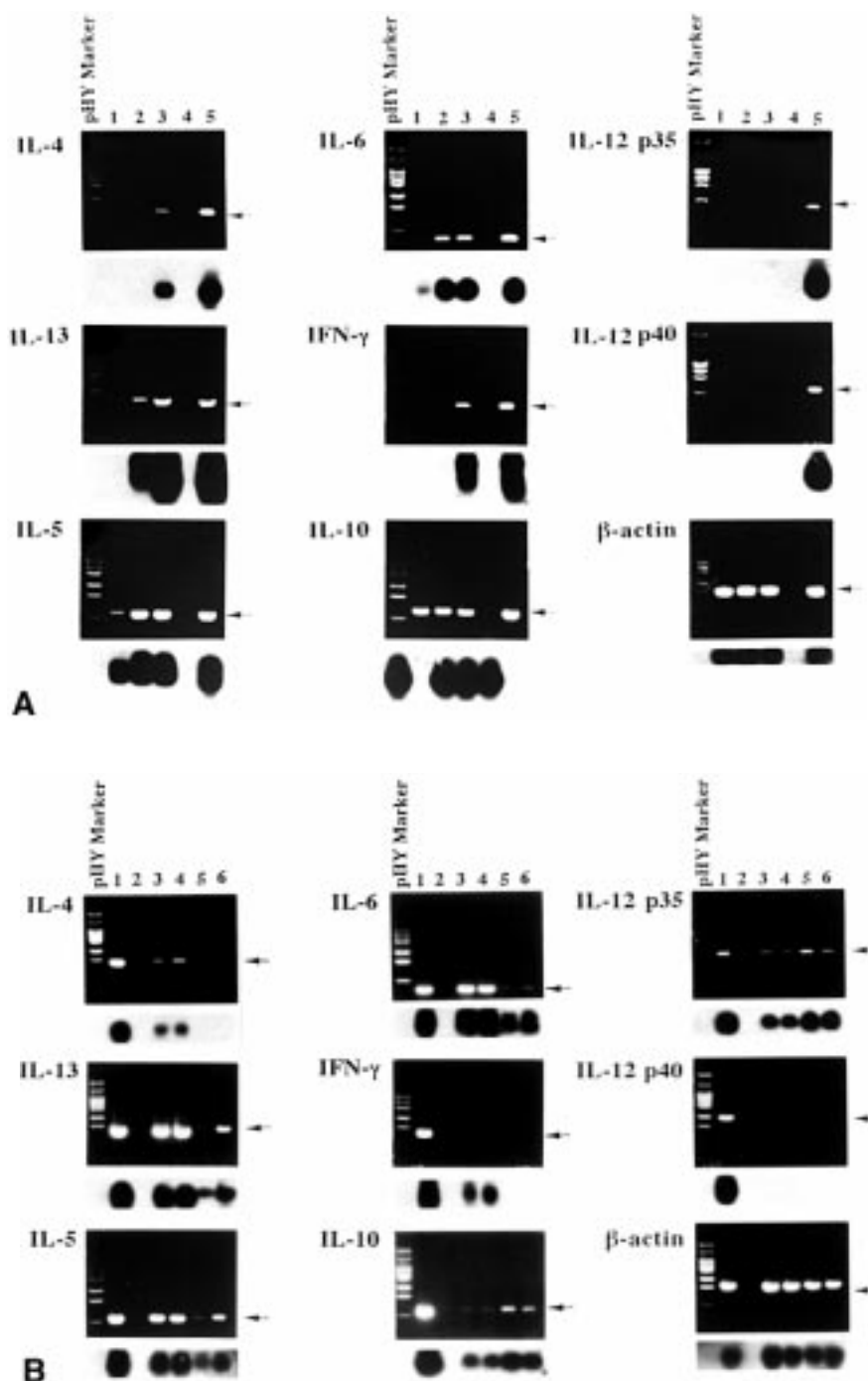


FIG 2. A, Cytokine mRNA expression in HCMCs. RNA extracted from HCMCs stimulated with anti-IgE antibody (1 μ g/mL) after incubation with IgE (2 μ g/mL) or PMA (20 ng/mL) + A23187 (0.5 μ mol/L) was subjected to reverse transcription and amplified by PCR by using primers specific for each cytokine listed in Table I. Southern blot analyses with specific probes showed the specificity of RT-PCR. Marker, pHY size marker (Takara Co, Ltd, Kyoto, Japan); lane 1, HCMCs without stimulation; lane 2, HCMCs stimulated with IgE + anti-IgE for 3 hours; lane 3, HCMCs stimulated with PMA + A23187 for 3 hours; lane 4, distilled water for the template as a negative control; lane 5, PBMCs stimulated with PMA + A23187 for 3 hours as a positive control. The result is 1 representative out of 3. **B,** Cytokine mRNA expression in KU812F cells and HMC-1 cells. Marker, pHY size marker; lane 1, PBMCs stimulated with PMA + A23187 for 3 hours as a positive control; lane 2, distilled water used as a negative control; lane 3, unstimulated KU812F cells; lane 4, KU812F cells stimulated with PMA + A23187 for 3 hours; lane 5, unstimulated HMC-1 cells; lane 6, HMC-1 cells stimulated with PMA + A23187 for 3 hours. The result is 1 representative out of 3.

tion with human myeloma IgE (2 μ g/mL) or with PMA (20 ng/mL) + A23187 (0.5 μ mol/L) in α -MEM supplemented with 20% FCS in the presence of SCF (100 ng/mL) and IL-6 (80 ng/mL). After the indicated period of culture, the supernatant samples were stored at -80° C until assayed. The supernatant samples for measurement of histamine were collected 30 minutes after each stimulation.

Measurement of histamine

The histamine content in the supernatant was measured by HPLC (TOSOH HPLC; TOSOH Co, Ltd, Tokyo, Japan). For measurement of total histamine content, the supernatant of the cells lysed with Triton X (1%) (Sigma Chemical Co, St Louis, Mo) was used.

Measurement of IL-13 and IL-4 by ELISA

IL-13 and IL-4 in the supernatants were detected by commercially available ELISA kits: Cytoscreen Immunoassay Kit for human IL-13 (BioSource International, Inc) and the Quantikine Human IL-4 Immunoassay for IL-4 (R & D Systems, Inc).

Flow cytometry for analysis of ICAM-1, LFA-1, Fc ϵ RI α -chain, and c-kit expression in HCMCs

The 10-week cultured HCMCs were further cultured with IL-4 (10 ng/mL) or IL-13 (100 ng/mL) (generous gifts from DNAX) for 5 days in the presence of SCF (100 ng/mL) and IL-6 (80 ng/mL) in α -MEM supplemented with 20% FCS. The surface molecule expression was examined by flow cytometry with specific mAbs as previously described.^{18,19} Briefly, the cells (2×10^5) were incubated with the relevant mAb (2 μ g/mL) and then washed with PBS. Subsequently, the cells were stained by FITC-conjugated goat anti-mouse IgG (Silenus, Victoria, Australia) and analyzed by a flow cytometer (CytoACE; JASCO, Tokyo, Japan). Mouse IgG1 or IgG2b (Coulter Immunology, Hialeah, Fla) was used as isotype-matched control antibodies.

Statistical analysis

Statistical analysis was performed by a paired 2-way Student's *t* test. Data were expressed as mean $\pm$ SD. A *P* value of less than .05 was considered statistically significant.

RESULTS

HCMCs

Ten-week cultured HCMCs had many granules in their cytoplasm as shown in Fig. 1, A. Histamine (5.62 ± 1.88 μ g/ 10^6 cells) was contained in the granules. More than 99% of the cultured cells were c-kit positive as determined by a flow cytometric analysis and were tryptase positive as detected by immunohistochemistry with anti-human tryptase mAb (Fig. 1, B). Less than 30% of the cells were chymase positive as detected by immunohistochemistry with anti-human chymase mAb (data not shown). Over half of the cells had a poly-lobed nucleus (Fig. 1, A, B, and C), which is different from a round to oval-shaped nucleus, in *in vivo* mast cells. The lobulation of the nuclei was more remarkable at higher concentrations of IL-6 than when the mast cells were grown at lower concentrations of IL-6 (Nakahata T et al., unpublished data). Therefore the lobulation of the nuclei may be attributed to IL-6, which is an important factor for mast cell growth, by enhancing the expansion of primitive hemopoietic cells in cord blood cells.

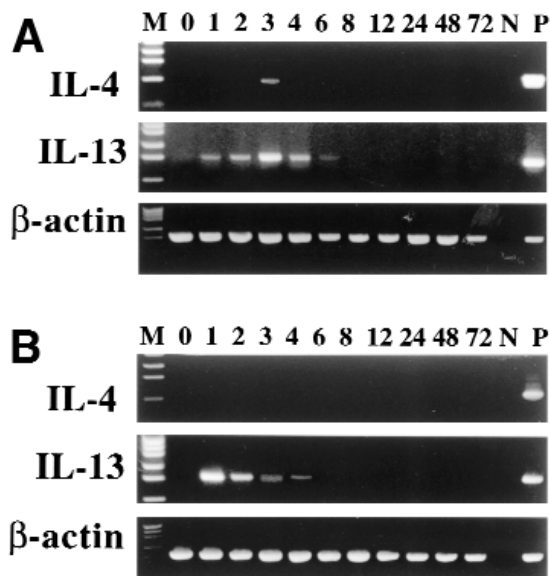


FIG 3. Time kinetics of IL-13 and IL-4 mRNA expression in HCMCs. IL-4 and IL-13 mRNA in HCMCs stimulated with PMA + A23187 (A), or IgE + anti-IgE (B) were detected by RT-PCR at indicated time after stimulation. Numbers represent hour or hours after stimulation. Lane N, Distilled water for the template as a negative control; lane P, PBMCs stimulated with PMA + A23187 for 3 hours as a positive control. The result is 1 representative out of 3.

Cytokine mRNA expression by HCMCs, KU812F, and HMC-1

As shown in Fig. 2, A, IL-13 mRNA was not constitutively expressed by HCMCs but was clearly detected after stimulation by cross-linking of Fc ϵ RI with IgE and anti-IgE antibodies. IL-13 mRNA was also detectable in mast cells stimulated with PMA + A23187. IL-4 mRNA was also not constitutively expressed by HCMCs, but in contrast to IL-13, IL-4 mRNA was not detectable even after stimulation with IgE and anti-IgE. IL-4 mRNA expression was only detected after stimulation of HCMCs with PMA + A23187. These results indicate that both IL-4 and IL-13 are not constitutively expressed by HCMCs and that the stimuli for expression for these cytokines are different.

The mRNA expression of other T_H2 -type cytokines in HCMCs was also examined. Unlike IL-4 and IL-13, IL-5, IL-6, and IL-10 were constitutively expressed by HCMCs. Stimulation with IgE and anti-IgE enhanced the expression of IL-5 and IL-6, but not IL-10. Stimulation with PMA + A23187 showed similar results to those obtained by stimulation with IgE and anti-IgE. mRNA expression for T_H1 -type cytokines, such as IFN- γ , were neither constitutively expressed nor expressed after stimulation by means of Fc ϵ RI. However, IFN- γ mRNA expression was detected when the cells were treated with PMA + A23187. Although murine mast cells were reported to express IL-12,²⁵ HCMCs did not express mRNA for either the p35 or p40 subunits of IL-12 even after maximal stimulation with PMA + A23187.

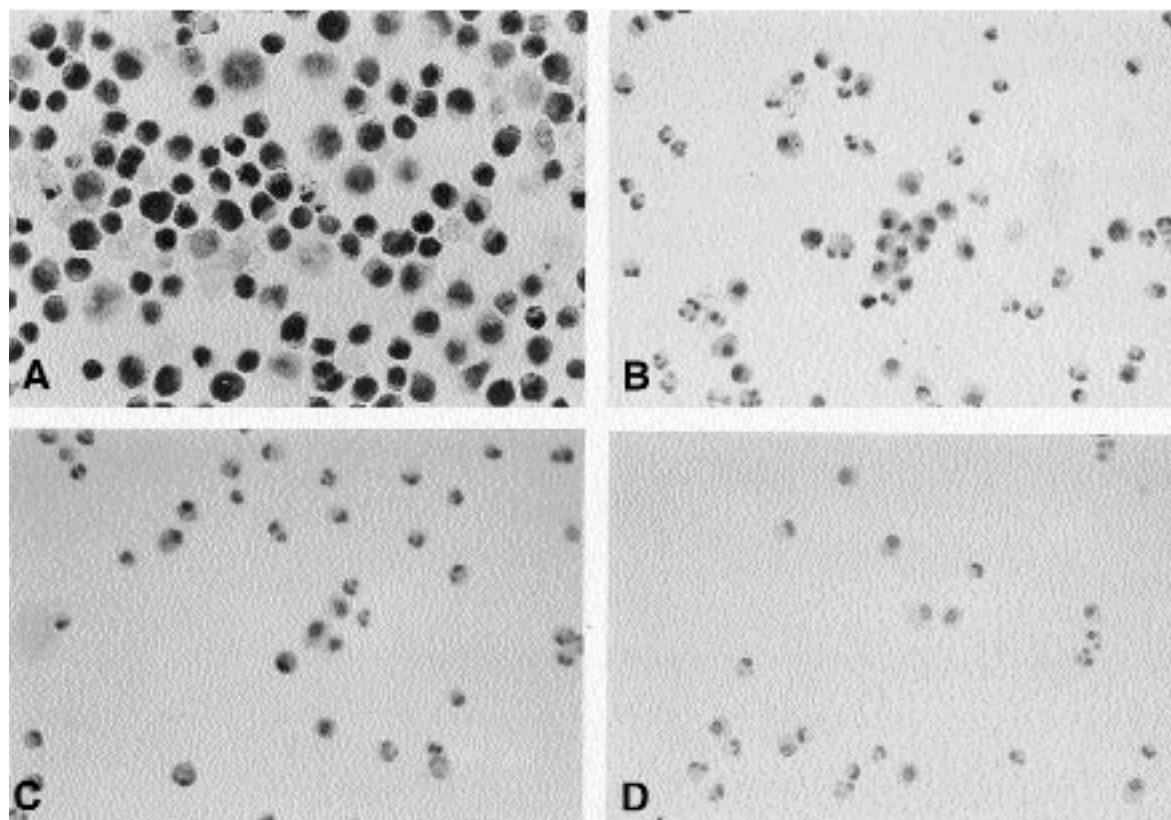


FIG 4. Detection of IL-13 protein in HMCs and KU812F cells. HMCs and KU812F cells were stained with mAb specific for IL-13. KU812F cells stimulated with PMA + A23187 for 3 hours (**A**); HMCs stimulated with PMA + A23187 for 3 hours (**B**); HMCs stimulated with IgE and anti-IgE for 4 hours (**C**); and HMCs stimulated with IgE and anti-IgE for 4 hours and stained with isotype matched control antibody as a control (**D**).

We next examined the cytokine mRNA expression in the human basophilic leukemia cell line, KU812F cells, and a mast cell leukemia cell line, HMC-1 cells (Fig. 2, *B*). Both KU812F cells and HMC-1 cells spontaneously expressed IL-13 mRNA. Stimulation with PMA + A23187 increased the expression of IL-13 mRNA in HMC-1 cells. IL-4 was not detected in HMC-1 cells, and stimulation with PMA + A23187 also failed to induce IL-4 mRNA expression. In contrast, KU812F cells constitutively expressed IL-4 mRNA. As expected from the observation that HMC-1 cells do not express the FcεRI,^{13,14} stimulation of the cells by IgE and anti-IgE did not induce the expression of IL-4 or IL-13 mRNA (data not shown).

The pattern of mRNA expression of cytokines other than IL-4 and IL-13 was further examined in KU812F cells and HMC-1 cells. KU812F cells spontaneously expressed all T_{H2} cytokines tested, including IL-5, IL-6, and IL-10. The T_{H1} cytokine IFN-γ was also expressed in KU812F cells. HMC-1 cells spontaneously expressed IL-5, IL-6, and IL-10, although IFN-γ mRNA expression was not detected even when stimulated with PMA + A23187. The p35 subunit of IL-12 was constitutively expressed in both cell lines; however, p40 subunit was not expressed even when stimulated with PMA + A23187, indicating that physiologically active IL-12 was

not produced because IL-12 is active only when both p35 and p40 subunits are expressed.²⁶⁻²⁸

Kinetics of IL-13 and IL-4 mRNA expression in HMCs

We examined the time kinetics of IL-13 and IL-4 mRNA expression by HMCs stimulated with PMA + A23187 or with IgE and anti-IgE antibodies. When HMCs were stimulated with PMA + A23187, IL-13 mRNA could be detected at 1 hour after stimulation. The peak expression was at 3 hours and could be detected until 6 hours after stimulation (Fig. 3, *A*). IL-4 mRNA could be detected at 3 hours when the cells were stimulated with PMA + A23187 (Fig. 3, *A*). When the HMCs were stimulated with IgE and anti-IgE, IL-13 mRNA was detectable at 1 hour after stimulation, and then the expression gradually decreased by 4 hours (Fig. 3, *B*). IL-4 mRNA was not detected when HMCs were stimulated by FcεRI cross-linking (Fig. 3, *B*).

Detection of intracytoplasmic IL-13 and IL-4 protein expression in HMCs

We further studied intracytoplasmic IL-13 and IL-4 protein expression in HMCs by immunohistochemistry. Both IL-13 and IL-4 protein expression was detectable in

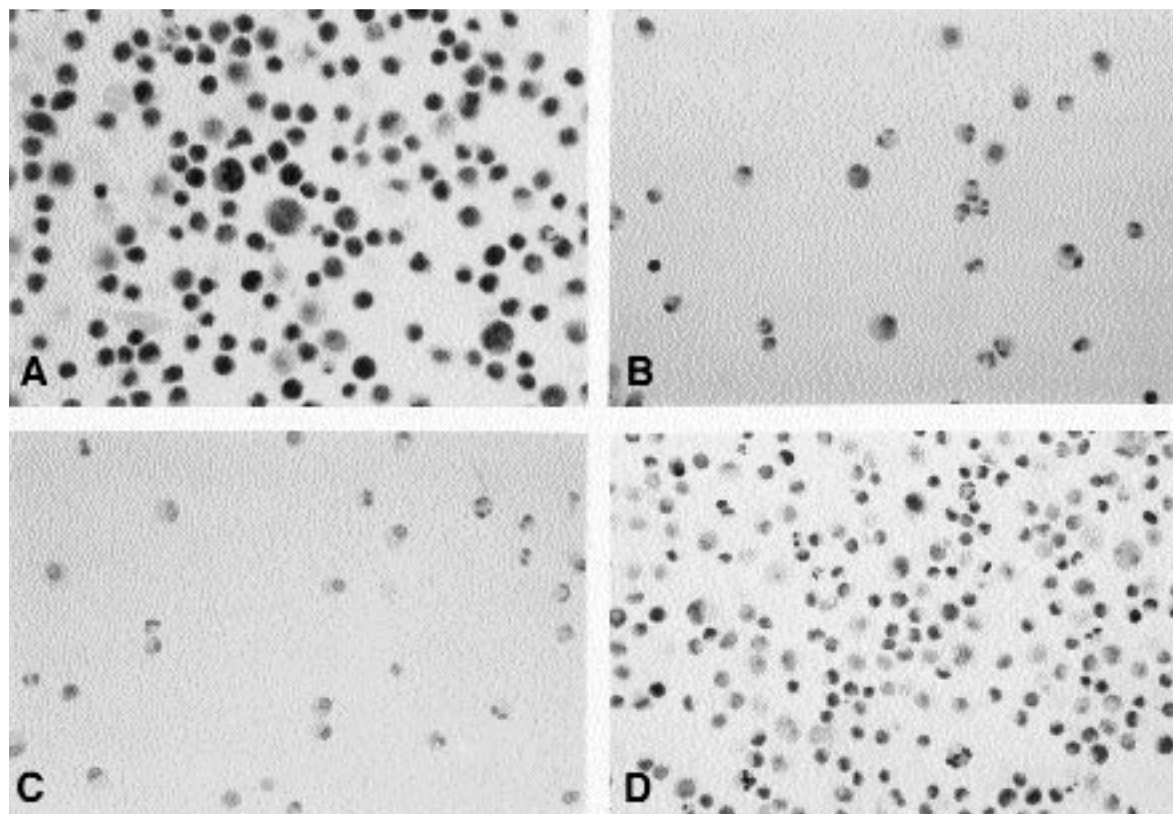


FIG 5. Detection of IL-4 protein in HCMCs and KU812F cells. HCMCs and KU812F cells were stained with mAb specific for IL-4. KU812F cells stimulated with PMA + A23187 for 3 hours (**A**); HCMCs stimulated with PMA + A23187 for 4 hours (**B**); HCMCs stimulated with IgE and anti-IgE for 4 hours (**C**); and KU812F cells stimulated with PMA + A23187 for 3 hours and stained with isotype-matched control antibody as a control (**D**).

the cytoplasm of KU812F cells stimulated with PMA + A23187 (Figs. 4, A and 5, A). As shown in Figs. 4, B and 5, B, IL-13 and IL-4 protein was detected in the cytoplasm of HCMCs stimulated with PMA + A23187, although the percentage of the cells positive for IL-4 was far less than that for IL-13. In addition, IL-13, but not IL-4, was detected in HCMCs stimulated with IgE and anti-IgE antibodies (Figs. 4, C and 5, C). Unstimulated HCMCs did not express either IL-4 or IL-13 (data not shown).

Kinetics of IL-13 and IL-4 protein production in HCMCs

When HCMCs were stimulated with PMA + A23187, the percentage of the IL-13–positive cells reached the maximum level ($28.3\% \pm 3.3\%$) at 3 hours after stimulation and could be detected until 8 hours after stimulation (Fig. 6, A). IL-4–producing cells reached the maximum level ($3.5\% \pm 1.5\%$) at 4 hours after the same stimulation (Fig. 6, B). When HCMCs were stimulated by FcεRI cross-linking, IL-13 protein expression was detectable from 3 to 8 hours after stimulation (Fig. 6, A), whereas IL-4 was not detectable at any time point tested (Fig. 6, B). These findings agree with the results obtained by RT-

PCR and suggest that HCMCs do not spontaneously express IL-4 or IL-13 and that IL-13, but not IL-4, protein is produced by FcεRI stimulation.

We also measured the concentration of these 2 cytokines secreted in the supernatants. Although it is difficult to know exactly when the stimulated cells start to secrete the cytokines, because of the sensitivity of the ELISA (IL-13, ≥ 3.1 pg/mL, IL-4, ≥ 15.6 pg/mL), when HCMCs were stimulated by FcεRI cross-linking, IL-13 was within detectable levels as early as 12 hours after stimulation, and the production reached plateau by 72 hours (20.7 ± 1.6 pg/mL by 1×10^5 cells) (Fig. 6, C). When HCMCs were stimulated with PMA + A23187, IL-13 was detectable at 24 hours after stimulation and, at 48 hours, the production reached the plateau (406.4 ± 13.4 pg/mL by 1×10^5 cells) (Fig. 6, D), which was 20 times as high as that observed when the cells were stimulated with IgE and anti-IgE. In either PMA + A23187–stimulated or IgE + anti-IgE–stimulated HCMCs, IL-4 was not detectable (<15.6 pg/mL) in the supernatant at any time points (data not shown), although IL-4 mRNA was expressed in PMA + A23187–stimulated HCMCs, and a few cells expressed IL-4 protein in their cytoplasm by immunohistochemistry. This may be attributable to a low amount of IL-4 produced by HCMCs.

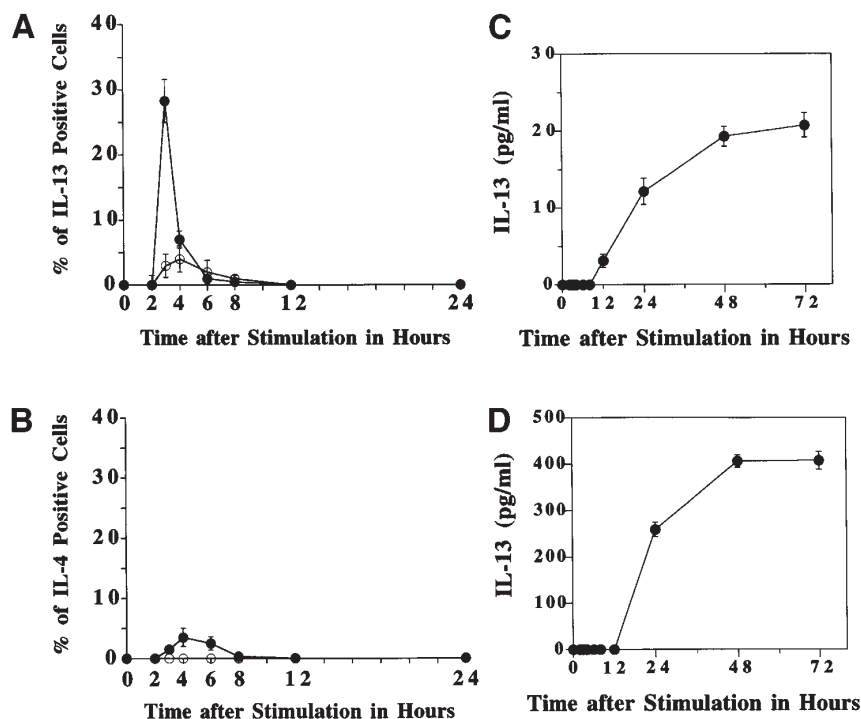


FIG 6. **A** and **B**, Time kinetics of intracytoplasmic IL-4 and IL-13 protein expression in HCMCs. HCMCs were stimulated with PMA + A23187 (filled circles) or with IgE and anti-IgE antibodies (open circles) for indicated periods and stained with mAb to IL-13 (**A**) or IL-4 (**B**). **C** and **D**, Time kinetics of IL-13 secretion. IL-13 secreted by HCMCs (10^5 cells) stimulated with IgE and anti-IgE (**C**) or with PMA + A23187 (**D**) was measured by ELISA. Results are shown as mean $\pm$ SD ($n = 3$). These experiments have been repeated 3 times with similar results.

Effect of IL-4 priming on IL-4 and IL-13 production in HCMCs

On the basis of our previous data that IL-4 can upregulate the Fc ϵ RI expression in HCMCs,¹⁸ we examined whether these IL-4-primed HCMCs could express IL-4 on cross-linking of Fc ϵ RI and whether these cells upregulated IL-13 production. When HCMCs were cultured in the presence of IL-4 (10 ng/mL) for 5 days and then were stimulated with IgE and anti-IgE, IL-4 mRNA expression was induced, and IL-13 mRNA expression was upregulated as compared with the respective β -actin mRNA expression (Fig. 7). IL-4 priming caused significantly increased histamine release on Fc ϵ RI cross-linking (Fig. 8, A), which may be attributed to increased Fc ϵ RI expression induced by IL-4.¹⁸ However IL-4 protein was not expressed either in the cytoplasm of IL-4-primed HCMCs or in the supernatant samples when the cells were stimulated with IgE and anti-IgE (data not shown). No significant increase was observed in the percentage of the intracytoplasmic IL-4-positive cells in IL-4-primed HCMCs stimulated with PMA + A23187, and IL-4 was still undetectable in the supernatant (data not shown). On the other hand, remarkable differences were found in IL-13 production (Fig. 8, B). IL-4-primed HCMCs (10^5 cells) produced 336.8 ± 30.3 pg/mL IL-13, whereas 21.3 ± 1.1 pg/mL IL-13 was produced by IL-4-nonprimed HCMCs (10^5 cells) on Fc ϵ RI cross-linking ($P < .005$)

(Fig. 8, B). Interestingly, when IL-4-primed HCMCs (10^5 cells) were stimulated with PMA + A23187, a significantly higher concentration of IL-13 (1830.0 ± 108.2 pg/mL) was detected compared with the concentration of IL-13 (418.4 ± 13.8 pg/mL) ($P < .005$) produced by IL-4-nonprimed HCMCs (10^5 cells) stimulated with PMA + A23187 (Fig. 8, B). Consistent with this, IL-4 priming increased IL-13-positive HCMCs in both IgE and anti-IgE and PMA + A23187 stimulation examined by immunohistochemistry ($8.2\% \pm 2.8\%$ in IL-4-primed mast cells and $3.5\% \pm 1.5\%$ in IL-4-nonprimed cells, both at 4 hours after stimulation with IgE and anti-IgE; and $43.2\% \pm 5.1\%$ in IL-4-primed mast cells and $28.3\% \pm 3.3\%$ in IL-4-nonprimed cells, both at 3 hours after stimulation with PMA + A23187). Because IL-4 priming caused increased IL-13 production by HCMCs not only after stimulation by Fc ϵ RI cross-linking but also after PMA + A23187 stimulation, it is possible that IL-4 priming augmented IL-13 production by some other mechanisms besides induction of Fc ϵ RI expression in HCMCs.

Effect of IL-4 and IL-13 on ICAM-1, LFA-1, Fc ϵ RI, and c-kit expression in HCMCs

In previous reports we have demonstrated that IL-4 is a potent regulator of surface molecule expression in HCMCs, upregulating ICAM-1, LFA-1, and Fc ϵ RI and downregulating c-kit (Fig. 9).^{18,19} Therefore we examined whether IL-13 was also effective on the expression

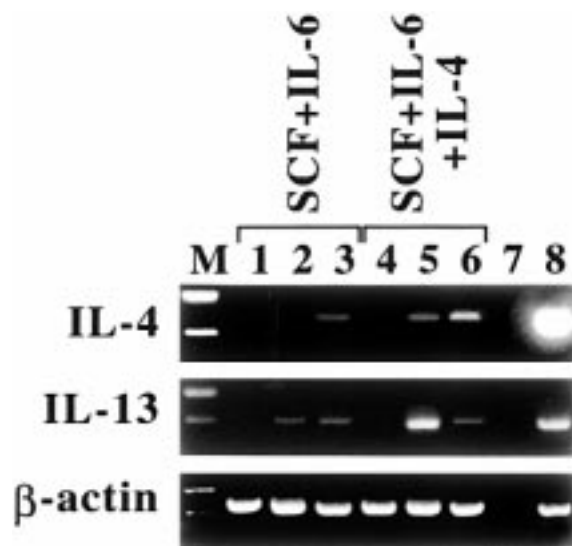


FIG 7. IL-4 and IL-13 mRNA expression in HCMCs cultured with IL-4 and then stimulated by FcεRI cross-linking. The 10-week cultured HCMCs were divided into 2 aliquots; 1 aliquot was cultured with IL-4 (10 ng/mL), and the other aliquot was cultured without IL-4, both in the presence of SCF (100 ng/mL) and IL-6 (80 ng/mL) for 5 days. HCMCs from each aliquot were stimulated with IgE and anti-IgE antibody or PMA + A23187, and RNA extracted from each sample was subjected to reverse transcription and amplified by PCR by using primers specific for each cytokine. Lane M, pHY size marker; lanes 1 to 3, HCMCs cultured without IL-4; lanes 4 to 6, HCMCs cultured with IL-4. Lanes 1 and 4, HCMCs without stimulation; lanes 2 and 5, HCMCs stimulated with IgE and anti-IgE antibodies for 3 hours; lanes 3 and 6, HCMCs stimulated with PMA + A23187 for 3 hours; Lane 7, distilled water for the template as a negative control; lane 8, PBMCs stimulated with PMA + A23187 for 3 hours as a positive control. This result is 1 representative out of 3.

of these molecules. However, no significant differences were found in these surface molecules in the cells cultured in the presence of IL-13 (Fig. 9). These data indicated that there were differences between IL-4 and IL-13 in the activities on HCMCs.

DISCUSSION

Here we report that IL-13 mRNA and protein are not spontaneously expressed in HCMCs, and that IL-13, but not IL-4, proteins are expressed on cross-linking of FcεRI. In addition, IL-4-primed HCMCs showed enhanced IL-13 production on cross-linking of FcεRI. Other T_{H2} cytokines, including IL-5, IL-6, and IL-10, were spontaneously expressed without stimulation, and T_{H1} cytokines, such as IL-12 and IFN-γ, failed to be induced by the FcεRI cross-linking.

Recently, IL-13 protein expression was detected in mast cells of the nasal mucosa of patients with perennial allergic rhinitis, but not in those of patients with chronic infective rhinitis or normal subjects, and a strong positive correlation was detected between the IL-13 gene expression in the nasal mucosa of patients with perennial allergic rhinitis and their serum-specific IgE.¹¹ In this context our observations that IL-13 was not spontaneously

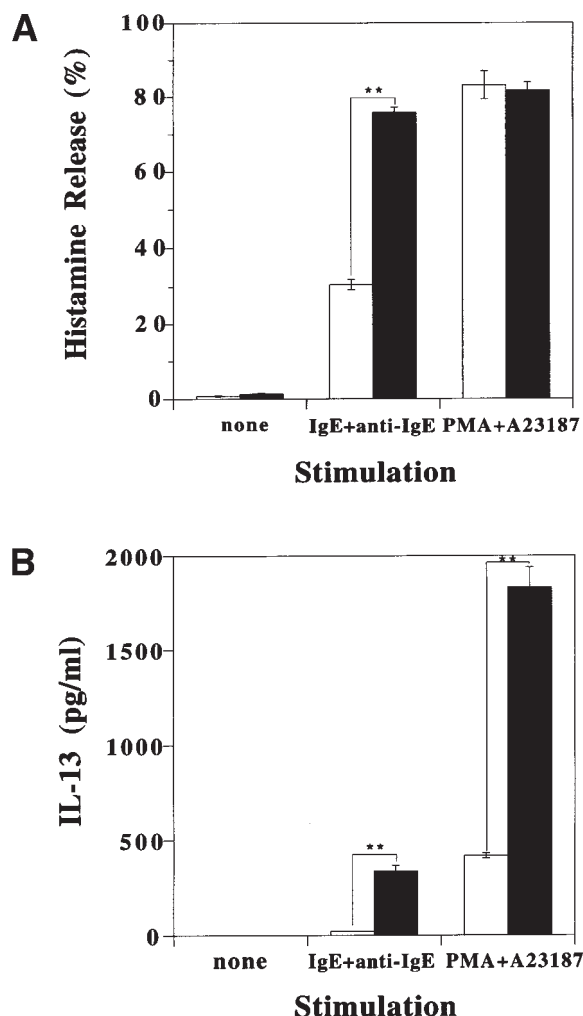


FIG 8. Enhanced IL-13 production by IL-4-primed HCMCs. HCMCs were cultured with IL-4 (10 ng/mL) or without IL-4 for 5 days both in the presence of SCF (100 ng/mL) and IL-6 (80 ng/mL). Then both IL-4-primed HCMCs (filled bars) and IL-4-nonprimed HCMCs (open bars) (10^5 cells/well respectively) were stimulated with IgE and anti-IgE or PMA + A23187. **A**, Histamine released by HCMCs was measured by HPLC, and histamine-releasing activity was shown as percentage of released histamine to total histamine content. Total histamine content was not significantly different between IL-4-primed and IL-4-nonprimed HCMCs. **B**, IL-13 in the supernatant at 72 hours after stimulation was measured by ELISA. Results are shown as mean $\pm$ SD ($n = 3$). ** $P < .005$. This result is 1 representative out of 3.

expressed by HCMCs and was produced through FcεRI cross-linking may be of interest because it indicates that FcεRI cross-linking by an allergen and its specific IgE plays an important role in inducing IL-13 production in mast cells in vivo. In addition, it is suggested the mast cell-derived IL-13 may contribute to amplification of the allergic reaction because IL-13, like IL-4, promotes IgE synthesis by B cells.²⁹⁻³⁶ Furthermore, because IL-13 has been reported to induce vascular cell adhesion molecule-1 expression on endothelial cells,³⁷ it is suggested that IL-13 produced by mast cells may be involved in the

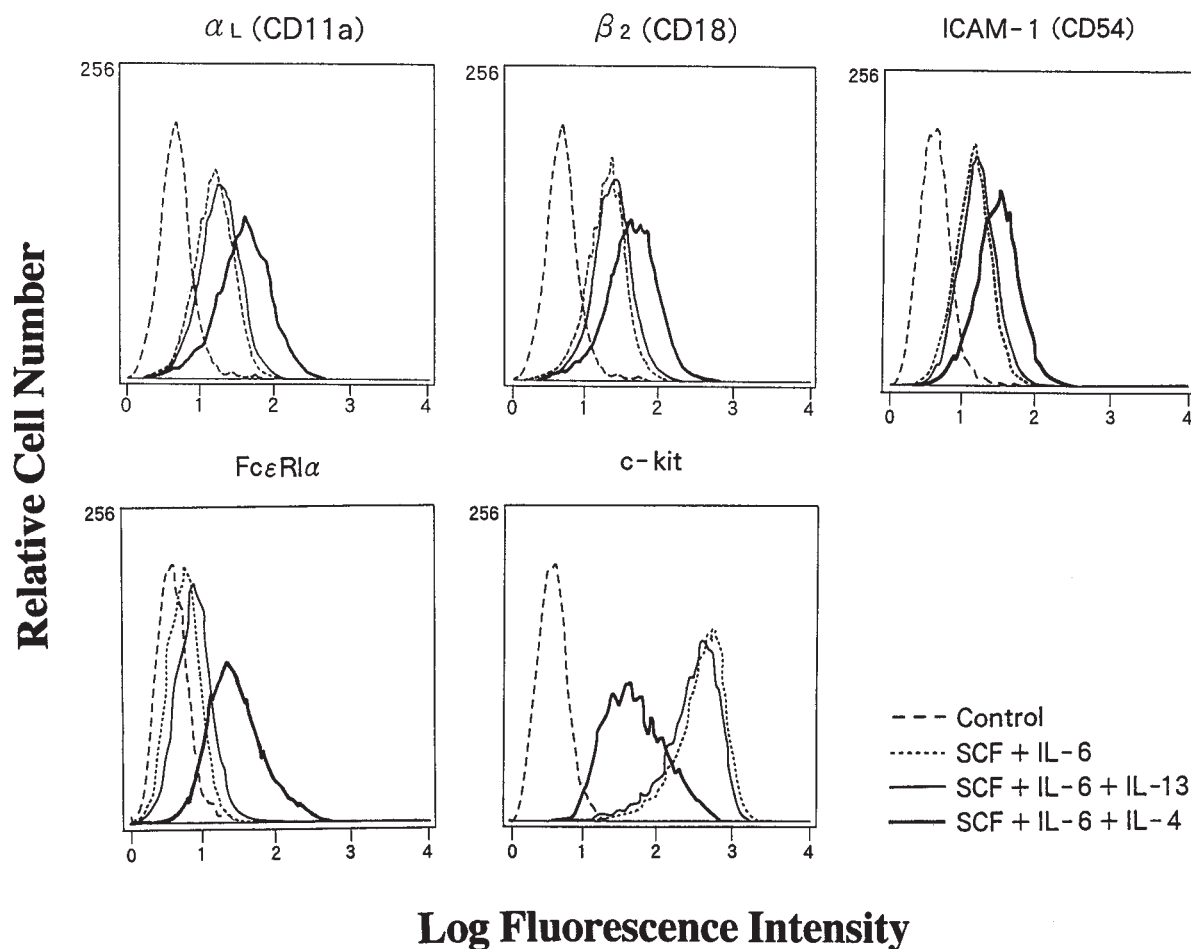


FIG 9. Effect of IL-4 and IL-13 on ICAM-1, LFA-1, FcεRI, and c-kit expression in cultured human mast cells analyzed by flow cytometry. The 10-week cultured HCMCs were further cultured with IL-4 (10 ng/mL) or IL-13 (100 ng/mL) for 5 days in the presence of SCF (100 ng/mL) and IL-6 (80 ng/mL) or cultured only with SCF and IL-6. Then surface molecules were analyzed by a flow cytometer with mAbs specific for ICAM-1 (RR1/1); α-chain of LFA-1 (αL) (TS1/22); β-chain of LFA-1 (β2) (TS1/18); FcεRI α-chain (CRA-1), and c-kit. As a control, each sample was stained with mouse isotype-matched antibody (IgG1 or IgG2b). This result is 1 representative out of 3.

initiation of the late phase of the allergic reaction. The time kinetics of intracytoplasmic expression of IL-13 in HCMCs, which reached a maximum within 3 to 4 hours after stimulation, may support the involvement of mast cell-derived IL-13 in the initiation of the late phase of the allergic reaction.

There have been controversial reports concerning IL-4 production by human mast cells.^{1,2,17} In this report we demonstrated that HCMCs, unlike HMC-1 cells, were capable of expressing IL-4 mRNA and protein if maximally stimulated with PMA + A23187. The level of IL-4 production, however, was quite low as indicated by the observation that only 3.5% of PMA + A23187-stimulated HCMCs were positive for intracytoplasmic IL-4 as determined by immunohistochemistry, and IL-4 in the supernatant was below detectable level. However, 28% of the mast cells were positive for IL-13, and a high amount of IL-13 was detected in the supernatant. In contrast to stimulation with PMA + A23187, FcεRI cross-

linking failed to induce IL-4 mRNA and protein synthesis in HCMCs. The inability of these cells to produce IL-4 may be dependent on the difference in signals transduced by FcεRI cross-linking and PMA + A23187. Alternatively, insufficient stimulation by means of FcεRI in HCMCs may be possible because of the relatively low expression of FcεRI on HCMCs.¹⁸

HCMCs have many characters, and therefore they could be regarded as an adequate model for human mast cells. Over 99% of the cells express c-kit, and they contain significant amounts of histamine and tryptase in the cytoplasmic granules. However, as we have reported previously,^{16,18} they are relatively immature mast cells because they do not express enough FcεRI to induce a high amount of histamine release.¹⁸ Furthermore, they show immature ultrastructure whether or not the nucleus is lobulated. Most of the cells contain a few granules that are empty or partially filled with particles or discrete scrolls along with lamellae, which shows immaturity on

the basis of granule-filling criteria,³⁸ but crystal granules, which appear in mature mast cells, are rare.¹⁶ Consistent with our observation, other investigators have reported that cord blood-derived mast cells cultured in the presence of SCF or SCF + IL-3 were morphologically and functionally immature even after 14 weeks of culture.^{39,40} It was also reported that fetal liver mast cells cultured only in the presence of SCF had less developed morphology.⁴¹ These observations suggest that human mast cells grown only in the presence of SCF (with or without IL-6 or IL-3) do not reach full maturity and that additional factors are required to generate mature mast cells. One of the factors that promotes human mast cell maturation, as we have reported recently,^{16,18} is IL-4. This is because addition of IL-4 to HCMCs significantly increased the surface FcεRI expression¹⁸ and promoted morphologic maturation, including increased numbers of crystal granules.¹⁶ Other investigators have also reported that IL-4 is a cytokine that enhances IgE-mediated mast cell activation in part by upregulation of FcεRI both in the human and murine system.^{42,43} As shown in this study, when we stimulated IL-4-primed HCMCs by FcεRI cross-linking, IL-4 mRNA was induced, and IL-13 mRNA and protein production was upregulated as compared with IL-4-nonprimed cells. These changes may be attributed to the increased mast cell activation that occurred because of the increased FcεRI expression in IL-4-primed HCMCs. In addition, IL-4 priming enhanced IL-13 production by HCMCs not only when the cells were stimulated by FcεRI cross-linking but also when they were activated by PMA + A23187. Thus it is possible that IL-4 priming augments IL-13 production by some other mechanisms besides increasing FcεRI expression in HCMCs. Further studies are necessary to evaluate the mechanism or mechanisms of IL-4-induced enhancement of IL-13 production and the conditions under which IL-4 protein can be expressed in HCMCs.

The T_{H1} cytokine IFN-γ was produced by HCMCs when the cells were maximally stimulated with PMA + A23187. Physiologic stimulation with FcεRI failed to induce IFN-γ production. It was the same pattern with their IL-4 production. As we have shown in this report, HCMCs were able to produce IL-4 mRNA on FcεRI cross-linking when they were primed with IL-4. In this context it is possible that HCMCs could produce IFN-γ on FcεRI cross-linking under some conditions that induce IFN-γ production. In addition, it was reported previously that freshly isolated mast cells from Brown Norway rat peritoneum that were stimulated ex vivo with recombinant IL-12 were able to secrete substantial amounts of biologically active IFN-γ,⁴⁴ suggesting the possibility that mast cells may potentially produce both T_{H1} and T_{H2} cytokines, and which type of cytokine is produced may depend on the microenvironment.

IL-13 shares biologic activities with IL-4 on monocytes and B cells²⁹⁻³⁶; however, unlike IL-4, IL-13 does not act on T cells.^{36,45} Although IL-4 and IL-13 have a partly common receptor and a common pathway in signal transduction, some differences have been reported.

^{36,45-54} The common component of both the IL-4 and IL-13 receptors is IL-4R α-chain, and IL-4R α-chain can associate either with γc chain to form an IL-4 receptor that does not bind IL-13 or with the IL-13R α-chain, which binds both IL-13 and IL-4. Cells that express only IL-4R α-chain/γc chain, such as human T cells, can respond to IL-4 but not to IL-13.⁴⁵ As we have shown in this report, IL-4, but not IL-13, showed surface molecule-regulating activity in HCMCs. These results that IL-13 did not act on HCMCs may be attributed to the receptors expressed on HCMCs. We have observed that HCMCs expressed IL-4R α-chain.¹⁸ We are currently studying whether HCMCs express IL-13R α-chain, γc chain, or both.

IL-13 has many important roles in the allergic reaction, including the induction of IgE synthesis by B cells and the induction of VCAM-1 expression on endothelial cells.²⁹⁻³⁷ Our observation that human mast cells produce IL-13 on cross-linking of FcεRI proposes an important role of mast cells in upregulating allergic inflammation.

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