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High-resolution tracking of cell division demonstrates differential effects of T_H1 and T_H2 cytokines on SCF-dependent human mast cell production in vitro: correlation with apoptosis and Kit expression

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T-helper 1 (T_H1) (interferon-γ [IFN-γ]) and T_H2 (interleukin-4 [IL-4] and IL-5) cytokines have been variably reported to alter human mast cell numbers in complex culture systems. The effects of these cytokines on the kinetics of cell division and cell death are unknown, and their effect on mast cell behavior is relevant to anticipate the consequences of in vivo strategies that alter cytokine levels. To determine the effect of these cytokines on stem cell factor (SCF)–dependent human mast cell production, we used high-resolution tracking of cell division and correlated the results with cell apoptosis, expression of Kit, and mast cell degranulation. When IFN-γ, IL-5, or IL-4 was administered over 8 weeks, we found each cytokine decreased the mast number through a different mechanism. IFN-γ inhibited early progenitor cell division, IL-4 down-regulated early Kit expression, and IL-5 blocked later cell division. Further, IL-4 and IFN-γ had the greatest suppressive effect on degranulation and FcεRI expression. When these cytokines were administered to mature mast cells, IFN-γ and IL-5 had no effect on degranulation and cell division, but IL-4 induced division and potentiated FcεRI-mediated degranulation. Thus, exposure of human mast cells to IL-4, IL-5, and IFN-γ during growth and differentiation generally down-regulated mast cell number and function, whereas IL-4 increased mature mast cell division and degranulation. (Blood. 2005; 105:592-599)

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Introduction

Human mast cells, unlike those of rodent origin, cannot be supported in interleukin-3 (IL-3) but rather have an apparent obligatory requirement for stem cell factor (SCF). The ability of other growth factors to influence SCF-dependent mast cell cultures has focused on T-helper 1 (T_H1) and T-helper 2 (T_H2) cytokines because mast cells play a key role in allergic diseases and may facilitate T_H1 inflammation. T_H1 cytokines are generally thought to inhibit, whereas T_H2 cytokines are reported to enhance, human mast cell number and responsiveness, though the results are inconsistent. IL-4 has been stated to increase human intestinal mast cell number[1] and to decrease,[2] increase,[3] or have no effect[4] on human cord blood mast cells. IL-4 is also reported to induce apoptosis of immature mast cell progenitors[2] and to have no effect on mast cells grown from human bone marrow.[5] IL-5 has been reported to prolong survival of cord blood–derived mast cells after the withdrawal of SCF.[6] IL-5 has also been shown to slightly inhibit the growth of an actively dividing SCF-dependent human mast cell line.[7] The few reports on the effects of interferon-γ (IFN-γ) on human mast cells show that IFN-γ decreases [^{3}H]thymidine incorporation by cord blood–derived mast cells[3] and inhibits mast cell growth from human bone marrow.[8] It is unclear whether IFN-γ inhibits cell division or induces apoptosis of mast cell progenitors.

Because of these inconsistencies, we hypothesized that there are cytokine-specific effects on SCF-dependent mast cell cultures and that such effects vary depending on whether exposure to a given cytokine occurs as cultures develop, thus exerting its effects on mast cell development, or is added to cultures of differentiated mast cells. We chose to examine the effects of IFN-γ, IL-4, and IL-5 on human mast cells derived from $CD34^+$ peripheral blood under identical conditions and over the duration of culture or when added to mature mast cells. To directly examine cell division, we used carboxyfluorescein succinimidyl ester (CFSE) dye for high-resolution cell tracking and simultaneously examined the kinetics of apoptosis and Kit expression. The data to be presented demonstrate that these cytokines, added throughout the duration of culture, inhibit mast cell number, albeit by different mechanisms. Only IL-4 was capable of affecting cell division and enhancing degranulation of mature mast cells. Such data are critical for anticipating the effects of cytokine manipulation on mast cell function and are sometimes counterintuitive in that, for instance, IL-4, which potentiates T_H2 responses, also inhibits mast cell growth if added throughout the process of mast cell proliferation and maturation.

Materials and methods

Materials and antibodies

Anti–CD34 antigen-presenting cell (APC), anti–IL-4Rα phycoerythrin (PE), IL-5Rα-PE, anti–FcεRI fluorescein isothiocyanate (FITC), and

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anti–Kit-PE antibodies were obtained from BD PharMingen (San Diego, CA). Anti–IFN-γRα was obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and antirabbit-PE was obtained from Caltag Laboratories (Burlingame, CA). Negative controls included immunoglobulin G1 (IgG1) (BD Biosciences, San Jose, CA) and irrelevant mouse IgG2a or IgG2b (PharMingen). Propidium iodide, *p*-nitrophenyl *N*-acetyl-β-D-glucosamide, 4-hydroxy-3-nitrophenylacetyl (NP)–bovine serum albumin (BSA) antigen, and benzoyl-DL-arginine-*p*-nitroaniline (BAPNA) were from Sigma-Aldrich (St Louis, MO). CFSE was from Molecular Probes (Eugene, OR). SCF, IFN-γ, IL-4, and IL-5 were obtained from PeproTech (Rocky Hill, NJ). Human IgE anti-NP was from Serotec (Raleigh, NC).

Human mast cell culture and cell counts

Human peripheral blood CD34$^+$ progenitor cells were collected from healthy donors after informed consent and were processed by affinity column apheresis. CD34$^+$ cells were seeded at a density of 10^5 cells/mL in serum-free media (StemPro-34 SFM; Life Technologies, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 50 μg/mL streptomycin, and 100 ng/mL SCF. Some cultures also contained 10 ng/mL IFN-γ, 10 ng/mL IL-4, or 10 ng/mL IL-5. The concentration of cytokine was selected to agree with previous studies.[3,4,8,9] All cultures were maintained at 37°C and 5% CO_2. Half the cytokine-supplemented medium was replaced weekly, and cells were transferred to a new flask to remove adherent cells. At days 3, 5, 7, 10, and 14 and every week thereafter, cell numbers were determined by staining with trypan blue and counting on a hemocytometer. Aliquots of 2×10^4 cultured cells were spun onto glass slides in a cytocentrifuge (Cytospin 2; Shandon Scientific, Cheshire, United Kingdom) and stained with toluidine blue, as described.[10]

CFSE labeling and flow cytometry

Cells were washed once with 0.1% bovine serum albumin/phosphate buffered saline (BSA/PBS) and resuspended to 5×10^7 cells/mL in 0.1% BSA/PBS. An equal volume of 10 μM CFSE in 0.1% BSA/PBS was added to the cell suspension and incubated at room temperature for 30 minutes.[11] Cells were washed first with 1% BSA/PBS and then with StemPro medium (with SCF) and were checked for viability (trypan blue staining) and intensity of CFSE staining (flow cytometry) before culture in StemPro medium (with SCF) supplemented with IFN-γ, IL-4, or IL-5 for the indicated number of days.

All flow cytometric analyses were carried out in cold 0.1% BSA/PBS. Cells (10^5) were incubated with appropriate antibody for 30 minutes on ice, washed twice with 0.1% BSA/PBS, and incubated with 0.1 μg/mL propidium iodide[11] for 5 minutes and analyzed on a FACScalibur (Becton Dickinson, San Diego, CA). Flow Jo (Tree Star, San Carlos, CA) was used to analyze proliferation data obtained with CFSE.

For analysis of apoptosis, unlabeled cells were washed once with PBS and stained with annexin V–FITC and propidium iodide using the TACS apoptosis kit (R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

β-Hexosaminidase release assay

Human cultured mast cells were sensitized with human IgE anti-NP (1 μg/mL) overnight. A total of 2×10^5 cells were resuspended in buffer (10 mM HEPES [*N*-2-hydroxyethylenepiperazine-*N'*-2-ethanesulfonic acid], 137 mM NaCl, 2.7 mM KCl, 0.38 mM $Na_2HPO_4 \cdot 7\ H_2O$, 5.6 mM glucose, 1.8 mM $CaCl_2 \cdot 2\ H_2O$, 1.3 mM $MgSO_4 \cdot 7\ H_2O$, 0.04% BSA, pH 7.4) and were stimulated with NP-BSA at various concentrations.[12] The β-hexosaminidase in the supernatants and cell lysates was quantified by hydrolysis of *p*-nitrophenyl *N*-acetyl-β-D-glucosamide in 0.1 M sodium citrate buffer (pH 4.5) for 90 minutes at 37°C. The percentage of β-hexosaminidase release was calculated as a percentage of the total.

Histamine and tryptase assays

Cells grown in SCF and IFN-γ, IL-4, or IL-5 for 8 weeks were washed once in HEPES buffer, as described, and 1×10^3 cells were lysed by 2 cycles of freeze/thaw. Histamine content was determined using an enzyme-linked immunosorbent assay (ELISA) (Beckman Coulter, Fullerton, CA) per the manufacturer's instructions.

Tryptase content in cell-free supernatants was measured using a colorimetric assay as described.[13] Briefly, 50 μL mast cell supernatants were mixed with 100 μL 0.8 mM BAPNA in Tris buffer (0.1 M Tris/1 M glycerol, pH 7.8) and were incubated for 4 hours at 37°C. The appearance of nitroaniline was measured at 410 nm. To quantitate tryptase, samples were compared with a standard curve obtained using purified lung tryptase (Cortex Biochemical, San Leandro, CA).

Statistical analysis

Experiments were conducted using CD34$^+$ cells from 5 separate donors unless otherwise stated. Each ELISA experiment was performed in duplicate, and values represent a mean of $n = 5 \pm$ SEM. Each release experiment was performed in triplicate, and values represent mean of $n = 5 \pm$ SEM. *P* values were determined by 1-way analysis of variance (ANOVA) (between groups) or by Student *t* test. All proliferation data (division index, proliferation index, and percentage divided) were calculated using Flow Jo software (Tree Star, San Carlos, CA). The Flow Jo proliferation software constructs a series of Gaussian curves—the sum of which equals the distribution found in the data file—and uses this model to calculate the division index (average number of divisions of a cell in the starting population), proliferation index (average number of divisions cells that divided have undergone), and percentage of cells that have divided in the original population.

Results

CD34$^+$ progenitors and human mature mast cells express receptors for IFN-γ, IL-4, IL-5, and SCF

We first determined whether CD34$^+$ cells and mature human mast cells express receptors for IFN-γ, IL-4, and IL-5. CD34$^+$ progenitors and mature mast cells (cultured for 8 weeks in SCF) were tested for the expression of Kit (CD117), IL-5Rα (CDw125), IL-4Rα (CD124), and IFN-γRα by flow cytometry. CD34$^+$ progenitor cells and mature mast cells expressed IL-5Rα, IL-4Rα, IFN-γRα, and Kit (Figure 1). CD34$^+$ progenitors expressed high levels of IL-5Rα, IL-4Rα, and IFN-γRα, whereas mast cells express high levels of Kit but low levels of IL-5Rα, IL-4Rα, and IFN-γRα. Therefore, both CD34$^+$ and mature human mast cells have the receptors for these cytokines that are required to initiate a response.

IFN-γ, IL-4, and IL-5 decrease cell number in peripheral blood CD34$^+$ cultures

To determine the effect of IFN-γ, IL-4, and IL-5 on total cell number and the number of mast cells observed when each was present throughout culture, CD34$^+$ progenitors were purified from human peripheral blood, and 1×10^6 cells were cultured in serum-free growth media containing SCF (100 ng/mL) and IFN-γ (10 ng/mL), IL-4 (10 ng/mL), or IL-5 (10 ng/mL). Each week, total cells were counted, and an aliquot was stained with toluidine blue to determine mast cell number.

CD34$^+$ cell–derived cultures grown in SCF alone increased rapidly in number within the first 10 days ($43.5 \pm 2.2 \times 10^6$ cells; Figure 2A). IFN-γ, IL-4, and IL-5 had no effect on SCF-dependent cell growth in cell number within the first 10 days. CD34$^+$ cells grown in IFN-γ, IL-4, or IL-5 in the absence of SCF did not proliferate, and, at 10 days, all cells were dead (data not shown).

By week 3, cell numbers in cultures containing SCF + IL-4 approximated cell numbers in cultures containing SCF + IFN-γ. Cell numbers in cultures containing SCF alone or SCF + IL-5 remained similar until week 5, after which there was a decrease in

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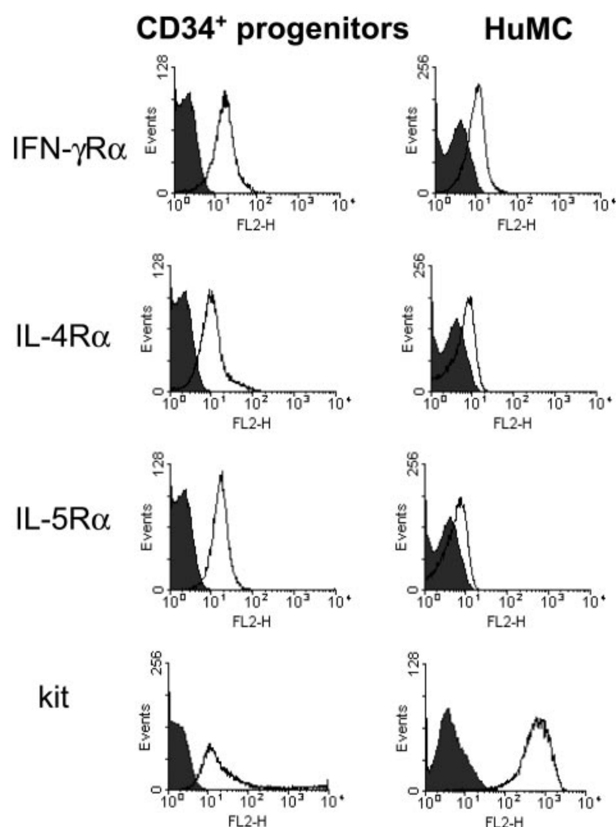

Figure 1. Receptor expression of CD34+ progenitors and mature human mast cells. Mature mast cells were grown for 8 weeks in 100 ng/mL SCF. Cells were stained with anti–IL-4R-PE, anti–IL-5R-PE, and anti–Kit-PE and were analyzed by flow cytometry. For IFN-γR, cells were stained with anti–IFN-γR (α chain) mAb and then with antirabbit-PE. Results shown were verified in the examination of 3 separate cultures from separate donors. No cell growth occurred in the absence of SCF.

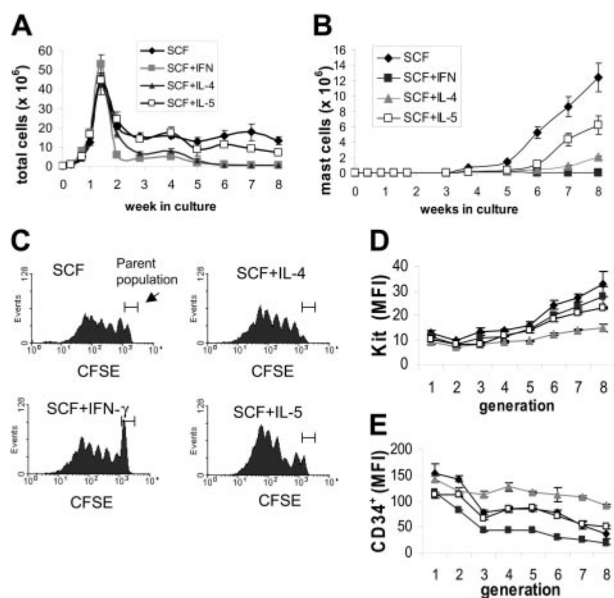

Figure 2. Effect of T_H1 and T_H2 cytokines on CD34+ growth and differentiation. Peripheral blood CD34+ cells were grown in medium supplemented with SCF (100 ng/mL) in combination with IFN-γ (10 ng/mL), IL-4 (10 ng/mL), or IL-5 (10 ng/mL) for 8 weeks. Total cell (A) and mast cell (B) numbers were monitored throughout the culture. Data represent mean ± SEM from 5 donors. (C) CD34+ cells were stained with CFSE (5 μM) and were cultured in medium containing SCF (100 ng/mL) and IFN-γ (10 ng/mL), IL-4 (10 ng/mL), or IL-5 (10 ng/mL) for 7 days. Division was analyzed by flow cytometry. Data are representative of 5 donors. (D-E) Receptor expression of CD34+ cells during cell division in the first week of culture. CD34+ cells were stained with CFSE (5 μM) and were cultured in medium containing SCF (100 ng/mL) and IFN-γ (10 ng/mL), IL-4 (10 ng/mL), or IL-5 (10 ng/mL) for 7 days. Cells were labeled with anti–Kit-PE, anti-CD34+, and propidium iodide (PI), and the mean fluorescence intensity (MFI) of Kit (D) and CD34+(E) expression of PI-negative cells in each generation was analyzed by flow cytometry. Data are representative of 5 donors. Data in graphs are presented as mean ± SEM (n = 5).

cell number in cultures containing IL-5 compared with SCF alone. However, the mast cell number in IL-5–containing cultures remained lower than in cultures containing SCF alone. By 8 weeks, SCF alone and SCF + IL-5 cultures contained significantly more total cells ($13.2 \pm 4.7 \times 10^6$ and $7.2 \pm 1.4 \times 10^6$ cells respectively; $P < .01$) than the SCF + IFN-γ and SCF + IL-4 cultures ($< 4 \times 10^5$).

Human mast cells, as identified by definitive metachromatic staining, were first obvious in the SCF-alone culture at approximately week 3 (Figure 2B) and reached a maximum at 8 weeks of culture. Total mast cell numbers in the presence of SCF + IL-5 ($6.2 \pm 1.4 \times 10^6$ cells) were lower than those observed in SCF alone ($11.2 \pm 1.3 \times 10^6$ cells). The SCF + IL-4 culture had considerably fewer mast cells through 8 weeks of culture ($4.0 \pm 2.3 \times 10^5$). SCF + IFN-γ cultures contained only 1×10^4 mast cells. Therefore, cells cultured in the presence of SCF and these T_H1 or T_H2 cytokines showed a rapid initial proliferation followed by a gradual decrease in total cell number. Mast cell numbers in cultures containing SCF + IFN-γ, IL-4, or IL-5 were 5%, 16%, and 60%, respectively, of those reached in SCF alone. Therefore, all cytokines examined decreased mast cell numbers when present over 8 weeks of culture.

Dynamics of cell division and receptor expression in the first week of culture

CFSE is a semipermeant dye that covalently binds to cytoplasmic components within the cell. On division, the dye is distributed equally between daughter cells. Therefore, each generation of cell division can be visualized as individual peaks of fluorescence (Figure 2C). After 1 week of culture, CD34+ cells grown in the presence of SCF underwent 6 divisions, giving rise to 7 distinct generations (Figure 2C). Approximately 62.4% of the progenitor cells (parent generation) underwent division (Table 1). Although fewer progenitor cells divided in the SCF + IFN-γ culture (44.2%), the cells that divided underwent 8 divisions compared with 7 in the SCF, SCF + IL-4, and SCF + IL-5 cultures (Figure 2C; Table 1). IL-4 and IL-5 induced more progenitor cells to divide (79.2% and 70.6%, respectively) than did IFN-γ, but in the IL-4 and IL-5

Table 1. CD34+ cell proliferation when cultured in SCF and IFN-γ, IL-4, or IL-5 for 7 days and 56 days

Week 1	No. generations	Division index	Proliferation index	Divided, %
Untreated	7	1.89	3.02	62.4
IFN-γ	8	1.62	3.66	44.2
IL-4	7	2.59	3.27	79.2
IL-5	7	2.13	3.02	70.6
Weeks 2-8				
Untreated	19	5.83	2.49	95.3
IFN-γ	15	1.21	2.12	57.2
IL-4	14	1.29	2.28	56.7
IL-5	17	1.5	2.43	61.7

Data presented are from 1 representative experiment (n = 5). Division index is the average number of divisions that a cell (present in the starting population) has undergone. Proliferation index is the average number of divisions cells that divided underwent. Divided percentage is the percentage of cells of the original sample that divided (assuming no cell death).

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cultures, cells underwent fewer divisions.[6] Therefore, IFN-γ inhibited progenitor cell division, but, because these cells underwent more divisions, the net result was similar to numbers of cells at week 1 cultured in SCF ± IL-4 or IL-5.

We similarly tracked the effect of IFN-γ, IL-4, and IL-5 on Kit and $CD34^+$ expression during cell division. $CD34^+$ cells were labeled with CFSE and were cultured in conditions as described here and then were isolated and stained with anti–Kit-PE and anti–CD34-APC (Figure 2D-E). Kit expression increased during 7 divisions in cultures under all conditions; however, the increase in Kit expression was significantly lower in cultures containing IL-4. Thus, IL-4 down-regulated early Kit expression.

CD34 expression in cultures containing IFN-γ rapidly decreased for 2 cell divisions, after which it continued to slowly decrease. Thus, IFN-γ led to a pronounced decrease in CD34 expression, again suggesting an effect on progenitor cells. Although IL-4 inhibited total and mast cell numbers in culture, it seemed to preserve expression of CD34.

Cell division: 2 to 8 weeks

At the end of week 1 of culture, cell numbers were increasing under all conditions. During weeks 1 to 2, cells rapidly increased in number and then decreased to numbers that roughly remained constant to 8 weeks. It was during this plateau phase that mast cells could be clearly identified at 3 to 4 weeks. To examine the complex dynamics of cultures during this period, we analyzed cell division sequentially over 1-week time periods by adding CFSE at the beginning of the time period and measuring division for the next 7 days.

Because dividing cells become increasingly autofluorescent as they mature, the coefficient of variation within each fluorescent peak increases. Statistical software was used to account for such variations and to mathematically calculate each division (approximated by lines in Figure 3A). Within the second week of culture, cells in SCF alone underwent 5 divisions, whereas cells grown in SCF + IL-5 underwent 4 divisions and cells grown in SCF + IFN-γ and SCF + IL-4 underwent only 3 divisions. Therefore, beginning in the second week of culture, IFN-γ, IL-4, and IL-5 (in the presence of SCF) inhibited division.

Cell division in each subsequent week of culture was similarly determined. These data over 8 weeks of culture were pooled and are represented in Table 1. Ninety-five percent of the cells grown in SCF alone divided, giving rise to at least 19 generations, or 18 divisions over the entire 8-week period. However, only 56% of the cells grown in SCF + IFN-γ and 57% of the cells grown in SCF + IL-4 divided, and cells grown in IL-4 gave rise to only 14 generations, though most divisions occurred within the first 2 weeks of culture. Approximately 62% of the cells grown in SCF + IL-5 divided and gave rise to 17 generations, and though most division occurred in the first 2 weeks of culture, there were 4 subsequent divisions in weeks 3 to 8. The average division index and proliferation index of cell cultures in the presence of various cytokines best reflects the rate of cell division and total cell numbers seen in Figure 2. Thus, for SCF alone, the average division and proliferation indexes were 5.83 and 2.49, respectively. For IFN-γ, the average division and proliferation indexes were 1.21 and 2.12, respectively. For IL-4, the average division index and proliferation index were 1.5 and 2.43, respectively. In every case, the average division and proliferation indexes were lower in the presence of IFN-γ, IL-4, and IL-5 compared with cells grown in SCF alone.

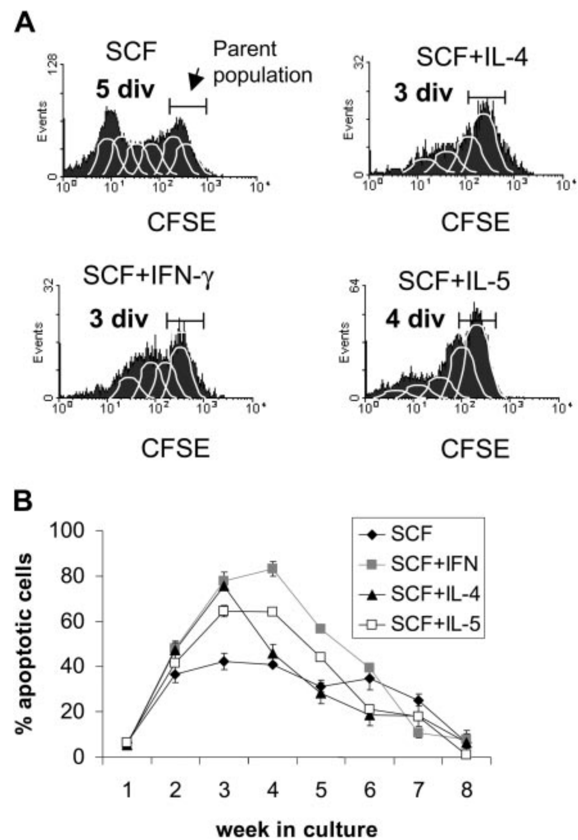

Figure 3. Analysis of cell division and apoptosis through weeks 2 to 8. (A) Cell division in the second week of culture. One-week-old $CD34^+$ cells were stained with CFSE (5 μM) and were cultured in medium containing SCF (100 ng/mL) and IFN-γ (10 ng/mL), IL-4 (10 ng/mL), or IL-5 (10 ng/mL) for 7 days. Division was analyzed by flow cytometry (n = 5). Because dividing cells become increasingly autofluorescent as they mature, the coefficient of variation within each fluorescence peak increases; statistical software was used to account for such variations and to mathematically calculate each division (represented by white lines). (B) Apoptosis of cells during entire 8-week culture. $CD34^+$ cells were cultured in the presence of SCF and IFN-γ (10 ng/mL), IL-4 (10 ng/mL), or IL-5 (10 ng/mL) for 8 weeks. Apoptosis was analyzed by propidium iodide and annexin V–FITC labeling and was analyzed on a FACScalibur. Data in graphs are presented as the mean ± SEM (n = 5).

Apoptosis: 1 to 8 weeks

Because cells continued to divide under all conditions, the stabilization of cell numbers after 2 weeks would best be explained by concurrent cell death. Thus, we determined the percentage of cells undergoing apoptosis through 8 weeks. As can be seen in Figure 3B, the number of cells undergoing apoptosis dramatically increased between 1 week and 2 weeks. The continued high rate of apoptosis through 7 weeks of culture is the most likely explanation for overall cell numbers remaining constant in the face of demonstrable proliferation (Table 1). The highest rate of apoptosis is seen in cultures containing IFN-γ or IL-4, again consistent with the observed effect of IFN-γ and IL-4 on cell number, as seen in Figure 2A. Further, in every instance, the number of apoptotic cells was greater when T_H1 and T_H2 cytokines were added to SCF-dependent cultures. The greatest number of apoptotic cells are seen in the cultures containing IFN-γ, followed by cultures containing IL-4 and IL-5.

T_H1 and T_H2 cytokines present over the term of culture differentially modulate mast cell phenotype and responsiveness

After examining how these cytokines affected long-term cultures, we asked whether the mast cell releasability at the end of culture

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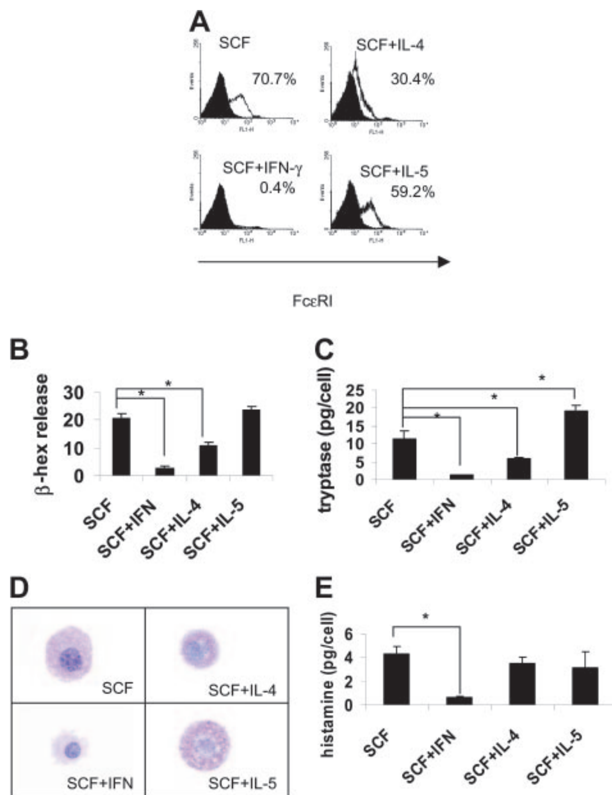

Figure 4. Receptor expression, mediator release, histamine content, and morphology of cells cultured in SCF and IFN-γ, IL-4, or IL-5. (A) CD34+ cells were cultured for 8 weeks, then stained with anti–FcεRI-FITC mAb. (Numbers indicate percentage of positive cells from 1 representative experiment; n = 5.) (B) Cells cultured in SCF (black bars), SCF + IFN-γ (light gray bars), SCF + IL-4 (white bars), and SCF + IL-5 (dark gray bars) were sensitized with IgE overnight, then stimulated with increasing doses of antigen and β-hexosaminidase (B), and tryptase (C) release was measured. (D) Cytospin preparations were stained with toluidine blue to observe mast cell morphology (from 1 representative experiment; n = 5). (E) Histamine content was measured using ELISA. Asterisks indicate significance as measured by paired 2 sample *t* test ($P > .01$). Data in graphs are presented as the mean ± SEM (n = 5).

would vary. To characterize the phenotypes of these cells, we measured FcεRI expression, mediator release, and histamine content of viable cells present in 8-week cultures that had been exposed to T_H1 and T_H2 cytokines over the entire culture period. More than 70% of cells grown in SCF alone were FcεRI positive by fluorescent-activated cell sorting (FACS) analysis (Figure 4A) and released 20% β-hexosaminidase after sensitization with antigen-specific IgE and challenge with antigen (Figure 4B). Less than 31% of cells in cultures containing IL-4 and less than 1% of cells in cultures containing IFN-γ were FcεRI positive. Cells cultured in IL-4 and IFN-γ released significantly less β-hexosaminidase (3.3% ± 1.3% and 12.6% ± 2.5%, respectively) when stimulated with antigen compared with cells cultured in IL-5, which released the same amount of β-hexosaminidase (30% ± 2.1%) as cells cultured in SCF only (27.8% ± 3.5%; $P < .01$). The tryptase content of cells cultured in SCF + IL-5 was greater than that of cells cultured in SCF alone. The tryptase content of cells cultured in IFN-γ and IL-4 was significantly less than the tryptase content of cells cultured in SCF alone ($P < .01$; Figure 4C).

Toluidine blue staining revealed that cells cultured in SCF + IFN-γ contained fewer granules and were smaller than cells cultured in SCF alone (Figure 4D). Cells grown in IL-4 were also smaller but contained some granules (Figure 4D). More than 90% of cells cultured in SCF + IL-5 were toluidine blue positive and were morphologically similar to cells cultured in SCF alone (Figure 4D). Cells cultured in SCF alone contained approximately 4.3 ± 0.6 pg histamine per cell (Figure 4E), and cells cultured in SCF + IL-4 and SCF + IL-5 also contained histamine (3.5 ± 0.5 pg/cell and 3.1 ± 1.4 pg/cell, respectively). However, cells cultured in SCF + IFN-γ contained significantly less histamine (0.6 ± 0.1 pg/cell; $P > .01$). Thus, mast cells grown in the presence of SCF + IL-4 or IFN-γ were of a different phenotype than cells cultured in SCF or SCF + IL-5. Cells grown over 8 weeks in the presence of IL-4 or IFN-γ were smaller, expressed less FcεRI, had diminished tryptase content, released less β-hexosaminidase (β-hex) when activated, and, in the case of cells grown in IFN-γ, contained fewer toluidine-positive granules. The effect of IFN-γ, IL-4, and IL-5 on mast cell numbers and phenotype are summarized in Table 2.

IL-4 potentiates division of mature mast cells and mast cell degranulation

Having examined the effect of cytokines on mast cell development throughout 8 weeks of culture, we next determined the effect of these cytokines when they were first added to mature mast cell cultures at 8 weeks. It has been reported that IL-4 potentiates mediator release and cell division when added later to mast cell cultures.[9] To test the effect of IFN-γ, IL-4, and IL-5 on mature 8-week-old mast cell cultures, we analyzed cell division and antigen-stimulated β-hexosaminidase release. Eight-week-old mast cells were stained with CFSE and were grown in SCF, SCF + IFN-γ, SCF + IL-4, or SCF + IL-5 for 7 days. Approximately 28.5% of the cells in the SCF alone or SCF + IL-5 culture divided once in this 7-day period (Figure 5A), and approximately 19.3% of the cells grown in SCF + IFN-γ divided once. However,

Table 2. Effect of SCF and IFN-γ, IL-4, or IL-5 on human mast cells after 8 weeks of culture

After 8-wk culture	SCF	SCF + IFN-γ	SCF + IL-4	SCF + IL-5
No. cells	13.2 ± 2.1 × 10^6	0.4 ± 0.23 × 10^6	2.0 ± 0.2 × 10^6	7.2 ± 0.8 × 10^6
No. mast cells	12.4 ± 3.1 × 10^6	0	1.8 ± 0.5 × 10^6	6.2 ± 0.7 × 10^6
Total divisions	19	15	14	17
CD34 expression MFI	11.2 ± 2.3	11.5 ± 2.1	12.3 ± 2.5	11.3 ± 3.1
Kit expression MFI	665.8 ± 89.9	22.9 ± 5.3	10.0 ± 3.4	653 ± 92.8
FcγRI expression % positive	70.7	0.4	30.4	59.2
β-hex release % of total	27.8 ± 3.5	3.3 ± 1.3	12.6 ± 2.5	30.0 ± 2.1
Tryptase content pg/cell	11.3 ± 2.3	1.2 ± 0.2	5.8 ± 0.4	19.3 ± 1.6
Histamine content pg/cell	4.3 ± 0.6	0.6 ± 0.1	3.5 ± 0.5	3.1 ± 1.4
Morphology	Toluidine positive many granules	Toluidine negative few granules	Toluidine positive some granules	Toluidine positive many granules
Size (diameter)	10-12 μm	3-5 μm	5-7 μm	10-12 μm

Data presented are mean ± SEM (n = 5).

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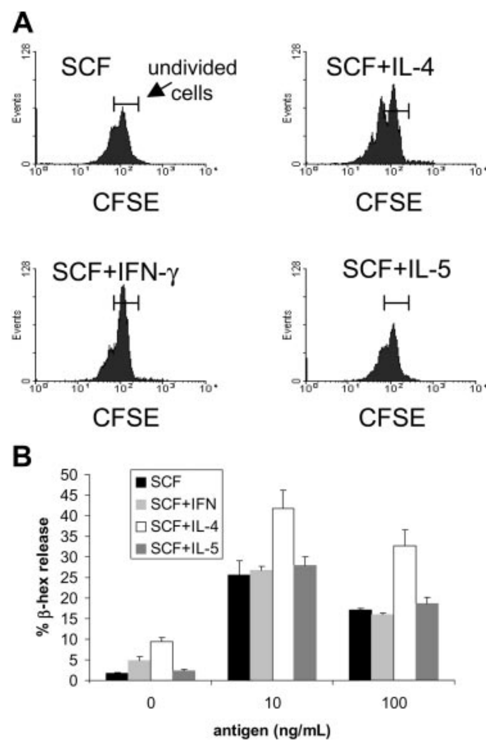

Figure 5. Effect of T_H1 and T_H2 cytokines on mature human mast cells. Mature mast cells were generated by culturing CD34+ cells for 8 weeks in 100 ng/mL SCF. (A) Mature mast cells were stained with CFSE and cultured in medium containing SCF (100 ng/mL) and IFN-γ, IL-4, or IL-5 (all at 10 ng/mL) for 7 days. Division was assessed by flow cytometry. (B) Cells grown in medium containing SCF and IFN-γ, IL-4, or IL-5 for 7 days were sensitized with IgE overnight, then stimulated with antigen and analyzed for β-hexosaminidase release. Data in graphs are presented as the mean ± SEM (n = 5).

55.3% ± 4.6% of cells treated with SCF + IL-4 divided at least twice (Figure 5A). Concurrent flow cytometry analysis of Kit expression showed that IL-4 had no effect on Kit expression (data not shown).

To examine releasability, 8-week-old mast cells pretreated with SCF, SCF + IFN-γ, SCF + IL-4, and SCF + IL-5 for 7 days were sensitized with IgE and stimulated with antigen, and β-hexosaminidase release was measured (Figure 5B). Although IFN-γ and IL-5 had no effect on human mast cell mediator release, IL-4 potentiated spontaneous and antigen-stimulated β-hexosaminidase release by approximately 40% (Figure 5B). The increase in releasability seen in cultures exposed to IL-4 was accompanied by an increase in FcεRI expression, consistent with previous reports (data not shown;[14]). Thus, of the T_H1 and T_H2 cytokines examined, IL-4 was one cytokine that clearly enhanced mast cell function when added for the first time late in cultures.

Discussion

Because mast cells are known to be critical effector cells in T_H2-dependent inflammation, it is important to understand the different effects of cytokines associated with these inflammatory states on the biologic characteristics of mast cells. Previous studies on the effect of the T_H1 cytokine IFN-γ and the T_H2 cytokines IL-4 and IL-5 in individual studies have generally reported that IFN-γ inhibits mast cell function and that IL-4 and perhaps IL-5 generally potentiate mast cell function. However, the effects of IL-4 appear to vary from study to study. Of particular difficulty is the determination of the effect of these cytokines on complex mast cell cultures in which the effects are often analyzed largely on the basis of whether mast cells can be identified by the presence of granules with characteristic staining.[4,9] To better understand the effects of T_H1 and T_H2 cytokines on mast cell growth and development, we examined the effect of each cytokine under identical conditions when added over the course of SCF-dependent mast cell cultures or to mature mast cells. The in vivo parallel would be determining whether altering specific cytokines occurs over weeks, which would alter the eventual mast cell number, and determining the effects of these cytokines on mature mast cell responses. As the data shows, the effect of these cytokines on proliferating and undifferentiated human mast cells is considerably different from their effect on mast cell proliferation and differentiation or on mature mast cells, particularly for IL-4.

The approaches we selected to determine the effects of these cytokines included a variety of techniques conventionally used to examine mast cell biology, such as determining mast cell number based on histochemical characteristics, determining expression of Kit and FcεRI and apoptosis by flow cytometry, determining cell-associated tryptase, and examining the degree of activation after aggregation of FcεRI. We also used CFSE for high-resolution trafficking of cell division within these cultures, which yielded data that included the number of cell generations, the division index, the proliferation index, and the number of cells divided. The simultaneous application of these varied technical approaches has allowed us to more carefully document the effects of cytokines on mast cells and the possible basis for the biologic effects observed.

The findings presented in this paper clarify the basis for the reports on the effect of IL-4 on mast cells that variously describe it as potentiating or inhibiting mast cell function.[2-4,9,15] Clearly, IL-4, when added throughout the process of mast cell growth and differentiation, inhibits SCF-dependent cell division (Table 1) and decreases final mast cell numbers (Figure 2). IL-4 further changes the mast cell phenotype in that mast cells cultured in the presence of IL-4 were smaller, expressed 50% less kit, and released fewer granule-associated enzymes when activated through FcεRI (Figure 4B). This is in marked contrast to the effect of IL-4 when it is added to a later culture containing differentiated mast cells. Here, IL-4 was unique among the cytokines examined in stimulating further mast cell division (Figure 5A), up-regulating FcεRI (data not shown), and potentiating mast cell degranulation (Figure 5B). These data clearly establish that it cannot be assumed that a T_H2 cytokine will under various conditions always enhance mast cell function, and they further underscore the importance of IL-4 in potentiating the mast cell function of mature mast cells.

IFN-γ is reported to inhibit mast cell outgrowth in cultures if present throughout the period of culture. Our data are consistent with these observations. We found that IFN-γ inhibits mast cell numbers and influences mast cell phenotype and that the few hypogranular, small mast cells that remain release few granule mediators after receptor aggregation (Figure 4). However, the effect of IFN-γ on mature mast cells was unexpected. Here, IFN-γ had relatively little effect (Figure 5), even though mature mast cells expressed IFN-γR (Figure 1). This observation, however, could mean that mast cell function could be expected to be maintained in type 1 inflammation, as others have suggested.[12,16,17]

Among the cytokines tested, IL-5 in essence served as a control in all parameters measured, including receptor expression, β-hex release, histamine content, and tryptase content. Results with IL-5 present differed only slightly from results when only SCF was present. It is tempting to correlate this lack of effect of IL-5 with the

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observation that human mast cells themselves make IL-5 but synthesize and release little IL-4 or IFN-γ.[18,19]

By comparison, IFN-γ and IL-4 had profound effects on cell division. IFN-γ inhibited the division of early CD34$^+$ progenitors within the first week of culture (Figure 2C) and subsequently promoted the apoptosis of older Kit$^+$ cells (Figure 3B). Specifically, IFN-γ inhibited the division of a CD34$^+$/Kit^{low} subpopulation in the first week of culture and blocked all cell division at 2 weeks of culture. Whereas it has been shown that mature 9-week-old cord blood–derived mast cells incorporate a substantial amount of thymidine in response to SCF,[3] CFSE measurements showed that mature 8-week-old peripheral blood–derived human mast cell divided only once in 7 days in response to SCF (Figure 5A). This may explain why mature cord blood–derived mast cell SCF-driven proliferation is inhibited by IFN-γ but mature peripheral blood derived mast cell proliferation is not. Therefore, IFN-γ inhibited peripheral blood–derived mast cell growth primarily by blocking early cell division.

In contrast to IFN-γ, IL-4 inhibited mast cell differentiation and survival by decreasing early Kit expression and promoted an immature CD34$^+$ phenotype. Some reports have suggested that IL-4 has an opposing effect on human mast cells, depending on their source and age. For example, IL-4 promotes SCF-driven proliferation of 4-week-old cord blood–derived mast cells but has no affect on 9- to 10-week-old cord blood–derived mast cells.[2-4] Yet, IL-4 enhances SCF-driven proliferation of intestinal mast cells.[20] We have also observed the differential effects of IL-4 on progenitor and mature mast cells because IL-4 inhibited early progenitor division but promoted the division of 8-week-old mast cells (Figure 3A). These differential effects were apparently mediated by decreasing Kit expression because IL-4 decreased Kit expression of CD34$^+$ progenitors (Figure 2D) but had no effect on mature mast cell Kit expression (data not shown).

There is an increasing appreciation of the biologic role of mast cells in T_H1- and T_H2-dependent inflammation. The data presented in this paper clearly show that the behavior and characteristics of mast cells are altered when they are exposed to IFN-γ or IL-4. When IFN-γ and IL-4 are continuously present during mast cell development, both cytokines lead to a decrease in mast cell number and mast cell releasability, though here the effect of IFN-γ is more dramatic. If mast cells are of benefit to inducing[21,22] and resolving[23] inflammation, the loss of mast cell function in chronic inflammation, whether T_H1 or T_H2 dependent, might be considered to be particularly detrimental to the host and raises the possibility that strategies to increase mast cell number and function in chronic inflammation may be paradoxically beneficial. Conversely, if mature mast cells are exposed to IL-4, the beneficial effect would be to counteract the increase in mast cell number and function.

The data presented in this paper may further explain why IL-4 receptor antagonists inhibit allergic symptoms in murine asthma models[24] and clinical trials in which persistent IL-4 production may sustain elevated mast cell numbers in the lung. Thus, administering IL-4 receptor antagonists over weeks to months might, from the data presented in this paper, be expected to decrease mast cell proliferation from progenitors and to decrease pulmonary mast cells and responses. Blocking IL-4 signaling by administering IL-4 mutant molecules or soluble IL-4 receptors also ablates asthma symptoms in patients with mild to moderate asthma.[25] In this instance, the effect may be on mature tissue mast cells and may decrease their proliferation. Similarly, IFN-γ has been shown to inhibit pulmonary allergic responses.[26] These effects of IFN-γ may be mediated, in part, by a decrease in mast cell numbers, consistent with the data presented herein. Some evidence suggests that recombinant human IFN-γ may similarly decrease mast cell numbers in patients with mastocytosis, although these patients often have relapses because of anti–rhIFN-γ production.[27]

The multitechnical approach applied in this report, including high resolution tracking of cell division, thus appears to yield data that anticipate possible outcomes of in vivo strategies that manipulate the effects and/or levels of cytokines, and that differ from the simple concept that T_H1 cytokines down-regulate T_H2 inflammation, while T_H2 cytokines potentiate such a response.

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