Neutralizing Endogenous IL-6 Renders Mast Cells of the MC \( \text{T} \) Type from Lung, but Not the MC \( \text{TC} \) Type from Skin and Lung, Susceptible to Human Recombinant IL-4-Induced Apoptosis

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Neutralizing Endogenous IL-6 Renders Mast Cells of the MC\textsubscript{T}\textsuperscript{\textsubscript{1}} Type from Lung, but Not the MC\textsubscript{T\textsubscript{C}} Type from Skin and Lung, Susceptible to Human Recombinant IL-4-Induced Apoptosis\textsuperscript{1}

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Human cord blood-derived mast cells undergo apoptosis upon exposure to recombinant human (rh)IL-4 and become resistant to rhIL-4-induced apoptosis when cultured in the presence of rhIL-6. The current study extends these effects of rhIL-4 to different populations of human mast cells, namely fetal liver-derived mast cells, lung-derived mast cells, and skin-derived mast cells. Endogenous production of IL-6 appears to protect fetal liver-derived mast cells and those of the MC\textsubscript{T}\textsuperscript{\textsubscript{1}} phenotype from rhIL-4-mediated apoptosis, because neutralization of IL-6 renders these mast cells sensitive. In contrast, mast cells of the MC\textsubscript{T\textsubscript{C}} phenotype from skin and lung were resistant to IL-4-mediated apoptosis, even after neutralization of endogenous IL-6. MC\textsubscript{T\textsubscript{C}} cells were CD124\textsuperscript{high} 1, whereas those of the MC\textsubscript{T} cells were CD124\textsuperscript{low}. These observations extend the phenotypic differences between MC\textsubscript{T} and MC\textsubscript{T\textsubscript{C}} types of human mast cells to include different functional responses to IL-4. The Journal of Immunology, 2004, 172: 593–600.

\textsuperscript{1} Abbreviations used in this paper: CB-MC, cord blood-derived mast cells; FL-MC, fetal liver-derived mast cells; LM-MC, lung-derived mast cells; SK-MC, skin-derived mast cells; FL-MC, fetal liver-derived mast cells; SCF, stem cell factor; TRITC, tetramethylrhodamine isothiocyanate; CPSR, controlled process serum replacement.

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0.1 mM nonessential amino acids, 10 mM HEPES, 50 μM 2-ME, 200 μM penicillin, and 100 μg/ml streptomycin. Cells were dispensed into 24-well plastic tissue culture plates (Costar, Cambridge, MA) at 10⁵ cells/2 ml/well in the presence of rhSCF (100 ng/ml; a gift from Aigen, Thousand Oaks, CA) alone or with different concentrations of rhIL-4 (a gift from Aigen). Half of the culture medium was replaced once a week.

Human fetal livers, 16–21 wk of gestational age, were obtained from therapeutic abortions. Cells were dispersed and placed into culture as described (28). Each liver was washed with sterile PBS, minced, and sieved (80 mesh). Dispersed cells were separated by density-dependent sedimentation, and resuspended at 2–4 × 10⁶ cells/ml of complete DMEM, which is DMEM supplemented with 2 mM L-glutamine, 0.1 mM nonessential amino acids, 10 mM HEPES, 50 μM 2-ME, penicillin (100 U/ml), streptomycin (50 μg/ml), and 10% CRSP-3, and plated in 24-well tissue culture plates with rhSCF (50 ng/ml). Half of the culture medium was replaced weekly during the first 2 wk of culture and biweekly thereafter.

Surgical lung tissue samples were obtained through the Cooperative Human Tissue Network (Columbus, OH), the National Disease Research Interchange Center (Philadelphia, PA), or from the Departments of Surgery and Pathology at Virginia Commonwealth University. Cells were prepared as previously described (29). Briefly, samples were minced, digested twice using a combination of type IA collagenase and type I-S hyaluronidase (Sigma-Aldrich), and sieved (80 mesh).

Pellet suspensions of HMC-1 were obtained after breast resection, mastectomy, or abdominoplasty through the Cooperative Human Tissue Network, the National Disease Research Interchange Center or from the Departments of Surgery and Pathology at Virginia Commonwealth University. Mast cells were isolated as previously described (30). Briefly, after removal of the s.c. fat, skin tissue was cut into fragments and incubated in a solution of HBBS containing 2 mg/ml collagenase (1.5 mg/ml), type 1-S hyaluronidase (0.7 mg/ml), type 1 D Nase (0.3 mg/ml; Sigma-Aldrich), 1% FCS, and 1 mM CaCl₂ for 2 h at 37°C with constant stirring. The dispersed cells were separated from residual tissue by filtration through an 80 mesh sieve and suspended in HBBS containing 1% FCS and 10 mM HEPES (washing buffer). The remaining tissue was subjected to an additional digestion, as previously described.

Erythrocytes were eliminated from the skin and lung cell preparations by centrifugation over a 75% and 65% Percoll cushion, respectively. To reduce the likelihood of any microbial contamination, cells were typically layered over a solution of PBS containing 20% human AB serum (Sigma-Aldrich) and centrifuged at low speed (500 x g) for 20 min at room temperature. Pelleted lung cells (15–20% mast cells) were then cultured in the same medium used to culture cord blood cells, whereas skin cells were cultured in AIM-V medium. All cells were cultured in a Nuaire incubator (Nuaire, Plymouth, MN) at 37°C in 6% CO₂, harvested and counted (viability by trypan blue dye exclusion), and then subjected to FACs analysis, cytotoxic centrifugation for immunocytochemistry or apoptosis determination.

In some experiments in which IL-6 neutralizing mAb was used, cells were first incubated in the presence of rhSCF alone, in the presence or absence of mouse anti-human IL-6 mAb (5 μg/ml; BioSource International, Camarillo, CA), or in the presence or absence of the isotype-matched negative control (5 μg/ml; MOPC-31C, mouse IgG1; Sigma-Aldrich). After 5 days, cells from each lot were counted (viability as previously mentioned) and then placed back into culture at 10⁵ cells/ml/well for an additional period of 4 days in the presence or absence of the anti-IL-6 or MOPC-31C mAbs as previously described, and in the presence or absence of rhIL-4 (20 ng/ml). At the end of the culture period, cells from each lot were counted (viability as previously mentioned), and then subjected to immunocytochemistry to identify MC₆ and MC₇ cells, to acidic toluidine blue staining, and to an assessment of apoptosis. In three experiments performed with different preparations of Sk-MC, soybean trypsin inhibitor (Sigma-Aldrich) was included in the cultures at 100 μg/ml, both during the IL-6 neutralization and during the IL-4 challenge phases of the experiment. No effect of soybean trypsin inhibitor on the viability or growth of Sk-MC was apparent during the next 9 days of culture.

Immunocytochemistry
Cytocentrifuge preparations of cells were fixed in methanol containing 0.6% formaldehyde at room temperature with H₂O₂ for 30 min at 4°C until used. Slides were labeled with biotin-conjugated B7 (or biotin-MOPC-31C, as an isotype-matched negative control), a mouse IgG1 anti-chymase mAb, and alkaline phosphatase-conjugated G3 (or alkaline phosphatase-conjugated MOPC-31C), as previously described (31), to identify MC₆ and MC₇ cells (chymase-positive and tryptase-positive) that stain reddish brown with 3-aminobenzyl carbazole, and MC₆ cells (tryptase-positive and chymase-negative) that stain blue with Fast Blue RR (Sigma-Aldrich).

Detection of apoptotic mast cells
The identification of apoptosis at a single cell level on slides was performed using the In Situ Apoptosis Detection kit according to the manufacturer (TACS TdT kit; R&D Systems, Minneapolis, MN) as described (1). The principle of the technique is to detect DNA fragments generated during apoptosis. Biotinylated nucleotides are incorporated into the ends of DNA fragments using TdT. Labeled ends are detected using streptavidin–HRP and the peroxidase substrate, TACS Blue Label, with which nuclei from apoptotic cells stain blue. Pelleted cells were gently suspended in 10% neutral-buffered formalin at a concentration of 10⁶ cells/ml and incubated for 10 min at room temperature. Fixed cells were centrifuged and resuspended in 80% ethanol. At room temperature, 50 μl of this cell suspension was dropped onto a glass slide, air-dried for 5 to 10 min, incubated with 70% ethanol for 5 min, air-dried for 1 h, and treated with 1 μg of proteinase K for 5 min after rehydration. To quench endogenous peroxidase activity, slides were placed in a Coplin jar containing a solution of 2% H₂O₂ for 5 min at room temperature. Slides were then transferred to a solution of 1× labeling buffer containing TdT dNTPs (1× final concentration) and adjusted to 1 mM Co²⁺. Labeling was initiated by addition of 15 U of TdT per sample. The labeling reaction was allowed to proceed for 15 min at 37°C, and was then stopped by transferring the slides into a solution of TdT stop buffer. Slides were then rinsed for 1 min at room temperature in water, incubated with 10 ng of Streptavidin-HRP for 10 min at room temperature, washed twice with water, and incubated with 50 μl of TACS Blue Label. In the case of human lung mast cells, an amplification step was added to the regular protocol: slides were first incubated in the presence of mouse monoclonal anti-peroxidase asciites fluid for 1 h at room temperature (2 μg/ml; Sigma-Aldrich). After two washes in water of 1.5 min each, goat anti-mouse IgG Ab-peroxidase at a concentration of 4 μg/ml was added to the slide for 1 h at room temperature (TAGO, Burlingame, CA). After two washes in water, slides were incubated with 50 μl of TACS Blue Label. Reactions were stopped when the parallel staining of a control slide provided by the manufacturer and monitored microscopically became positive, typically after 7 min at room temperature. Slides then were washed four times in water, processed for the immunofluorescence staining of tryptase using tetracyethylrhodamine isothiocyanate (TRITC)-G3 and analyzed. For each experiment, four different slides of each culture condition were processed and a total of 100–200 cells were analyzed per slide. DNase-free deionized water was used throughout the procedure.

Immunofluorescent staining
After the apoptosis staining, slides were processed for the immunofluorescence staining of tryptase using TRITC-G3 (or a TRITC-MOPC-31C isotype-matched negative control), as described (1) or staining for chymase using Alexa 488-B7 (or an Alexa 488-MOPC-31C isotype-matched negative control) at a concentration of 10 μg/ml. The Alexa 488 conjugates were prepared as recommended by the manufacturer (Molecular Probes, Eugene, OR). After an overnight incubation at 4°C, or 1 h at 37°C, slides were washed in Tween 20 plus TBS and analyzed using a BX50 fluorescence microscope with a PM-30 exposure control attachment (Olympus Optical, Tokyo, Japan).

Measurement of immunoreactive IL-6 in supernatants
IL-6 was quantified by a sandwich ELISA, according to the manufacturer’s instructions (BioSource International). The sensitivity of the assay was 7.8 pg/ml.

Flow cytometry
Human Lu-MC and Sk-MC were analyzed for expression of surface Kit using the mouse PE-labeled YB5.B8 (IgG1) mAb and a mouse biotin-labeled anti-human IL-4 (r-chain) IgG1 mAb (BD PharMingen, San Diego, CA). Cells (10⁶) were washed once in PBS and incubated in DMEM containing 10% human AB serum for 30 min at 4°C. Cells were washed twice at 4°C in 1% BSA and were incubated at 4°C for 30 min in 100 μl of PBS containing 1% BSA and YB5.B8-PE (1 μg/ml) and anti-IL-4-biotin (10 μg/ml), or an IgG1 isotype-matched, labeled negative control mAb, MOPC-31C (1 or 10 μg/ml). Cells were washed twice and incubated for 30 min at 4°C in 100 μl of streptavidin-fluorescein (1:1000; DAKO, Copenhagen, Denmark). After two washes, cells were analyzed with a FACScan, using the CellQuest software (BD Biosciences, San Jose, CA).
Results

RhIL-4 induces apoptosis of human CB-MC, but not of human FL-MC, Lu-MC, or Sk-MC

IL-4 was previously shown to cause apoptosis of rhSCF-dependent CB-MC, but not of rhSCF-dependent FL-MC and Lu-MC, because FL-MC and Lu-MC numbers were not altered by exposure to rhIL-4 (1). These data have been extended and confirmed as shown in Fig. 1. CB-MC, FL-MC, Lu-MC, and Sk-MC were exposed to rhSCF (100 ng/ml) in the presence and absence of rhIL-4 (20 ng/ml) for 6 days, and assessed for survival and mast cell apoptosis by double-labeling the cells for DNA fragmentation and tryptase. CB-MC exposed to rhIL-4 declined in number by over 90% compared with those not exposed to rhIL-4 and to those just before addition of rhIL-4. Furthermore, 94% of the tryptase-positive cells remaining were apoptotic. In contrast, rhIL-4 did not cause a significant loss of FL-MC, Lu-MC, or Sk-MC, and no increases in the percentages of apoptotic cells were evident.

FL-MC, Lu-MC, and Sk-MC preparations spontaneously secrete IL-6

Because rhIL-6 protects CB-MC against IL-4-mediated apoptosis (1), differences in endogenous IL-6 production between different mast cell preparations could explain different susceptibilities to IL-4. Media from cultures of CB-MC, FL-MC, Lu-MC, and Sk-MC were assessed for the presence of immunoreactive IL-6 as shown in Fig. 2. IL-6 was undetectable (<7.8 pg/5 x 10⁶ cells/ml) in medium from CB-MC cultures collected 6 days after a medium change, both in the presence of rhSCF alone or in combination with rhIL-4. In contrast, substantial levels of immunoreactive IL-6 were found in the medium from cultures of FL-MC (median = 65 pg/ml, n = 54), and addition of rhIL-4 to these cultures induced an ~8-fold increase in the mean level of IL-6 (median = 1030 pg/ml; p < 0.001, Mann-Whitney Rank Sum, n = 44). Freshly dispersed Lu-MC and Sk-MC (20–30% mast cells) also contained substantial amounts of immunoreactive IL-6, the median values being 7300 ng/ml (n = 4) and 1995 ng/ml (n = 4) (Fig. 2). RhIL-4 had no significant effect on IL-6 levels produced by these mast cell preparations. These results raised the possibility that endogenous IL-6 was protecting FL-MC, Lu-MC, and Sk-MC from rhIL-4-induced apoptosis.

Neutralizing endogenous IL-6 makes FL-MC and Lu-MC, but not Sk-MC, more susceptible to IL-4-mediated apoptosis

To test whether endogenous IL-6 affected the apoptotic response of FL-MC and Lu-MC to rhIL-4, mast cells in culture with rhSCF were first treated for 5 days with a neutralizing anti-IL-6 mAb or the isotype-matched negative control mAb. Medium from the cultures in which IL-6 was neutralized did not display detectable levels of IL-6 by ELISA (data not shown), indicating that free IL-6 had been bound by the neutralizing anti-IL-6 mAb. Cells from the different experimental groups were then placed back into culture with rhSCF, the same mAb used during the previous 5 days of culture and in the presence or the absence of rhIL-4 for an additional period of 4 days. As shown for Lu-MC preparations in Fig. 3, neutralizing IL-6 had no significant effect on the total numbers of viable cells recovered (upper panel). However, IL-6 neutralization led to a significant decline in numbers of mast cells detected after exposure to rhIL-4 (Fig. 3, middle panel), apparently by rendering these Lu-MC responsive to the apoptotic effect of rhIL-4 (Fig. 3, lower panel). By the 4th day of culture with rhIL-4, a 69% decline in the total number of mast cells was observed, and among the remaining mast cells, >70% were apoptotic. Examples of an apoptotic Lu-MC are shown in Fig. 4, A and B, and of nonapoptotic mast cells in Fig. 4, C and D.

Similar experiments were performed using FL-MC (~95% purity). Likewise, neutralization of IL-6 in cultures of FL-MC sensitized these cells to the apoptotic effect of rhIL-4 (n = 2). In two independent cultures, 50 and 55% of the FL-MC remaining after exposure to rhIL-4 for 4 days were apoptotic when IL-6 had been neutralized, but <5% were apoptotic in the absence of IL-6 neutralization (non-immune Ab).

Freshly dispersed and partially purified Sk-MC also were challenged with rhIL-4 following neutralization of endogenous IL-6. As shown in Fig. 5, total cell numbers (upper panel) and Sk-MC numbers (middle panel) failed to significantly decrease, and the percentage of mast cell apoptosis (lower panel) failed to increase in response to rhIL-4 after IL-6 neutralization. These results, using anti-tryptase mAb to identify mast cells, were confirmed using an Alexa 488 conjugate of anti-chymase mAb in three independent cultures of Sk-MC preparations in which neutralizing anti-IL-6 mAb or an irrelevant isotype-matched negative control was used for 5 days, following which the presence or absence of rhIL-4 was included for an additional 4 days. Light microscopy was used to
neutralizing endogenous IL-6 renders Lu-MC sensitive to rhIL-4-induced apoptosis. Lu-MC of 20–30% purity were placed into culture for 5 days with either anti-IL-6 Ab, control IgG or no Ab, and then recultured for 4 days at $5 \times 10^5$ cells/ml under the same condition with rhIL-4 (20 ng/ml) being either included or omitted. RhSCF (100 ng/ml) was added to all cultures. Each bar and error bar show mean and SD values ($n = 4$). An ANOVA on Mann-Whitney Rank Sum Test was used to compare total cell numbers, mast cell numbers, and the percentages of mast cells that were apoptotic; followed by an all pairwise multiple comparison procedure (Student-Newman-Keuls Method) to evaluate significant results. $*, p < 0.05$.

Lu-MC of the MC<sub>T</sub> type become sensitive to IL-4-mediated apoptosis after IL-6 neutralization, whereas those of the MC<sub>TC</sub> type remain viable

In the experiments described in this study with Lu-MC, each experimental group of cells was also subjected to cytocentrifugation and sequential immunocytochemical staining for chymase and tryptase. As shown in Fig. 6, exposure to rhIL-4 after neutralizing endogenous IL-6 did not significantly change the numbers of total cells (Fig. 6A) and the numbers of viable lung-derived MC<sub>TC</sub> cells (Fig. 6C), whereas the percentage of mast cells (Fig. 6B) decreased by 66% and the numbers of lung-derived MC<sub>T</sub> cells (Fig. 6D) dramatically decreased by ~99%. Thus, MC<sub>T</sub> cells, whether in lung or skin, resist the apoptogenic effect of rhIL-4 regardless as to whether IL-6 was neutralized.

**Discussion**

The novel finding of the current study is that the MC<sub>T</sub> type of mast cell from lung is sensitive to IL-4-induced apoptosis, whereas MC<sub>TC</sub> cells from both skin and lung are resistant. Most in vitro derived mast cells from cord blood and fetal liver progenitors also are sensitive. However, endogenously produced IL-6 by preparations of FL-MC and Lu-MC must be neutralized before this response to rhIL-4 occurs in vitro, a second important finding that explains why apoptosis of these mast cells is not observed when exposed to rhIL-4 without first neutralizing IL-6. In contrast, preparations of CB-MC do not produce detectable levels of IL-6; most CB-MC become apoptotic when exposed directly to rhIL-4, but are protected if first treated with exogenous rhIL-6 (1). IL-6 also attenuates apoptosis occurring after withdrawal of rhSCF (32).
Human mast cells also are capable of producing IL-4. Both mRNA and protein for IL-4 have been localized to mast cells in skin (41, 45-47), upper (36, 48, 49) and lower (41, 46, 50-53) airways, conjunctiva (39), and intestine (54), and in mast cells derived from cord blood progenitors with rhSCF and rhIL-6 after their activation with phorbol myristic acid and A23187 (55). IL-4 production by nasal mast cells during perennial allergic rhinitis has been associated with elevated production of IgE by B cells (48). Release of IL-4 by an activated mast cell, in theory, could induce apoptosis in that mast cell or in neighboring ones in the absence of IL-6. Thus, the time course of production of IL-4 and IL-6 by mast cells and by other cell types nearby would influence whether apoptosis by this pathway occurs in vivo.

Of potential interest to the IL-4-mediated pathway of mast cell apoptosis is the report that rhIL-4 down-regulates IL-6 production by preparations of intestinal mast cells (40). This contrasts with our finding that rhIL-4 up-regulated IL-6 production by FL-MC and had no significant effect on IL-6 production in preparations of Lu-MC or Sk-MC. However, in the intestinal preparations was the cell or cells producing IL-6 precisely determined. Preliminary data obtained with Sk-MC and Lu-MC preparations show anti-trypsin and anti-IL-6 double-labeling of a portion of the mast cells, indicating that mast cells are at least one source of this cytokine (data not shown). The effect of IL-4 on IL-6 production also has been studied in cells other than mast cells. RhIL-4 has been shown to up-regulate IL-6 production in keratinocytes (56), endothelial cells (57), fibroblasts (58), bronchial epithelial cells (59), and LPS-activated HUVEC cells (60). The IL-1β-induced IL-6 synthesis by human bone marrow stromal cells is also up-regulated by IL-4 (61). By increasing local levels of IL-6, IL-4 might modulate the susceptibility of mast cells to apoptosis.

RhlIL-4 was previously demonstrated to down-regulate IL-4R on CB-MC, converting them from IL-4Rhigh to IL-4Rlow (1). Interestingly, MCTC skin cells also are IL-4Rlow, unlike MC T lung cells that are IL-4Rhigh. This differential expression of IL-4R on Lu-MCT cells vs Sk-MCT cells might reflect their differential susceptibility to rhIL-4. However, why neutralization of endogenous IL-6 did not render MCTC cells from skin and lung susceptible to rhIL-4-induced apoptosis is uncertain. Chymase has been reported to be able to alter the biological activity of different cytokines, such as TGF-β and SCF (62, 63). Whether destruction of rhIL-4 activity by released chymase might explain the resistance of MCTC cells to rhIL-4-mediated apoptosis is one possibility to consider. However, it seems unlikely that destruction of IL-4 by secreted chymase would account for this selectivity. Only a minor percentage of Lu-MC in suspension culture is of the chymase-containing MCTC type, yet resistance to rhIL-4 was selective for MCTC cells. Further, inclusion of soybean trypsin inhibitor to neutralize released chymase (and cathepsin G) during the 9 day culture of the MCTC cells from skin with anti-IL-6 failed to result in apoptosis when IL-4 was added. Also, no alteration in surface IL-4R levels was detected by flow cytometry after IL-6 was neutralized. Thus, whether the difference in surface IL-4R levels between MCT and

Whether the small portion of FL-MC or CB-MC that resist rhIL-4-mediated apoptosis are enriched for MCTC cells or their progenitors was not addressed in this study. However, surviving Lu-MC are almost entirely those of the MCTC type, as are essentially all mast cells from normal skin. The fact that MCTC cells from lung resist rhIL-4 whereas lung MCT cells succumb to rhIL-4 indicates an intrinsic difference between these types of mast cells. This functional difference between MCT and MCTC types of human mast cells extends the phenotypic distinctions between MCT and MCTC cells previously described (33-35).

Human mast cells are a known source of IL-6. Immunoreactive IL-6 has been detected in situ in many of the mast cells located in the upper (36, 37) and lower (38) airways, conjunctiva (39), and intestine (40), but not in those of skin (41, 42). In both the upper and lower airways, IL-6 immunoreactivity was detected selectively in mast cells lacking chymase, i.e., the MCT type of mast cell (41). Whether this distribution of IL-6 reflected a fundamental difference between the MCT and MCTC types of mast cells, or was a result of the activation state of the cells in vivo was uncertain. For example, IL-6 was detected in Sk-MC after their IgE-dependent activation (42). The mast cell leukemia cell line, HMC-1, expresses no IL-6 mRNA and protein during standard culture conditions (42-44), but does express IL-6 mRNA and protein after stimulation with phorbol myristic acid and the calcium ionophore A23187 (42). Thus, all human mast cells may be capable of producing IL-6, which in turn can provide autocrine and paracrine protection from IL-4-induced apoptosis.
MC<sub>TC</sub> cells explains their different response to rhIL-4 remains to be fully understood.

MC<sub>T</sub> mast cells appear to be selectively recruited to sites of inflammation in mucosal surfaces such as the conjunctiva (64), in atopic dermatitis skin (65) and in rheumatoid synovium (66), whereas the MC<sub>TC</sub> type normally predominates at these sites and has been associated with fibrosis in rheumatoid synovium (66). The potential susceptibility of MC<sub>T</sub> cells at sites of inflammation to feedback regulation by IL-4 produced by mast cells or by other cell types, e.g., basophils and T lymphocytes, is of interest. Although considered in this study only in the context of mast cells, cross-talk between IL-4 and IL-6 may represent an important regulatory loop for IgE-mediated allergy. IL-4 facilitates development of Th2 cells and is important for B cell switching to IgE production, whereas IL-6 can augment IgE production by B cells. Thus, IL-6 and IL-4 released by mast cells may orchestrate various facets of immunity and hypersensitivity, as well as affect mast cells themselves.

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