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Inhibition of Kit Expression by IL-4 and IL-10 in Murine Mast Cells: Role of STAT6 and Phosphatidylinositol 3’-Kinase

Paria Mirmonsef, Christopher P. Shelburne, C. Fitzhugh Yeatman II, Hey Jin Chong, and John J. Ryan

The c-kit protooncogene encodes a receptor tyrosine kinase that is known to play a critical role in hemopoiesis and is essential for mast cell growth, differentiation, and cytokine production. Studies have shown that the Th2 cytokine IL-4 can down-regulate Kit expression on human and murine mast cells, but the mechanism of this down-regulation remains unresolved. Using mouse bone marrow-derived mast cells, we demonstrate that IL-4-mediated Kit down-regulation requires STAT6 expression and phosphotidylinositol-3’-kinase activation. We also find that the Th2 cytokine IL-10 potently down-regulates Kit expression. IL-4 enhances IL-10-mediated inhibition in a manner that is STAT6 independent and phosphotidylinositol-3’-kinase dependent. Both IL-4- and IL-10-mediated Kit down-regulation were coupled with little or no change in c-kit mRNA levels, no significant change in Kit protein stability, but decreased total Kit protein expression. Inhibition of Kit expression by IL-4 and IL-10 resulted in a loss of Kit-mediated signaling, as evidenced by reduced IL-13 and TNF-α mRNA induction after stem cell factor stimulation. These data offer a role for STAT6 and phosphotidylinositol-3’-kinase in IL-4-mediated Kit down-regulation, coupled with the novel observation that IL-10 is a potent inhibitor of Kit expression and function. Regulating Kit expression and signaling may be essential to controlling mast cell-mediated inflammatory responses. The Journal of Immunology, 1999, 163: 2530–2539.

Mature mast cells are distributed throughout all vascularized tissues, including skin, gastrointestinal, respiratory, and urogenital tracts. They are known to play a pivotal role in allergic disease and in inflammatory responses to some bacterial pathogens (reviewed in Refs. 1 and 2) and intestinal helminths (3). Mast cells have been hypothesized to act as “sentinel” cells for early innate immune responses (reviewed in Ref. 1).

Mast cell development, proliferation, and function require proper expression and signaling of several cytokine receptors, among which the Kit tyrosine kinase is perhaps best studied. Mast cell development is drastically reduced in animals with mutations in genes encoding Kit or its ligand, stem cell factor (SCF). These animals also suffer from macrocytic anemia, loss of melanocyte migration, and sterility (4–7). Thus Kit expression and signaling play an essential role in the development of multiple organs.

In addition to its role in mast cell ontogeny, Kit expression is regulated on mature mast cells. Binding by its ligand, SCF, triggers ubiquitin-mediated degradation of Kit expression (8). IL-4 is also known to inhibit Kit expression on mature mast cells (9–12). However, neither the mechanism nor the functional significance of IL-4-mediated Kit down-regulation has been fully determined. Previous reports have argued for either loss of Kit mRNA expression or loss of Kit protein following IL-4 stimulation (11, 12).

IL-4 is produced by T lymphocytes (13), mast cells (14), and basophils (15) following receptor-mediated activation. It exerts a number of biological activities within the hemopoietic system. Previous studies have shown that IL-4 is a potent regulator of myeloid progenitor and mast cell growth, proliferation, and gene regulation (13, 16–18). It also directs Th2 cell development and induces Ig class switching and IgE production by B cells (reviewed in Ref. 19). IL-4 mediates its biological responses by binding to a high-affinity receptor complex, which has a wide distribution on hemopoietic and nonhemopoietic cells (reviewed in Ref. 20).

Signaling via the IL-4R occurs through distinct pathways, which predominantly promote growth or gene expression (21–24). Many IL-4-mediated effects on gene expression require activation of the transcription factor STAT6. Animals genetically deficient in STAT6 fail to develop Th2 cells, synthesize IgE, or induce the expression of CD23 and MHC class II molecules in response to IL-4 (25–27). Thus, STAT6 is considered an essential positive gene regulator of IL-4-mediated responses (reviewed in 28–30). However, recent data also support a role for STAT6 as a negative regulator of gene expression (31, 32).

In addition to STAT6, IL-4-mediated responses through activation of other pathways including phosphotydylinositide-3’-kinase (PI3K) (reviewed in Refs. 33 and 34). PI3Ks are ubiquitously expressed enzymes that phosphorylate the D3 position of inositol rings (reviewed in Ref. 35). Several classes of PI3Ks have been identified based on their sequence similarities and substrate selectivity. The best studied of PI3Ks consist of a 110-kDa catalytic subunit and a tightly associated regulatory subunit of 85, 55, or 50 kDa. PI3K has been proven to be a critical signaling intermediate in response to a wide variety of extracellular stimuli and is activated by a number of proteins containing intrinsic or associated tyrosine kinase activities. PI3K expression has been demonstrated to be essential in many cellular processes.

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2 Address correspondence and reprint requests to Dr. John J. Ryan, Department of Biology, Box 842012, Virginia Commonwealth University, Richmond, VA 23284-2012. E-mail address: jjryan@saturn.vcu.edu
3 Abbreviations used in this paper: SCF, stem cell factor; PI3K, phosphotydylinositide-3’-kinase; BMNC, bone marrow-derived mast cells; cRPMI, complete RPMI 1640 medium; CML, conditioned medium; WT, wild type; RPA, RNA protection assay; IRS, insulin receptor substrate; MFI, mean fluorescent intensity; HBS, HEPES-buffered saline.

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responses, such as rearrangement of cytoskeletal actin, movement of organelle membranes, and chemotaxis (36). It has recently been shown that disruptions in the p85α subunit, the most abundantly expressed regulatory isoform of PI3K, severely affect B cell development and proliferation while disruption of the entire p85α gene (which also encodes p55κ and p50α) can be lethal (37, 38).

Like IL-4, IL-10 is also produced by Th2 cells, as well as activated macrophages, B cells, activated mast cells, and keratinocytes (reviewed in Ref. 39). IL-10 possesses potent negative regulatory activities, inhibiting the production of IFN-γ by lymphocytes, TNF-α and IL-6 by macrophages, monocytes, and peritoneal mast cells, and TNF-α and GM-CSF production by eosinophils (40–46). However, IL-10 has also been reported to synergize with IL-3 and IL-4 in supporting mast cell development and proliferation (47, 48).

The current study demonstrates that IL-4-mediated murine bone marrow-derived mast cell (BMMC) Kit down-regulation is STAT6 and PI3K dependent. Additionally, we find that IL-10 has potent inhibitory effects on BMMC Kit expression and that the effects of IL-10 on Kit expression are enhanced by IL-4 in a STAT6-independent, PI3K-dependent manner. Our data also demonstrate that IL-4- and IL-10-mediated Kit down-regulation is transient and requires constant stimulation with IL-4 and/or IL-10. Loss of Kit expression is not explained by a reduction in steady-state c-kit mRNA levels, but rather by a loss of Kit protein expression without changes in protein stability. We also report that Kit stimulation induces IL-13 transcription and that Kit-mediated induction of both IL-13 and TNF-α mRNAs is greatly reduced by IL-4 and IL-10.

Materials and Methods

Cells and reagents

Cells were maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY), supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate (all from Biofluids, Rockville, MD) (cRPMI), supplemented with 10–20% WEHI-3-conditioned medium (cRPMI/WEHI-3-3CM), BMMC were derived from femurs of adult C57BL/6, CB57BL/6 STAT6-deficient (26) mice by culture in cRPMI with 20–25% WEHI-3 (cRPMI–IL-3) CM. BMMC were treated with IL-3 alone or combinations of IL-3, IL-4, and/or IL-10. For long-term incubations, indicated times in cRPMI containing 5 ng/ml mouse IL-3 and the indicated cytokines. LY294002, wortmannin, PD98059, chelerythrine chloride, or DMSO (also purchased from Sigma) were added to cell cultures at indicated concentrations of mouse IL-4 and/or IL-10. For long-term incubations, cells were then plated at 3 × 10^5 cells/ml in a 200-μl volume using a 96-well flat-bottom plates (Costar, Cambridge, MA). Cells were incubated for 30–60 min at 25°C or 37°C, after which time they were treated with IL-3, IL-4, and/or IL-10. Fresh inhibitors were added to cultures daily. Kit levels were determined by flow cytometry.

Flow cytometry analysis

To detect Kit expression on BMMC, cells were incubated with 0.3 μl 2,4G2 rat anti-mouse FcγRII/IRII asperites per 100 μl for 10 min at 4°C, followed by 10 μg/ml FITC-conjugated rat anti-mouse Kit for 30 min at 4°C in PBS/3% FBS/0.1% sodium azide (FACS buffer). Cells were then washed twice and analyzed in the presence of propidium iodide with a Becton Dickinson FACScan flow cytometer (Becton Dickinson, San Jose, CA). Control samples were identically stained with FITC-labeled rat anti-mouse CD4. Percent inhibition of Kit expression was determined using mean fluorescence intensities (MFI), with cells cultured in IL-3 alone as the baseline for all comparisons. FcεRI or CD13 expression was determined as described previously (31).

RNase protection assay

For each sample, −5 × 10^6 BMMC were stimulated with 5 ng/ml IL-3 with or without 10 ng/ml of IL-4 and/or IL-10, as described above for periods of 6 h to 7 days, and cells were fed every 4 days. Cells were then washed and resuspended at 1 × 10^6 cells/ml in cRPMI with 1 ng/ml IL-3 (cRPMI–IL-3). SCF was added to a final concentration of 100 ng/ml, and cells were incubated for 2 h at 37°C. RNA was harvested with Trizol (Biotecx, Friendswood, TX), and an RNase protection assay was performed using a custom-made probe set or the mCK-1 probe set from the RibonQuant System (Pharmingen) based on the manufacturer’s instructions. Pixel intensity was determined using the Phosphorimaging 445SI System (Molecular Dynamics, Sunnyvale, CA).

EMSA

BMMC (1 × 10^7) were resuspended in cRPMI without WEHI-3-3CM at 2 × 10^7/ml, incubated for 4 h at 37°C, washed, and resuspended in cRPMI at 2 × 10^7/ml for 1.5 h at 37°C. Cells were incubated with DMSO, LY294002, or wortmannin at indicated concentrations for 30 min at 37°C. Cells were washed with 10 ng/ml IL-4 which were added to cells at 100 ng/ml for 10 min at room temperature. Total cell lysates were obtained as previously described (31), and 7.5 μg of lysate was subjected to EMSA analysis using a double-stranded oligonucleotide derived from the mouse IL-4 promoter (4GL3): gatc AACACC TGCCTACAGGAA TGCCTTTAA TCT gac as described previously (31). 4GL3 has been shown to specifically bind to STAT6 (31, 51).

Western blot analysis

BMMC were treated with IL-3 alone or combinations of IL-3, IL-4, and IL-10 for 3 days. Cells were then collected and washed. Pellets were lysed in lysis buffer (50 mM Tris, pH 7.2, 150 mM NaCl, 1% glycerol, 1.5 mM MgCl2, 0.5% Triton X-100, 10 mM EDTA plus protease inhibitors (Boehringer Mannheim, Indianapolis, IN)). Total protein lysates (25 μg) were subjected to SDS-PAGE on a 10% polyacrylamide gel and transferred to Nitran Membrane (Bio-Rad, Hercules, CA). Blots were blocked with 5% nonfat dry milk in HEPES-buffered saline (HBS: 50 mM HEPES, pH 7.2, 150 mM NaCl, and 10 mM EDTA) for 30 min at 37°C. Polyclonal Anti-Kit (Oncogene Science, Uniondale, NY) was added at 0.5 μg/ml in 5% nonfat dry milk in HBS and rocked overnight at 4°C. Blots were then washed three times, for 10 min each, with HBS. A secondary F(ab′)2, HRP-conjugated donkey anti-rabbit IgG (Amersham, Arlington Heights, IL), was then added at a 1:1000 dilution in 5% nonfat dry milk in HBS for 1 h at room temperature. Blots were then washed three times, for 10 min each, in HBS. Kit expression was detected using enhanced chemiluminescence reagents (Pierce, Rockford, IL) as directed by the manufacturer. Quantitation of Kit band intensity was achieved using a personal densitometer SI (Molecular Dynamics, Sunnyvale, CA).

Results

STAT6 expression is required for IL-4-mediated inhibition of BMMC Kit expression

To assess the regulation of Kit expression, BMMC were washed to remove WEHI-3-3CM and were incubated for 4–6 h at 37°C in cRPMI. BMMC were then plated at ~300,000 cells/ml in a 200-μl volume using 96-well flat-bottom plates (Costar, Cambridge, MA). Cells were incubated for the indicated times in cRPMI containing 5 ng/ml mouse IL-3 and the indicated concentrations of mouse IL-4 and/or IL-10. For long-term incubations, cultures were fed every 4 days by replacement of half the medium and cytokines. LY294002, wortmannin, PD98059, chelerythrine chloride, or DMSO (also purchased from Sigma) were added to cell cultures at indicated concentrations. Cells were then incubated for 30–60 min at 25°C or 37°C, after which time they were treated with IL-3, IL-4, and/or IL-10. Fresh inhibitors were added to cultures daily. Kit levels were determined by flow cytometry.
IL-4-mediated down-regulation of Kit was greatly diminished in these cells (Fig. 1). The lack of IL-4 responsiveness in STAT6−/− BMMC did not appear to be due to a deficiency in IL-4R expression. These cells have previously been shown to express IL-4R at levels similar to WT BMMC populations and to exhibit similar proliferative responses to IL-4 in combination with suboptimal doses of IL-3 or SCF (31).

**IL-4-mediated inhibition of Kit expression is PI3K dependent**

We assessed the role of PI3K in the IL-4-mediated inhibition of Kit activity using the PI3K inhibitor, LY294002. As shown in Fig. 2, IL-4-mediated inhibition of Kit expression was diminished in cells treated with LY294002, but not in cells treated with DMSO, the vehicle control. This effect was dose dependent, with optimal inhibition of IL-4 effects observed when 10 μM LY294002 was used. Similar inhibitory effects were noted when LY294002 was added daily or as a single dose at the initiation of cultures (data not shown).

**STAT6 DNA binding ability is not affected by PI3K**

PI3K is known to activate the serine/threonine kinase protein kinase B (Akt), with subsequent downstream activation of other kinases (reviewed in Ref. 52). Because serine phosphorylation has been demonstrated to be necessary for full STAT functionality in some systems (53–55), we wished to determine whether the PI3K and STAT6 pathways intersect during IL-4-mediated Kit down-regulation. As shown in Fig. 3, the PI3K inhibitors, LY294002 or wortmannin, had no effect on STAT6 DNA binding activity.

**IL-10 inhibits Kit expression in mouse BMMC**

IL-10 has been shown to regulate mast cell growth, mediator release, and cytokine production, and synergizes with IL-4 in some of these activities (46–48). In an effort to determine the role of this Th2 cytokine on Kit expression, we treated BMMC with IL-10 and assessed Kit levels by flow cytometry. As shown in Fig. 4, cells cultured in the presence of IL-3 and IL-10 showed significantly reduced Kit levels compared with those cultured in IL-3 alone. IL-10-mediated inhibition of Kit expression did not appear to be due to intrinsic IL-4 production, as BMMC derived from IL-4−/− or IL-4R−/− mice (49–50) also showed reduced Kit levels when cultured in medium containing IL-3 and IL-10 (Table I). Furthermore, STAT6 was not required for this effect, as STAT6−/− BMMC responded normally to IL-10 (Fig. 5).

**IL-4 enhances IL-10-mediated inhibition of Kit expression in a PI3K-dependent, STAT6-independent manner**

Because IL-10 has been demonstrated to synergize with IL-3 and IL-4 to enhance mast cell development (47, 48), we examined the
combined effects of IL-4 and IL-10 on Kit expression. As shown in Fig. 5A, stimulation with IL-4 or IL-10 decreased Kit levels an average of 55%, while combined stimulation with IL-4 and IL-10 reduced Kit levels nearly 80% compared with cells cultured in IL-3 alone ($p < 0.05$; Tukey test). Given that STAT6 expression is required for full IL-4-mediated Kit inhibition, we examined the role of STAT6 in Kit inhibition mediated by costimulation with IL-4 and IL-10. Treatment of STAT6$^{-/-}$ BMMC with IL-3 plus IL-4 and IL-10 led to a greater reduction in Kit expression than did culture in IL-3 and IL-10 (Fig. 5). Thus, the ability of IL-4 to enhance IL-10-mediated Kit inhibition is not STAT6 dependent. This effect was also independent of endogenously produced IL-4, but required expression of the IL-4R$\alpha$. As demonstrated in Table I, IL-4$^{-/-}$, but not IL-4R$^{-/-}$ BMMC demonstrated IL-4 enhancement of IL-10-mediated Kit inhibition.

Because PI3K activity is required for IL-4-mediated Kit down-regulation, we determined the role of PI3K in the enhanced Kit down-regulation observed when BMMC are stimulated with IL-4 and IL-10. As shown in Fig. 5B, the ability of IL-4 to enhance IL-10-mediated Kit down-regulation was diminished in both the WT and STAT6$^{-/-}$ BMMC upon treatment with LY294002. Thus, PI3K activation is essential for the IL-4-mediated enhancement of IL-10 signaling.

The effect of IL-4 and IL-10, alone or combined, was sensitive and transient. IL-4-mediated inhibition of Kit expression could be observed at concentrations as low as 1.25 ng/ml IL-4. IL-10 gave comparable results at 0.4 ng/ml (Fig. 5C). While inhibition increased over time, peaking at days 2–3, Kit expression returned to normal levels by day 7 of culture, even with continuous cytokine stimulation (Fig. 5D). Cells stimulated with IL-10 alone or with IL-4 and IL-10 demonstrated an insignificant second peak of inhibition on day 14 of culture ($p \geq 0.05$). It should be noted that IL-4 and/or IL-10 stimulation of BMMC did not appear to mediate a decrease in expression of all surface proteins, as CD13 expression was not decreased by IL-4 or IL-10 stimulation (data not shown).

Inhibition of Kit expression requires constant stimulation with IL-4 and IL-10

To further investigate the kinetics of IL-4- and IL-10-mediated inhibition of Kit expression, BMMC were cultured in the presence of IL-3, with or without IL-4 and/or IL-10, for 2 days, washed extensively, and recultured in IL-3 alone for 16 h (Fig. 6, bottom). Kit levels on BMMC deprived of IL-4 and/or IL-10 for 16 h were
comparable to cells cultured in IL-3 alone. Control cultures stimulated with IL-3 plus IL-4 and/or IL-10 for 3 (Fig. 6, top) or 2 days (Fig. 6, middle) demonstrated Kit repression, as expected. Therefore, IL-4- and IL-10-mediated Kit down-regulation was not only transient, but also rapidly reversible.

**Effects of IL-4 and IL-10 on steady-state c-kit mRNA and total Kit protein levels in mouse BMMC**

To determine whether IL-4- and/or IL-10-mediated Kit down-regulation correlated with a loss of c-kit mRNA production in murine BMMC, cells were cultured in the presence of IL-3, with or without IL-4 and/or IL-10 for periods of 6 h to 7 days, before assessing c-kit mRNA expression by RNase protection assay (RPA). Murine BMMC showed no significant reduction of c-kit mRNA expression following treatment with IL-3 plus IL-4 or IL-3 plus IL-10. However, stimulation with all three cytokines did reduce steady-state c-kit mRNA levels by an average of 30% (mean of four independent experiments) compared with cells cultured in IL-3 alone (Fig. 7A). These data indicate that loss of c-kit mRNA alone fails to explain IL-4- or IL-10-mediated Kit repression and that combined stimulation with IL-4 and IL-10 may alter Kit expression by a mechanism not employed by either cytokine alone.

We next assessed Kit protein expression and stability following stimulation with IL-4 and/or IL-10. In keeping with a previous study (12), we found that BMMC cultured with IL-3 plus IL-4 had a 62% reduction in total Kit levels, compared with cells cultured in IL-3 alone (Fig. 7B). Similarly, culturing BMMC in IL-3 plus IL-10 yielded a 49% reduction in Kit protein levels, where stimulation with IL-3 plus IL-4 plus IL-10 resulted in a drastic 84% loss of Kit expression, relative to IL-3-treated control BMMC. These data correlate well with our observed loss in surface Kit staining.

Loss of Kit expression could occur due to changes in translation or stability of the protein. To assess changes in stability, we cultured BMMC in IL-3 with or without IL-4 and/or IL-10, followed by treatment with the translational inhibitor, cycloheximide (Fig. 7B). Western blot analysis, coupled with densitometry measurements, indicated that treatment with IL-4 and/or IL-10 failed to decrease the half life of Kit protein. BMMC cultured in IL-3 alone had a half life of 3.5 h; those treated with IL-3 plus IL-4, IL-3 plus IL-10, or IL-3 plus IL-4 plus IL-10 had half lives of 4.3 h, 5.0 h, and 5.8 h, respectively (all measurements are means of two experiments, comparing cycloheximide-treated to DMSO-treated cells). Thus IL-4 and IL-10 reduce Kit protein expression without increasing degradation.

**IL-4- and IL-10-mediated inhibition of Kit expression affects production of SCF-induced cytokine mRNA**

We wished to determine whether the IL-4- and IL-10-mediated reduction in Kit expression levels translated to a loss of Kit functionality. As SCF stimulation has been shown to induce production of TNF-α and IL-6 from BMMC (56), RPA analysis was used to assess induction of multiple cytokine mRNAs following SCF stimulation (Fig. 8). In addition to TNF-α production, we noted a significant induction of IL-13 mRNA following SCF stimulation. IL-6 mRNA was constitutively expressed by these BMMC populations and showed little increase after SCF stimulation (data not shown). Culturing BMMC in the presence of IL-3 plus IL-4 and/or IL-10 greatly reduced their ability to induce TNF-α or IL-13 mRNA following SCF stimulation, compared with BMMC cultured in IL-3 alone. In fact, TNF-α and IL-13 mRNA levels were reduced by nearly 70% in cells cultured in IL-3 plus IL-4 plus IL-10 before SCF-stimulation. Interestingly, culturing BMMC in IL-3 plus IL-4 plus IL-10 led to constitutive IL-13 mRNA transcription, a result observed in two experiments (data not shown).

### Table 1. Effects of IL-4 and IL-10 on BALB/c-derived BMMC

<table>
<thead>
<tr>
<th>BMMC</th>
<th>IL-3 + IL-4 % Inhibition (±SE)</th>
<th>IL-3 + IL-10 % Inhibition (±SE)</th>
<th>IL-3 + IL-4 + IL-10 % Inhibition (±SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c WT</td>
<td>33.8 (4.7)</td>
<td>52 (5.8)</td>
<td>79.2 (2.3)</td>
</tr>
<tr>
<td>IL-4⁻/⁻</td>
<td>39.3 (4.9)</td>
<td>46.2 (3.5)</td>
<td>81.5 (1.9)</td>
</tr>
<tr>
<td>IL-4R⁻/⁻</td>
<td>23.4 (2.4)</td>
<td>44.3 (4.8)</td>
<td>49.3 (4.2)</td>
</tr>
</tbody>
</table>

*a* IL-10-mediated inhibition of Kit expression does not require expression of IL-4 or IL-4Rα-chain. BMMC were cultured in media containing IL-3 (5 ng/ml) with or without IL-4 and/or IL-10 (each at 10 ng/ml) for 3 days. Percent inhibition was determined as in Fig. 1. Numbers in parentheses represent SE. Data and SE are representative of six independent experiments. Kit inhibition mediated by IL-3 + IL-4 + IL-10 was significantly greater than IL-3 + IL-10 in BALB/c WT and IL-4⁻/⁻, but not in IL-4R⁻/⁻ (values of *p* are significant at the 0.05 level, as determined by Tukey test).
Taken together, these data suggest that costimulation with IL-4 and IL-10 not only leads to reduced Kit expression, but that the remaining surface Kit has lost functionality.

Discussion

IL-4-mediated Kit down-regulation has been noted in several systems, but the mechanistic and functional aspects of this regulation have remained unresolved. IL-4R is known to signal through multiple pathways, broadly categorized as those that regulate growth or gene expression activities. The ability to regulate these activities has been mapped to distinct regions of the IL-4Rα-chain and is thought to be due to the selective interaction of IL-4Rα with secondary signaling proteins (21, 23, 29). The IL-4Rα gene regulation domain is known to bind and activate STAT6, while the growth domain binds the adapter proteins insulin receptor substrate.
[IRS]-1 and IRS-2, which allow for subsequent activation of PI3K, protein kinase C, and/or the Ras/mitogen-activated protein kinase pathways (reviewed in Ref. 30).

STAT6 has been known to act as a positive regulator of transcription. However, recent evidence shows that STAT6 is necessary for inhibiting FcεRI expression on mast cells, E-selection expression on endothelial cells, and macrophage cytokine production (31, 32, 57, 58). Our data indicate that STAT6 expression is essential for IL-4-mediated Kit down-regulation, as STAT6−/− BMMC decrease Kit levels only slightly following IL-4 stimulation.

We also assessed the role of other signal transduction pathways known to be activated by the IL-4Rα growth domain by using selective inhibitors of PI3K, protein kinase C, mitogen-activated protein kinase/extracellular signal-related kinase, or p70 S6 kinase. The PI3K inhibitor LY294002 completely abrogated IL-4-mediated Kit down-regulation, while all other inhibitors had no effect (Fig. 2, and data not shown). Therefore, rather than the selective activation of either STAT6-related or IRS-related signaling pathways, our data demonstrate that both pathways are required for IL-4-mediated Kit down-regulation in murine BMMC. A molecular mechanism for the cooperation between these two pathways is not due to PI3K-dependent modifications in STAT6 DNA binding ability (Fig. 3). However, a potential explanation could involve PI3K-mediated alterations in STAT6 transcriptional activation functions. Taken together, our data indicate that both PI3K and STAT6 are required for IL-4-mediated Kit repression and that these pathways may operate separately.

Because IL-10 is both a Th2 cytokine and a known inhibitor of gene expression (reviewed in Ref. 59), we assessed its effect on BMMC Kit expression. Indeed, IL-10 potently down-regulated Kit expression in a sensitive, dose-responsive manner (Figs. 4 and 5). Inhibitors of PI3K, protein kinase C, mitogen-activated protein kinase/extracellular signal-related kinase, and p70 S6 kinase had no effect on IL-10-mediated Kit repression (data not shown). We also found that IL-4 enhanced this IL-10-mediated effect on Kit expression through a mechanism that required PI3K function but was independent of STAT6 expression. Both cytokines appeared to act in a strain-independent manner, as we obtained similar results using BMMC populations derived from C57BL/6, C57BL/6 × 129, and BALB/c mice (Figs. 1 and 2, also Table I). A role for both IL-4 and IL-10 in Kit regulation emphasizes the importance of Th2 function in allergic diseases involving mast cell activation. Coupled with our earlier observation of IL-4-mediated FcεRI down-regulation, these data argue for an ability of Th2 cytokines
to negatively regulate mast cell function. In fact, preliminary evidence indicates that IL-10 is also capable of inhibiting FcεRI expression (C.P.S. and J.J.R., unpublished observations).

Unlike data obtained using the human mast cell line HMC-1 (11), we found that IL-4- and IL-10-mediated Kit repression occurs largely at a posttranscriptional level. Although mast cells stimulated with IL-3 plus IL-4 or IL-3 plus IL-10 demonstrated a 50% reduction in Kit surface expression, there was no change in c-kit mRNA levels. Similarly, BMMC cultured in IL-3 plus IL-4 or IL-3 plus IL-4 plus IL-10 Cells were then treated with 50 μM cycloheximide (CHX) for the indicated times (h). DMSO (D) and untreated controls are included. Kit levels were analyzed by Western blot and enhanced chemiluminescence, followed by densitometric analysis of individual bands as described in Materials and Methods.

IL-10 or IL-3 plus IL-4 also led to loss of Kit protein expression. This reduction in protein expression was not coupled with increased degradation, as shown by an overall increase, rather than a decrease of Kit half-life (Fig. 7B). These data, coupled with the minimal changes in Kit mRNA levels we have observed, argue for cytokine-mediated control of Kit translation. This hypothesis fits well with our observed transient and reversible nature of Kit down-regulation. While the role of STAT6 and PI3K in this process remains to be determined, each could be proposed to function in expression or activation of translational cofactors.

Although IL-4- and IL-10-mediated Kit inhibition was transient, the functional effects of this regulation were evidenced by assessment of Kit-mediated cytokine production. SCF-stimulated BMMC induced transcription of TNF-α, as reported previously (56). We also detected an increase in IL-13 mRNA following SCF activation (Fig. 8). However, BMMC cultured in the presence of IL-3 plus IL-4 and/or IL-10 showed a remarkable loss of mRNAs encoding TNF-α and IL-13, compared with those cultured in IL-3 alone, following SCF activation (Fig. 8). IL-13 has recently been demonstrated to play an important role in allergic asthma (60, 61). Therefore, regulation of IL-13 transcription by IL-4 and IL-10 could be an important mechanism in the asthmatic response.
As Kit is known to be important for mast cell survival and cytokine production, the decrease in both Kit expression and function argues for key roles of IL-4 and IL-10 in mast cell-related allergic disease. Together, with our previous data demonstrating IL-4-mediated FcεRI down-regulation, we postulate the use of IL-4 and IL-10 as homeostatic factors to limit mast cell-mediated inflammatory responses.

**Note added in proof.** While this manuscript was in publication, Kanbe et al. reported that cord-blood-derived human cultured mast cells produce IL-13 in the presence of SCF (62).

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