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Differential Regulation of Human Eosinophil IL-3, IL-5, and GM-CSF Receptor α-Chain Expression by Cytokines: IL-3, IL-5, and GM-CSF Down-Regulate IL-5 Receptor α Expression with Loss of IL-5 Responsiveness, but Up-Regulate IL-3 Receptor α Expression[1]

Bernard Gregory,* Antje Kirchem,† Simon Phipps,* Phillipe Gevaert,‡ Carol Pridgeon,*† Sara M. Rankin,† and Douglas S. Robinson[2]*†

Our recent data suggested that tissue eosinophils may be relatively insensitive to anti-IL-5 treatment. We examined cross-regulation and functional consequences of modulation of eosinophil cytokine receptor expression by IL-3, IL-5 GM-CSF, and eotaxin. Incubation of eosinophils with IL-3, IL-5, or GM-CSF led to reduced expression of IL-5Rα, which was sustained for up to 5 days. Eosinophils incubated with IL-5 or IL-3 showed diminished respiratory burst and mitogen-activated protein kinase kinase phosphorylation in response to further IL-5 stimulation. In contrast to these findings, eosinophil expression of IL-3Rα was increased by IL-3, IL-5, and GM-CSF, whereas GM-CSF receptor α was down-regulated by GM-CSF, but was not affected by IL-3 or IL-5. CCR3 expression was down-regulated by IL-3 and was transiently reduced by IL-5 and GM-CSF, but rapidly returned toward baseline. Eotaxin had no effect on receptor expression for IL-3, IL-5, or GM-CSF. Up-regulation of IL-3Rα by cytokines was prevented by a phosphoinositol 3-kinase inhibitor, whereas this and other signaling inhibitors had no effect on IL-5Rα down-regulation. These data suggest dynamic and differential regulation of eosinophil receptors for IL-3, IL-5, and GM-CSF by the cytokine ligands. Since these cytokines are thought to be involved in eosinophil development and mobilization from the bone marrow and are present at sites of allergic inflammation, tissue eosinophils may have reduced IL-5R expression and responsiveness, and this may explain the disappointing effect of anti-IL-5 therapy in reducing airway eosinophilia in asthma. ***The Journal of Immunology*****, 2003, 170: 5359–5366.**

The eosinophil has long been associated with asthma and allergic diseases, since increased numbers of cells are present in blood and airway samples, and these numbers can be related to disease severity (1, 2). Eosinophils may contribute to airway hyper-responsiveness in asthma through the release of leukotrienes and by epithelial damage caused by their highly basic granule proteins.

IL-5 is a key cytokine in eosinophil development, endothelial adhesion, activation, and survival (3). These actions are largely shared with IL-3 and GM-CSF, which share a common signaling receptor β-chain with IL-5, and responsiveness to these cytokines is controlled by cell surface expression of the cytokine-associated receptor α-chain (4, 5). We have previously shown that during eosinophil development, IL-5 up-regulated the surface expression of its own receptor α-chain by inducing gene switching to produce the membrane-associated mRNA isoform, but that this activity was not shared by IL-3 and GM-CSF (6). In contrast, it has been reported that IL-3, IL-5, and GM-CSF all down-regulate mRNA for IL-5Rα in mature eosinophils (7).

IL-5 has been suggested as a specific therapeutic target for asthma therapy, and in animal models of allergen challenge mAb blockade and gene deletion have been effective in reducing airway eosinophilia and hyper-responsiveness (8, 9). However, recent clinical studies of a humanized mAb to IL-5 have been disappointing, with no effect on baseline lung function or allergen-induced changes in a small study despite a dramatic reduction in blood and sputum eosinophils (10). We have recently completed a study showing that anti-IL-5 therapy did not fully deplete bronchial mucosal or bone marrow eosinophils in asthma (11). We hypothesized that mature eosinophils that have been exposed to IL-5 might down-regulate IL-5R and thus be less dependent on this cytokine in airway tissue. Although it has previously been reported that eosinophil CCR3 expression is down-regulated by IL-3, but not by IL-5 and GM-CSF, the effect of eotaxin on IL-3, IL-5, and GM-CSF receptor expression has not been reported (12). Here we show differential regulation of eosinophil receptors for IL-3, IL-5, and GM-CSF, with prolonged reduction in surface expression of IL-5Rα by all three cytokines, increased expression of IL-3Rα, and regulation of GM-CSF receptor α (GM-CSFRα)[3] only by its cognate cytokine.

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[3] Abbreviations used in this paper: GM-CSFRα, GM-CSF receptor α; MEK, mitogen-activated protein kinase kinase; PB, peripheral blood; PI-3 kinase, phosphoinositol 3-kinase.

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Materials and Methods

Eosinophil isolation

The study was approved by the ethics committee of Royal Brompton Hospital, and all volunteers gave written informed consent. Eosinophils were isolated from the peripheral blood (PB) of atopic donors by a combination of dextran sedimentation and density gradient centrifugation on Histopaque (Sigma-Aldrich, Poole, U.K.). Blood was collected in heparinized syringes, mixed with a 0.2 vol of 6% (w/v) dextran T500 (Pharmacia Biotech, Piscataway, NJ) in HBSS (Sigma-Aldrich), and left to stand for 45 min at room temperature to allow sedimentation of erythrocytes. The leukocyte-rich supernatant was decanted and layered onto 18-ml cushions of Ficoll-Hypaque (1.077 g/ml; Sigma-Aldrich) in 50-ml conical polypropylene tubes and centrifuged at 400 × *g* for 20 min at 17–20°C. The granulocyte pellet was then washed once in HEPES-buffered RPMI 1640 containing 5 mM EDTA and 0.1% (w/v) BSA (Sigma-Aldrich). Residual erythrocytes were removed from the granulocyte fraction by hypotonic lysis in ice-cold sterile deionized H_2O for 30–45 s, followed by restoration of osmolarity with HEPES-buffered RPMI 1640. Eosinophils were purified from the granulocyte fraction using a modification of the method of Hansel (13). Briefly granulocytes were incubated with anti-CD16-, anti-CD3-, and anti-CD14-coated microbeads (Miltenyi Biotech, Bisley, U.K.) before being passed through steel fiber CS columns, and eosinophils were separated by negative immunomagnetic selection to >97% purity as determined by Kimura staining.

Cytokines, Abs, and signaling inhibitors

Recombinant human cytokines used were recombinant human IL-5 (BD PharMingen, Oxford, U.K.), recombinant human IL-3 (Genzyme, West Malling, U.K.), and recombinant human GM-CSF (R&D Systems, Abingdon, U.K.). Stock solutions were prepared in eosinophil culture medium, stored at −80°C, and thawed just before use.

For Western blotting, anti-phospho-mitogen-activated protein kinase kinase 1/2 (anti-phospho-MEK1/2) was purchased from New England Biolabs (Beverly, MA). Anti-IL-5Rα (α16) used for FACS analysis was generously provided by Prof. J. Tavernier, and anti-common β-chain (ICI Biomedicals, Costa Mesa, CA)), anti-IL-3Rα (6H6), and anti-GM-CSFRα (8G6) were all gifts from Prof. A. Lopez (Hanson Institute, Adelaide, Australia). These Abs had previously been confirmed as noncompetitive with the cytokines for receptor binding (14) (J. Tavernier, unpublished observations). Anti-CCR3 Ab (7B11) was a gift from Dr. W. Newman (Leukocyte, Inc., Boston, MA). Anti-CD9-labeled FITC and anti-CD69-labeled CyChrome were obtained from BD PharMingen (Oxford, U.K.), IgG1 isotype control was puchased from DAKO (High Wycombe, U.K.), and fluorescent-conjugated goat anti-mouse IgG secondary Abs were purchased from Caltag (Northampton, U.K.).

The pharmacologic signaling inhibitors, SB 203580, PD 98059, LY 294002, and SU 6656, were obtained from Calbiochem-Novabiochem (Nottingham, U.K.). Stock solutions were solubilized in DMSO and added to eosinophil cultures at the appropriate dilution (with DMSO only controls) 1 h before the addition of cytokine.

Eosinophil culture

Isolated eosinophils were cultured at 1×10^6/ml in 24-well plates in RPMI 1640 (without HEPES) supplemented with 10% (v/v) FBS (Sigma-Aldrich), 1 mM L-glutamine, antibiotic/antimycotic cocktail (Life Technologies, Paisley, Scotland), and different cytokines in 5% CO_2 at 37°C (eosinophil culture medium).

Isolation of RNA

Total RNA was isolated from 10^6 eosinophils/treatment using the RNeasy kit (Qiagen, Crawley, U.K.) according to the manufacturer's instructions. Total RNA was eluted in 50 μl of RNase-free H_2O and stored at −80°C until required for RT-PCR analysis.

RT-PCR

The expression patterns of soluble vs transmembrane IL-5Rα isoforms were determined using a competitive RT-PCR as described previously (6). This allows estimation of the ratios of expression of both human IL-5Rα isoforms using a forward primer designed on the extracellular part of the receptor at position 1033–1056 (5′-GTGTCTGCTTTTCCAATCCAT TGC-3′) and two reverse primers specific for the transcripts encoding either the secreted (position 1279–1298, 5′-TCAGATACGGTGT GGGGCAG-3′) or the membrane-anchored (position 1539–1561, 5′-TTTTGGTGCTGGAATTGGTGG-3′) isoform. Predicted PCR reaction products are 245 and 527 bp for the soluble and transmembrane transcripts, respectively. Fifteen microlitersof total RNA per sample was reverse transcribed using SuperScript RNase H reverse transcriptase (Life Technologies), and 4 μl of the first-strand synthesis reaction mix was used in the subsequent PCR reaction using Platinum *Taq* DNA polymerase (Life Technologies). Expression of β-actin was measured to normalize between samples.

Quantitative PCR

mRNA for IL-5Ra transmembrane and soluble isoforms was analyzed by a quantitative PCR protocol developed in Gent,[4] while total IL-5Rα was quantified using TaqMan analysis. Briefly, 8 μl of total RNA/sample was reverse transcribed using the SuperScript First Strand Synthesis system (Life Technologies) with both oligo(dT) and random hexamer primers. Twenty-five-microliter PCRs were preformed in triplicate on the ABI PRISM 7700 SDS machine (PE Biosystems, Foster City, CA). Reactions included 300 nM (final concentration) of primers (forward, 5′-GGTTG CAGCCACGAGCATA-3′; reverse, 5′-AATCTAAAACAATCTGTAGC CTGTCTACA-3′), 175 nM (final concentration) dual-labeled probe (5′-FAM-ACGACAGACACGGTCCTCGCCATC-TAMRA-3′), and TaqMan Universal PCR MasterMix (part 4304437; PE Applied Biosystems). Samples were normalized to 18S (part 4310875; PE Applied Biosystems), and IL5Rα message was quantified using a relative standard curve.

Flow cytometry

Eosinophils were stained for surface Ags at 0.5–1.0 × 10^5/100 μl in V-bottom, 96-well plates in PBS with 0.1% azide and 0.5% BSA. Abs stocks were constituted in 10% (v/v), normal human serum in PBS with 0.1% sodium azide and 0.5% BSA (Sigma-Aldrich) such that the final concentration of normal human serum was 2% after addition of primary Ab to the cells. Cells were stained by incubation at 4°C for 30 min, followed by two washes in PAB (Sigma-Aldrich) and developing with a second layer Ab.

Measurement of respiratory burst activity

Respiratory burst activity was measured by oxidation of nonfluorescent dihydrorhodamine 123 (Calbiochem, Nottingham, U.K.) to fluorescent rhodamine 123 according to established methods (15–17).

Eosinophils were pretreated by overnight culture in the presence or the absence of cytokine at 1×10^6/ml. Nineteen hours later cells were resuspended in assay buffer (HBSS and 0.2% BSA) at 2×10^6/ml and preincubated for 15 min at 37°C before being mixed 1/1 (v/v) with 2× dihydrorhodamine 123 (2 μM) and sodium azide (200 μM) and incubated for an additional 30 min at 37°C. This cell mix was then added at 50 μl/well (1×10^5 cells) to a range of cytokine concentrations (2× in assay buffer) or control (no cytokine) plated at 50 μl/well on a 96-well plate. The cells were then incubated at 37°C, and the increase in fluorescence at 530 nm after excitation at 485 nm was monitored continuously every 3 min for up to 60 min at 37°C using a CytoFluor multiwell plate reader (PerSeptive Biosystems, Framingham, MA).

Western blotting

For MEK phosphorylation analysis, 1×10^6 eosinophils/treatment were lysed in 30 μl of 1× HBSS (with Ca^{2+}/Mg^{2+}), pH 7.4, containing 10 mM HEPES and 0.2% BSA (w/v). To this 10 μl of 5× Laemmli buffer (25% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-ME, and 0.1% (w/v) bromophenol blue) was added, and the volume was reduced to 20–25 μl by heating at 95°C. Extracts were analyzed on a 10% SDS-PAGE gel run in Tris-glycine buffer. Following electrophoresis, proteins were transferred to Hybond-P transfer membranes. The membranes were blocked for 1 h at room temperature in 5% nonfat dried milk made in PBS/0.1% Tween (PBS-T). This solution was replaced, and primary Ab was added at a 1/1000 dilution and incubated overnight at 4°C. Membranes were then washed in PBS/0.1% Tween (three times, 5 min each time) and incubated for 1 h at room temperature with rabbit anti-mouse HRP-conjugated secondary Ab. Immunoreactive bands were visualized using the luminol ECL system (Pierce-Warriner, Chester, U.K.) according to the manufacturer's instructions. For quantification, blots were scanned using laser scanning densitometry software (Psion, London, U.K.).

[4] P. G. A. Gevaert. C. Bachert, G. Holtappels, C. Perez, P. Howarth, L. Fransen, J. Tavemier, and P. Cauwenberge. Eosinophilic inflammation and interleukin 5 receptor α isoform expression in eosinophilic airway diseases. *Submitted for publication.*

Results

Incubation of mature PB eosinophils with IL-5 leads to prolonged down-regulation of surface IL-5Rα and down-regulation of IL-5Ra mRNA expression

Incubation of PB eosinophils with IL-5 led to a rapid dose-dependent reduction in surface staining for IL-5Rα, which was detectable by 1 h and maximal at 19 h (Fig. 1*a*). Quantitative PCR confirmed a reduction in mRNA expression for IL-5Ra, which was for both membrane-bound and soluble mRNA isoforms (Fig. 1*b*).

IL-3 and GM-CSF also down-regulate eosinophil IL-5Rα expression, and there is no recovery of IL-5Rα

To examine cross-regulation of receptors in the IL-3, IL-5, and GM-CSF cytokine families, we incubated eosinophils with IL-3 or GM-CSF. Both cytokines caused a dose-dependent down-regulation of surface IL-5Rα on eosinophils (Fig. 2*a*). For all three cytokines this down-regulation of IL-5Rα remained for up to 5 days in culture (Fig. 2*b*). In contrast, all three cytokines caused a rapid reduction of surface expression of the shared β-chain, which returned to baseline levels by 19 h of culture for IL-5 and GM-CSF, but not for IL-3 (Fig. 3).

Down-regulation of surface IL-5Rα reduces eosinophil responsiveness to IL-5, while up-regulation of IL-3Rα increases response to IL-3

To examine the functional consequences of the marked reduction in surface expression of IL-5Rα on eosinophils by IL-5 or IL-3, we examined respiratory burst and MEK signaling. Superoxide generation by eosinophils in response to IL-5 was reduced in a dose-dependent manner by prior overnight incubation in IL-3 or IL-5

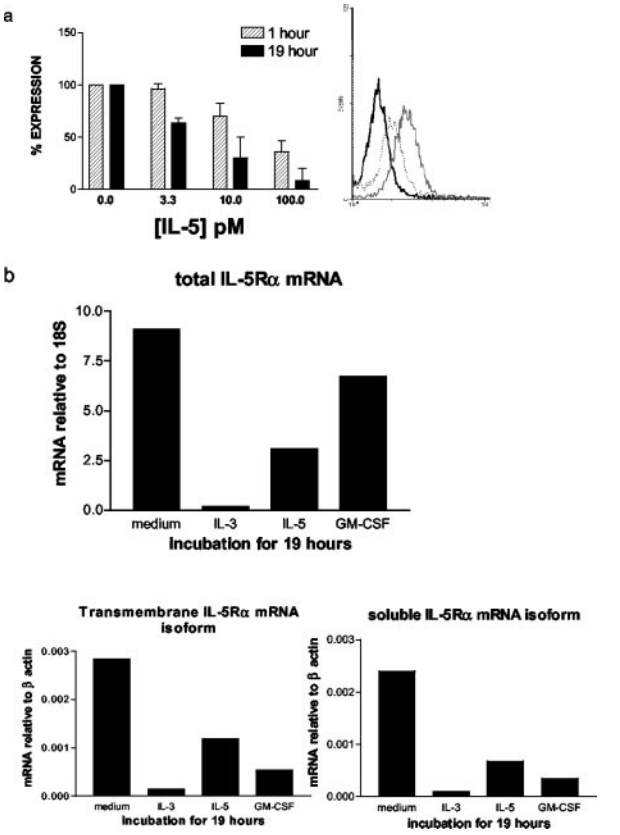

FIGURE 1. Expression of surface IL-5Rα by isolated PB eosinophils. *a*, Dose-response effect of IL-5 incubation for 1 or 19 h on surface expression of IL-5Rα by PB eosinophils. Cells were stained with 5 μg/ml anti-IL-5Rα mAb (α16) or an equivalent mouse IgG1 isotype control and then labeled with goat anti-mouse IgG-TRI-Color PE-Cy5 conjugate for analysis by flow cytometry. Surface expression is shown as the percentage of specific mean fluorescence detected with mAb α16 in IL-5-treated cultures compared with incubation in medium alone ($n = 6$; mean specific mean fluorescence for IL-5Rα, 2.25; range, 0.82–3.98). A representative flow cytometry plot shows showing staining for IL-5Rα after 1-h incubation in medium (gray line), 100 pM IL-5 (dotted line), and isotype control (black line). *b*, IL-5, IL-3, and GM-CSF all down-regulate eosinophil mRNA expression for total IL-5Rα and equally for soluble and transmembrane isoforms. mRNA for total IL-5Rα was quantified by real-time TaqMan PCR after 19 h of incubation in medium alone, IL-5 (100 pM), IL-3 (200 pM), or GM-CSF (200 pM). Results are representative of two separate experiments. In addition, using a separate Syber Green real-time PCR protocol, mRNA for soluble and transmembrane isoforms of IL-5Rα was quantified in a separate experiment after incubation for 19 h in IL-5, IL-3, and GM-CSF at the concentrations described above, but the relative expression did not alter.

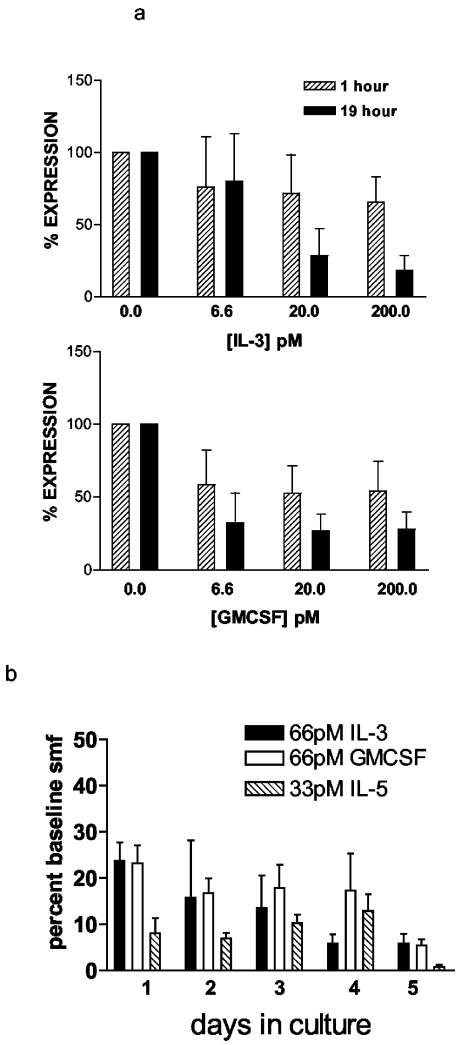

FIGURE 2. IL-3 and GM-CSF also induce dose-dependent down-regulation of IL-5Rα on eosinophils, and effects of IL-3, IL-5, and GM-CSF persist for up to 5 days in culture. Isolated PB eosinophils were cultured in different concentrations of IL-3 (*a*) and GM-CSF (*b*) or in medium only for 1 and 19 h at 37°C. Cells were stained with anti-IL-5Rα or anti-βC mAb or mouse IgG1 isotype control, labeled with TRI-Color (PE-Cy5) conjugated secondary Ab, and then analyzed by flow cytometry. Specific mean fluorescent intensities were normalized with respect to medium-only controls, and data ($n = 6$ individual experiments) are expressed as the mean ± SEM. *c*, Eosinophils cultured in IL-5, IL-3, or GM-CSF for 1–5 days in culture and analyzed by flow cytometry for IL-5Rα expression. Mean fluorescence is expressed as a percentage of that seen at time zero ($n = 5$).

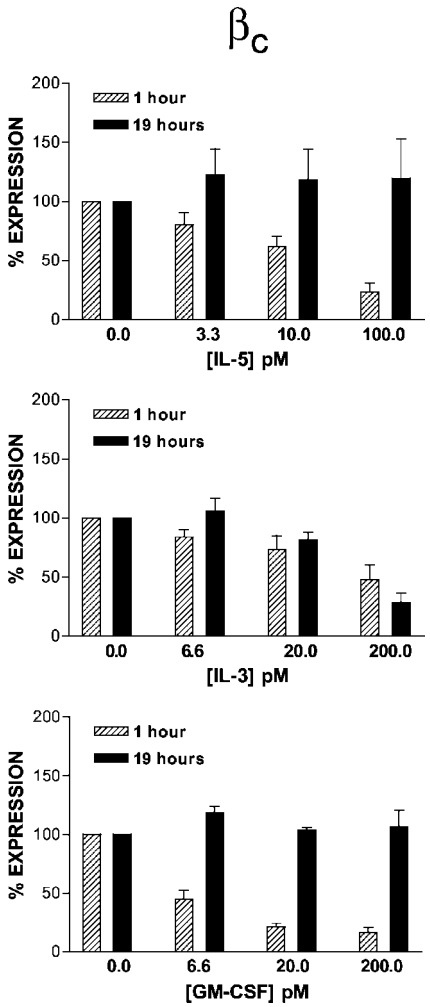

FIGURE 3. Effects of IL-3, IL-5, and GM-CSF on common β-chain (β_c). IL-5 and GM-CSF induce a dose-dependent and transient reduction in surface expression of the shared receptor β-chain, whereas the effect of IL-3 is sustained at 19 h ($n = 6$).

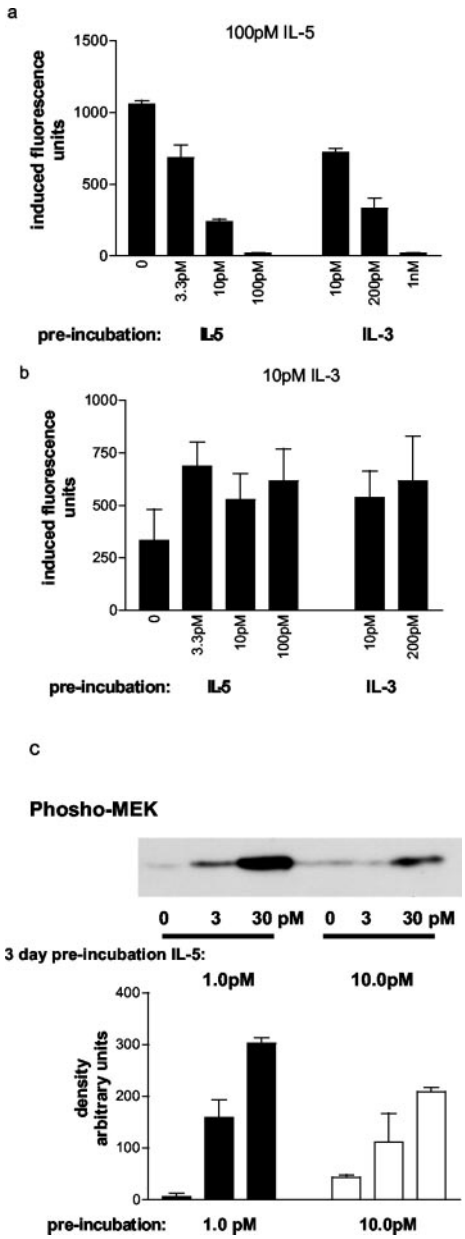

FIGURE 4. IL-5-induced respiratory burst and IL-5 signaling are attenuated in eosinophils pre-exposed to IL-5 and IL-3, whereas IL-3-stimulated respiratory burst is increased. *a*, Eosinophils were preincubated in culture medium plus various concentrations of IL-5 or IL-3. Following overnight culture, cells were washed, resuspended in assay medium, and restimulated with 100 pM IL-5 or left untreated for 1 h. Superoxide production in response to restimulation was measured as described in *Materials and Methods*. The data are expressed as the difference in fluorescence units between cytokine-stimulated and untreated cells and are shown as the mean of triplicate values ± SEM. Data are representative of three separate experiments. *b*, Eosinophils were preincubated in culture medium plus various concentrations of IL-5 or IL-3 as in *a*. Following overnight culture, cells were washed, then restimulated with 10 pM IL-3 or left in medium for 1 h, and superoxide generation was measured. Data are expressed as described in *a* and are representative of two experiments. *c*, Eosinophils were cultured for 3 days in 1.0 pM IL-5 (which maintained viability, but did not down-regulate IL-5Rα expression) or 10 pM IL-5 (which did reduce IL-5Rα expression) and then washed, resuspended at 1×10^6/ml, and restimulated for 15 min with medium or 3 or 30 pM IL-5. Total cellular protein extracts were analyzed by electrophoresis on a 10% SDS-PAGE gel. Western blotting for phosphorylated MEK was performed as described in *Materials and Methods*. Blots were scanned, and densitometry was performed. Data are shown from one representative blot, and densitometry results are the mean and SE from three separate experiments.

(Fig. 4*a*). Respiratory burst to IL-3 was retained or increased after incubation in IL-5 or IL-3, in keeping with up-regulation of IL-3Rα (Fig. 4*b*). These cells retained responsiveness to eotaxin (data not shown).

To further characterize the effect of receptor down-regulation on responsiveness to IL-5, we examined IL-5-induced MEK phosphorylation. We compared eosinophils incubated for 3 days in two doses of IL-5, one that maintained viability but did not significantly down-regulate IL-5Rα expression, and a higher concentration that reduced surface expression of IL-5Rα. There was a reduction in IL-5-induced phospho-MEK in cells preincubated at the higher dose (Fig. 4*c*). A 3-day culture was chosen after initial experiments that showed this to be the optimal time point for a return to basal levels of phospho-MEK after initial IL-5 exposure.

Eosinophil IL-3Rα is up-regulated by IL-3, GM-CSF, and IL-5, while GM-CSFRα is down-regulated only by GM-CSF

To determine whether α-chains for all three cytokines (IL-3, IL-5, and GM-CSF) were down-regulated by eosinophil signaling via the common β-chain, we incubated eosinophils with different doses of each cytokine and examined surface expression of receptors by flow cytometry. In contrast to down-regulation of IL-5Rα, eosinophil surface expression of IL-3Rα was increased by incubation in IL-5, GM-CSF, and IL-3, although IL-3 did cause an

initial (1 h) reduction in expression (Fig. 5, *a–c*). This was accompanied by maintained responsiveness of cells to IL-3-induced respiratory burst (Fig. 4*b*). In contrast to both IL-3Rα and IL-5Rα, eosinophil expression of GM-CSFRα was down-regulated by GM-CSF alone and was not affected by IL-3 or IL-5 (Fig. 5, *d–f*). To confirm the specificity of the observed effects on cytokine and chemokine receptors, we examined CD9, which was not affected by any of the three cytokines. In contrast, CD69 expression was increased by all three cytokines, reflecting cellular activation (Fig. 6).

Eosinophil expression of CCR3 is down-regulated by IL-3, but is only transiently reduced by IL-5 and GM-CSF

IL-3 has previously been reported to induce down-regulation of eosinophil CCR3 expression. In our experiments we saw a less rapid, but sustained, effect of IL-3, while incubation of eosinophils with IL-5 and GM-CSF lead to a rapid and transient down-regulation of surface CCR3, which, like common β-chain, was restored toward basal levels by 19 h of culture (Fig. 7). In contrast, eotaxin did not affect the surface expression of IL-3Rα, IL-5Rα, or GM-CSFRα (data not shown).

Up-regulation of IL-3Rα, but not down-regulation of IL-5Rα, is phosphoinositol 3-kinase (PI-3 kinase) dependent

To examine the signaling pathways involved in cytokine-induced changes in receptor expression by human eosinophils, we used a variety of inhibitors. IL-3-induced IL-3Rα up-regulation was prevented by the PI-3 kinase inhibitor LY294002 at a concentration

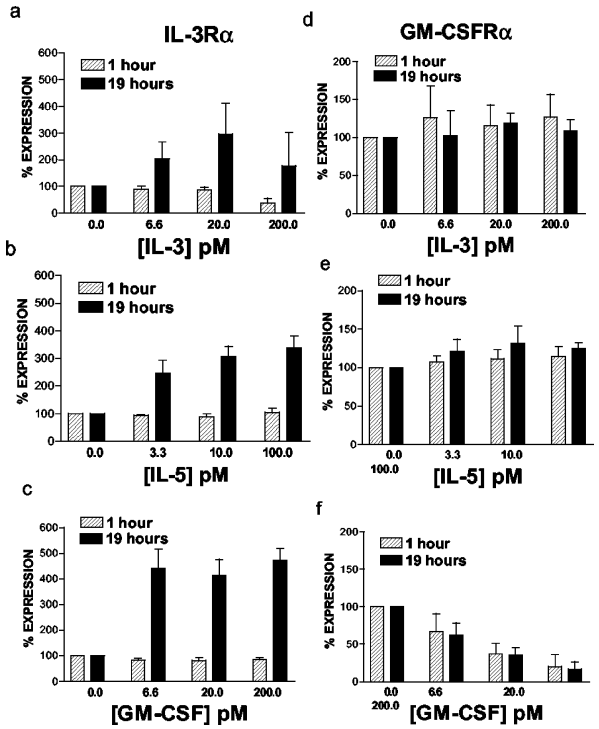

FIGURE 5. Regulation of GM-CSFRα and IL-3Rα. Eosinophils were incubated in the same concentrations of IL-5, IL-3, and GM-CSF used to study IL-5Rα regulation (Figs. 1 and 2). Cells were harvested at 1 and 19 h and then stained with mAbs IL-3Rα (1 μg/ml 6H6; *a–c*) or GM-CSFRα (1 μg/ml 8G6; *d–f*) or mouse IgG1 isotype control. Specific staining with primary Abs was visualized using goat anti-mouse IgG-PE conjugated (for IL-3Rα staining) or goat anti-mouse IgG1-TRI-Color (PE-Cy5) (for GM-CSFRα staining). Specific mean fluorescent intensities were determined by flow cytometry and normalized as described in Fig. 1. Data (n = 6) are expressed as the mean ± SEM.

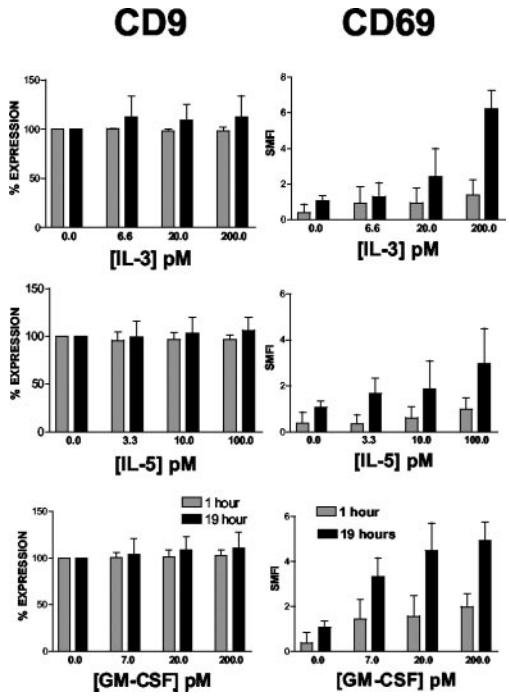

FIGURE 6. Specificity of cytokine receptor changes. CD9 expression is not affected on PB eosinophils by IL-5, IL-3, and GM-CSF, but CD69 expression is up-regulated by these cytokines. Eosinophils were cultured as described for receptor regulation analysis with IL-5Rα common β-chain (β_c), GM-CSFRα, and IL-3Rα. Thereafter, cells were stained with directly conjugated anti-CD69-CyChrome or anti-CD9-FITC and the relevant isotype controls. Since CD69 expression is negligible on freshly isolated cells, data (n = 3) are expressed as the specific mean fluorescent intensity (SMFI) ± SEM. CD9 data (n = 3) were normalized as described in Fig. 1 and are presented as the mean ± SEM.

that had no effect on cell survival (18, 19) (data not shown), whereas this compound had no effect on the regulation of IL-5Rα (Fig. 8). Inhibitors of Src kinases, p38 kinase, and MEK had no significant effect on IL-3Rα or IL-5Rα expression, suggesting that other signaling pathways may be involved in IL-5Rα down-regulation (Table I).

Discussion

In this study we examined regulation of IL-3Rα, IL-5Rα, and GM-CSFRα on mature eosinophils by their cytokine ligands. The prolonged reduction of IL-5Rα by IL-3, IL-5, and GM-CSF was accompanied by diminished biological responsiveness to IL-5. In contrast, the expression of IL-3Rα was increased by all three cytokines via a separate signaling pathway, and GM-CSFRα was down-regulated by GM-CSF alone. Thus, receptors for IL-3, GM-CSF, and IL-5 are differentially regulated by these cytokines, and eosinophils that have previously seen these cytokines may have diminished dependence on IL-5. These data may explain the failure of mAb to IL-5 to fully deplete bronchial mucosal eosinophils.

Cytokine receptor internalization upon ligand binding is the expected norm, but generally receptors are either recycled to the cell surface or targeted for proteosome degradation, and new receptors are synthesized. The IL-3, IL-5, and GM-CSF receptor family appears unusual in this respect. Martinez-Moczygemba et al. (20) reported that the shared receptor β-chain is internalized upon activation, partially degraded, and then re-expressed in a nonfunctional truncated form. Over time new receptor synthesis was seen.

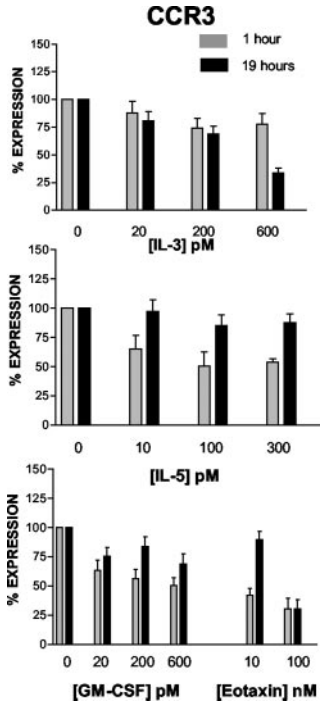

FIGURE 7. IL-3 reduces eosinophil CCR3 expression, while IL-5 and GM-CSF induce rapid, but reversible, down-regulation of CCR3. PB eosinophils were incubated in various concentrations of IL-3, IL-5, GM-CSF, and eotaxin, then surface expression of CCR3 was examined by flow cytometry using Ab 7B11. Expression is shown as a percentage of the specific mean fluorescence for cells cultured in medium alone ($n = 3$).

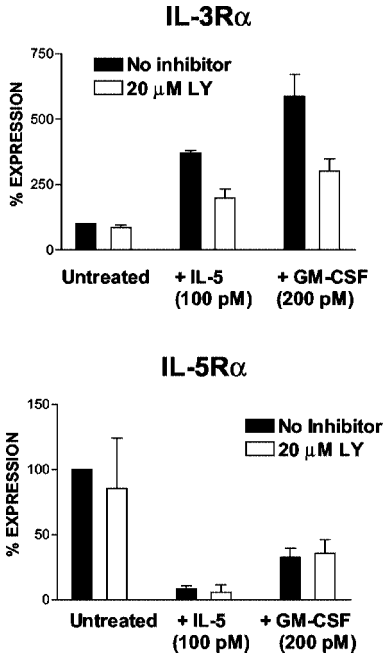

FIGURE 8. IL-3Rα up-regulation by IL-5 or GM-CSF is PI-3 kinase mediated, but IL-5Rα down-regulation is not. *a*, Eosinophils preincubated with 20 μM LY 294002 for 30 min before and during overnight culture with IL-5 or GM-CSF show reduced up-regulation of IL-3Rα (by a mean of 46% for IL-5 stimulation and 49% for GM-CSF; $n = 3$; $p < 0.05$ for each cytokine). *b*, Under the same conditions, IL-5Rα up-regulation was unaffected in eosinophils pretreated with the PI-3 kinase inhibitor ($n = 3$).

Our data conform to these findings with rapid down-regulation of surface common β-chain, which was restored to baseline by 24 h for IL-5 and GM-CSF. These authors also reported down-regulation of IL-5Rα on ligand binding. One mechanism for such reduction in surface-associated IL-5Ra was recently described by Liu et al. (21), who showed evidence for increased soluble IL-5Rα after exposure of eosinophils to IL-5, which was due to proteolytic cleavage. It is of note that protease inhibitors prevented ~50% of the down-regulation of receptor by IL-5, suggesting that other mechanisms could also be involved, particularly over longer time periods (21). These authors found no reduction in mRNA expression for IL-5Rα, although our data are in agreement with Wang et al. (7), who previously showed reduced expression of mRNA for the membrane-associated IL-5Rα isoform when eosinophils were incubated with IL-3, IL-5, or GM-CSF at 24 h, with reduced binding of radiolabeled cytokine. Thus, cleavage appears to be part of the mechanism for receptor down-regulation by IL-5, but there may be an additional effect on mRNA expression. Additional mechanisms might include effects on protein synthesis, post-transcriptional modification, mobilization of receptor to cell surface, or internalization, although the prolonged duration of down-regulation might argue against the last two.

We have previously reported that IL-5 (but not IL-3 or GM-CSF) up-regulates the relative expression of mRNA for the transmembrane isoform of IL-5Rα during eosinophil development, and that this precedes surface expression of the receptor by progenitors (6). Here we used quantitative PCR and showed reduced expression of IL-5Rα (both isoforms) when mature eosinophils were incubated with IL-5. These findings are in agreement with those reported by Wang et al. (7) and argue for differential regulation of IL-5Rα at different stages of eosinophil development.

Some of the effects of IL-3, IL-5, and GM-CSF could be due to activation of the cell and general phenotypic changes. Thus, the expression of CD69 was increased by all three cytokines (22). However, there was no modulation of CD9, showing that this was not a global reduction in surface receptors, and the initial reduction in expression of both common β-chain and CCR3 by IL-5 was short-lived, with rapid restoration of baseline expression. Furthermore, we show differential receptor regulation by the three cytokines. In contrast to IL-5Rα, IL-3Rα expression was increased by all three cytokines, whereas GM-CSFRα was down-regulated by GM-CSF alone and was not affected by IL-3 and IL-5. This argues

Table I. *Effect of signaling inhibitors on IL-5 and GM-CSF-induced down-regulation of IL-5Rα and up-regulation of IL-3Rα on human eosinophils*[a]

Condition	Receptor		
	IL-5Rα, 1 h	IL-5Rα, 19 h	IL-3Rα, 19 h
IL-5	32.9 (2.1)	4.7 (1.1)	439 (34)
+SB203580	25.8 (3.6)	6.6 (1.0)	280 (10.5)
+PD98059	36.6 (5.4)	6.7 (1.7)	391 (30.4)
+LY294002	31.1 (2.2)	5.7 (1.8)	201 (32.4)[b]
+SU6656	42.4 (6.8)	6.7 (3.8)	515 (91)
GM-CSF	74.5 (3.2)	32.8 (2.7)	774 (91)
+SB203580	67.4 (3.5)	34.9 (4.5)	427 (218)
+PD98059	73.6 (7.5)	33.2 (8.6)	512 (45)
+LY294002	72.5 (3.0)	33.0 (3.4)	388 (86)[b]
+SU6656	87.0 (9.5)	25.2 (7.3)	606 (122)

[a] Values shown are the percent expression (as specific mean fluorescence) of untreated cells at either 1 or 19 h of culture. The PI-3 kinase inhibitor LY294002 significantly reduced up-regulation of IL-3Rα ([b] $p < 0.05$, by paired t test), but did not affect IL-5Rα down-regulation. The trend for SB203580 (p38 kinase inhibitor) to reduce IL-3Rα up-regulation was not significant, and the MEK inhibitor (PD98059) and the Src kinase inhibitor (SU 6656) had no effect on either IL5Rα or IL-3Rα modulation. $n = 3$ for all experiments.

for both shared and separate signaling by these three cytokines, which may involve the cytokine-specific α-chain (for GM-CSF) as well as the shared common receptor β- chain (for IL-5Rα and IL-3Rα). Our previous findings in eosinophil progenitors raised a similar possibility (but with IL-5Rα-specific signaling regulating its own expression), and this is supported by other reports (23, 24). We used kinase inhibitors to examine the pathways involved and showed that while IL-3Rα up-regulation was at least in part PI-3 kinase dependent, IL-5Rα reduction was not affected by blocking PI-3 kinases, MEK, p38 kinase, or Src kinases. It has been shown that IL-5Rα associates with syntenin (24) and can signal via Lyn kinase (25); both of these may be involved in specific up-regulation of IL-5Rα expression during eosinophil development, but down-regulation of IL-5Rα on mature eosinophils was seen with IL-3, IL-5, and GM-CSF and thus presumably involves common β-chain events. Further work will be required to determine the signaling pathways controlling IL-5Rα expression.

Our demonstration that eosinophils that have been exposed to IL-5 show diminished respiratory burst or MEK phosphorylation on rechallenge suggests that the observed surface down-regulation of IL-5Rα is functionally significant. The recent confirmation, since our paper was submitted, that airway eosinophils have reduced IL-5Rα expression compared with PB eosinophils confirms the likely relevance of our findings (26). Here we confirmed our previous observation that IL-5 increases CD69 expression by PB eosinophils, and we have previously shown that CD69 is increased in bronchoalveolar lavage eosinophils relative to blood cells, which may reflect in vivo IL-5 exposure (22). This raises the possibility that airway or tissue eosinophils have down-regulated IL-5Rα and IL-5 responsiveness due to exposure to IL-5 (and IL-3 and GM-CSF), which we have previously shown to be expressed in asthmatic airways. Since IL-3Rα expression was increased by all three cytokines, and the cells retained IL-3-induced respiratory burst after culture in IL-3 or IL-5, it is possible that airway eosinophils are dependent on this cytokine or others rather than IL-5 for survival and activation, although this remains to be confirmed. Analysis of two mouse models of allergen challenge suggested that persistence of airway eosinophils and bronchial hyper-responsiveness was associated with persistence of IL-3 expression rather than IL-4 or IL-5 (27). It is also of note that mice made deficient in IL-5 or even the shared β-chain and murine IL-3 β-chain have reduced, but not absent, eosinophils and eosinophilic responses to airway challenge, suggesting additional potential routes to eosinophil development and activation that may not involve IL-3, IL-5, or GM-CSF (28). Taken together with the recent papers by Liu et al. (21, 26), our findings add to a comprehensive analysis of eosinophil cytokine receptor regulation. These data provide a likely explanation for our recently published finding that anti-IL-5 mAb therapy does not deplete airway or bone marrow eosinophils as effectively as it does blood eosinophils in asthmatics (11).

In our studies CCR3 was initially down-regulated, but returned to baseline expression levels in eosinophils incubated with IL-5 or GM-CSF, while IL-3 down-regulated CCR3 expression at 19 h. These data are largely in agreement with those of Dulkys et al. (12), who reported down-regulation by IL-3 and no significant effect of IL-5 or GM-CSF on CCR3 at 24 h. We have previously shown that airway eosinophils express CCR3 in asthma (29), and that eotaxin as well as IL-5 can mobilize eosinophils from bone marrow (30). Together with mast cell, basophil, and Th2 expression of CCR3, this may make this receptor system a more effective target for asthma therapy.

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