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Cytokines and chemokines are responsible for the attraction and activation of eosinophils in allergic and inflammatory diseases. Whereas cytokines such as IL-3, IL-5, and GM-CSF activate eosinophils via heterodimeric receptors containing a distinct α-chain (binding domain) and a common β-chain (signaling domain), chemokines such as eotaxin activate eosinophils via seven-transmembrane G protein-coupled CCRs. Recent studies have demonstrated the importance of CCR3 on human eosinophils that undergo receptor recycling after chemokine activation, but the modulation of this receptor by cytokines has not yet been addressed. In this study, we demonstrate that IL-3 induces a dose- and time-dependent down-regulation of CCR3 from the surface of human eosinophils comparable to the CCR3-specific ligand eotaxin, whereas IL-5, GM-CSF, IL-4, IL-10, IL-13, IFN-γ, and TNF-α had no effect. Maximal down-regulation of CCR3 in response to IL-3 was reached at 24 h. Reduction of CCR3 surface protein in response to IL-3 could be prevented by an anti-IL-3 mAb and was neither due to the release of CC chemokines nor to nonspecific binding of IL-3 to CCR3. Moreover, down-regulation was prevented by phenylarsine oxide, a nonspecific inhibitor of receptor internalization. After 24 h, IL-3-induced decrease of CCR3 surface expression correlated with diminished mRNA expression, suggesting a transcriptional regulation mechanism. Since wortmannin partially inhibited IL-3- but not eotaxin-induced CCR3 down-regulation, receptor down-modulation seems to underlie different signaling events. Therefore, these data suggest a novel role for the cytokine IL-3 in the activation process of eosinophils and its predominant chemokine receptor CCR3. The Journal of Immunology, 2001, 167: 3443–3453.

Human eosinophils play an important role in the pathogenesis of allergic diseases such as allergic asthma. Locally generated chemokines and cytokines regulate the accumulation of eosinophils in tissues. At sites of inflammation, eosinophils are believed to be responsible for tissue damage by the release of reactive oxygen species and toxic granule proteins (1, 2). Moreover, eosinophils contribute to the propagation of the inflammatory response through the secretion of cytokines (3, 4) and chemokines (5, 6).

Chemokines are chemotactic proteins which play a central role in immune and inflammatory responses by the attraction and activation of leukocytes (7, 8). Chemokines differ from other cytokines in that they activate seven-transmembrane spanning G protein-coupled receptors (GPCR),³ and receptors for members of the two major subclasses (CC and CXC) of the chemokine family have been found to be expressed on eosinophils. The major chemokine receptor on eosinophils from normal patients is CCR3 (9, 10) but CCR1 is also expressed at a lower level, and there is evidence that eosinophils from atopic patients also express other CCRs (11).

Cytokines, in particular IL-3, IL-5, and GM-CSF, are not only responsible for the generation of eosinophils from the bone marrow, but also for their activation. Human eosinophils have prolonged survival, enhanced functional properties, such as the release of toxic proteins and reactive oxygen species, and become hypodense when exposed to these cytokines (12, 13). The high-affinity receptors for IL-3, IL-5, and GM-CSF are heterodimers consisting of a ligand-specific α-chain and a common β-chain, with the three α-chains being more closely related to each other in primary sequence than to other members of the cytokine family (14, 15). Although binding of IL-3, IL-5, and GM-CSF to their respective receptor α-chains is the first step in receptor activation, it is the recruitment of the β-chain that allows high-affinity irreversible binding and leads to full receptor activation and in consequence to signal transduction.

From in vitro experiments it has been established that eosinophil functions, as well as expression of cell surface proteins, can be induced or increased by several cytokines such as IL-2, IL-3, IL-4, IL-5, GM-CSF, TNF-α, or IFN-γ (2). More recently, cytokines have been shown to be responsible for the up-regulation of chemokines receptors on lymphocytes, mononuclear phagocytes, and neutrophils (16–18). Only few reports exist about the regulation of chemokine receptor expression regulated by cytokines on eosinophils such as CXCR4 and CXCR3 (19, 20). However, to date, there are no reports about the influence of cytokines on the regulation of the dominant CCR on human eosinophils, CCR3.

The relationship between cytokines and chemokines in the activation process is poorly understood. Down-regulation and recycling of receptors, particularly chemokine receptors, during the migratory response is very important for the adaptation and desensitization of cells to the inflammatory environment (21). Recent studies have demonstrated that chemokine activation of receptors does not always result in the same trafficking pattern. RANTES
induction of CCR3 down-regulation allows its recycling to the cell surface, whereas the modified RANTES protein AOP-RANTES prevents the re-expression of cell surface CCR5 (22). On the other hand, CCR1 does not recycle after RANTES stimulation (23). CXCR2 has been shown to be degraded in the lysosomal compartment (24) whereas CCR3 appears to follow both fates; it is partially degraded in the lysosomes, but ~70% of the receptors re-cycle (23, 25).

In this study, we report that a cytokine, IL-3, which is not known to be a ligand for CCR3, very efficiently induces CCR3 down-regulation. This activity was not shared by other cytokines, IL-5 and GM-CSF, which signal through the common β-chain of the receptor for these three cytokines. The mechanism for this regulation of CCR3 expression remains to be elucidated.

Materials and Methods

Chemokines, cytokines, and ELISA kits

Eotaxin was obtained from Peprotech (London, U.K.). C5a was obtained from Sigma (Deisenhofen, Germany). IL-3, IL-4, IL-5, GM-CSF, IFN-γ, TNF-α, and IL-13 were obtained from R&D Systems (Wiesbaden, Germany). ELISA kits for eotaxin and RANTES were obtained from R&D Systems.

Monoclonal Abs and protein inhibitors

The mAb against CCR3 (clone 7B11, mouse IgG2a) was a kind gift from Dr. Ponath. Since this Ab was no more obtainable, we used a rat mAb against human CCR3 (clone 61828.11; IgG2a) from R&D Systems which shows a similar binding pattern and inhibitory features than 7B11. The mouse and the rat IgG2a isotype control Ab were obtained from Immunotech (Hamburg, Germany). The PE-conjugated anti-CD16 mAb was obtained from Immunotech; anti-IL-3, anti-RANTES, and anti-eotaxin mAbs were obtained from R&D Systems. The protein inhibitors wortmannin, staurosporin, and genistein were obtained from Calbiochem (San Diego, CA).

Eosinophil isolation

Human granulocytes were isolated from heparin-anticoagulated venous blood from normal nonatopic healthy or atopic donors using Ficoll (Pharmacia, Erlangen, Germany) density gradient centrifugation as described previously (23). For further purification, granulocytes were resuspended in HEPES-buffered HBSS (Life Technologies, Grand Island, N.Y.), pH 7.4, containing 1 mg/ml BSA (HBSS plus BSA). The granulocyte pellets were harvested and washed with PBS; the remaining erythrocytes were lysed using brief exposure to hypotonic solution (0.85% NaCl); and the eosinophils were harvested and washed with PBS containing 1 mg/ml BSA (HBSS plus BSA). The granulocyte pellets were centrifuged (500 × g, 10 min) to remove neutrophils following the methods described by the manufacturer (26). The resulting eosinophil purity was 99% as determined by microscopic examination with Kimura staining and flow cytometric analysis (FACSscan; BD Biosciences, Heidelberg, Germany) using anti-CD16. There were no differences in viability of the cells (<95%) between the fractions, as judged by trypan blue exclusion.

Immunofluorescence of eosinophils

Immunofluorescence of eosinophils was conducted as described previously (23). Aliquots (10 µl) containing 1 × 10⁶ cells were incubated at 4°C for 30 min with the indicated mAb, stained in a second step by a FITC-conjugated goat anti-mouse (for clone 7B11) or goat anti-rat Ab (for clone 61828.111) (Immunotech) and analyzed by flow cytometry. Data are expressed as original plot (specific mAb vs isotype control), ratio of fluorescence intensity of specific mAb (median) and isotype control (median), or fluorescence intensity (percent) calculated as: (median channel fluorescence (stimulus) – median channel fluorescence (isotype control))/median channel fluorescence (isotype control) × 100%.

Down-modulation and re-expression of chemokine receptors

These experiments were conducted as described in detail previously (23). Eosinophils were incubated in a total volume of 100 µl of RPMI 1640 at 37°C for different time periods at 37°C in an incubator or on ice with the indicated cytokine, various concentrations of IL-3, IL-5, GM-CSF, and eotaxin. RPMI 1640 medium-cultured eosinophils were used as the control. Aliquots were taken at the times indicated and stained for receptor surface expression as described above. For re-expression experiments, cells were incubated for 24 h with IL-3 or eotaxin to induce maximal down-regulation of the receptor. Thereafter, eosinophils were washed three times with RPMI 1640 and further incubated in IL-3 and eotaxin-free medium (RPMI 1640) for the indicated time at 37°C. For data analysis, only cells were taken with a viability of <95%, as judged by trypan blue exclusion.

Inhibition of internalization

Eosinophils were treated for 5 min with 8 µM phenylarsine oxide (PAO; Sigma) at 37°C. Thereafter, cells were further incubated in the presence of PAO with IL-3 (5.7 × 10⁻⁷ M), eotaxin (1.25 × 10⁻⁷ M), or medium for the indicated time periods. After washing, immunofluorescence staining was conducted as described above. There were no differences in viability of the cells (<95%) between the fractions, as judged by trypan blue exclusion.

Inhibition of protein phosphorylation

Eosinophils were treated with the indicated inhibitor or medium in the presence or absence of eotaxin and IL-3 for 24 h at 37°C. Thereafter, cells were washed and immunofluorescence staining was conducted as described above. There were no differences in viability of the cells (<95%) between the fractions, as judged by trypan blue exclusion.

Real-time quantitative RT-PCR assay

RT-PCR analysis of eosinophil mRNA was performed with a LightCycler Instrument (Roche Molecular Biochemicals, Mannheim, Germany) which allows the quantification of mRNA as described previously (27). In brief, poly(A)+ RNA was isolated from 10⁶ eosinophils using a mRNA isolation kit (Roche Molecular Biochemicals). The isolated mRNA was used for cDNA synthesis with the first-strand cDNA synthesis kit (Roche Molecular Biochemicals) following the instruction manual as described previously (27). Specific PCR primers for human mRNA of CCR3 and β-actin were designed and conventional PCR was performed under primer-specific conditions. The primers used for PCR were CCR3 (sense, 5′-ATG CGT GTC ACA GAG GTG AT-3′ and antisense, 5′-AGG GTA GTC TGG AAC CTT TA-3′; expected bp length 354), eotaxin (sense, 5′-TGA AGC TGG GCC CAG CTT CTG TTC CAA CC-3′ and antisense, 5′-TGG TAC GTG GGA GTT GGA GAT TTT TGG TC-3′), and β-actin (sense, 5′-GAG CGA ATC ATG CGT CGA ATT TC-3′ and antisense, 5′-GAA GGT AGT TTC GTG GAC CCC-3′; expected bp length 204) and were designed according to the published sequences (GenBank accession nos.: CCR3, AF026355, eotaxin, AJ223461, and β-actin, AB004047). The standards and unknown samples were added in a total volume of 20 µl reaction as described in detail previously (27). The standards used in this experiment were obtained from a serial dilution of cDNA from medium-stimulated eosinophils (24 h). The following experimental protocol was adapted to use CCR3 and β-actin: After an initial denaturation step at 95°C for 30 s, 40 cycles (CCR3) and 30 cycles (β-actin) of amplification were performed, respectively, followed by a final elongation step at 95°C, followed by primer-specific annealing (55°C for CCR3; 60°C for β-actin), the elongation was conducted at 72°C with an ampiclon-depending incubation time (15 s for CCR3; 9 s for β-actin). Finally, the standard curve allowed calculation of the template concentrations in the unknown samples using LightCycler software. Agarose gel electrophoresis of the probes obtained by the LightCycler revealed the predicted size for CCR3.

Measurement of intracellular Ca²⁺ concentration ([Ca²⁺]i)

Eosinophils were incubated in a total volume of 100 µl of RPMI 1640 at 37°C for 24 h in an incubator with IL-3 and eotaxin to induce a down-regulation of CCR3. RPMI 1640 alone was used as the control. After 24 h, all samples were washed with PBS/0.1% BSA and loaded for 30 min with 2 µM fura 2 (Molecular Probes, Eugene, OR). Thereafter, cells were washed three times with PBS/0.1% BSA at room temperature and further incubated at 37°C. Aliquots were taken at the times indicated and [Ca²⁺]i was measured at 37°C in an Aminco Bowman series 2 spectrofluorometer (SLM-Aminco, Urbana, IL) as described previously (23). Data are expressed as the original plots or as the ratio of chemokine-stimulated maximal [Ca²⁺]i level and baseline [Ca²⁺]i level as described previously (28). The increase in magnitude of intracellular calcium basal level as well as C5a-stimulated maximal calcium concentration can be explained by a longer resting period of the cells in PBS/0.1% BSA.

Lucigenin-dependent chemiluminescence

Lucigenin-dependent chemiluminescence was measured using a single-photon imaging system with a two-dimensional photon counting system which allows the simultaneous measurement and analysis of 96-well (MTP
reader; Hamamatsu Photonics, Herrsching, Germany) as described previously (23). In brief, eosinophils were suspended at a density of 5 × 10⁶ cells/ml in HBSS plus BSA containing 200 μM lucigenin (Sigma). Aliquots (100 μl) containing 5 × 10⁶ eosinophils were placed into flat-bottom white microtiter plates (Microfluor; Dynatech, Denkendorf, Germany). Cells were preincubated in the presence and absence of the anti-CCR3 mAb 61828.111 and subsequently stimulated by IL-3 or medium as a control. Measurements were performed in triplicate at 37°C.

Statistical analysis

The number of experiments is stated in the legends of the figures as n and represents different donors. Unless otherwise stated, the data in the text and figures are expressed as mean ± SEM as determined by SigmaStat (SPSS, Chicago, IL) analysis. Values of p > 0.05 were accepted as significant using Student’s t test: *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Results

IL-3 induced a down-regulation of CCR3 on human eosinophils

In the first set of experiments, eosinophils were incubated for 24 h with different cytokines at concentrations that have been shown to modulate receptor expression on eosinophils (29). From all cytokines tested (IL-3, IL-5, GM-CSF, IL-13, IFN-γ, TNF-α), only IL-3 was able to modulate CCR3 expression. Surprisingly, it down-regulated CCR3 expression on the surface of human eosinophils (Table I). Therefore, we decided to investigate the effect of IL-3 on CCR3 expression in comparison to the CCR3-specific ligand eotaxin, which has recently been shown to induce internalization of CCR3 on human eosinophils (23). Since IL-3, IL-5, and GM-CSF are members of the same cytokine family sharing a common receptor chain, we also examined CCR3 expression following stimulation with these cytokines in different doses.

Human eosinophils were incubated for 24 h with medium and different doses of IL-3, IL-5, and GM-CSF in a range between 10⁻¹¹ M and 10⁻⁸ M. In comparison to these non-CCR3 ligands we used eotaxin as natural ligand for CCR3. Neither IL-5 nor GM-CSF was able to significantly modulate CCR3 surface expression (Fig. 1). However, CCR3 surface expression decreased to 40% (compared with medium) following stimulation with IL-3 at a concentration of 5.7 × 10⁻¹¹ M. Maximal down-regulation of CCR3 down to 30% was reached at 5.7 × 10⁻⁹ M (Fig. 1). In comparison, maximal eotaxin-induced CCR3 down-regulation was observed at 4 × 10⁻⁸ M. At optimal concentrations, IL-3 was just as efficient as eotaxin to induce CCR3 down-regulation. However, at lower concentrations IL-3 was more potent than eotaxin to induce CCR3 down-regulation (Fig. 1).

To determine whether IL-3 and eotaxin behave synergistically, cells were cultured with a submaximal concentration of each stimulus (100 ng/ml eotaxin and 10 ng/ml IL-3) for 30 min and 24 h, respectively. We found that CCR3 down-modulation was not intensified by the addition of eotaxin in comparison to eosinophils incubated with IL-3 alone (data not shown). These data suggest no synergistic or additive effect regarding CCR3 down-regulation due to IL-3 incubation in combination with eotaxin.

To clarify whether the reduced cell surface expression in response to IL-3 and eotaxin detected by binding of the anti-CCR3 mAb is temperature dependent, eosinophils were incubated at 4°C. At this temperature, the binding of the anti-CCR3 mAb was only slightly but not significantly reduced after incubation with IL-3 (Fig. 2A). These data indicate that IL-3 binding to IL-3R induced a temperature-dependent down-modulation of CCR3.

Table I. IL-3-induced CCR3 down-regulation

<table>
<thead>
<tr>
<th>Medium</th>
<th>IL-1β (1.8 × 10⁻⁸ M)</th>
<th>IL-3 (5.7 × 10⁻¹⁰ M)</th>
<th>IL-5 (1.5 × 10⁻⁹ M)</th>
<th>GM-CSF (7.1 × 10⁻⁸ M)</th>
<th>IL-4 (7.1 × 10⁻⁸ M)</th>
<th>IL-10 (5.4 × 10⁻⁹ M)</th>
<th>IL-13 (8.3 × 10⁻⁹ M)</th>
<th>IFN-γ (8.3 × 10⁻⁸ M)</th>
<th>TNF-α (8.3 × 10⁻⁸ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constitutive</td>
<td>15.01 ± 1.89</td>
<td>8.93 ± 2.25</td>
<td>9.03 ± 2.26</td>
<td>14.69 ± 2.46</td>
<td>12.52 ± 4.85</td>
<td>13.70 ± 1.87</td>
<td>16.21 ± 2.81</td>
<td>14.32 ± 2.53</td>
<td>14.34 ± 3.10</td>
</tr>
<tr>
<td>Anti-Eotaxin mAb</td>
<td>6.45 ± 2.33</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Eotaxin ELISA</td>
<td>74.3 pg/ml</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>RANTES ELISA</td>
<td>3.3 pg/ml</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Eotaxin mRNA</td>
<td>Constitutive</td>
<td>No up-regulation</td>
<td>No up-regulation</td>
<td>No up-regulation</td>
<td>No up-regulation</td>
<td>No up-regulation</td>
<td>No up-regulation</td>
<td>No up-regulation</td>
<td>No up-regulation</td>
</tr>
</tbody>
</table>

* Eosinophils were preincubated for 24 h with medium or the indicated cytokine. Data (n = 8) are expressed as the mean ± SD of the ratio of fluorescence intensity between specific anti-CCR3 mAb and isotype control.

† Eosinophils were incubated with medium, IL-3 (5.7 × 10⁻¹⁰ M), eotaxin (1.25 × 10⁻⁷ M), IL-3 (5.7 × 10⁻¹⁰ M) plus anti-IL-3 mAb (5 μg/ml), or eotaxin (1.25 × 10⁻⁷ M) plus anti-eotaxin mAb (10 μg/ml) for 24 h at 37°C. Data (n = 6 for anti-IL-3; n = 1 for anti-eotaxin mAb) are expressed as the mean ± SD of the ratio of fluorescence intensity between specific anti-CCR3 mAb and isotype control.

ND, not done.

‡ Eosinophils were stimulated with medium or IL-3 (5.7 × 10⁻¹⁰ M) for 24 h at 37°C and thereafter the supernatant was measured for eotaxin or RANTES using a standard ELISA technique. One representative experiment of three is shown. Note: These concentrations were not sufficient to induce CCR3 down-regulation (data not shown).

§ RT-PCR analysis was carried out as described in Materials and Methods.
IL-3-induced CCR3 down-regulation was specific and neither due to release of eotaxin and RANTES from eosinophils nor due to binding to CCR3.

To investigate whether the IL-3-induced CCR3 down-regulation was directly mediated by IL-3, eosinophils were incubated with a mixture of IL-3 and a neutralizing anti-IL-3 mAb. Flow cytometric analysis revealed that anti-IL-3 mAb prevented IL-3-induced down-regulation of CCR3 (Fig. 2B and Table I), further supporting the receptor dependence of this action. To exclude the possibility that the IL-3-induced down-regulation could be caused by the release of CCR3 ligands, such as eotaxin and RANTES, from eosinophils in response to IL-3, down-regulation experiments were performed in the presence of the neutralizing anti-eotaxin mAb 10C11. However, this mAb (at concentrations of 1, 5, and 10 ng/ml) was not able to prevent the IL-3-induced but could inhibit the eotaxin-induced CCR3 down-regulation of human eosinophils (Table I). No increased protein expression for eotaxin and RANTES was detectable in IL-3-treated eosinophils when compared with medium-treated cells as determined by ELISA (Table I). Pretreatment of eosinophils with the blocking anti-CCR3 mAb, 61828.111 did not prevent the release of reactive oxygen species from eosinophils in response to IL-3 (data not shown). Therefore, IL-3 induces a specific down-regulation of CCR3 on human eosinophils which could not be explained by an IL-3-induced release of eotaxin and RANTES from eosinophils or a direct receptor interaction to CCR3.

Kinetics of CCR3 down-regulation and CCR3 re-expression

To further characterize the IL-3-induced down-modulation, we first focused our interest on the time dependence of the process. As seen in Fig. 3A, we could show that IL-3-induced CCR3 down-regulation occurred significantly after 30 min ($p > 0.05$) and 60 min ($p > 0.01$). In contrast, at 10, 15, 120, and 180 min no significant down-regulation of IL-3-treated eosinophils could be detected (Fig. 3A). The strongest down-regulation, however, could be observed after 24 h ($p > 0.001$; Fig. 3A). On the other hand, eotaxin, which is well known to induce an internalization of CCR3 (23, 25), induced a strong down-regulation of CCR3 after 10 min, with significance after 15 min ($p > 0.001$; Fig. 3A).

To address the question whether different re-expression patterns occurred after IL-3 and eotaxin treatment, we measured the reappearance of CCR3 on stimulated eosinophils. We therefore analyzed CCR3 expression at different time periods from 0 to 120 min after 24-h stimulation with IL-3 or eotaxin, respectively (Fig. 3B).
Interestingly, flow cytometric analysis showed that the re-expression of CCR3 on eotaxin- and IL-3-stimulated cells followed the same time course (Fig. 3B). Re-expression of CCR3 slowly occurred after 30 min of culturing in medium and even after 120 min in medium only 40% of the receptors were detectable on the surface. After 24 h of reculturing in pure medium, the viability of human eosinophils was too low to further investigate CCR3 re-expression.

**PAO prevented IL-3-induced CCR3 down-regulation**

Previous studies revealed that eotaxin induces CCR3 internalization (23, 25). Therefore, we next asked whether short-term IL-3 treatment induces internalization of CCR3 and incubated eosinophils with IL-3 and eotaxin, respectively, in the presence of PAO. PAO inhibits the protein tyrosine phosphatase and has widely been used as a general inhibitor of receptor internalization (30, 31). Treatment of human eosinophils with 8 μM PAO prevented IL-3-induced as well as eotaxin-induced CCR3 down-regulation as shown in Fig. 4 and Table II. In cells incubated for 15 min with IL-3, CCR3 expression decreased down to 62.3%. Whereas in cells additionally treated with PAO, down-regulation was almost completely inhibited (98.2%; Fig. 4 and Table II). These data indicate that IL-3 induces a quick receptor-mediated endocytosis of CCR3 comparable to the eotaxin-induced internalization of CCR3.

**Reduced CCR3 mRNA expression in eosinophils by IL-3**

To prove whether transcription is a regulatory step leading to reduced surface CCR3 expression, we examined the mRNA expression of medium- and cytokine-stimulated eosinophils using real-time quantitative RT-PCR. Therefore, eosinophils were stimulated for 24 h with cytokines or incubated with medium. After isolation of the mRNA, real-time quantitative RT-PCR was performed. The melting curves in Fig. 5A indicate that mRNA for CCR3 was present in nonstimulated human eosinophils as well as in eosinophils incubated for 24 h with different cytokines. Agarose gel electrophoresis of the probes obtained by the LightCycler revealed the predicted size for CCR3 (Fig. 5A). During amplification, the fluorescence signal for CCR3 cDNA in IL-3-incubated eosinophils increased later than for the other cytokines tested, indicating lower CCR3 mRNA amounts in IL-3-treated cells (Fig. 5B). Comparing the amplification of standard cDNA template (24-h medium-incubated eosinophils) and cytokine-treated cDNA with the cDNA of a housekeeping gene (β-actin), we could detect a significant decrease in CCR3 cDNA. Whereas after 60 min of stimulation, CCR3 mRNA was only lowered to 80%; after 24 h of incubation, CCR3 mRNA was dramatically diminished (p < 0.001) down to 35% compared with medium-cultured cells (Fig. 5C). The other cytokines tested (IL-5, GM-CSF, and IFN-γ) had no significant effect on CCR3 mRNA expression. Therefore, the down-regulation

![Graph](https://via.placeholder.com/150)

**FIGURE 3.** Time-course of IL-3- and eotaxin-induced CCR3 down-modulation and re-expression on human eosinophils. A, Eosinophils were incubated with IL-3 (5.7 × 10⁻⁹ M) and eotaxin (1.25 × 10⁻⁷ M) at 37°C for different time periods. Thereafter, cells were stained with anti-CCR3 mAb and analyzed by flow cytometry. B, Eosinophils were incubated with IL-3 and eotaxin at 37°C to induce maximal CCR3 internalization. Thereafter, cells were washed and resuspended in IL-3- or eotaxin-free medium for different time periods. Cells were stained with anti-CCR3 mAb and analyzed by flow cytometry. Data (n = 7) are expressed as the mean ± SEM of relative fluorescence intensity.

![Graph](https://via.placeholder.com/150)

**FIGURE 4.** PAO prevented IL-3- and eotaxin-induced CCR3 down-regulation. Eosinophils were incubated with IL-3 (5.7 × 10⁻⁹ M), eotaxin (1.25 × 10⁻⁷ M), or medium at 37°C for different time periods in the presence or absence of the unspecific internalization inhibitor PAO. Cells were washed and stained with anti-CCR3 mAb and analyzed by flow cytometry. Data are expressed as original plot of the histogram analysis of seven different experiments.
of CCR3 surface protein by IL-3 parallels with decreased CCR3 mRNA expression.

**Wortmannin partly inhibited the IL-3-induced CCR3 down-regulation on human eosinophils**

The data presented in this study so far implicate that IL-3 induces CCR3 down-regulation via a pathway not directly mediated by CCR3 signaling. Kinase inhibitors are a sensitive way to assess signal transduction pathways and the method of choice when only low numbers of cells are available, as in the present study. Pretreatment of eosinophils with wortmannin was able to prevent in part the IL-3-induced down-regulation of CCR3 in a dose-dependent manner (Fig. 6, A and B). On the contrary, wortmannin was not able to prevent the eotaxin-induced CCR3 down-regulation (Fig. 6, A and C). In contrast to wortmannin, other kinase inhibitors such as genistein, a tyrosine kinase inhibitor, and staurosporin, a protein kinase C inhibitor, were not able to prevent IL-3-induced down-regulation (data not shown).

**IL-3 induced a functional desensitization to eotaxin**

To address the question whether the CCR3 down-regulation parallels with eosinophil effector functions, [Ca\(^{2+}\)]\(_i\) assays were performed. Eosinophils were preincubated with IL-3 (5.77 × 10\(^{-8}\) M) or eotaxin (1.25 × 10\(^{-7}\) M) for 24 h to induce maximal CCR3 down-regulation and subsequently resuspended in fresh medium without IL-3 or eotaxin. After reincubation in ligand-free medium for various periods of time, eosinophils were exposed to different doses of eotaxin and [Ca\(^{2+}\)]\(_i\) was measured using spectrofluorometry.

Immediately after eotaxin-induced CCR3 down-regulation and washing steps eosinophils did not show a [Ca\(^{2+}\)]\(_i\) increase in response to eotaxin (Fig. 7, 0 min). A 10-min incubation time in eotaxin-free medium was sufficient to induce a small but detectable [Ca\(^{2+}\)]\(_i\), rise following stimulation with eotaxin. Prolonged incubation time (20, 30, or 60 min) in eotaxin-free medium resulted in an enhanced [Ca\(^{2+}\)]\(_i\) signal after exposure to eotaxin (Fig. 7).

To investigate whether IL-3 acts directly via CCR3, freshly isolated eosinophils were prestimulated with IL-3 and subsequently stimulated with eotaxin to monitor [Ca\(^{2+}\)]\(_i\) transients (Fig. 8, Control). However, IL-3 was not able to block [Ca\(^{2+}\)]\(_i\) in response to eotaxin, indicating that IL-3 did not bind to a region of the CCR3 close to the binding side of the CCR3-specific ligand eotaxin (Fig. 8, Control). Moreover, short-term IL-3 treatment did not desensitize freshly eosinophils to eotaxin (Fig. 8, Control).

To determine whether long-term IL-3 treatment affects eosinophil effector functions, human eosinophils were incubated for 24 h with IL-3 (5.77 × 10\(^{-9}\) M) and medium, respectively. Thereafter, cells were stimulated with eotaxin in different doses (1.25 × 10\(^{-9}\), 1.25 × 10\(^{-8}\), and 1.25 × 10\(^{-7}\) M). IL-3-pretreated (24-h) eosinophils did not show significant [Ca\(^{2+}\)]\(_i\) transients when cells were immediately resuspended in IL-3-free medium and stimulated with eotaxin (Fig. 8, 0 min, and Fig. 9). The insufficient resensitization to eotaxin at this early time period (0 min) in IL-3-treated cells differed significantly (p > 0.01) from that of medium-treated cells and was dose and time dependent of eotaxin (Fig. 9). Longer resuspension of eosinophils in IL-3-free medium resulted in a resensitization to eotaxin and did not differ from that of medium-pretreated cells (Figs. 8 and 9). Interestingly, whereas the eotaxin-induced magnitude of [Ca\(^{2+}\)]\(_i\) increased during longer resuspension time in IL-3-free medium, the C5a-induced [Ca\(^{2+}\)]\(_i\) magnitude was not significantly affected by preincubation of eosinophils with IL-3 (Fig. 8). Taken together, we could clearly demonstrate that besides the down-regulation of CCR3 surface protein and mRNA the effector functions of eosinophils are markedly decreased in response to the CCR3 ligand eotaxin.

**Discussion**

Although chemokines and their receptors play an important role in trafficking, recruitment, and activation of eosinophils during inflammation (2, 8), cytokines such as IL-5, GM-CSF, and IL-3 are also pivotal in their role in the production of eosinophils from the bone marrow and the induction of their adhesion capacity, cytotoxic activity, and survival (13, 14). Although cytokines such as IL-2 (18), IL-3 (32), IL-10 (33), and IFN-γ (16, 17) are able to modulate chemokine receptor expression on lymphocytes, murine bone marrow macrophages, monocytes, and neutrophils, to date only little data exist on cytokine-induced chemokine receptor regulation on human eosinophils such as CXCR4 and CXCR3, respectively (19, 20). Therefore, the aim of this study was to investigate the role of cytokines in the regulation of CCR3 on human eosinophils, the predominant chemokine receptor on these leukocytes.

In this study, we have found that a dose-dependent decrease in cell surface CCR3 levels occurs in IL-3-treated eosinophils, while none of the other cytokines tested (IL-5, GM-CSF, IL-1β, IL-4, IL-10, IL-13, IFN-γ, and TNF-α) modified the CCR3 expression. This suggests a central role of IL-3 in CCR3-mediated eosinophil activation. The question that IL-3 binds directly to CCR3 could be raised. Although we have not addressed this question by examining the ability of IL-3 to bind to CCR3, for instance in CCR3-stable transfectants using iodinated IL-3, we have investigated the heterologous desensitization of eotaxin by IL-3 in freshly isolated eosinophils. IL-3 was not able to block the calcium signal in response to eotaxin, indicating that IL-3 did not functionally inactivate the response of CCR3 to eotaxin. A blocking anti-CCR3 mAb did not inhibit the release of reactive oxygen species from human eosinophils in response to IL-3. IL-3 was not able to reduce the binding of a neutralizing anti-CCR3 mAb which blocks eotaxin binding at 4°C, a temperature at which a receptor binding is efficient, demonstrating that IL-3 does not impair eotaxin binding. We therefore proposed that IL-3-induced down-regulation of CCR3 could be due to IL-3-induced release of CCR3 ligands such as eotaxin and RANTES. However, no significant amount of eotaxin and RANTES could be detected in the supernatant of IL-3-stimulated eosinophils. An anti-IL-3 mAb but not an anti-eotaxin mAb prevented IL-3-induced down-regulation of CCR3.
although Schroder and Mochizuki (1) have demonstrated that eosinophils under certain circumstances can release CC chemokines such as eotaxin and RANTES, only picomolar amounts are released which are not sufficient to induce CCR3 down-regulation.

**FIGURE 5.** Real-time quantitative RT-PCR assay revealed IL-3-induced down-regulation of CCR3 mRNA in human eosinophils. Eosinophils were cultured for 24 h in medium or with the indicated cytokines ($5.7 \times 10^{-9}$ M), respectively. Isolation of mRNA and real-time quantitative RT-PCR of eosinophils were performed as described in detail in Materials and Methods. A, Melting curves of the indicated PCR products are illustrated (temperature $88.4 \pm 0.5 \degree C$). Electrophoresis of RT-PCR products and visualization with ethidium bromide indicated the predicted size for CCR3 cDNA (M, 50-bp DNA size marker; bright band, 350 bp). One representative experiment of four is shown. B, Fluorescence (F1) signal vs cycle number during amplification for CCR3-specific cDNA of the indicated stimulated eosinophils are illustrated. C, The standard curve allows calculation of the template concentrations in the unknown samples using LightCycler software. CCR3 mRNA expression is calculated by the ratio of concentration of CCR3 cDNA and concentration of $\beta$-actin cDNA $\times 100\%$ in untreated or cytokine-treated eosinophils ($n = 4$), respectively. *** $p > 0.001$.

**FIGURE 6.** Wortmannin partly prevented the IL-3-induced CCR3 down-regulation on eosinophils. A, Eosinophils were treated with IL-3 ($5.7 \times 10^{-9}$ M) and eotaxin ($1.25 \times 10^{-7}$ M) in the presence or absence of different concentrations of wortmannin for 24 h at $37\degree C$. Thereafter, cells were stained with anti-CCR3 mAb and analyzed by flow cytometry. Data ($n = 5$) are expressed as the mean $\pm$ SEM of relative fluorescence intensity. B, Eosinophils were treated with medium (a) and IL-3 ($5.7 \times 10^{-9}$ M) in the presence (c) or absence (b) of wortmannin ($10^{-6}$ M) for 24 h at $37\degree C$. d, Isotype control. C, Eosinophils were incubated with medium (a) and eotaxin ($1.25 \times 10^{-7}$ M) in the presence (c) or absence (b) of wortmannin ($10^{-6}$ M) for 24 h at $37\degree C$. d, Isotype control. After incubation, cells were stained with anti-CCR3 mAb and analyzed by flow cytometry. Data are expressed as original plot of the histogram analysis of six different experiments.
Thus, the possibility that IL-3-induced down-regulation of CCR3 on human eosinophils could be due to a nonspecific binding to CCR3 or due to IL-3-induced release of eotaxin or RANTES from eosinophils can be excluded. Down-regulation of chemokine receptors is induced by ligand activation which has been shown for CXCR1/CXCR2 (34), CCR2 (35), CCR5 (22), and CCR3 (23, 25). However, the results presented here show that a cytokine, IL-3, which is not a ligand for the chemokine receptor CCR3, is able to induce down-regulation of this receptor.

It is well known that eotaxin and other CC chemokines induce an internalization of CCR3 from the surface of human eosinophils (36, 37). Again, in this study eotaxin induced a strong internalization of CCR3 after 10 min. There exist several possiblities of cellular events leading to the modulation of receptor expression, e.g., endocytosis, exocytosis, shedding, modifications of the transcription of the gene, etc. (38). To find out the mechanism responsible for the marked reduction of CCR3 in response to IL-3, we conducted different approaches. In this report, we provide several lines of evidence that IL-3 has the capacity to activate the endocytosis of CCR3. First, the mechanism leading to the down-regulation of CCR3 after IL-3 treatment was temperature sensitive because IL-3-induced down-modulation did not occur at 4°C. Second, we could show that pharmacological treatment with PAO that blocks GPCR endocytosis prevented eotaxin- as well as IL-3-induced down-regulation of CCR3. Therefore, short-term exposure of human eosinophils to eotaxin as well as to IL-3 led to receptor down-regulation via the endosomal pathway.

Cytokines have been shown to modulate mRNA expression for certain chemokine receptors. The first demonstration was that of Loetscher et al. (18), who showed that IL-2 modulated the mRNA levels of CCR1 and CCR2. It has also been shown that the expression of chemokine receptors on particular leukocytes may be modified by proinflammatory cytokines—neutrophils normally express the CXC chemokine receptors CXCR1 and CXCR2 and respond to their ligands, but after treatment with IFN-γ, there is induction of mRNA for the CCRs CCR1 and CCR3 and the cells acquire responsiveness to the CC chemokines that activate these receptors (16). Cytokine regulation of receptors is not restricted to chemokine receptors; for example, the mRNA for IL-5R is down-regulated in human eosinophils by the cytokines IL-5, IL-3, and GM-CSF (39). More recently, Nagase et al. (19) found intense expression of CXCR4 surface protein after 24-h culturing of human eosinophils which could be prevented and down-regulated by several cytokines such as IL-3, IL-4, IL-5, and GM-CSF. In another preliminary study from our laboratory, we could confirm these results; however, there exists a different regulation profile between CXCR4 and CCR3 in human eosinophils. In contrast to CXCR4, CCR3 could only be down-regulated by IL-3 and not by other cytokines, indicating a more specific interaction between the IL-3R and CCR3.

To get more insight into the IL-3-induced CCR3 down-regulation, real-time quantitative RT-PCR was conducted. Studying the mRNA level of CCR3 after IL-3 stimulation, we could demonstrate that IL-3 diminished the amount of CCR3 mRNA. Here, we
could distinguish between the early effect (60 min) and the late effect (24 h) of IL-3 on CCR3 mRNA amounts. In contrast to IL-3, no changes in mRNA levels of IL-5-, GM-CSF- and IFN-γ-treated eosinophils could be detected. Therefore, the down-regulation of CCR3 surface protein after long-term incubation with IL-3 parallels with drastically diminished mRNA for CCR3. Changes in the steady-state level of RNA expression of a gene result from alterations in either the rate of transcription, the rate of degradations of the message, or a combination of both (40). Our data suggest that CCR3 mRNA expression in eosinophils is dependent on continuous transcription of the CCR3 gene, and, furthermore, that at least the late IL-3 effect is dependent upon diminished transcriptional activity of the CCR3 gene.

Since IL-3 but not IL-5 and GM-CSF induced CCR3 down-regulation in human eosinophils raised the question as to whether a different signaling between these cytokines and a common cascade of signaling molecules between IL-3 and eotaxin may occur. Protein inhibitors are only indirect tools; however, they are suitable to investigate the signal transduction pathway particularly when the number of cells of interest is low (41). Here, we have demonstrated that neither genistein, a tyrosine kinase inhibitor, nor staurosporin, a protein kinase C inhibitor, were able to prevent IL-3-induced CCR3 down-regulation. In contrast, the phosphatidylinositol 3-kinase (PI3-kinase) inhibitor wortmannin prevented in part the IL-3-induced but not eotaxin-induced down-regulation of CCR3. Recently, Coffer et al. (42) reported that both cytokines such as IL-3 and chemottractants such as RANTES and C5a induced PI3-kinase activity in human eosinophils. However, inhibition of IL-3-induced CCR3 down-regulation after wortmannin treatment is limited on micromolar doses of wortmannin. Other kinases such as myosin L chain kinase (43), phosphatidylinositol 4-kinase (43), and phospholipase A (44) are likely to be inhibited at these doses. To the best of our knowledge, up to now no data exist about an association between myosin L chain kinase or phosphatidylinositol 4-kinases or phospholipase A, respectively, and IL-3 signaling as detected by searching National Library of Medicine’s PubMed. Therefore, it appears that PI3-kinase may be partly responsible for the IL-3-induced down-regulation of CCR3. Furthermore, we showed that the protein tyrosine phosphatase inhibitor PAO almost completely prevented IL-3-induced CCR3 down-regulation. Tyrosine phosphorylation plays a major role in transmembrane signal transduction through most cell surface receptors. Binding of IL-3 to its cell surface receptor induces tyrosine phosphorylation of a set of protein substrates (45). Since PAO is also known as an inhibitor of PI3-kinase (46), our results indicate another hint for the involvement of PI3-kinase in IL-3-induced CCR3 down-regulation.

To more directly address the question as to whether the IL-3-induced CCR3 down-regulation parallels with eosinophil effector functions, [Ca\(^{2+}\)]\(_i\) assays were performed. Whereas short-term treatment of freshly isolated eosinophils with IL-3 did not desensitize cells to eotaxin, long-term IL-3 treatment for 24 h desensitized eosinophils to eotaxin. Resuspension of eosinophils in IL-3-free medium for 10 min resulted in a resensitization to eotaxin. This fast resensitization could be explained by quick mobilization of a few functional CCR3 from intracellular stores, since even 30
Thereafter, cells were resuspended in IL-3-free medium and measured directly and 30 min after resuspension using spectrofluorometry. Cells were stimulated with eosinax in different doses. Data (n = 5) are expressed as the ratio of chemokine-stimulated maximal [Ca^2+]_{i} level and baseline [Ca^2+]_{i}, level. *p > 0.05.

min after IL-3 removal only 30% of the receptors are re-expressed as shown by FACS analysis. The delayed CCR3 surface protein re-expression suggests a transcriptional control, which requires more time than the more “classical” resensitization due to the release of the receptor from intracellular stores. However, our data clearly demonstrate a loss of functional properties, concerning the major chemokine receptor CCR3, in eosinophils stimulated for a longer time with IL-3.

Cellular responses to GPCRs are usually rapidly attenuated. Mechanisms of signal attenuation include removal of agonists from the extracellular fluid, receptor desensitization, endocytosis, and down-regulation (21, 47). This process, termed desensitization, is the consequence of a combination of different mechanisms. These mechanism include the uncoupling of the receptor from heterotrimeric G proteins in response to receptor phosphorylation by intracellular kinases, the internalization of receptors to intracellular membranous compartments, and the down-regulation of the total cellular complement of receptors due to reduced receptor mRNA and protein synthesis (38). In this case, it is known that protein kinases not only phosphorylate agonist-activated, but also indiscriminately phosphorylate receptors that have not been exposed to agonists (48, 49). Therefore, it seems possible that IL-3 binding to its receptor activates kinases (e.g., PI3-kinase) which phosphorylate CCR3 and lead to receptor internalization. However, the underlying IL-3 (and not IL-5 and GM-CSF)-specific signal transduction pathway leading to CCR3 internalization, an altered transcription of the CCR3 gene and therewith to reduced surface protein expression, has to be clarified in further studies.

Although the effects of IL-3, IL-5, and GM-CSF on eosinophils are very similar in regard to stimulation of the production of eosinophils from the bone marrow and inhibition of apoptosis, this study reveals fundamental differences between these cytokines with respect to the recruitment of eosinophils. This suggests a novel role of the cytokine IL-3 in the activation process of eosinophils and its predominant chemokine receptor CCR3. Therefore, IL-3 may be responsible for a negative feedback mechanism of human eosinophils with respect to actions mediated via the CCR3 in Th2-associated inflammation.

References
12. Zeck Kap, G., W. Czech, and A. Kapp. 1994. TNF alpha-induced activation of human eosinophils and its predominant chemokine receptor CCR3. Therefore, IL-3 may be responsible for a negative feedback mechanism of human eosinophils with respect to actions mediated via the CCR3 in Th2-associated inflammation.

FIGURE 9. IL-3-induced desensitization of eosinophils to eotaxin is dose and time dependent. Eosinophils were incubated with IL-3 (5.7 × 10^{-9} M or medium at 37°C for 24 h to induce CCR3 down-regulation. Thereafter, cells were resuspended in IL-3-free medium and [Ca^2+]_{i} was measured directly and 30 min after resuspension using spectrofluorometry. Cells were stimulated with eotaxin in different doses. Data (n = 5) are expressed as the ratio of chemokine-stimulated maximal [Ca^2+]_{i} level and baseline [Ca^2+]_{i}, level. *p > 0.05.
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