

***n*-Nonanoyl-CC Chemokine Ligand 14, a Potent CC Chemokine Ligand 14 Analogue That Prevents the Recruitment of Eosinophils in Allergic Airway Inflammation¹**

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CCR3 is responsible for tissue infiltration of eosinophils, basophils, mast cells, and Th2 cells, particularly in allergic diseases. In this context, CCR3 has emerged as a target for the treatment of allergic asthma. It is well known that the N-terminal domain of chemokines is crucial for receptor binding and, in particular, its activation. Based on this background, we investigated a number of N-terminally truncated or modified peptides derived from the chemokine CCL14/hemofiltrate CC chemokine-1 for their ability to modulate the activity of CCR3. Among 10 derivatives tested, *n*-nonanoyl (NNY)-CCL14[10–74] (NNY-CCL14) was the most potent at evoking the release of reactive oxygen species and inducing chemotaxis of human eosinophils. In contrast, NNY-CCL14 has inactivating properties on human eosinophils, because it is able to induce internalization of CCR3 and to desensitize CCR3-mediated intracellular calcium release and chemotaxis. In contrast to naturally occurring CCL11, NNY-CCL14 is resistant to degradation by CD26/dipeptidyl peptidase IV. Because inhibition of chemokine receptors through internalization is a reasonable therapeutic strategy being pursued for HIV infection, we tested a potential inhibitory effect of NNY-CCL14 in two murine models of allergic airway inflammation. In both OVA- and *Aspergillus fumigatus*-sensitized mice, i.v. treatment with NNY-CCL14 resulted in a significant reduction of eosinophils in the airways. Moreover, airway hyper-responsiveness was shown to be reduced by NNY-CCL14 in the OVA model. It therefore appears that an i.v. administered agonist internalizing and thereby inhibiting CCR3, such as NNY-CCL14, has the potential to alleviate CCR3-mediated diseases. *The Journal of Immunology*, 2004, 173: 3456–3466.

The role of inflammation in allergic diseases is widely recognized, especially in asthma (1, 2). The major effector cells in asthma contributing to the inflammatory response are eosinophils, mast cells, and Th2 lymphocytes (3–5). Together they contribute to a complex pathologic process that leads to asthma symptoms and impaired lung function. CCL11/eotaxin is the first specific chemokine described as an attractant for eosinophils in bronchoalveolar lavage fluid (BALF),³ a result obtained from an experimental model of allergen exposure of sensitized guinea pigs (6). Subsequently, it was also shown to exist in humans (7). The functionally related chemokines CCL24 and CCL26 were discovered later (8, 9). Besides the eotaxins, the MCPs, CCL5 and CCL14[9–74] attract the same type of inflammatory cells involved in asthma (10, 11). An important role of CCL11 for the attraction of eosinophils into the lung was recently shown by

several groups in human asthmatics. The migration of eosinophils correlates strongly with increased peptide levels and mRNA expression of CCL11 (12, 13).

A common feature of the eotaxins, the MCPs, and CCL5 is their ability to mediate chemotaxis via CCR3, which is shown to be expressed on eosinophils (14), mast cells (15), basophils (16), and Th2 cells (17, 18). The involvement of other chemokines was demonstrated in vivo, showing that different chemokines, mostly activators of CCR3, contribute to the complex pathophysiology of asthma (19). A major breakthrough, showing the impact of CCR3 in asthma, was achieved recently (20). Targeted disruption of CCR3 was successfully performed, demonstrating that eosinophils and other inflammatory cells are arrested in the subendothelial space of pulmonary vessels after bronchial allergen challenge in OVA-sensitized mice (20), suggesting that the local inflammatory response is abolished, targeting CCR3 already in the circulation. However, airway hyper-responsiveness (AHR) is enhanced in i.p. sensitized mice, which correlates to increased intraepithelial mast cells in the airways (20), whereas AHR is abrogated in CCR3-deficient mice sensitized by the epicutaneous route (21). Therefore, CCR3 appears to be particularly attractive as a drug target; its blockade has been proposed as a tool for the therapy of asthma (22).

Besides chemokines, cytokines such as IL-4, IL-5, and IL-13 are thought to be key molecules in the pathogenesis of allergic asthma (23). The first clinical trials to block IL-5 using mAbs showed a dramatic reduction of eosinophil counts in peripheral blood from patients suffering from allergic asthma (24–26). However, approximately half this cell type was still found in bone marrow and bronchial mucosa, which may contribute to the disappointing results of these studies regarding improvement of symptoms (24–26). With respect to these results, Kay and Menzies-Gow (27)

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Received for publication February 2, 2004. Accepted for publication June 11, 2004.

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¹ This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft (SFB 587, B2) and the Bundesministerium für Bildung und Forschung (FKZ 0311815).

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³ Abbreviations used in this paper: BALF, bronchoalveolar lavage fluid; AHR, airway hyper-responsiveness; AOP, amino-oxypentane; bis-NNY, bis-*n*-nonanoyl; [Ca²⁺]_i, intracellular calcium concentration; CD26/DPP IV, dipeptidylpeptidase IV; NNY, *n*-nonanoyl; PAO, phenylarsine oxide; ROS, reactive oxygen species; HCC-1, hemofiltrate CC chemokine-1.

speculate that a combination therapy to block the maturation of eosinophils using anti-IL-5 mAbs and to stop tissue accumulation using CCR3 antagonists may be more useful than just IL-5 blockade.

In this report we first describe the effects of NH₂-terminal modifications of CCL14/hemofiltrate CC chemokine-1 (HCC-1) (28, 29) on its biological activity mediated via CCR3. Chemical modification of the most active form was performed with the intention to generate a CCR3 ligand leading to the inactivation of CCR3. Truncation of amino acid residues 1–8 of CCL14 and replacement of the amino acid in position 9 by nonanoic acid led to the identification of a potent CCR3 agonist, termed *n*-nonanoyl (NNY)-CCL14[10–74] (NNY-CCL14), with inactivating properties and that is resistant to cleavage by dipeptidyl peptidase IV (CD26/DPP IV; EC 3.4.14.5). CD26/DPP IV is an abundant peptidase found in blood plasma, tissue, and on the cell surface of various cell types. It is involved in the regulation of chemokine activity by hydrolysis of peptides with NH₂-terminal Xaa-Pro and Xaa-Ala motifs (30). Most anti-inflammatory strategies are based on the mechanism that acts on target cells after they migrate to the site of inflammation (31). In contrast, we propose that cellular recruitment is prevented by receptor inactivation of inflammatory cells before they extravasate. We show that NNY-CCL14 impairs the migration of eosinophils into the airways of OVA-sensitized and *Aspergillus fumigatus*-sensitized mice. Our investigation presents a novel strategy for the treatment of CCR3-mediated diseases.

Materials and Methods

Chemokines

CCL11/eotaxin and CXCL12 were obtained from PeproTech (London, U.K.). C5a was obtained from Sigma-Aldrich (Taufkirchen, Germany). CCL14/HCC-1[1–74] was prepared as previously described (28).

Synthesis of CCL14 derivatives

CCL14/HCC-1[6/7/8/9/10/11/12–74] and NH₂-terminally modified derivatives were prepared by F-moc-based, solid phase peptide synthesis as previously described (32). The synthesis of CCL14 peptides was conducted on a 433A peptide synthesizer (Applied Biosystems, Weiterstadt, Germany) at a scale of 0.1 mmol with a 10-fold excess of F-moc amino acid using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium/1,1-hydroxybenzo-triazole activation. After peptide chain assembly, nonanoic acid was coupled as symmetrical anhydride (Sigma-Aldrich) in *N*-methylpyrrolidone (65 equivalents) to the obtained polypeptide. The resulting peptides were cleaved and deprotected in the presence of trifluoroacetic acid/H₂O/EDT/phenol (86/6/6/2, v/v/v/w; 15 ml/g), precipitated in cold *tert*-butylmethylether and purified chromatographically. The resulting chromatographically homogeneous peptides were analyzed by capillary zone electrophoresis and electrospray mass spectrometry. The purified derivatives were used for biological testing according to the net peptide content as determined by amino acid analysis.

Antibodies

The rat mAb against human CCR3 (clone 61828.111; IgG2a) and the murine mAb against human CCR1 (clone 53504.111; IgG2b) were obtained from R&D Systems (Wiesbaden, Germany). The rat IgG2a and mouse IgG2b isotype control Ab were obtained from Immunotech (Hamburg, Germany).

Eosinophil isolation

Eosinophils were purified from the venous blood of normal nonatopic healthy or atopic volunteers, all of whom had given their informed consent according to the local research ethics committee at Hannover Medical School, using Ficoll (Pharmacia, Erlangen, Germany) density gradient centrifugation as described previously (33). For further purification, granulocytes were resuspended in HEPES-buffered HBSS (Invitrogen, Karlsruhe, Germany), pH 7.4, containing 1 mg/ml BSA. Eosinophils were purified by negative selection using a MACS CD16 kit (Miltenyi Biotec, Auburn, CA) as described previously (33). The resulting eosinophil purity was >99% as determined by microscopic examination with Kimura staining.

Lucigenin-dependent chemiluminescence

The generation of reactive oxygen species (ROS) was measured by lucigenin-dependent chemiluminescence using a single-photon imaging system with a two-dimensional photon counting system that allows simultaneous measurement and analysis of 96 wells (MTP reader; Hamamatsu Photonics, Herrsching, Germany) as described previously (34). In brief, eosinophils were suspended at a density of 5×10^4 cells/ml in HEPES-buffered HBSS, pH 7.4, containing 1 mg/ml BSA with 200 μ M lucigenin (Sigma-Aldrich). Aliquots (100 μ l) containing 5×10^3 eosinophils were placed into flat-bottom, white microtiter plates (Microfluor; Dynatech, Denkendorf, Germany). Cells were stimulated with the indicated stimuli or with medium as a control. Measurements were performed in triplicate at 37°C. Data are expressed as the ratio between stimulus-induced intensity integral counts and medium-induced intensity integral counts (34).

Flow cytometric measurement of CCR3 internalization

These experiments were performed as previously described in detail (33, 34). For internalization of CCR3, the cells were preincubated for 30 min at 37°C with the indicated stimuli in a total volume of 100 μ l of RPMI 1640 medium before staining. Thereafter, cells were fixed on ice and immediately washed with cold PBS. After the washing step, 10^5 eosinophils were stained by anti-CCR3 mAb as described below. The inhibition of CCR3 internalization was achieved by initial treatment of the cells for 5 min with 8 μ M phenylarsine oxide (PAO; Sigma-Aldrich) at 37°C and alternatively by treatment of cells with the stimuli at 4°C. Both strategies are suitable to inhibit internalization of seven-transmembraneous receptors from the cell surfaces as described previously (33).

For flow cytometry (FACScan; BD Biosciences, Heidelberg, Germany) analysis, 10^5 eosinophils were incubated at 4°C for 30 min with anti-chemokine receptor mAb or isotype control at the concentrations recommended by the supplier. In a second step, the cells were stained by FITC-conjugated, goat anti-rat or goat anti-mouse Ab (Immunotech) and thereafter analyzed by flow cytometry. Data are expressed as an original plot (specific mAb vs isotype control) or as relative fluorescence intensity (percentage), which was calculated using the following equation: (median channel fluorescence [stimulus] – median channel fluorescence [isotype control]) / (median channel fluorescence [medium] – median channel fluorescence [isotype control]) $\times$ 100% (33).

In vitro chemotaxis

Chemotaxis was assessed in 48-well chambers (NeuroProbe, Cabin John, MD) using polyvinylpyrrolidone-free polycarbonate membranes with 5- μ m pores (Nucleopore, NeuroProbe) for 5×10^4 eosinophils/well as previously described (8). Cell suspension and chemokine dilution were conducted in RPMI 1640 containing 25 mM HEPES (pH 7.4) and 0.5% BSA. Migration was allowed to proceed for 60 min at 37°C in 5% CO₂. The membrane was then removed, washed on the upper side with PBS, fixed, and stained. All assays were performed in triplicate, and the migrated cells were counted in five randomly selected fields at 1000-fold magnification. Spontaneous migration was determined in the absence of chemoattractant. For inhibition of CCL11/eotaxin-induced chemotaxis, eosinophils were preincubated for 15 min at room temperature with 10^{-7} M NNY-CCL14/HCC-1 and thereafter directly placed in the upper compartment of the chemotaxis chamber.

Measurement of intracellular calcium concentration ([Ca²⁺]_i)

Eosinophils were loaded with 2 μ M fura 2-AM (Molecular Probes, Eugene, OR), and [Ca²⁺]_i was measured at 37°C in a Bowman series 2 spectrofluorometer (SLM-Aminco, Urbana, IL) as described previously (34). Autofluorescence represents ~10% of the total fluorescence of cells loaded with fura 2 and is slightly greater at 340 nm than at 380 nm. The fluorescence of unloaded cells was therefore subtracted from an equivalent density of cells loaded with fura 2 to correct the signal for autofluorescence. The autofluorescence of the cells was virtually unchanged upon addition of stimulus. Dual excitation spectra were collected at 340 and 380 nm, and emission was fixed at 510 nm as previously described (35). Receptor desensitization was tested by monitoring [Ca²⁺]_i changes in response to sequential stimulation with chemokines as previously described (34).

Kinetics of CD26/DPP IV processing

To analyze the processing of the naturally occurring chemokine CCL14/HCC-1[9–74] (29) and the modified derivative NNY-CCL14, an in vitro kinetic study was performed by incubating 10^{-5} M chemokine with 6.6×10^{-4} U of porcine kidney DPP IV (lot 100K38002; Sigma-Aldrich) in Tris-HCl, pH 7.5, at 37°C. At specific time intervals, the reactions were stopped with 0.1% trifluoroacetic acid and placed on ice. For comparison,

the DPP IV processing of the chemokines CCL11/eotaxin and CXCL12 (36) was examined in parallel. The composition of the reactions was determined offline with a MALDI mass spectrometer (Voyager DE-Pro; Applied Biosystems) in linear mode accumulating eight spectra of 100 shots each. α -Cyano-4-hydroxycinnamic acid (Sigma-Aldrich) was used as the matrix. The instrument uses a high potential acceleration source (20 kV), and other parameters were optimized for the measurement of chemokines.

Animals

Female BALB/c mice, obtained from Charles River (Sulzfeld, Germany) at 8 wk of age and an average weight of 19 g, were used in the experiments as described previously (37). Mice were maintained on laboratory food and tap water ad libitum under pathogen-free conditions in a regular 12-h dark, 12-h light cycle at a temperature of 22°C and were allowed to become acclimated to their environment for a period of 7 days before the experiment.

Protocol of allergic sensitization with OVA or with *A. fumigatus* extract and NNY-CCL14 treatment

The animal experiment was approved by the Bezirksregierung Hannover and was conducted according to the guidelines of the institutional animal care and use committee. For OVA sensitization, animals were divided into two groups of four. Sensitization of the animals was conducted via the i.p. route on days 0, 14, and 21, each with 10 μ g of OVA (chicken OVA grade VI; Sigma-Aldrich) together with 1 mg of Al(OH)₃ (Alum Inject; Pierce, Rockford, IL) as adjuvant dissolved in sterile saline (37). To provoke an allergic airway response, aerosol challenge was performed using a Pari Master nebulization system (mass median aerodynamic diameter, 2.5 μ m) and a 1% OVA solution. Animals were exposed to allergen on day 28 for 10 min, yielding a calculated airway allergen deposition of ~10 μ g of OVA/mouse. To examine the inhibitory effect of NNY-CCL14, four mice were treated with 10 nmol/kg NNY-CCL14/HCC-1 diluted in sterile saline (applied via the tail vein as a bolus) 30 min before and 3 and 8 h after the challenge. The other group (positive control) was injected with sterile saline at identical time points. For lung function measurement, the protocol was modified, and two allergen challenges on days 28 and 29 were performed. Subsequently, animals were treated four times with 3 nmol/kg NNY-CCL14 (30 min before and 6 h after each challenge). AHR in response to methacholine was assessed on day 30 by head-out plethysmography as described previously (38).

For *A. fumigatus* sensitization, animals were divided into three groups of eight to 12 mice. Allergic airway inflammation was induced using a modified allergy model described by Hogaboam et al. (39). Mice were sensitized s.c. and i.p. with an equal volume of 0.1 ml using a mixture of *A. fumigatus* extract (Greiner Laboratories, Lenoir, NC; lot XPM3A3) in sterile saline emulsified with IFA. Sensitization was performed using an allergen dose of 5.4 μ g of *A. fumigatus*/mouse. Control animals received sterile saline (negative control). Fourteen days later, animals (NNY-CCL14 group and positive controls) were challenged with aerosolized *A. fumigatus* extract using the Pari Master system and an *A. fumigatus* solution at a concentration of 5.4 mg/ml for 12 min, resulting in a final lung dose of ~5 μ g/mouse. The negative control animals received aerosolized saline. The second challenge was performed on day 21 exactly as the first challenge, except that the negative control animals received *A. fumigatus* aerosol as well. To examine the inhibitory effect of NNY-CCL14, one group of mice was treated on days 14 and 21 with 30 nmol/kg NNY-CCL14 30 min before and 6 h after the challenge (NNY-CCL14 group). The control groups were injected with sterile saline at identical time points.

To investigate inappropriate cellular activation by NNY-CCL14, non-allergen-sensitized/challenged mice were treated i.v. with 10 nmol/kg NNY-CCL14 or sterile saline (each group, $n = 10$). Five animals of each group were killed 30 min or 24 h after i.v. treatment and subjected to histological evaluation of the lung.

BALF and histological evaluation

Animals were killed 24 h after allergen challenge by injecting an overdose of sodium pentobarbital (Narcoren, Merial, Hallbergmoos, Germany) i.p. The trachea was catheterized, airways were lavaged with 0.8 ml of cold 0.9% NaCl, and lung dissection was performed. Total cell numbers in BALF were counted, and cytopspins were evaluated (37). The left lungs of the mice were dissected and fixed in formalin for additional histological examination using H&E staining.

Statistical analysis

The number of experiments is given in the figures as n and represents different donors. Unless otherwise stated, the data in the text and figures

Chemokine analog

Chemokine analog	Amino terminus
CCL14[1-74]	1 5 10 15 TKTESSSRGPYPHPSECC
CCL14[6-74]	SSRGPYPHPSECC
CCL14[7-74]	SRGPYPHPSECC
CCL14[8-74]	RGPYPHPSECC
CCL14[9-74]	GPYPHPSECC
CCL11 (eotaxin)	GPASVPTCC
CCL14[10-74]	PYPHPSECC
CCL14[11-74]	YPHPSECC
CCL14[12-74]	HPSECC
NNY-CCL14[10-74]	CH ₃ (CH ₂) ₇ CO-PYPHPSECC
Bis-NNY-CCL14	CH ₃ (CH ₂) ₇ CO-Orn [CH ₃ (CH ₂) ₇ CO] -PYPHPSECC

FIGURE 1. Alignment of NH₂-terminal sequences of CCL14 derivatives and CCL11.

were expressed as the mean $\pm$ SEM, as determined by SigmaStat (SPSS, Erkrath, Germany) analysis. Values of $p \leq 0.05$ were accepted as significant using Student's t test.

Results

Derivatives of CCL14

To characterize the functional importance of the NH₂-terminal domain of CCL14/HCC-1, the biological activities of 10 different CCL14 analogues were investigated. These included NNY-CCL14 (NNY-CCL14[10–74]), bis-NNY-CCL14[10–74], full-size CCL14[1–74], and seven NH₂-terminally truncated variants. The NH₂-terminal sequences of these derivatives and that of CCL11 are shown in Fig. 1.

Human blood eosinophils as a natural system to study the effects of CCL14 derivatives on CCR3

To study the effects of the CCL14/HCC-1 derivatives on CCR3, we used freshly isolated human blood eosinophils, a natural cell population expressing high surface levels of CCR3. We screened 80 donors for CCR1 expression and found only two individuals with significant CCR1 surface expression (data not shown), who were excluded from the subsequent experiments.

The CCL14 analog NNY-CCL14 is a potent activator of the respiratory burst mediated by CCR3

First, we compared the effects of all CCL14/HCC-1 derivatives on the release of ROS using lucigenin-dependent chemiluminescence,

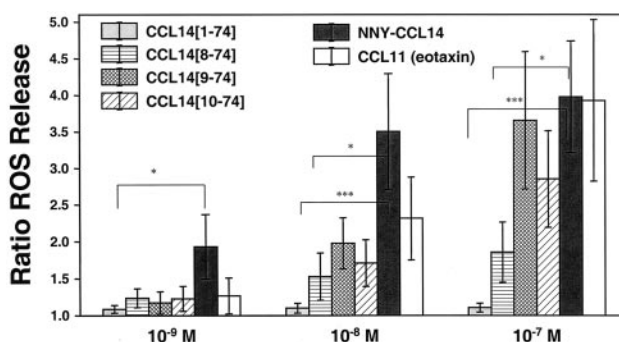


FIGURE 2. NNY-CCL14/HCC-1 induces the release of ROS from human eosinophils at lower concentrations than CCL11/eotaxin. The release of ROS was measured using lucigenin-dependent chemiluminescence. Human eosinophils were stimulated with different concentrations of the indicated chemokine. Data ($n = 7$) are expressed as relative ROS release, which is calculated as the ratio of stimulus-treated to medium-treated cells. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with the matched stimuli indicated by the line.

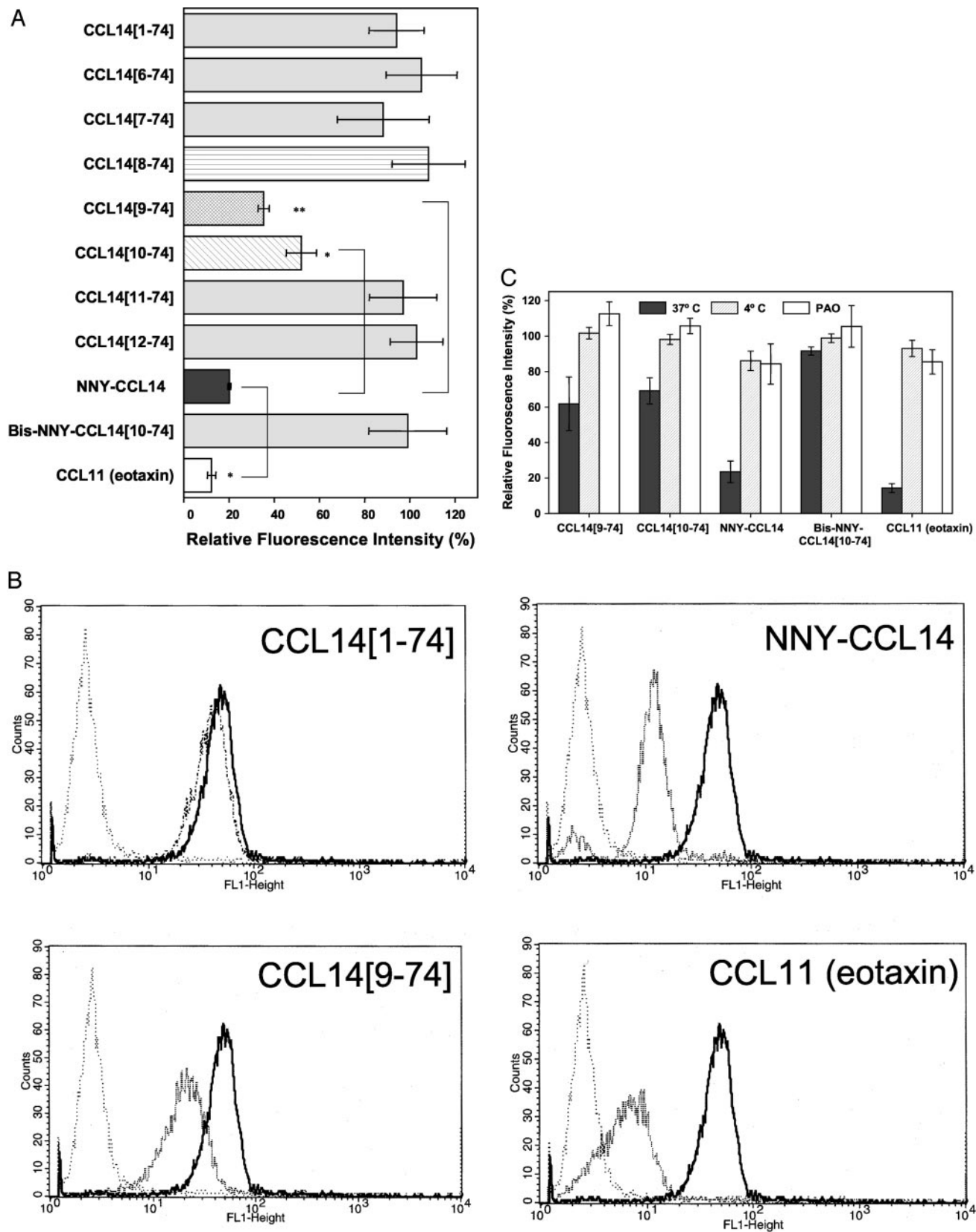


FIGURE 3. NNY-CCL14 induces internalization of CCR3 from human eosinophils in the same range as CCL11. **A**, Human eosinophils were treated for 30 min at 37°C with the indicated CCL14/HCC-1 derivatives (10^{-7} M) and CCL11/eotaxin (10^{-7} M). Thereafter, cells were stained with anti-CCR3 mAb and analyzed by flow cytometry. Data ($n = 4$) are expressed as the mean $\pm$ SEM relative fluorescence intensity as described in *Materials and Methods*. *, $p < 0.05$; **, $p < 0.01$ (compared with the matched stimuli indicated by the line). **B**, Histogram analysis of one representative experiment shown in **A**. Bold line, anti-CCR3 staining before chemokine treatment; dotted line, isotype control; broken line, anti-CCR3 staining after chemokine treatment. **C**, Cells were incubated with the indicated chemokine (10^{-7} M), at 37 or 4°C or were pretreated with PAO (8 μ M). Data ($n = 4$) are expressed as the mean $\pm$ SEM relative fluorescence intensity.

which has been established as a sensitive method to study effector functions mediated by chemokine receptors on human eosinophils (34). Of all the derivatives studied, only CCL14[8–74], CCL14[9–74], CCL14[10–74], and NNY-CCL14 induced a significant release of ROS at concentrations up to 10^{-7} M (Fig. 2). These derivatives were compared at different doses with CCL11/eotaxin, which has been described as the most potent activator of the respiratory burst in eosinophils (40), and CCL14[1–74] as the naturally abundantly occurring form. As shown in Fig. 2, CCL14[9–74] and NNY-CCL14 were as efficacious as CCL11 in inducing the release of an identical amount of ROS at a concentration of 10^{-7} M, whereas CCL14[8–74] and [10–74] were less efficacious, and full-length CCL14[1–74] was virtually inactive. NNY-CCL14 was the most potent stimulus tested, being significantly active starting at concentrations of 10^{-9} M and almost reaching its maximal effect at 10^{-8} M (Fig. 2). The inactive analogues were further analyzed for antagonistic effects. Pretreatment of human eosinophils with 10^{-7} M CCL14[1–74], [6–74], [7–74], [11–74], or [12–74] or bis-NNY-CCL14[10–74] did not result in significant inhibition of ROS release after stimulation with CCL11 at identical concentrations (data not shown).

NNY-CCL14 induces internalization of CCR3 as efficiently as CCL11

In the next set of experiments, human eosinophils were incubated for 30 min with the CCL14/HCC-1 derivatives or CCL11/eotaxin as positive control at a concentration of 10^{-7} M at 37°C. The cells were stained with anti-CCR3 mAb, and receptor expression was measured by flow cytometry. Preincubation of human eosinophils with CCL14[9–74], CCL14[10–74], and NNY-CCL14 led to a significant down-regulation of CCR3 (Fig. 3, A and B). The other derivatives, including CCL14[8–74], which is a weak inducer of ROS, did not affect CCR3 expression (Fig. 3A). At optimal doses, NNY-CCL14 and CCL11 both removed 80% of cell surface CCR3 and were significantly more effective than CCL14[9–74] (65%) and CCL14[10–74] (50%; Fig. 3A).

As reduced staining intensity in flow cytometry may depend on altered receptor accessibility of the Ab after preincubation with ligands, we performed the same type of experiments with the active ligands at 4°C, a temperature at which receptor internalization is prevented (41). In the same set of experiments we also studied the influence of PAO on the expression of CCR3. PAO inhibits protein tyrosine phosphatases and has been widely used as a general inhibitor of receptor internalization (33). In both treatments, the disappearance of CCR3 is prevented for the active CCL14 derivatives in the same experimental setting (Fig. 3C). These data clearly show that the induced decrease in cell surface CCR3 on human eosinophils was due to receptor internalization and that NNY-CCL14-induced internalization of CCR3 was as efficient as that induced by CCL11.

NNY-CCL14 is the most potent eosinophil chemoattractant among CCL14 derivatives and inhibits CCL11-induced chemotaxis

To evaluate whether the most active CCL14/HCC-1 derivatives induce chemotaxis of CCR3⁺ cells, migration assays were conducted in 48-well microchemotaxis chambers. As shown in Fig. 4A, CCL14 derivatives were effective chemoattractants for human eosinophils. The activity of the derivatives tested was similar in terms of efficacy, as indicated by the number of migrated cells. Maximal responses to CCL14[9–74] and CCL14[10–74] were reached at 3×10^{-7} and 10^{-6} M, respectively. NNY-CCL14 was more potent than the other derivatives, as its maximal effect was observed at 10^{-8} M, which is in the range of CCL11/eotaxin, as

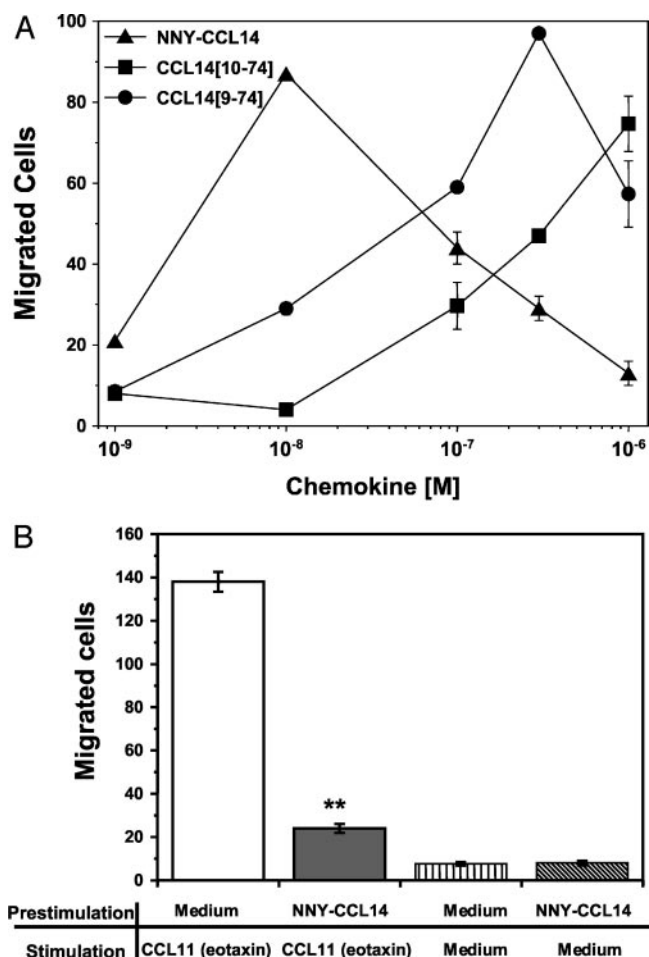


FIGURE 4. NNY-CCL14 induces chemotaxis of eosinophils, but also inhibits CCL11. **A**, CCL14/HCC-1 derivatives induce *in vitro* chemotaxis of human eosinophils. The numbers of migrating cells per five high power fields ($\times 1000$) are given. **B**, Pretreatment of eosinophils with 10^{-7} M NNY-CCL14 15 min before loading onto the chemotaxis chamber dramatically inhibits CCL11/eotaxin-induced migration. One of three similar experiments performed with cells from different donors is shown for each experimental setting. **, $p < 0.01$ compared with prestimulation with medium and subsequent stimulation with CCL11.

shown previously (8). When eosinophils were treated with 10^{-7} M NNY-CCL14 before transfer to the migration chamber, it almost abolished the migratory response to CCL11 (Fig. 4B).

NNY-CCL14 induces a functional desensitization of CCR3

CCL11/eotaxin-induced $[Ca^{2+}]_i$ changes in human eosinophils are mediated exclusively via CCR3 (42). To study the potential of the active CCL14/HCC-1 derivatives to desensitize CCR3, we performed desensitization experiments with the most active CCL14 derivatives and CCL11. Stimulation of eosinophils with NNY-CCL14 or CCL14[9–74] at 10^{-7} M completely desensitized the cells to CCL11 at the same dose (Fig. 5, A and C). The less active form, CCL14[10–74], did not desensitize eotaxin at equal doses (Fig. 5E), in agreement with the results obtained for the release of ROS and the moderate CCR3 internalization after preincubation with this ligand. Stimulation of eosinophils with CCL11 completely desensitized the active CCL14 derivatives (Fig. 5, B, D, and F). These results demonstrate that NNY-CCL14 desensitizes CCR3 for CCL11 stimulation. The specificity for desensitization of CCR3 given as NNY-CCL14 is not able to desensitize the response of eosinophils toward C5a (Fig. 5A). The C5a peak after

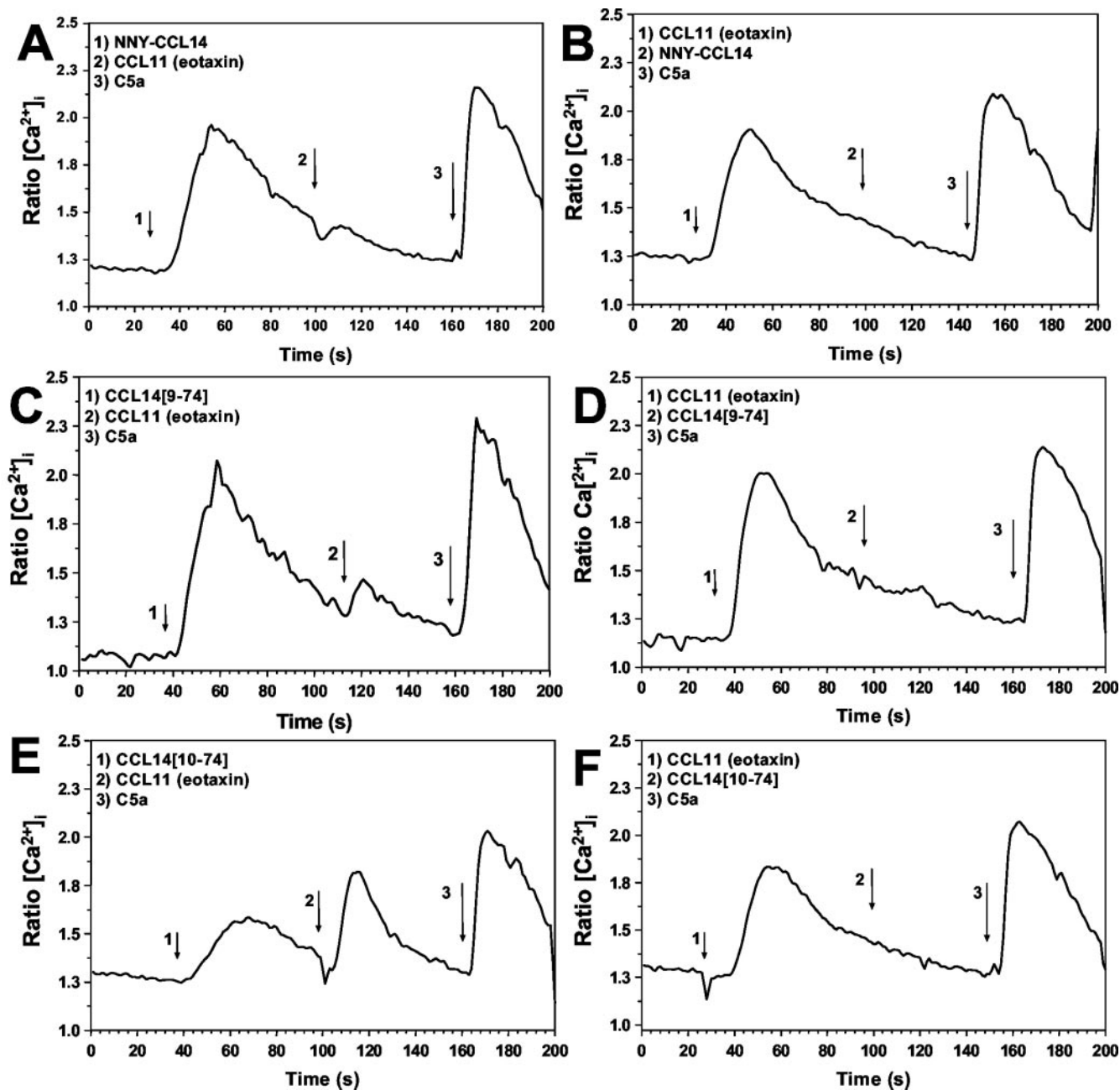


FIGURE 5. NNY-CCL14/HCC-1 induces a functional desensitization of CCR3. Human eosinophils were loaded with Fura 2AM, and $[Ca^{2+}]_i$ was measured using spectrofluometry. Cells were stimulated with the indicated chemokines (10^{-7} M) and the anaphylatoxin C5a (10^{-8} M). Data are presented as an original plot of one representative experiment of five.

NNY-CCL14 stimulation had the same intensity as if the cells were stimulated with C5a alone (data not shown).

NNY-CCL14 is resistant to CD26/DPP IV processing

CD26/DPP IV processing of CCL11/eotaxin, CCL14[9-74], and NNY-CCL14/HCC-1 was analyzed in vitro, essentially as previously described (36). First the amount of enzyme applied was optimized to give a kinetic profile for CCL11 and CXCL12 comparable to that previously reported (Fig. 6A). CXCL12 was completely processed within 10 min, and CCL11[1-74] was fully converted into CCL11[3-74] after 1 h at 37°C. Using these conditions, the processing of CCL14[9-74] was performed similarly over a period of time. Although significantly slower than for CCL11 and CXCL12, a virtually complete conversion of

CCL14[9-74] into CCL14[11-74] was achieved within 12 h (Fig. 6, B and C). In contrast to these chemokines, NNY-CCL14 remained completely resistant to CD26/DPP IV treatment after 24 h (Fig. 6, B and C) and even after 90 h of incubation (data not shown).

NNY-CCL14 is an effective inhibitor of eosinophil infiltration in murine models of allergic airway inflammation

To test the hypothesis that NNY-CCL14/HCC-1 may influence the influx of eosinophils in vivo, we used two murine models of allergic airway inflammation. The i.v. application of 10 nmol/kg NNY-CCL14 30 min before and 3 and 8 h after OVA-aerosol provocation significantly reduced the infiltration of eosinophils

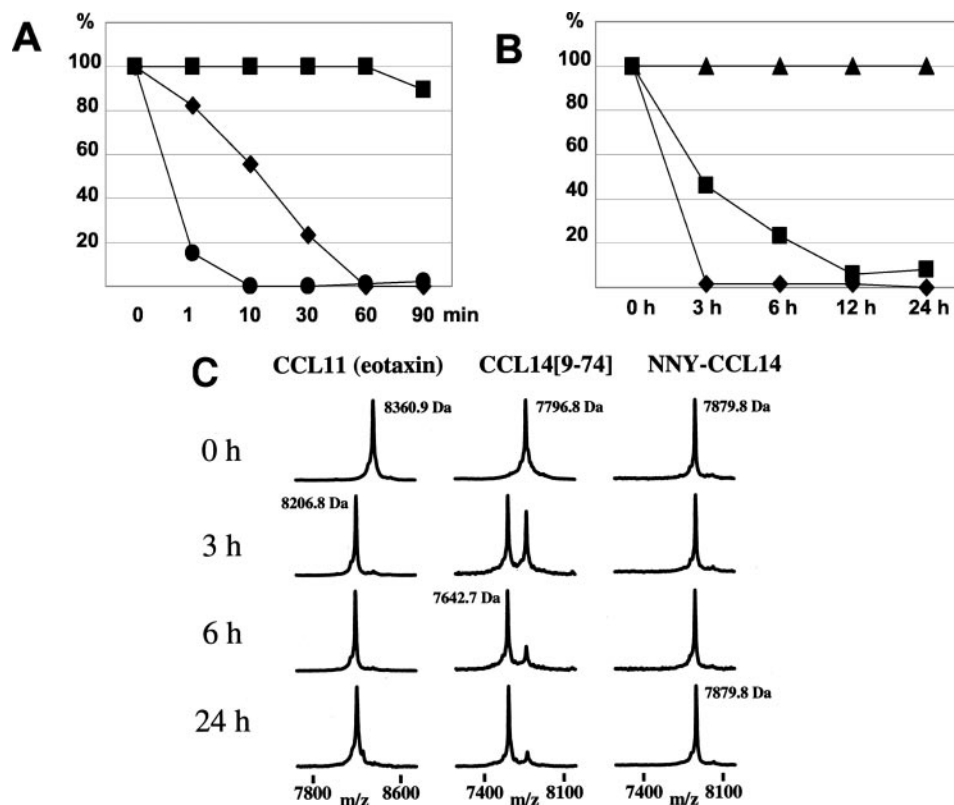


FIGURE 6. NNY-CCL14 is not processed by CD26/DPP IV. A and B, CCL11/eotaxin[1–74] (◆), CXCL12/SDF-1α[22–89] (●), CCL14/HCC-1[9–74] (■), and NNY-CCL14 (▲; 10^{-5} M each) were incubated for the indicated times with porcine kidney CD26/DPP IV as described in *Materials and Methods* and were analyzed using mass spectrometry. Processing was calculated as the amount of full-length chemokine related to the total amount of the full-length and processed forms, as defined by the peak heights. C, Partial MALDI mass spectrometry spectra of chemokines after incubation for different periods. The relative molecular masses of unprocessed chemokines are indicated on the right of the peaks, and those of processed chemokines (minus two NH_2 -terminal amino acids) are given on the left.

into the airways of OVA-treated mice compared with the saline-treated positive control group. Eosinophil infiltration into the lung tissue was demonstrated by standard staining procedures and was clearly reduced in the NNY-CCL14-treated animals (Fig. 7). A reduced diapedesis and tissue infiltration of eosinophils was observed in the NNY-CCL14-treated animals, as shown in Fig. 7A. In the saline-treated group, a diffuse infiltrate consisting mainly of eosinophils was found (Fig. 7B). To exclude inappropriate cellular activation that might lead to trapping of eosinophils within the lung, nonallergen-sensitized/challenged mice were treated with NNY-CCL14 i.v. once at a dose of 10 nmol/kg vs NaCl i.v. NNY-CCL14-treated mice did not exhibit any trapping or sludging of cells in the vessels of the lung, as observed in those mice killed 30 min or 24 h after NNY-CCL14 treatment (Fig. 7, C and D). The inconspicuous image was identical in the saline-treated animals (data not shown). For quantification of the effect on inflammation, differential cell counts were performed on cytopins of BALF. Application of NNY-CCL14 significantly reduced the influx of eosinophils into the airways compared with the saline-treated group (0.2 vs 1.3×10^4 cells/ml BALF; $p = 0.007$; Fig. 8). To evaluate the effect of NNY-CCL14 treatment on AHR, lung function measurements were additionally performed. Application of NNY-CCL14 significantly reduced AHR, measured as the effective dose of methacholine to decrease midexpiratory flow to 50% (ED_{50}). The AHR in saline-treated and OVA-challenged animals was $29.6 \pm 7.6 \mu\text{g}$ of methacholine (positive control) and improved in NNY-CCL14-treated animals to 65.1 ± 13.8 ($p = 0.016$). For comparison, nonsensitized animals had an ED_{50} of $61.3 \pm 15.1 \mu\text{g}$ of methacholine.

As OVA is not a naturally occurring allergen, extracts of the more relevant *A. fumigatus* were used in a second model of allergic airway inflammation (43). In this model the dose of NNY-CCL14 was increased, but the frequency of dosing around the challenges was decreased. In the experimental setting the peptide compound

significantly reduced the number of eosinophils in BALF compared with saline-treated mice (positive control; 1.1 vs $5.1 \times 10^4/\text{ml}$ BALF; $p = 0.004$), reaching levels similar to those in negative control mice, which were not sensitized with *A. fumigatus* (Fig. 9). Furthermore, a significant reduction of BALF lymphocytes was observed after NNY-CCL14 treatment (1.2 vs $2.9 \times 10^4/\text{ml}$ BALF; $p = 0.011$; Fig. 9).

Discussion

Chemokine receptors are attractive targets for therapeutic intervention in inflammation, allergic diseases, and infection by viruses such as HIV. In this context, several kinds of chemokine receptor antagonists derived from peptides have been developed (44). It is well known that the N-terminal region is crucial for the biological activity of chemokines; therefore, modifications of this region can profoundly alter the activity of chemokines on leukocytes (44). Thus, modified chemokines, such as Met-RANTES (45), aminoxy-pentane (AOP)-RANTES (46), RANTES[3–68] (47), and MCP-1[9–68] (48), were constructed by deletion or extension of amino acids or by chemical modification of the N terminus. In this study we demonstrate that NNY-CCL14/HCC-1 is the most potent CCL14 derivative, with the ability to block CCR3 by internalization and desensitization. Substitution of the N-terminal Gly-Pro motif of CCL14[9–74] by the NNY-Pro motif leads to a peptide compound that is resistant to biological degradation. Interestingly, this agonistic peptide reduces accumulation of eosinophils in allergic airway inflammation, as demonstrated in two different murine models.

Recently, the importance of CCR3 in allergic asthma has been highlighted. This receptor is expressed constitutively or upon activation of cytokines on eosinophils, Th2 cells, basophils, and mast cells (49). All these cells contribute to the inflammatory infiltrate in allergic asthma (1). In contrast to eosinophils and basophils, the expression of CCR3 on a subpopulation of Th2 cells has been

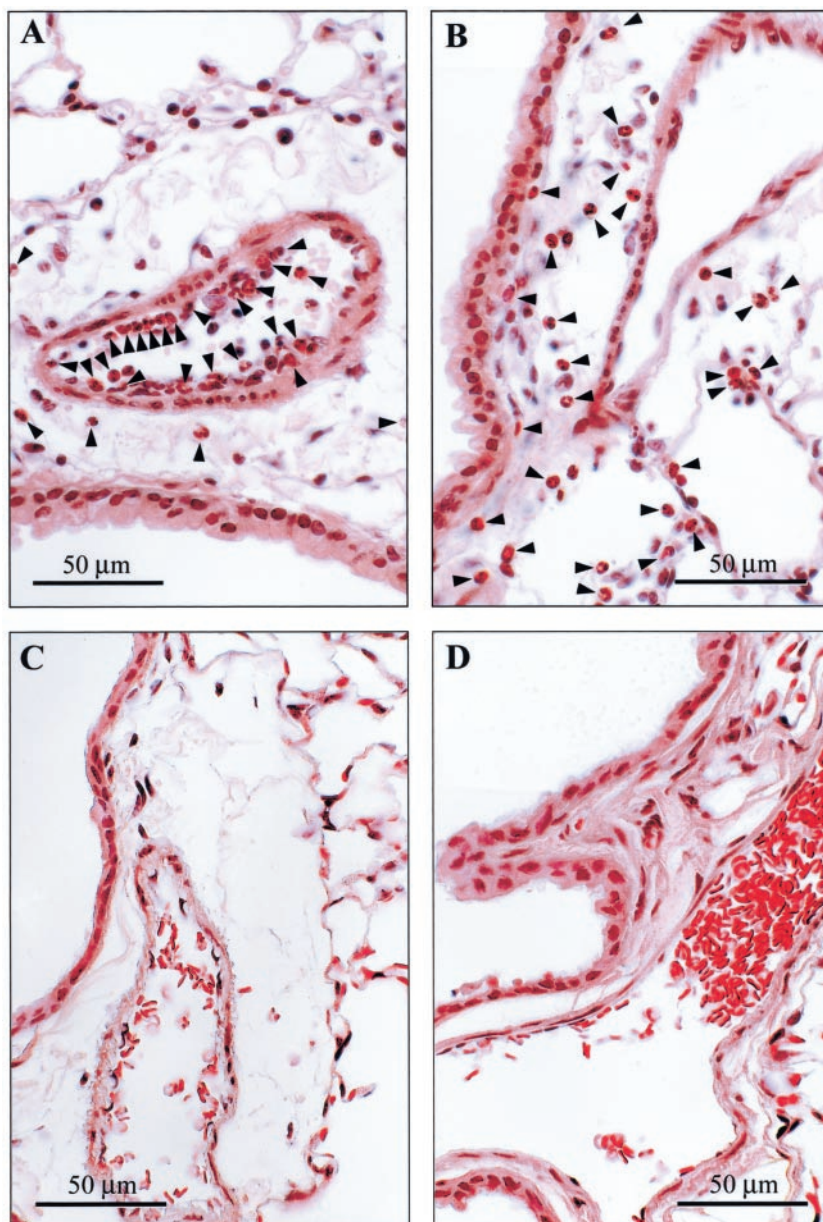


FIGURE 7. NNY-CCL14 prevents migration of eosinophils in vivo. *A* and *B*, NNY-CCL14/HCC-1 prevents the diapedesis of eosinophils (indicated by arrows) into the lung tissue of OVA-sensitized mice. The photographs represent the reduced diapedesis of eosinophils arrested at the endothelium in NNY-CCL14-treated mice (*A*) compared with the saline-treated group that had marked peribronchial infiltration primarily consisting of eosinophils (*B*). *C* and *D*, Prevention of eosinophil migration by NNY-CCL14 is not caused by trapping or sludging, because there are no eosinophils captured in the vessel walls of nonallergen-sensitized/challenged mice that have been treated only with NNY-CCL14 and killed either 30 min (*C*) or 24 h (*D*) thereafter for study. Original magnification, $\times 630$.

controversially discussed (17, 50). A recent study using CCR3 knockout mice demonstrated that the expression of the Th2 cytokine, IL-4, in OVA-sensitized skin was comparable in *CCR3*^{-/-} mice and wild-type controls, suggesting that CCR3 does not play an important role in the recruitment of Th2 cells to sites of allergic inflammation (21). In contrast to mice, in skin lesions of patients with atopic dermatitis, there is evidence of a role for CCR3 in the recruitment of a subpopulation of T cells (51). However, the in vivo relevance of CCR3 for the attraction of T cells to the site of inflammation has to be evaluated in additional studies. The deletion of the CCR3 locus in the germline of mice gave new insight into the role of this receptor especially in the trafficking of eosinophil cells into the lung (20, 21). In a model of allergen-induced airway inflammation, these studies showed that allergen challenge results in subendothelial trapping of eosinophils in CCR3-deficient mice, whereas wild-type controls had an impressive infiltration of the lung accompanied by lymphocytes, neither of which was found in *CCR3*^{-/-} mice. In addition, the CCR3-deficient mice are protected from allergen-induced AHR if the epicutaneous route is used for sensitization instead of the i.p. route, where an enhanced

AHR was observed that correlated with increased intraepithelial mast cells, underlining the involvement of CCR3 in several phases of asthma (20, 21).

A previous study by our group demonstrated that the N-terminally truncated form of CCL14, CCL14[9–74], is an agonist of CCR3 (29). According to these data, we investigated a greater series of N-terminally truncated or modified peptides derived from the chemokine CCL14 to test their activity profile on CCR3 using human eosinophils expressing CCR3, but not CCR1. The derivatives CCL14[6–74/7–74/8–74/11–74/12–74] and bis-NNY-CCL14[10–74] show modest activity or are inactive on eosinophils expressing CCR3 and do not have antagonistic properties on this receptor. In contrast to these derivatives, CCL14[9–74], CCL14[10–74], and NNY-CCL14 evoke the release of ROS and chemotaxis of human eosinophils. The rank order of activity of these peptides was NNY-CCL14 > CCL14[9–74] > CCL14[10–74]. The activity of NNY-CCL14 on CCR3 was confirmed, because it induced mobilization of intracellular calcium in the nanomolar range in 300.19 cells transfected with human CCR3 (data not shown).

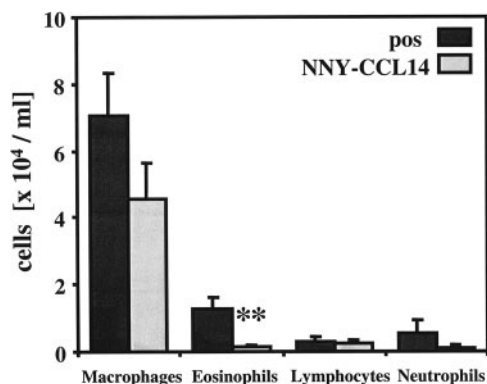


FIGURE 8. NNY-CCL14 prevents the migration of eosinophils into the lung lumen of OVA-challenged mice. OVA-challenged mice were treated with NNY-CCL14/HCC-1 (3×10 nmol/kg) or saline (positive control), respectively. Cell composition in BALF 24 h after allergen challenge was analyzed, differentiating 500 cells/cytospin, and is expressed as cell number per milliliter of BALF. **, $p < 0.01$ compared with positive control of the same cell type.

The inhibition of chemokine receptors through internalization is a reasonable therapeutic strategy being pursued for HIV and its interaction with CCR5. In this context, it has been shown that AOP-RANTES and NNY-RANTES induce a powerful internalization that improves effective inhibition of HIV entry in CCR5-transfected cell lines (52, 53). In this study we show that the active CCL14 derivatives are able to induce internalization of CCR3 with the same rank order as that for the release of ROS and chemotaxis. Moreover, of the active CCL14 derivatives, NNY-CCL14 was the most efficient to desensitize CCR3, as shown by its inhibition of CCL11-induced intracellular calcium release of human eosinophils. Additionally, pretreatment of human eosinophils with NNY-CCL14 inhibits CCL11/eotaxin-induced chemotaxis, overlapping with its capacity of CCR3 internalization. In this context, data on other modified chemokines strongly support the idea that receptor internalization plays a central role in chemokine-mediated inhibition of receptor function (34, 52, 54).

The enzyme CD26/DPP IV is an abundant peptidase found in blood plasma and tissue and on the cell surface of various cell types (30, 36). It is involved in the regulation of chemokine activity by hydrolysis of peptides with N-terminal Xaa-Pro and Xaa-Ala motifs (30). Truncation of chemokines by CD26/DPP IV, such as CCL5, CCL11, CXCL11, and CXCL12, leads to rapid inactivation and reduced binding capacity to their corresponding chemokine receptors (36). The most potent CCR3 peptide antagonist yet described, I-Tac/E₀H1, contains the eight N-terminal amino acids of CXCL11 (I-TAC) (55). As CXCL11 is efficiently cleaved by CD26/DPP IV within 2 min (56), I-TAC/E₀H1 is likely to share a similar fate. In this study we demonstrate that, in contrast to CCL11, the most potent of the CCL14 derivatives, NNY-CCL14 is resistant to degradation by CD26/DPP IV even after incubation for up to 90 h. Thus, substitution of the N-terminal Gly-Pro motif by the NNY-Pro motif protects NNY-CCL14 from degradation. This is a relevant difference in comparison with CCL11, which is processed rapidly into its inactive form, CCL11[3–74], resulting in a reduced interaction of CCL11 with CCR3 (57). In contrast, NNY-CCL14 remains in its active form to induce CCR3 internalization, desensitization, and inhibition of chemotaxis. In this context, latest experiments show that serum incubation of NNY-CCL14 for 48 h does not result in the loss of biological activity, with respect to CCR3 in vitro internalization, whereas CCL11's activity is clearly affected (U. Forssmann, unpublished observations).

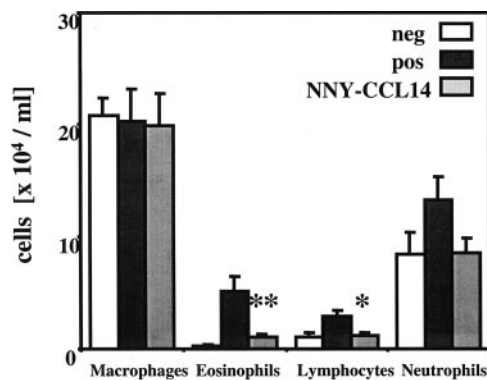


FIGURE 9. NNY-CCL14 prevents the migration of eosinophils and lymphocytes into the lung lumen of *A. fumigatus*-challenged mice. *A. fumigatus*-treated mice were injected with NNY-CCL14/HCC-1 (twice, 30 nmol/kg) or saline (positive control) on days 14 and 21, respectively. Non-sensitized mice were used as negative controls. For details, see *Materials and Methods*. Cell composition in BALF 24 h after allergen challenge was analyzed, differentiating 500 cells/cytospin, and is expressed as cell number per milliliter of BALF. **, $p < 0.01$; *, $p < 0.05$ (compared with positive control of the same cell type).

The therapeutic use of chemokines or their derivatives with antagonistic or agonistic properties, such as Met-RANTES, AOP-RANTES, or NNY-RANTES, has been intensively discussed in the literature, particularly for the inhibition of HIV infection (44). The treatment of asthma is one rationale for the development of CCR3 antagonists such as I-TAC-eotaxin hybrid-1 (55). Besides peptides, small m.w. antagonists are also considered potential drugs for the blockade of chemokine receptors. Because the latter compounds, derived from piperazine and piperidine, may exhibit unexpected side effects (particularly in the heart and CNS), peptide ligands inactivating chemokine receptors may represent an alternative due to better tolerance. There are already agonistic drugs, which are applied to induce inhibitory effects, such as luteinizing hormone-releasing hormone analogues, which are used to down-regulate hormone release (58). Hence, we propose the concept of an agonistic receptor inactivator mediating its effects through desensitization and internalization, thereby rendering target cells insensitive to further activation through the inactivated receptors.

A previous study focusing on the in vivo role of IL-8 in normal healthy NZW rabbits demonstrated that i.v. application of IL-8 blocked PMN extravasation (elicited 30 min after i.v. injection by s.c. IL-8 or FMLP application) during a 120-min interval. The authors of this study explained their results by adhesion inhibition rather than by desensitization (59). The transient fall of neutrophils during the first 30 min as described by Hechtman et al. (59) seems to be a feature of neutrophils, but not eosinophils. It has been shown that in guinea pigs, circulating eosinophil numbers increased after 5 min, with maximal levels at ~30 min after i.v. eotaxin injection (60). Beyond the study by Hechtman et al. (59), we used two murine models of allergic airway inflammation, which are relevant for allergic lung diseases, to prove whether NNY-CCL14 is able to block the infiltration of eosinophils into the lung tissue. Intravenous administration of NNY-CCL14 before and after allergen challenge was well tolerated, indicating that a potential intravascular activation of eosinophils was not harmful for the animals. Furthermore, the treatment of nonallergen-sensitized/challenged mice with NNY-CCL14 did not induce trapping of cells in lung vessels after 30 min, the time point at which such a phenomenon would be most likely expected. An identical observation was made after 24 h. In a separate experiment three non-sensitized mice were injected with a single dose of 1.000 nmol/kg

NNY-CCL14, which was very well tolerated during an observation period of 1 wk (U. Forssmann and A. Heitland, unpublished observations). Remarkably, the number of eosinophils in BALF as well as lung tissue decreased dramatically in the NNY-CCL14-treated group. Both OVA and *A. fumigatus* are frequently used allergens for pharmacological efficacy studies (61–63). However, these allergens clearly differ in the picture of inflammation they induce. OVA induces a Th2 phenotype associated with an almost exclusive influx of eosinophils in this model (62). In contrast, *A. fumigatus* induces a more mixed immune response, with a proinflammatory and a Th2 component (61). According to this different phenotype of allergic inflammation, systemic treatment with NNY-CCL14 resulted in a reduction of eosinophil numbers in the OVA model and the reduction of eosinophil and lymphocyte numbers in the *Aspergillus* model. Moreover, AHR to methacholine was tested by head-out plethysmography in the OVA model. The reduced number of eosinophils in the NNY-CCL14 treatment group was associated with the prevention of AHR. Eosinophil numbers are well-established markers of allergic airway inflammation, but their contribution to AHR is controversially discussed (64, 65). Therefore, it remains open whether the effect of NNY-CCL14 on AHR is mediated by eosinophils only, or if other mechanisms are involved as well.

We suggest from the presented study that the blood represents an ideal compartment for application of such an agonistic receptor inactivator, as generated toxic substances such as reactive oxygen species accumulate much more slowly than in inflamed tissues. Therefore, peptides would be interesting candidates because they are mainly administered parenterally, are usually highly specific, and consequently have relatively low systemic toxicity (66). Finally, we speculate that CD26/DPP IV-resistant agonists, such as NNY-CCL14, which is able to efficiently internalize its receptors, can be given at significantly lower doses than antagonists such as Met-RANTES to inhibit extravasation of effector cells (19). Additional studies are necessary to demonstrate the proposed mechanism in vivo of NNY-CCL14 and to exclude an involvement of other receptors.

Acknowledgments

We thank Ines Barg, Sabine Knöss, Birgit Eilers, and Wolfgang Posselt for their excellent technical assistance.

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