

Intravascular inactivation of CCR5 by *n*-Nonanoyl-CC chemokine ligand 14 and inhibition of allergic airway inflammation

Shipra Gupta,* Barbara Fuchs,[†] Sandra Schulz-Maronde,* Aleksandra Heitland,* Sylvia E. Escher,* Matthias Mack,[‡] Hanns-Christian Tillmann,[§] Armin Braun,[†] Wolf-Georg Forssmann,* Jörn Elsner,^{||} and Ulf Forssmann*,^{||,1}

*Center of Pharmacology and Toxicology, Hannover Medical School, Hannover, Germany; [†]Fraunhofer Institute of Toxicology and Experimental Medicine, Hannover, Germany; [‡]Department of Internal Medicine, University of Regensburg, Regensburg, Germany; [§]VIRO Pharmaceuticals GmbH & Co. KG, Hannover, Germany; ^{||}Department of Dermatology and Allergology, Fachklinik Bad Bentheim, Germany; and ^{||}IPF PharmaCeuticals GmbH, Affiliated Institute of Hannover Medical School, Hannover, Germany

Abstract: Modulation of leukocyte recruitment through intervention with chemokine receptors is an attractive, therapeutic strategy. Recently, we have shown that *n*-Nonanoyl (NNY)-CCL14 internalizes and desensitizes human (h)CCR3, resulting in the inactivation of eosinophils. In this study, we investigated the interaction of NNY-CCL14 with CCR1 and CCR5 and the relevance of these NNY-CCL14 receptors on its *in vivo* effects in allergic airway inflammation. NNY-CCL14 has inactivating properties on CCR1⁺ and CCR5⁺ cell lines and primary leukocytes. It desensitizes hCCR1- and hCCR5-mediated calcium release and internalizes these receptors from the cellular surface. Treatment of OVA-sensitized BALB/c mice with NNY-CCL14 resulted in reduced pulmonary inflammation. Above all, it is demonstrated that systemic treatment with NNY-CCL14 down-modulates CCR5 from the surface of lymphocytes *in vivo*. Although NNY-CCL14 acts on murine lymphocytes and internalizes CCR5, it does not internalize CCR3 on mouse eosinophils, showing species selectivity regarding this particular receptor. Therefore, the inhibitory effects of NNY-CCL14 in murine models of allergic airway inflammation can be assigned to its interaction with CCR5. The presented results substantiate the relevance of CCR5 as a target for allergic airway inflammation. *J. Leukoc. Biol.* 83: 000–000; 2008.

Key Words: allergy · chemokines · human · lymphocytes · mouse

INTRODUCTION

Allergic asthma is characterized by an influx of eosinophils, mast cells, and T lymphocytes contributing to a complex pathological process [1–3]. The recruitment of leukocytes to the sites of inflammation is mediated by chemokines, which exert their effects through the activation of G protein-coupled receptors

present on the cell surface and play an important role in various allergic diseases, in particular, asthma [4, 5].

The prevention of leukocyte recruitment through intervention with chemokine receptors is an attractive, therapeutic strategy [6]. The concept of using chemokine-derived agonists for anti-inflammatory therapy has been proposed recently [7, 8]. The blockade of CCR3 is being explored as an approach for asthma therapy [7]. Besides CCR3, chemokine receptors CCR1, CCR2, CCR4, CCR5, CCR6, CCR7, and CXCR4 have been shown to be potential targets, playing a role in the development and maintenance of the diverse stages of allergic asthma [9–15]. Moreover, in the case of allergic lung inflammation, it has also been demonstrated that the agonistic CCL5 analog amino-oxypentane (AOP)-CCL5 inhibits bronchoalveolar lavage (BAL) eosinophilia and T cell recruitment via a CCR1- and CCR5-dependent pathway [16]. In mouse models of allergic airway disease, blockade of CCL5 has been shown to have a marked effect on recruitment and activation of eosinophils, independent of CCR3 [16, 17], reinforcing that CCR1 and/or CCR5 represent potential targets for therapy of asthma.

Most anti-inflammatory strategies are based on the mechanisms that act on target cells during extravasation and after they migrate to the site of inflammation. In contrast, we proposed that cellular recruitment can be prevented by receptor inactivation of inflammatory cells before they extravasate [18]. 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, in a previous study, 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 [18]. Among the modified peptides studied, *n*-Nonanoyl (NNY)-

¹ Correspondence: Center of Pharmacology and Toxicology, Hannover Medical School, Feodor-Lynen-Strasse 31, 30625 Hannover, Germany. E-mail: u.forssmann@gmx.de

Received June 25, 2007; revised October 30, 2007; accepted November 1, 2007.

doi: 10.1189/jlb.0607429

CCL14 was described as a potent CCR3 agonist, which has inactivating properties for human eosinophils [18].

As CCL14(9–74), the natural precursor of NNY-CCL14, has been shown to act with high affinity on CCR1 and CCR5 [19], which are of relevance in allergic airway inflammation, in the present study, we focus on the interaction of NNY-CCL14 with these receptors. We describe that the agonist NNY-CCL14 can act as an inactivator of human (h)CCR1 and hCCR5 as well as murine (m)CCR5, as it is able to efficiently desensitize these receptors and remove them from the cell surface. Remarkably, systemic NNY-CCL14 treatment in OVA-sensitized and -challenged BALB/c mice abrogates airway hyper-responsiveness (AHR) and inhibits infiltration of inflammatory cells into the lung. CCR5 internalization of circulating lymphocytes could be demonstrated as a part of the complex mechanism of action of NNY-CCL14. This study substantiates the strategy of treating airway inflammation by preventing cellular recruitment as a result of inactivation of chemokine receptors on inflammatory cells before they extravasate.

MATERIALS AND METHODS

Chemokines and cytokines

hCCL3 and hCCL11 were purchased from ImmunoTools (Friesoythe, Germany); hCXCL12, hCCL5, and mCCL3 from Peprotech (Rocky Hill, NJ, USA); and mL-2 from Roche Applied Science (Mannheim, Germany). AOP-CCL5 was provided by Oliver Hartley (Department of Structural Biology and Bioinformatics, Centre Médical Universitaire, Geneva, Switzerland), and CCL14(9–74) and NNY-CCL14 were produced using N-(9-fluorenyl)-methoxycarbonyl (F-Moc) chemistry as described previously [18].

Antibodies

The mAb against hCCR1 (clone 53504.111) and mCCR3-FITC (clone 83101.111) were purchased from R&D Systems (Wiesbaden, Germany), and hCCR5 (clone 2D7/CCR5), mPan-NK-FITC (clone DX-5), mCD8a-FITC (clone 53-6.7), and mCD4-FITC (clone RM4-5; isotype recombinant IgG_{2a} κ) were from BD Biosciences (Heidelberg, Germany). The mAb against mCCR5 (clone MC68) was generated as described previously [20]. The FITC/PE-conjugated anti-mIgG or CyTM5-conjugated mouse anti-rat IgG secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (Cambridge, UK).

Cell lines

Stably transfected murine pre-B 300.19 cells expressing hCCR1 or hCCR5 were obtained from Bernhard Moser (Theodor Kocher Institute, University of Berne, Switzerland). Stably transfected human embryonic kidney (HEK) cell line HEK293 expressing mCCR1 or mCCR5 was prepared as described previously [21].

PBMC isolation

PBMCs were purified from the venous blood of normal, nonatopic, healthy volunteers using Ficoll (Pharmacia, Erlangen, Germany) density gradient centrifugation as described previously [22]. All blood donors had given their informed consent, according to the local research ethics committee at Hannover Medical School (Germany).

Functional assays for ligand-induced intracellular calcium mobilization

The increase of intracellular calcium induced by stimulation with ligands in the different cell types used was performed on a fluorescence image plate

reader (FLIPR) system (Molecular Devices, Munich, Germany) as described previously [21].

Flow cytometric measurement of CCR1 and CCR5 internalization

In vitro internalization experiments were performed as described previously in detail [22, 23]. The cell surface expression of the chemokine receptors is expressed 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)] × 100% [22]. For in vivo internalization experiments, BALB/c mice were i.v.-injected with NNY-CCL14 or saline. Blood was drawn at indicated time-points from the retro-orbital plexus and transferred immediately on ice, and staining of CCR5⁺DX5⁺ cells was performed.

Allergic sensitization with OVA and NNY-CCL14 treatment

Female BALB/c mice, obtained from Charles River (Sulzfeld, Germany) at 8 weeks of age and an average weight of 20 g, were used in the experiments as described previously [24]. The animal experiment was approved by the government of Lower Saxony (Hannover, Germany) and was conducted according to international guidelines for the use of laboratory animals. For OVA sensitization, animals were divided into four groups of 12. Sensitization of the animals was performed via the i.p. route on Days 0, 14, and 21, each with 10 µg OVA (chicken OVA grade VI, Sigma-Aldrich, St. Louis, MO, USA), together with 1 mg Al(OH)₃ (Alum Inject, Pierce, Rockford, IL, USA) as adjuvant dissolved in sterile saline [24]. Animals were exposed to allergen on Days 28 and 29 for 10 min, yielding a calculated airway allergen deposition of ~10 µg OVA/mouse. Subsequently, animals were treated four times with 3 nmol/kg NNY-CCL14 or 0.5 mg/kg dexamethasone (30 min before and 6 h after each challenge). The positive control group is represented by animals in which a maximum infiltration of cells in BAL can be observed, i.e., OVA-sensitized and -challenged mice, which are not drug-treated at Days 28 and 29 but injected with sterile saline at identical time-points. The negative control group in these experiments is nonallergen-sensitized/challenged animals representing the baseline of BAL cells in “healthy animals”. The dexamethasone represents the control for an effective treatment. For the evaluation of BAL, the animals were killed 24 h after the last allergen challenge by injecting an overdose of sodium pentobarbital (Narcoren, Merial, Hallbergmoos, Germany) i.p. The trachea was catheterized, and airways were lavaged twice with 0.8 ml cold 0.9% NaCl. Total cell numbers in BAL were counted, and cytopins were evaluated [24].

mCD4⁺ T cell preparation

Spleens from female BALB/c mice were macerated, and a single cell suspension was prepared. T lymphocytes were purified by negative selection using the Dynal[®] CD4 negative isolation kit (Invitrogen, Karlsruhe, Germany). The purity was >95%, as assessed by flow cytometry. The cells were stimulated for 8–10 days in culture medium (RPMI 1640 with L-glutamine, penicillin-streptomycin, and 10% FCS), supplemented with 10 U/ml mL-2 using the Dynabeads mCD3/mCD28 T cell expander kit (Invitrogen). Afterwards, the CD3 blasts were cultured without Dynabeads in culture medium (described above), additionally supplemented with mL-2 (50 U/ml), 1 mM MEM-sodium pyruvate, 1× MEM nonessential amino acids, and 50 µM 2-β-ME as described elsewhere [25, 26]. The cells were used for functional assays on Days 21–24.

Statistics

The number of experiments is stated in the legends of the figures as *n*. Unless mentioned otherwise, the data in the text and figures are expressed as mean ± SEM, determined by Prism 3.03 (GraphPad Software Inc., San Diego, CA, USA) software analysis. Values of *P* ≤ 0.05 were accepted as significant using Student's *t*-test.

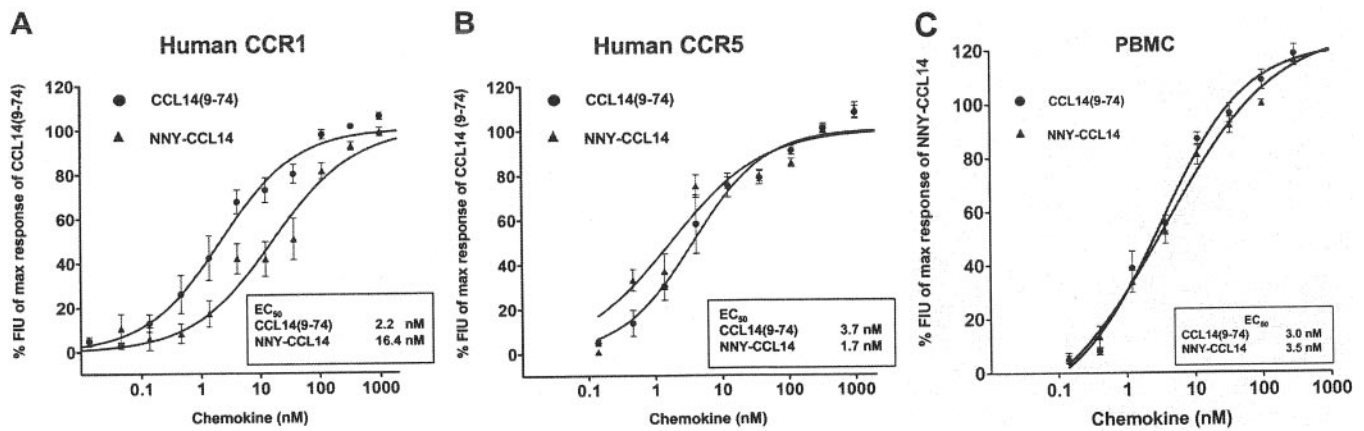


Fig. 1. NNY-CCL14 mobilizes intracellular calcium in B300.19-CCR1⁺ and B300.19-CCR5⁺ cell lines and PBMCs. hCCR1 (A)- or hCCR5 (B)-expressing B300.19 cell lines or PBMCs (C) were used to study the dose-dependent response of NNY-CCL14 by measuring intracellular calcium mobilization using the FLIPR. EC₅₀ values, expressed in nM concentrations, were calculated from the dose-response curves. All values are the mean $\pm$ SEM ($n \geq 8$). FIU, .

RESULTS

NNY-CCL14 is a potent inducer of intracellular calcium fluxes mediated by hCCR1 and hCCR5

To study the effects of NNY-CCL14 on CCR1 and CCR5, we analyzed ligand-induced intracellular calcium mobilization by FLIPR in B300.19-CCR1⁺ and B300.19-CCR5⁺ cell lines and PBMCs. For each ligand tested, EC₅₀ values were calculated from dose-response curves (**Fig. 1**). NNY-CCL14 acts on CCR1 and CCR5 in the nanomolar range with EC₅₀ values of 16.4 nM (Fig. 1A) and 1.7 nM (Fig. 1B), respectively. Thus, NNY-CCL14, like CCL14(9–74), is a potent agonist of CCR1 and CCR5. To investigate the efficacy and potency of NNY-CCL14 on primary leukocytes, we studied the intracellular calcium fluxes in PBMCs upon activation by NNY-CCL14. The potency of NNY-CCL14 observed on CCR1⁺ and CCR5⁺ cell lines was consistent with that on PBMCs (EC₅₀: 3.5 nM; Fig. 1C).

NNY-CCL14 induces efficient, dose-dependent internalization of hCCR1 and hCCR5

In the next set of experiments, B300.19-CCR1⁺ and -CCR5⁺ cell lines were incubated for 30 min at 37°C with various concentrations (1000–0.1 nM) of NNY-CCL14, CCL14(9–74),

or CXCL12 (only 100 nM) as negative control. The cells were stained with anti-hCCR1 mAb or anti-hCCR5 mAb, and receptor expression was measured by flow cytometry. The preincubation of 300.19-CCR1⁺ and -CCR5⁺ cell lines with NNY-CCL14 and CCL14(9–74) at 37°C led to an effective down-regulation of CCR1 and CCR5 in a dose-dependent manner, and no internalization was observed upon incubation with CXCL12 (**Fig. 2**). At a dose of 100 nM, NNY-CCL14 internalized 74% of cell surface CCR1 and was less effective than CCL14(9–74), which internalized 94% (Fig. 2A). CCR5 was efficiently internalized by 100 nM NNY-CCL14, removing 64% of cell surface CCR5. CCL14(9–74) was less efficient at 100 nM, removing only 42% of CCR5 from the cell surface (Fig. 2B). It is demonstrated that NNY-CCL14 is more potent and efficient than its natural ligand CCL14(9–74) on hCCR5, and it is less potent and less efficient than CCL14(9–74) on CCR1. To rule out reduced staining intensity in flow cytometry as a result of altered receptor accessibility of the antibody after preincubation with ligands, we performed the same type of experiment with 100 nM active ligands at 4°C, a temperature at which receptor internalization is prevented. In these experiments, receptor internalization was prevented for CCR1 and CCR5, as depicted in Figure 2, A and B.

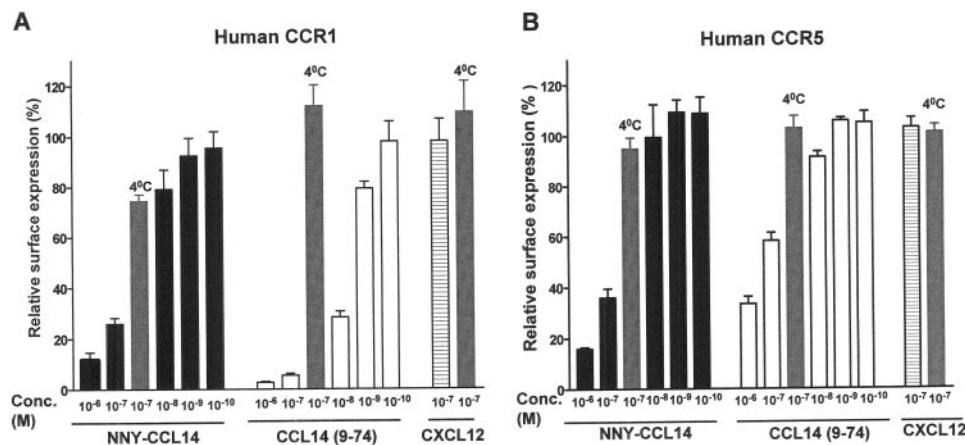
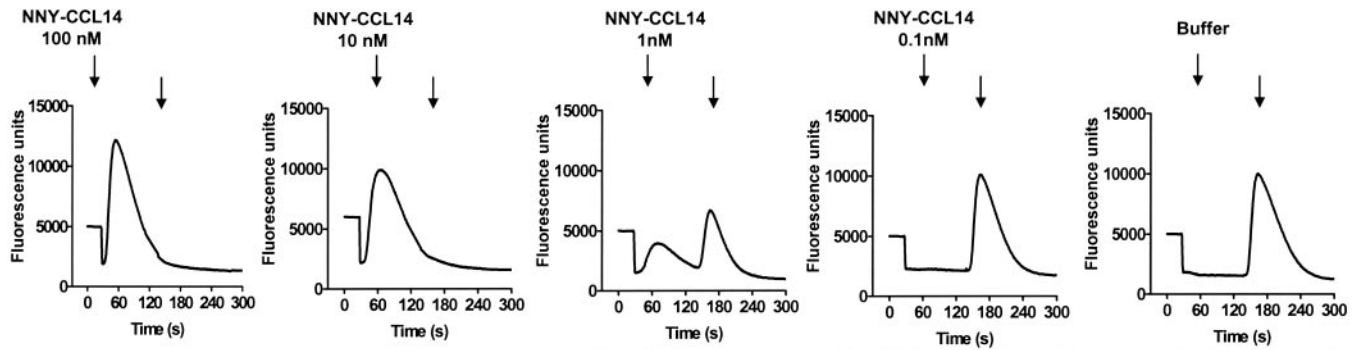


Fig. 2. Internalization of cell surface hCCR1 and hCCR5 by NNY-CCL14. B300.19 cells stably expressing hCCR1 or hCCR5 were treated for 30 min at 37°C (black bars) or 4°C (gray bars) with the indicated ligand concentrations for CCL14(9–74), NNY-CCL14, or CXCL12 (A and B). Thereafter, cells were stained with anti-hCCR1 or anti-hCCR5 mAb and analyzed by flow cytometry. Data ($n \geq 4$) are expressed as the mean $\pm$ SEM relative fluorescence intensity as described in Materials and Methods. (M), .

A Human CCR1



B Human CCR5

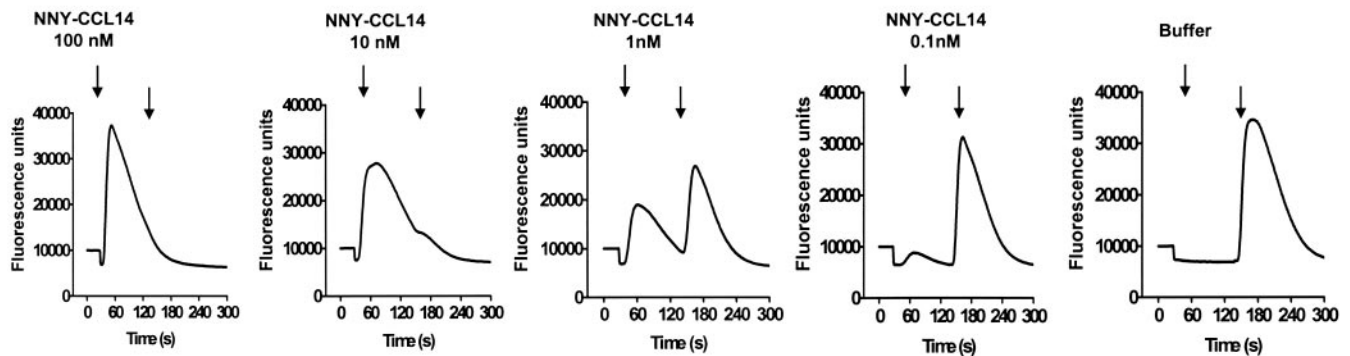


Fig. 3. NNY-CCL14 induces functional desensitization of hCCR1 and hCCR5. Cross-desensitization experiments were performed using B300.19-CCR1⁺ or B300.19-CCR5⁺ cell lines. Cells were sequentially stimulated at 90 s intervals with varying concentrations of NNY-CCL14 as indicated (first arrow) and 100 nM CCL14(9–74) (second arrow); intracellular calcium fluxes were recorded by FLIPR. The results are representative of three to five independent experiments for each ligand pair under identical conditions.

Desensitization of hCCR1 and hCCR5

The capability of NNY-CCL14 to desensitize hCCR1 and hCCR5 was studied in desensitization experiments performed with CCL14(9–74), which is a potent ligand of CCR1 and CCR5 [19]. The response to CCL14(9–74) was studied by calcium fluxes 90 s after prestimulation with NNY-CCL14. As indicated in **Figure 3A**, 10 nM NNY-CCL14 was able to completely desensitize 100 nM CCL14(9–74) on B300.19-CCR1⁺ cells. Similarly, on B300.19-CCR5⁺ cells, at a dose of 10 nM, NNY-CCL14 completely desensitized 100 nM CCL14(9–74; Fig. 3B). Using PBMC and CCR-selective ligand, i.e., CCL15 for CCR1 and CCL4 for CCR5, desensitization of these receptors by NNY-CCL14 could be confirmed (data not shown). Thus, these experiments demonstrate that NNY-CCL14 effectively desensitizes CCR1 and CCR5 to further stimulation by natural ligands.

NNY-CCL14 treatment inhibits inflammatory cell infiltration in a murine model of allergic inflammation

We next investigated the effect of NNY-CCL14 treatment on leukocyte migration to the lung in a murine model of allergic inflammation, where the mice were challenged twice with OVA to induce a stronger inflammation compared with our previous studies with a single OVA challenge or *Aspergillus fumigatus* challenge [18]. The application of NNY-CCL14 significantly

reduced the influx of lymphocytes in comparison with the saline-treated group (0.43 vs. 1.33×10^4 cells/ml BAL; $P < 0.001$; **Fig. 4A**). As shown in Figure 4B, a significant reduction of infiltrating eosinophils was also seen in the airways of NNY-CCL14-treated mice in comparison with the saline-treated group (2.65 vs. 6.23×10^4 cells/ml BAL; $P = 0.021$). Thus, NNY-CCL14 efficiently inhibits the airway infiltration of lymphocytes and eosinophils in this murine model of allergic inflammation. Using the same model, we have shown previously that application of NNY-CCL14 significantly reduced AHR [18], measured as the effective dose of methacholine to decrease midexpiratory flow to 50% (ED_{50}). Moreover, we now observe that the improvement in AHR in NNY-CCL14-treated animals (ED_{50} of 65.1 ± 13.8) [18] was comparable ($P > 0.05$) with a dexamethasone (0.5 mg/kg)-treated group (ED_{50} of 51.7 ± 11).

NNY-CCL14 activates HEK293-mCCR1⁺ and -mCCR5⁺ cells and mCD4⁺ T cells

To gain further insight into the mechanism of action of NNY-CCL14, we studied the activation of mCCR1 and mCCR5 by NNY-CCL14. To investigate the potency and efficacy of NNY-CCL14 on these murine chemokine receptors, we analyzed ligand-induced intracellular calcium mobilization by FLIPR on mCCR1⁺ and mCCR5⁺ cell lines. Comparative analysis revealed that NNY-CCL14 is more potent and efficacious on

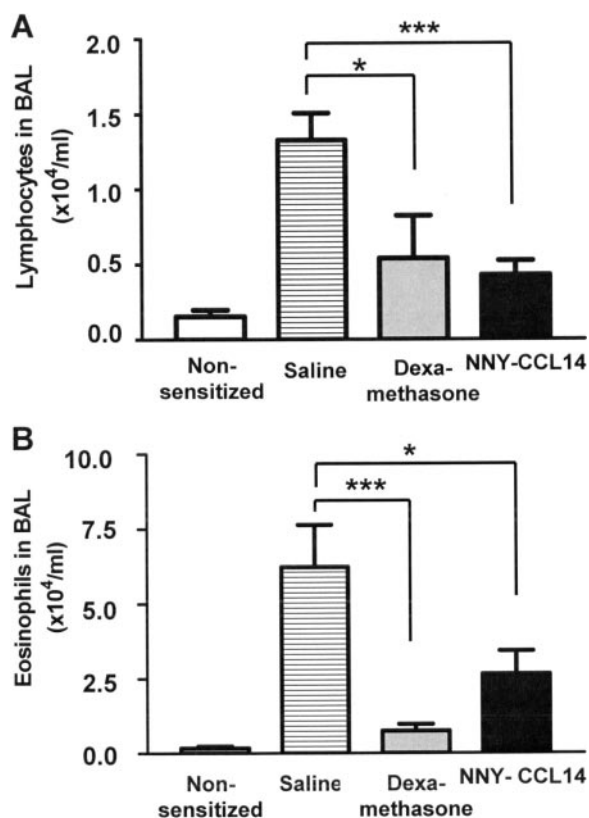


Fig. 4. NNY-CCL14 inhibits migration of inflammatory cells in a murine model of allergic airway inflammation. (A and B) Cell composition in BAL fluid was analyzed on cytospins. ***, $P < 0.001$; *, $P < 0.05$, compared with saline-treated group. Mean $\pm$ SEM are depicted; $n \geq 11$ mice/group.

mCCR5 than on mCCR1 (**Fig. 5, A and B**). As NNY-CCL14 activated mCCR1 and mCCR5, we investigated the role of NNY-CCL14 on calcium release in mCD4⁺ T cells. As depicted in Figure 5C, NNY-CCL14 induces a dose-dependent response on mCD4⁺ T cells. In addition, 100 nM NNY-CCL14 is able to considerably internalize surface CCR5 from cultured mCD4⁺ T cells (**Fig. 5D**). These observations suggest that NNY-CCL14 mediates its *in vivo* effects in mCD4⁺ T cells via CCR1 and CCR5, which are both expressed by these cells [26, 27].

NNY-CCL14 internalizes mCCR5 but not mCCR3

The potential of NNY-CCL14 to internalize mCCR5 and mCCR3 by incubation for 30 min at 37°C was studied on murine leukocytes. Different subsets of murine lymphocytes, namely CD4⁺ T cells, CD8⁺ T cells, and DX5⁺ NK cells, which express varying levels of CCR5, were stained to study NNY-CCL14-mediated internalization of CCR5 [20]. Eosinophils were used to study CCR3 internalization. In comparison with the medium control, 100 nM NNY-CCL14 substantially internalized surface CCR5 (**Fig. 6A**) from each of these cell types. In contrast, 100 nM NNY-CCL14 did not internalize surface CCR3 on eosinophils (**Fig. 6B**), and no remarkable internalization of CCR3 could be obtained at a concentration as high as 1000 nM (data not shown). Thus, these observations suggest that NNY-CCL14 is a potent ligand of mCCR5, but it shows species selectivity with respect to CCR3 and does not activate mCCR3 within the nanomolar range.

i.v.-Administered NNY-CCL14 induces internalization of mCCR5 *in vivo*

To verify the relevance of the effects of NNY-CCL14 on mCCR5 internalization, we were interested in investigating the capacity of NNY-CCL14 to exert these effects in the experi-

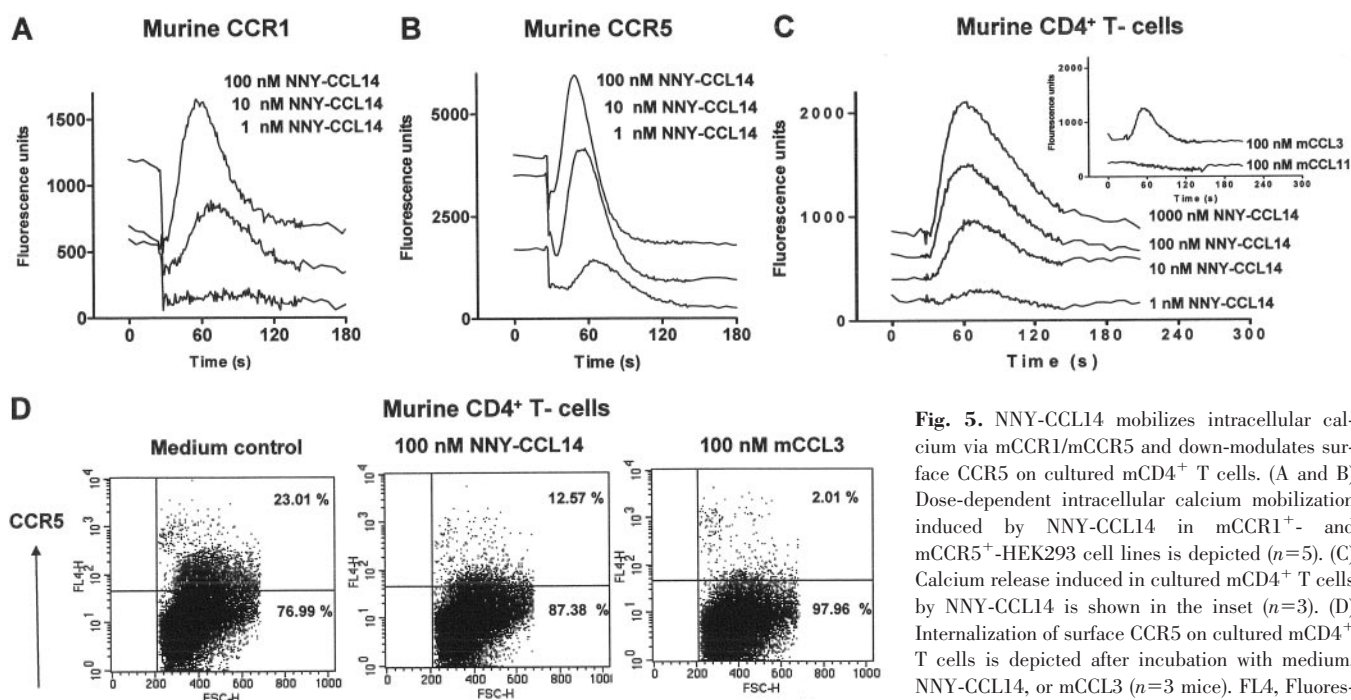


Fig. 5. NNY-CCL14 mobilizes intracellular calcium via mCCR1/mCCR5 and down-modulates surface CCR5 on cultured mCD4⁺ T cells. (A and B) Dose-dependent intracellular calcium mobilization induced by NNY-CCL14 in mCCR1⁺- and mCCR5⁺-HEK293 cell lines is depicted ($n=5$). (C) Calcium release induced in cultured mCD4⁺ T cells by NNY-CCL14 is shown in the inset ($n=3$). (D) Internalization of surface CCR5 on cultured mCD4⁺ T cells is depicted after incubation with medium, NNY-CCL14, or mCCL3 ($n=3$ mice). FL4, Fluorescence 4; FSC, forward-scatter.

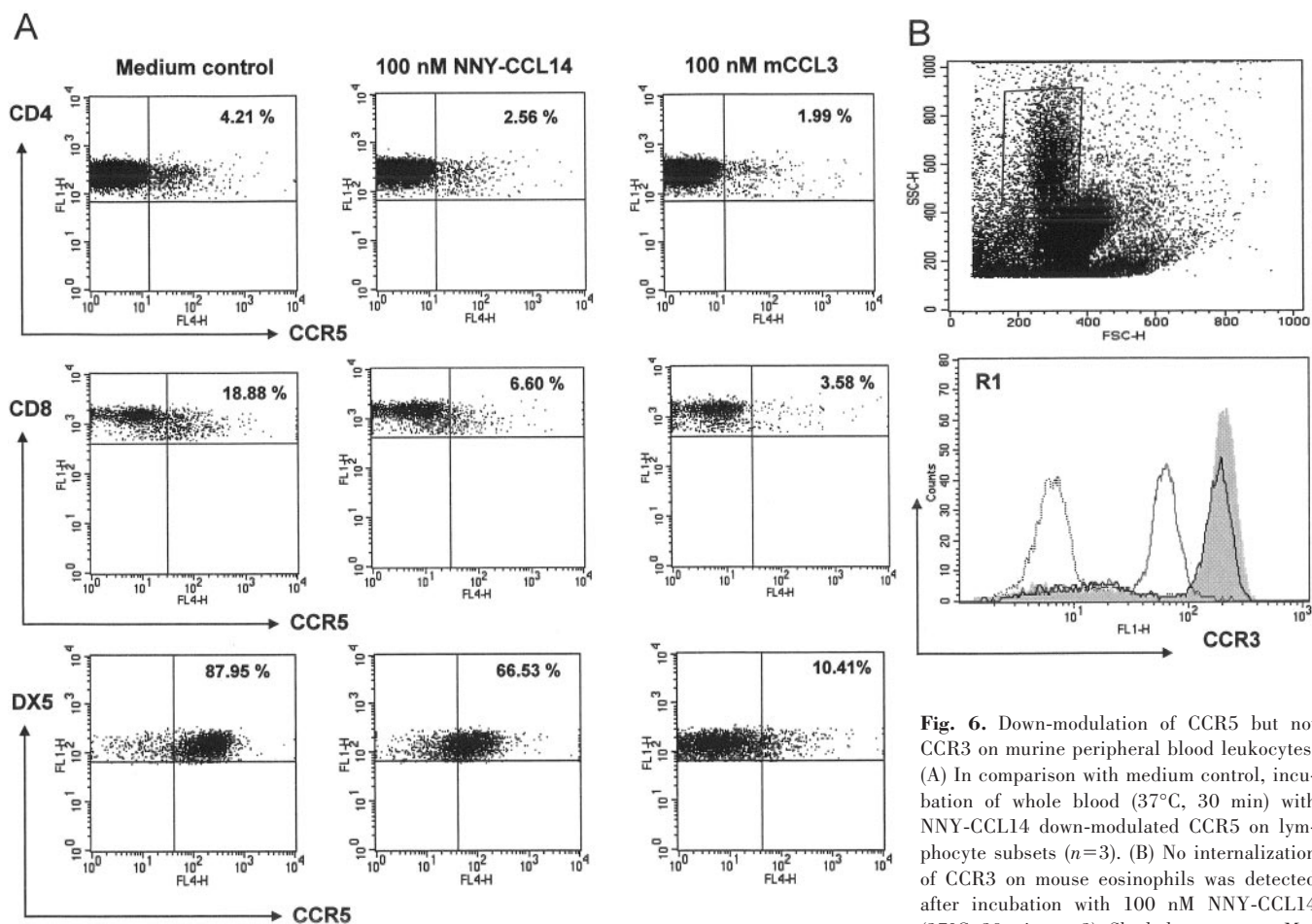


Fig. 6. Down-modulation of CCR5 but not CCR3 on murine peripheral blood leukocytes. (A) In comparison with medium control, incubation of whole blood (37°C, 30 min) with NNY-CCL14 down-modulated CCR5 on lymphocyte subsets ($n=3$). (B) No internalization of CCR3 on mouse eosinophils was detected after incubation with 100 nM NNY-CCL14 (37°C, 30 min; $n=3$). Shaded, gray curve, Medium control; black line, 100 nM NNY-CCL14; gray line, 100 nM CCL11; dotted line, isotype control. SSC, Side-scatter.

mental animal. As DX5⁺ NK cells homogenously express CCR5 in comparison with other lymphocyte subsets, as also previously demonstrated by Mack et al. [20], these cells were used to demonstrate the ability of NNY-CCL14 to internalize CCR5 in vivo in mouse blood. Therefore, in vivo internalization of mCCR5 and prevention of its recycling after systemic injection of the inhibitory peptide NNY-CCL14 were studied at the well-tolerated dose of 100 nmol/kg (0.79 mg/kg) [18]. Efficient receptor down-modulation was observed 15 min after systemic treatment, which persisted in the same range for at least 1 h (Fig. 7). A total re-expression of CCR5, equal to the level prior to the application of NNY-CCL14, was not observed, even after a time period of 180 min. Systemic treatment with saline alone does not affect the surface expression of mCCR5. These experiments present the in vivo mechanism of receptor inactivation after systemic treatment with NNY-CCL14.

DISCUSSION

Recent studies suggest a critical role for the chemokine receptors CCR1, CCR3, and CCR5 [14, 15, 28] in the pathogenesis and progression of allergic airway diseases. We have previously shown that NNY-CCL14 is a potent ligand of hCCR3, which can inactivate human eosinophils [18]. In this study, we demonstrate that NNY-CCL14 also interacts with CCR1 and

CCR5, and we reinforce the role of CCR5 in allergic airway inflammation.

First, we characterized the functional properties of NNY-CCL14 in comparison with CCL14(9–74) on B300.19-CCR1⁺ or -CCR5⁺ cell lines. CCL14(9–74) represents the naturally processed, biologically active form of hemofiltrate-derived hCCL14 and is a potent ligand for hCCR1 and hCCR5 [19, 29]. NNY-CCL14 is an aminoterminally modified derivative of CCL14(9–74) [18]. In this study, we demonstrate that NNY-CCL14 is as potent and efficacious as CCL14(9–74) in calcium flux assays but even more effective in internalizing cell surface CCR5. Additionally, NNY-CCL14 desensitizes CCR1- and CCR5-mediated responses to their natural ligands. Previously, many studies have shown that amino-terminal modifications alter the pharmacological properties of natural chemokines [23, 30]. Our results show that the amino terminal modification in NNY-CCL14 retains its strong, agonistic and desensitizing properties for CCR1 and CCR5, and it internalizes cell surface CCR5 more efficiently than its natural precursor.

This work supports the concept that NNY-CCL14 has inactivating properties for CCR1 and CCR5. This characteristic of NNY-CCL14 has been demonstrated previously for CCR3 [18]. NNY-CCL14 mediates its anti-inflammatory effects via desensitization and internalization, rendering the target cells unresponsive to further stimulation. A similar strategy of inhibiting CCR5 by internalization is being pursued for anti-HIV treat-

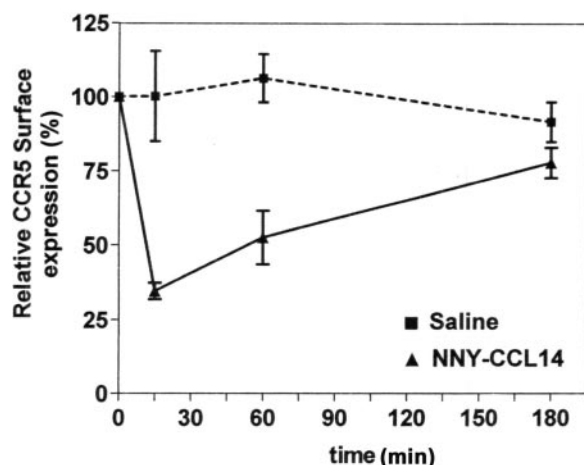


Fig. 7. In vivo internalization of CCR5 and re-expression in BALB/c mice, which were injected i.v. with NNY-CCL14 or saline. Blood was drawn from the retro-orbital plexus at time-points $t = 0$ (before injection) and 15, 60, and 180 min after injection. CCR5 expression on DX5⁺ cells was determined by flow cytometric analysis. The graph represents mean $\pm$ SEM values for relative percentage expression of CCR5 for $n = 5$ experiments.

ment [6, 31, 32], and it has been successfully demonstrated that high doses of PSC-RANTES prevent vaginal SIV/HIV transmission in macaques through inhibition of CCR5 [33]. Moreover, such a strategy of using agonistic inactivators has been proposed for anti-inflammatory agents as well [7, 8, 16, 34].

CCR1 and CCR5 are receptors of the inflammatory chemokines CCL3, CCL4, CCL5, CCL7, and CCL8, among other things controlling the maneuvers of lymphocytes, eosinophils, and monocytes in vivo [15]. Therefore, we further studied the in vivo effects of NNY-CCL14 in an OVA-dependent murine model of allergic inflammation. In the lung function tests previously performed, the treatment with NNY-CCL14 significantly reduced AHR [18]. Interestingly, NNY-CCL14 is as effective as dexamethasone in abrogating AHR. The effect of NNY-CCL14 on the migration of inflammatory cells to the lung was studied using the approach of a double challenge with OVA before assessment of BAL cell content. In this murine model of inflammation, a pronounced decrease in the number of migrated eosinophils and also lymphocytes was observed. In our previous study, where a model of allergic inflammation with a single OVA challenge was used, we demonstrated a decrease of migrated eosinophils upon NNY-CCL14 treatment with no significant effect on BAL lymphocyte numbers [18]. This difference can be attributed to the intensity of inflammation induced in each case, as assessed by the absolute numbers of migrated eosinophils and lymphocytes in the saline-treated group. The decrease in AHR previously observed may be a result of a decrease in eosinophil and lymphocyte numbers, although the role of eosinophils in AHR is controversially discussed [35–37]. There is evidence in T lymphocyte-deficient mice for prevention of lung eosinophilia and bronchial hyper-reactivity during antigen-induced inflammation [38]. This supports a critical role of the T cell cytokines IL-4 and IL-5 in eosinophil migration and AHR [39, 40]. In a chronic, fungus-induced asthma model using CCR5-deficient mice, a decrease in AHR, airway remodeling, peribronchial T cell, and

eosinophil accumulation was observed [13]. In another study using microarray and knockout mice, CCR5 has been revealed as a significant and complex mediator of AHR [41]. In the context of the in vivo studies, the activity of NNY-CCL14 on mCCR1, mCCR3, and mCCR5 was analyzed. NNY-CCL14 is an efficient activator of mCCR1 and mCCR5 but surprisingly, unable to induce the internalization of mCCR3. These results suggest a crucial role of CCR1 and CCR5 in inhibiting the influx of inflammatory cells, especially lymphocytes, and subsequently, AHR. As CD4⁺ lymphocytes expressing CCR1 and CCR5 are the key inflammatory lymphocytes present in human allergic airway disease [27, 42], the ability of NNY-CCL14 to activate these cells was tested. Potency and efficacy of NNY-CCL14 to induce calcium efflux and internalize cell surface CCR5 from cultured mCD4⁺ T lymphocytes and various blood lymphocyte subsets further substantiate its potential to inhibit airway inflammation. Moreover, we show the ability of i.v.-injected NNY-CCL14 to down-regulate CCR5 of circulating leukocytes. The extent of internalization and intracellular sequestration has been shown to depend on the activation state of the cells and their receptor profile, which is altered in inflammatory conditions [42, 43]. In consequence, the in vivo internalization of CCR5 represents a part of the complex mechanism of action of i.v.-injected NNY-CCL14, which contributes to its inhibitory effects in models of allergic airway inflammation. The internalization of mCCR1 could not be studied, as an appropriate commercial source of anti-mCCR1 antibody is currently not available. Nevertheless, the in vivo mechanism of action may depend on further specific details. Summing up, our study strengthens the concept that blocking the activation and migration of T lymphocytes may prevent the successive events that contribute to the pathophysiology of asthma.

The finding that NNY-CCL14 does not internalize mCCR3, and preincubation with NNY-CCL14 does not desensitize the internalization by the CCR3 ligand CCL11 (data not shown) suggests that inhibition of CCR5 and/or CCR1 is at least in part responsible for the observed in vivo effects. Thus, we propose an upstream role of T lymphocyte inactivation by NNY-CCL14 in the inhibition of migration of eosinophils into the airways in allergic airway inflammation. The inhibition of migration via CCR1 present on eosinophils [44] may also contribute to a decrease in the observed eosinophilia. Chvatchko et al. [16] have described previously modified CCL5 analogs to inhibit BAL eosinophilia and T cell recruitment, but these analogs were not able to reduce AHR. Further, we cannot rule out the possibility that NNY-CCL14 modulates other factors contributing to AHR and allergic inflammation, although the examination of the lung and liver of mice treated with NNY-CCL14 suggests that this compound does not lead to sequestration of leukocytes in the vessels as a result of trapping or sludging (data not shown). This study suggests that NNY-CCL14 is a potent compound to reduce infiltration of lung eosinophils and lymphocytes and abrogate AHR in allergic airway inflammation by inactivating CCR5 and most likely, CCR1. Thus, the important role of these receptors on T cells as therapeutic targets for treatment of allergic airway disease is substantiated [45].

Peptides have been described as interesting candidates for therapy because of their high specificity and low systemic

toxicity [46, 47], and there are studies that suggest the use of agonistic chemokines as potential anti-inflammatory agents acting as inactivators of their receptors to further stimulation [8]. In our previous study, NNY-CCL14 is described as a peptide, which is a CD26/dipeptidyl peptidase IV-resistant agonistic inactivator of CCR3 [18]. Moreover, in the present study, we describe NNY-CCL14 as a potential therapeutic compound capable of efficiently desensitizing and internalizing hCCR1 and hCCR5 and subsequently having inactivating properties for effector cells, namely, T lymphocytes. NNY-CCL14 significantly abrogates AHR and accumulation of lymphocytes and eosinophils in BAL in a murine model of allergic inflammation. Thus, NNY-CCL14, acting via multiple chemokine receptors, has the potential to alleviate allergic airway diseases. Additional studies, in regard to the effect of NNY-CCL14 in a therapeutic setting using a chronic model of allergic airway inflammation, will help to evaluate its in vivo potential and might further facilitate the understanding of its complex mechanism of action beyond the inactivation of CCR5, which has been demonstrated to occur in vivo.

ACKNOWLEDGMENTS

This work was supported by the Disabled Facilities Grant FO77/10-1. We thank Birgit Eilers and Wolfgang Posselt for their technical assistance. U. F. recently became an employee of Merck KGaA Darmstadt, Germany.

REFERENCES

- Robinson, D. S. (2004) The role of the mast cell in asthma: induction of airway hyperresponsiveness by interaction with smooth muscle? *J. Allergy Clin. Immunol.* **114**, 58–65.
- Gleich, G. J. (2000) Mechanisms of eosinophil-associated inflammation. *J. Allergy Clin. Immunol.* **105**, 651–663.
- Luster, A. D., Tager, A. M. (2004) T-cell trafficking in asthma: lipid mediators grease the way. *Nat. Rev. Immunol.* **4**, 711–724.
- Gerard, C., Rollins, B. J. (2001) Chemokines and disease. *Nat. Immunol.* **2**, 108–115.
- Zimmermann, N., Hershey, G. K., Foster, P. S., Rothenberg, M. E. (2003) Chemokines in asthma: cooperative interaction between chemokines and IL-13. *J. Allergy Clin. Immunol.* **111**, 227–242.
- Wells, T. N., Power, C. A., Shaw, J. P., Proudfoot, A. E. (2006) Chemokine blockers—therapeutics in the making? *Trends Pharmacol. Sci.* **27**, 41–47.
- Elsner, J., Escher, S. E., Forssmann, U. (2004) Chemokine receptor antagonists: a novel therapeutic approach in allergic diseases. *Allergy* **59**, 1243–1258.
- Ali, S., O'Boyle, G., Mellor, P., Kirby, J. A. (2007) An apparent paradox: chemokine receptor agonists can be used for anti-inflammatory therapy. *Mol. Immunol.* **44**, 1477–1482.
- Gutierrez-Ramos, J. C., Lloyd, C., Kapsenberg, M. L., Gonzalo, J. A., Coyle, A. J. (2000) Non-redundant functional groups of chemokines operate in a coordinate manner during the inflammatory response in the lung. *Immunol. Rev.* **177**, 31–42.
- Bromley, S. K., Thomas, S. Y., Luster, A. D. (2005) Chemokine receptor CCR7 guides T cell exit from peripheral tissues and entry into afferent lymphatics. *Nat. Immunol.* **6**, 895–901.
- Lukacs, N. W., Prosser, D. M., Wiekowski, M., Lira, S. A., Cook, D. N. (2001) Requirement for the chemokine receptor CCR6 in allergic pulmonary inflammation. *J. Exp. Med.* **194**, 551–555.
- Schuh, J. M., Power, C. A., Proudfoot, A. E., Kunkel, S. L., Lukacs, N. W., Hogaboam, C. M. (2002) Airway hyperresponsiveness, but not airway remodeling, is attenuated during chronic pulmonary allergic responses to *Aspergillus* in CCR4^{-/-} mice. *FASEB J.* **16**, 1313–1315.
- Schuh, J. M., Blease, K., Hogaboam, C. M. (2002) The role of CC chemokine receptor 5 (CCR5) and RANTES/CCL5 during chronic fungal asthma in mice. *FASEB J.* **16**, 228–230.
- Hogaboam, C. M., Carpenter, K. J., Schuh, J. M., Proudfoot, A. A., Bridger, G., Buckland, K. F. (2005) The therapeutic potential in targeting CCR5 and CXCR4 receptors in infectious and allergic pulmonary disease. *Pharmacol. Ther.* **107**, 314–328.
- Gonzalo, J. A., Lloyd, C. M., Wen, D., Albar, J. P., Wells, T. N., Proudfoot, A., Martinez-A, C., Dorf, M., Bjerke, T., Coyle, A. J., Gutierrez-Ramos, J. C. (1998) The coordinated action of CC chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J. Exp. Med.* **188**, 157–167.
- Chvatchko, Y., Proudfoot, A. E., Buser, R., Juillard, P., Alouani, S., Kosco-Vilbois, M., Coyle, A. J., Nibbs, R. J., Graham, G., Offord, R. E., Wells, T. N. (2003) Inhibition of airway inflammation by amino-terminally modified RANTES/CC chemokine ligand 5 analogues is not mediated through CCR3. *J. Immunol.* **171**, 5498–5506.
- Schuh, J. M., Blease, K., Bruhl, H., Mack, M., Hogaboam, C. M. (2003) Intrapulmonary targeting of RANTES/CCL5-responsive cells prevents chronic fungal asthma. *Eur. J. Immunol.* **33**, 3080–3090.
- Forssmann, U., Hartung, I., Balder, R., Fuchs, B., Escher, S. E., Spodsborg, N., Dulkys, Y., Walden, M., Heitland, A., Braun, A., Forssmann, W. G., Elsner, J. (2004) n-Nonanoyl-CC chemokine ligand 14, a potent CC chemokine ligand 14 analogue that prevents the recruitment of eosinophils in allergic airway inflammation. *J. Immunol.* **173**, 3456–3466.
- Detheux, M., Standker, L., Vakili, J., Munch, J., Forssmann, U., Adermann, K., Pohlmann, S., Vassart, G., Kirchhoff, F., Parmentier, M., Forssmann, W. G. (2000) Natural proteolytic processing of hemofiltrate CC chemokine 1 generates a potent CC chemokine receptor (CCR)1 and CCR5 agonist with anti-HIV properties. *J. Exp. Med.* **192**, 1501–1508.
- Mack, M., Cihak, J., Simonis, C., Luckow, B., Proudfoot, A. E., Plachy, J., Bruhl, H., Frink, M., Anders, H. J., Vielhauer, V., Pfistering, J., Stangassinger, M., Schlondorff, D. (2001) Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. *J. Immunol.* **166**, 4697–4704.
- Gupta, S., Schulz-Maronde, S., Kutzleb, C., Richter, R., Forssmann, W. G., Kapp, A., Forssmann, U., Elsner, J. (2005) Cloning, expression, and functional characterization of cynomolgus monkey (*Macaca fascicularis*) CC chemokine receptor 1. *J. Leukoc. Biol.* **78**, 1175–1184.
- Dulkys, Y., Kluthe, C., Buschermöhle, T., Barg, I., Knoss, S., Kapp, A., Proudfoot, A. E., Elsner, J. (2001) IL-3 induces down-regulation of CCR3 protein and mRNA in human eosinophils. *J. Immunol.* **167**, 3443–3453.
- Elsner, J., Mack, M., Bruhl, H., Dulkys, Y., Kimmig, D., Simmons, G., Clapham, P. R., Schlondorff, D., Kapp, A., Wells, T. N., Proudfoot, A. E. (2000) Differential activation of CC chemokine receptors by AOP-RANTES. *J. Biol. Chem.* **275**, 7787–7794.
- Braun, A., Appel, E., Baruch, R., Herz, U., Botchkarev, V., Paus, R., Brodie, C., Renz, H. (1998) Role of nerve growth factor in a mouse model of allergic airway inflammation and asthma. *Eur. J. Immunol.* **28**, 3240–3251.
- Sallusto, F., Lenig, D., Mackay, C. R., Lanzavecchia, A. (1998) Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med.* **187**, 875–883.
- Qin, S., Rottman, J. B., Myers, P., Kassam, N., Weinblatt, M., Loetscher, M., Koch, A. E., Moser, B., Mackay, C. R. (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J. Clin. Invest.* **101**, 746–754.
- Campbell, J. J., Brightling, C. E., Symon, F. A., Qin, S., Murphy, K. E., Hodge, M., Andrew, D. P., Wu, L., Butcher, E. C., Wardlaw, A. J. (2001) Expression of chemokine receptors by lung T cells from normal and asthmatic subjects. *J. Immunol.* **166**, 2842–2848.
- Ma, B., Zhu, Z., Homer, R. J., Gerard, C., Strieter, R., Elias, J. A. (2004) The C10/CCL6 chemokine and CCR1 play critical roles in the pathogenesis of IL-13-induced inflammation and remodeling. *J. Immunol.* **172**, 1872–1881.
- Forssmann, U., Magert, H. J., Adermann, K., Escher, S. E., Forssmann, W. G. (2001) Hemofiltrate CC chemokines with unique biochemical properties: HCC-1/CCL14a and HCC-2/CCL15. *J. Leukoc. Biol.* **70**, 357–366.
- Elsner, J., Petering, H., Hochstetter, R., Kimmig, D., Wells, T. N., Kapp, A., Proudfoot, A. E. (1997) The CC chemokine antagonist Met-RANTES inhibits eosinophil effector functions through the chemokine receptors CCR1 and CCR3. *Eur. J. Immunol.* **27**, 2892–2898.
- Mack, M., Luckow, B., Nelson, P. J., Cihak, J., Simmons, G., Clapham, P. R., Signorel, N., Marsh, M., Stangassinger, M., Borlat, F., Wells, T. N. C., Schlondorff, D., Proudfoot, A. E. I. (1998) Aminoxy-pentane-RANTES induces CCR5 internalization but inhibits recycling: a novel inhibitory mechanism of HIV infectivity. *J. Exp. Med.* **187**, 1215–1224.

32. Mack, M., Schlondorff, D. (2000) Downmodulation and recycling of chemokine receptors. *Methods Mol. Biol.* **138**, 191–195.
33. Lederman, M. M., Veazey, R. S., Offord, R., Mosier, D. E., Dufour, J., Mefford, M., Piatak Jr., M., Lifson, J. D., Salkowitz, J. R., Rodriguez, B., Blauvelt, A., Hartley, O. (2004) Prevention of vaginal SHIV transmission in rhesus macaques through inhibition of CCR5. *Science* **306**, 485–487.
34. Ali, S., Robertson, H., Wain, J. H., Isaacs, J. D., Malik, G., Kirby, J. A. (2005) A non-glycosaminoglycan-binding variant of CC chemokine ligand 7 (monocyte chemoattractant protein-3) antagonizes chemokine-mediated inflammation. *J. Immunol.* **175**, 1257–1266.
35. Lee, J. J., Dimina, D., Macias, M. P., Ochkur, S. I., McGarry, M. P., O'Neill, K. R., Protheroe, C., Pero, R., Nguyen, T., Cormier, S. A., Lenkiewicz, E., Colbert, D., Rinaldi, L., Ackerman, S. J., Irvin, C. G., Lee, N. A. (2004) Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* **305**, 1773–1776.
36. Humbles, A. A., Lloyd, C. M., McMillan, S. J., Friend, D. S., Xanthou, G., McKenna, E. E., Ghiran, S., Gerard, N. P., Yu, C., Orkin, S. H., Gerard, C. (2004) A critical role for eosinophils in allergic airways remodeling. *Science* **305**, 1776–1779.
37. Eum, S. Y., Maghni, K., Hamid, Q., Eidelman, D. H., Campbell, H., Isogai, S., Martin, J. G. (2003) Inhibition of allergic airways inflammation and airway hyperresponsiveness in mice by dexamethasone: role of eosinophils, IL-5, eotaxin, and IL-13. *J. Allergy Clin. Immunol.* **111**, 1049–1061.
38. Gavett, S. H., Chen, X., Finkelman, F., Wills-Karp, M. (1994) Depletion of murine CD4⁺ T lymphocytes prevents antigen-induced airway hyper-reactivity and pulmonary eosinophilia. *Am. J. Respir. Cell Mol. Biol.* **10**, 587–593.
39. Foster, P. S., Hogan, S. P., Ramsay, A. J., Matthaei, K. I., Young, I. G. (1996) Interleukin 5 deficiency abolishes eosinophilia, airways hyperactivity, and lung damage in a mouse asthma model. *J. Exp. Med.* **183**, 195–201.
40. Coyle, A. J., Le Gros, G., Bertrand, C., Tsuyuki, S., Heusser, C. H., Kopf, M., Anderson, G. P. (1995) Interleukin-4 is required for the induction of lung Th2 mucosal immunity. *Am. J. Respir. Cell Mol. Biol.* **13**, 54–59.
41. Walker, J. K., Ahumada, A., Frank, B., Gaspard, R., Berman, K., Quackenbush, J., Schwartz, D. A. (2006) Multistrain genetic comparisons reveal CCR5 as a receptor involved in airway hyperresponsiveness. *Am. J. Respir. Cell Mol. Biol.* **34**, 711–718.
42. Medoff, B. D., Thomas, S. Y., Banerji, A., Wain, J. C., Zhang, H., Lilly, C. M., Ginns, L. C., Luster, A. D. (2005) Pathogenic T-cell recruitment into the airway in human disease. *Ann. N. Y. Acad. Sci.* **1062**, 220–241.
43. Sabbe, R., Picchio, G. R., Pastore, C., Chaloin, O., Hartley, O., Offord, R., Mosier, D. E. (2001) Donor- and ligand-dependent differences in C-C chemokine receptor 5 reexpression. *J. Virol.* **75**, 661–671.
44. Elsner, J., Dulkys, Y., Gupta, S., Escher, S. E., Forssmann, W. G., Kapp, A., Forssmann, U. (2005) Differential pattern of CCR1 internalization in human eosinophils: prolonged internalization by CCL5 in contrast to CCL3. *Allergy* **60**, 1386–1393.
45. Coyle, A. J., Lloyd, C. M., Gutierrez-Ramos, J. C. (2000) Biotherapeutic targets for the treatment of allergic airway disease. *Am. J. Respir. Crit. Care Med.* **162**, S179–S184.
46. Loffet, A. (2002) Peptides as drugs: is there a market? *J. Pept. Sci.* **8**, 1–7.
47. Hauff, K., Zamzow, C., Law, W. J., De Melo, J., Kennedy, K., Los, M. (2005) Peptide-based approaches to treat asthma, arthritis, other autoimmune diseases and pathologies of the central nervous system. *Arch. Immunol. Ther. Exp. (Warsz.)* **53**, 308–320.