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CCR1- and CCR5-mediated inactivation of leukocytes by a nonglycosaminoglycan (non-GAG)-binding variant of n-Nonanoyl-CCL14 (NNY-CCL14)

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ABSTRACT

Intervention on chemokine receptors to prevent directional leukocyte migration is a potential therapeutic strategy. NNY-CCL14 is a CD26-resistant lead molecule, which exerts its effects on multiple chemokine receptors (CCR1, CCR2, CCR3, and CCR5). The inhibitory effects of NNY-CCL14 in murine models of allergic airway inflammation have been assigned to its interaction with CCR1 and CCR5. In this study, a non-GAG-binding variant of NNY-CCL14 was generated by mutating basic amino acids within the identified GAG-binding ^{49}BBXB^{52} motif. This CD26-resistant, non-GAG binding variant, NNY-CCL14(G,A), does not promote CCR1-dependent cell arrest on modeled endothelium. Its biological activity tested on human and murine chemokine receptors revealed distinguishing properties to NNY-CCL14. As suggested by EC_{50} values for intracellular calcium mobilization, NNY-CCL14(G,A) demonstrated a reduced ability to activate hCCR1, but internalization and desensitization of hCCR1 were unperturbed. Surprisingly, its activity on hCCR3 was strongly reduced, and it did not internalize mCCR3. A significantly reduced chemotactic activity of eosinophils and monocytes was observed. All biological effects mediated by NNY-CCL14(G,A) via hCCR5 and mCCR5 showed no difference to NNY-CCL14. In mice treated i.v. with NNY-CCL14(G,A), a sustained in vivo down-modulation of CCR5 was achieved over 3 h. Therefore, NNY-CCL14(G,A) inactivates leukocytes by desensitizing and internalizing multiple chemokine receptors, thus rendering them unresponsive to further stimulation by natural ligands. When administered systemically, NNY-CCL14(G,A) may modulate leukocyte functions prior to their interaction with other endothelium-bound chemokines expressed under pathophysiological conditions, such as allergic inflammation. *J. Leukoc. Biol.* 88: 383–392; 2010.

Introduction

The hallmark of inflammatory diseases, such as allergic asthma, is the massive infiltration of leukocytes, in particular, eosinophils, T lymphocytes, and mast cells, which contribute to a complex pathological process [1, 2]. This orchestration of inflammatory cells to specific sites of inflammation is mediated by chemokines [3]. During inflammation, the migration of blood-borne leukocytes across the vascular endothelium is a multi-faceted process, which includes the activation of leukocytes by GAG-bound chemokines at the vascular endothelium and extracellular matrix, mediating transmigration of cells along the chemotactic gradient [4, 5]. The main GAG expressed by endothelial cells is heparan sulfate, which binds and presents chemokines to circulating leukocytes on the luminal side of the endothelium, providing directional cues for migrating cells [6]. GAG has also been shown to play an important role in the oligomerization of chemokines, such as CCL2, CCL4, and CCL5, thus regulating their function [7].

Chemokine receptors, such as CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, and CXCR4, have been shown to play an important role in the development and maintenance of allergic asthma [8–14]. Modulation of leukocyte recruitment

Abbreviations: AHR=airway hyper responsiveness, FLIPR=fluorescence image plate reader, GAG=glycosaminoglycan, h=human, m=murine, NNY=*n*-Nonanoyl, TFA=trifluoroacetic acid

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through intervention with these chemokine receptors has been proposed as an attractive therapeutic strategy for the treatment of allergic asthma [15, 16]. Various strategies proposed to target chemokine receptors include peptide-chemokine receptor antagonists, such as Met-CCL5, CCL11(3-28), and I-Tac/E0H1A [16], and lipophilic nonpeptide compounds, i.e., small molecules that may be piperidine or pyrrolidine derivatives [16]. More recently, several strategies for using chemokine-derived agonists for anti-inflammatory therapy have been proposed [17–21].

In previous studies [17, 18, 21], the proposed strategy of treating airway inflammation by preventing cellular recruitment via inactivation of chemokine receptors on inflammatory cells before they extravasate was substantiated. The compound NNY-CCL14 [21] is an N-terminally truncated, modified derivative of the naturally occurring chemokine CCL14(9-74) [22]. It is resistant to degradation by the peptidase CD26/dipeptidyl aminopeptidase IV, which is found abundantly in blood plasma and tissues and on the cell surface of various cell types [21]. Moreover, it acts as an agonistic inactivator of CCR1, CCR2, CCR3, and CCR5 by desensitizing these receptors to further stimulation by natural ligands and internalizing these receptors from the cell surface, subsequently inactivating effector cells [17, 18, 21]. In addition, systemic administration of NNY-CCL14 significantly abrogates AHR and accumulation of lymphocytes and eosinophils in broncoalveolar lavage in a murine model of allergic inflammation [18]. The in vivo inhibitory effects of NNY-CCL14 in murine models have been assigned to its interaction with CCR1 and CCR5. Therefore, our previous studies validate NNY-CCL14 as a potential therapeutic for the alleviation of allergic airway inflammation [17, 18, 21].

In the present study, we identify the GAG-binding domain of NNY-CCL14 and generate the non-GAG-binding mutant, NNY-CCL14(G,A), which retains the potential to induce receptor desensitization and internalization but cannot be presented on the endothelium via heparin-binding sites. Surprisingly, there was a marked decrease in chemotaxis of eosinophils and monocytes in response to NNY-CCL14(G,A). In mice treated i.v. with this compound, a sustained in vivo down-modulation of CCR5 was observed. These properties suggest that unlike its parent compound, NNY-CCL14(G,A) may be administered via various systemic routes (e.g., i.v., s.c., or intrapulm.) without causing a local inflammatory reaction. This receptor agonist, present in the blood compartment, should be able to inactivate leukocyte functions prior to their interaction with other endothelium-bound chemokines expressed under inflammatory conditions.

MATERIALS AND METHODS

Chemokines and cytokines

hCCL11 was purchased from ImmunoTools (Friesoythe, Germany); hCXCL12, mCCL3, and mCCL5 from PeproTech (Rocky Hill, NJ, USA); and CCL14(9-74), NNY-CCL14, and NNY-CCL14(G,A) were produced using Fmoc chemistry, as described previously [21, 23]. In brief, peptides were synthesized on a preloaded Tentagel R-Trt-Asn(Trt) resin (0.15 mmol/g; Rapp Polymere, Tuebingen, Germany). Cysteine residues were Trt-protected. The N terminus was modified after solid-phase assembly and cleavage of the terminal Fmoc group by treatment with the symmetric anhydride of nonanoic acid in N-methyl pyrrolidinone. After cleavage and deprotection of the linear NNY peptides with TFA/water/ethanedithiol/phenol (86/6/6/2, v/v/v/w, 15 ml/g crude product), the product was precipitated with cold tert-butylmethylether and dried. The obtained product was purified by preparative HPLC [Vydac C18 column, 47×300 mm, 15–20 μm, flow rate 50 ml/min 0.5–1% B/min; eluent A: 0.1% TFA in water; eluent B: 0.07% TFA in CH_3CN/H_2O (4:1, v:v); detection at 214 and 230 nm], and pure fractions were subjected to oxidative folding in guanidinium hydrochloride and the presence of cysteine/cystine as a redox system as described earlier [23]. After completion of the folding, the major product was separated by semi-preparative HPLC [Vydac C18 column, 20×250 mm, 10 μm, flow rate 7 ml/min, 0.5–1% B/min; eluent A: 0.1% TFA in water; eluent B: 0.07% TFA in CH_3CN/H_2O (4:1, v:v); detection at 214 and 230 nm]. As reported earlier [23], oxidative folding of linear CCL14 peptides results in the native 1-3/2-4 disulfide pattern. The purified products were characterized by analytical HPLC [Vydac C18 column, 4.6×250 mm, 5 μm, flow rate 0.8 ml/min, 0.5–1% B/min; eluent A: 0.1% TFA in water; eluent B: 0.07% TFA in CH_3CN/H_2O (4:1, v:v); detection at 214 and 230 nm] and found to exhibit a purity of 98%. Molecular weight determined by electrospray ionization mass spectrometry was 7880.1 Da (NNY-CCL14; calculated 7880.7) and 7723.8 Da [NNY-CCL14(G,A); calculated 7724.6].

Antibodies

The mAb against hCCR1/hCCR2/hCCR3 and mouse CCR3 were purchased from R&D Systems (Wiesbaden, Germany) and hCCR5, mouse Pan-NK (clone DX-5), mouse CD8a, and mouse CD4 from BD Biosciences (Heidelberg, Germany). The mAb against mouse CCR5 (clone MC68) was generated as described previously [24]. The FITC/PE-conjugated anti-mouse IgG or Cy™5-conjugated mouse anti-rat IgG secondary antibodies were obtained from Jackson ImmunoResearch Laboratories (Cambridgeshire, UK).

Cells

Stably transfected murine pre-B 300.19 cells expressing hCCR1, hCCR2, hCCR3, or hCCR5 were obtained from Bernhard Moser (Theodor Kocher Institute, University of Berne, Switzerland). Venous blood was drawn from normal, nonatopic, healthy volunteers who had given their informed consent according to the requirements of the local research ethics committee at Hannover Medical School (Germany). PBMCs and granulocytes were purified using Ficoll (Pharmacia, Erlangen, Germany) density gradient centrifugation as described previously [25]. Monocytes were isolated from PBMCs using MACS Monocyte Isolation Kit II (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Purity of >90% was checked by flow cytometry and microscopic examination. 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, USA), as described previously [25]. The resulting eosinophil purity was >90%, as determined by microscopic examination.

Determination of heparin-binding affinity

The binding affinity of the BBXB motif-modified NNY-CCL14(G,A) was performed with a high trap heparin column (1 ml). The column was equilibrated in buffer A (0.0125 M NaCl/0.025 M Tris, pH=8) and injected with 10 μg of the chemokine (0.5 $\mu g/\mu l$ in H_2O). The chemokines were followed by elution with rising salt concentration of buffer B (2 M NaCl/0.025 M Tris, pH=8; gradient: 0–25–50% of buffer B in 0–50–70 min) and detected using UV absorption at 280 nm. Over the retention time, the salt concentration necessary for elution of each chemokine was determined.

The salt concentration is a measure of the strength of the ionic interaction between chemokine and GAG, in this case, heparin.

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 FLIPR system (Molecular Devices, Munich, Germany) as described previously [26].

Flow cytometric measurement of CCR1, CCR2, CCR3, and CCR5 internalization

In vitro internalization experiments were performed on cell lines, physiological cells, or whole blood, as described previously in detail. The cell surface expression of the chemokine receptors was expressed as relative fluorescence intensity (percentage), calculated using the following equation: [median channel fluorescence (stimulus)–median channel fluorescrence (isotype control)]/[median channel fluorescence (medium)–median channel fluorescence (isotype control)] × 100% [25, 27]. Purified eosinophils, as described above, and the monocyte fraction within the PBMCs were gated based on forward- and side-scatter properties [17, 18, 21]. The animal experiments were approved by the government of Lower Saxony (Hannover, Germany) and were conducted according to international guidelines for the use of laboratory animals. For in vivo internalization experiments, BALB/c mice were i.v.-injected with NNY-CCL14(G,A) or saline. Blood was drawn at indicated time-points from the retro-orbital plexus and transferred immediately on ice, and staining of various cell populations was performed. For in vitro re-expression analysis, the cells were incubated for 30 min at 37°C with the respective ligands, which were then washed twice with room temperature PBS, resuspended in prewarmed RPMI as described previously [27], and incubated at 37°C until the indicated time-points when they were stained for CCR1 or CCR5 expression. The cell viability at the respective time-points was analyzed by trypan blue staining.

In vitro chemotaxis

Chemotaxis was assessed in 48-well chambers (NeuroProbe, Cabin John, MD, USA) using polyvinylpyrrolidone-free polycarbonate membranes with 5 μm pores (Nucleopore, NeuroProbe) for 5×10^4 monocytes or eosinophils/well. Migration was allowed to proceed for 60 min at 37°C in response to 0.1–1000 nM CCL14(9-74) (positive control), NNY-CCL14, or NNY-CCL14(G,A). Spontaneous migration was determined in the absence of chemoattractant in media. The migration index is defined as the ratio of the number of cells migrated in response to the specific chemokine to spontaneous migration in the absence of the chemokine.

Modulation of adhesion of B300.19-CCR1$^+$ and -CCR5$^+$ cells to the HUVEC monolayer

HUVEC monolayers were grown in ibidi plastic flow chambers and preincubated overnight with 20 ng/ml hTNF-α [28]. Before subjecting the cells to the flow chamber, they were preincubated with 100 μl medium, with or without the CCL14 derivatives CCL14(9-74), NNY-CCL14, or NNY-CCL14(G,A) at a concentration of 5 μg/ml for 15 min. Subsequently, the chemokines were removed, and 10^5/100 μl medium of CCR1- or CCR5-transfected B300.19 cells was subjected to the flow chamber, as described previously [29]. The cells were allowed to adhere to the HUVEC monolayer for 5 min without flow. The shear stress was then increased to 2.0 dyn/cm^2. After each interval, a photograph was taken. One thousand to 2000 cells/viewed field were seen in the beginning. Counting of cells was performed using ImageJ free software.

Statistics

The number of experiments is stated in the legends of the figures as *n*. The data are expressed as mean ± SEM, as determined by Prism 3.03 (GraphPad Software Inc., San Diego, CA, USA) software analysis.

RESULTS

Identification of the GAG-binding motif in NNY-CCL14 and generation of the mutant variant NNY-CCL14(G,A)

BBXB motifs have been characterized as GAG-binding motifs in several chemokines such as CXCL12, CCL3, CCL4, CCL5, and CCL7 [6, 7, 30, 31]. We identified this motif as ^{49}KRGH^{52} in CCL14(9-74) and NNY-CCL14 (**Fig. 1A**). NNY-CCL14 is an N-terminally modified derivative of CCL14(9-74), which has been described previously as a CD26-resistant agonistic inactivator of CCR1, -2, -3, and -5 [17, 18, 21]. We synthesized the next generation of this compound, where the first two basic amino acids lysine and arginine in the identified BBXB (^{49}KRGH^{52}) motif were replaced with neutral amino acids glycine and alanine, respectively, to generate the derivative NNY-CCL14(G,A) (Fig. 1A). The heparin-binding assay was used in the screening for mutants. A major limitation of this assay is the use of heparin instead of the more physiologically relevant GAG heparin sulfate. However, many previous studies have demonstrated heparin to be structurally and chemically similar to heparin sulfate and a good substitute for preliminary screening assays for non-GAG-binding derivatives [31, 32]. Thus, the new derivative, NNY-CCL14(G,A), was tested for its ability to bind to the GAG heparin. It was observed that NNY-CCL14(G,A) was eluted at a five times lower salt concentration than its parent compounds [CCL14(9-74), NNY-CCL14] and CXCL12 (Fig. 1B), which have the intact GAG-binding motif, suggesting the low-binding affinity of NNY-CCL14(G,A) to heparin. Therefore, these findings suggest that ^{49}KRGH^{52} is the

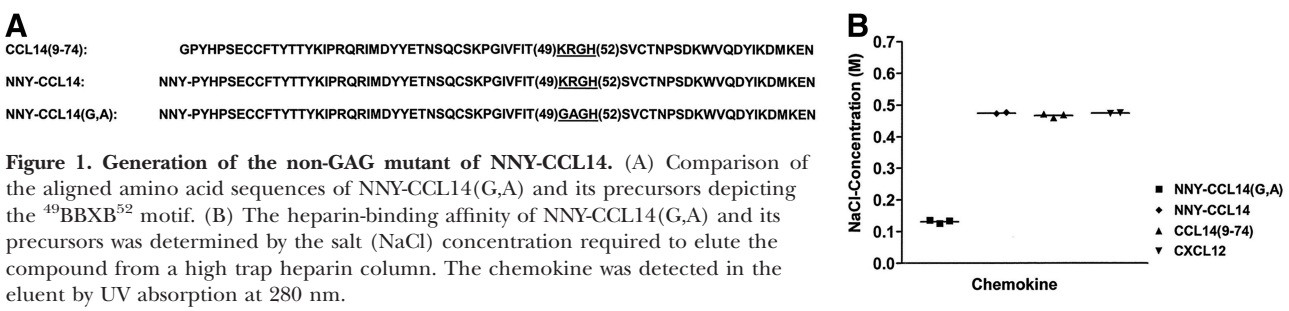

Figure 1. Generation of the non-GAG mutant of NNY-CCL14. (A) Comparison of the aligned amino acid sequences of NNY-CCL14(G,A) and its precursors depicting the ^{49}BBXB^{52} motif. (B) The heparin-binding affinity of NNY-CCL14(G,A) and its precursors was determined by the salt (NaCl) concentration required to elute the compound from a high trap heparin column. The chemokine was detected in the eluent by UV absorption at 280 nm.

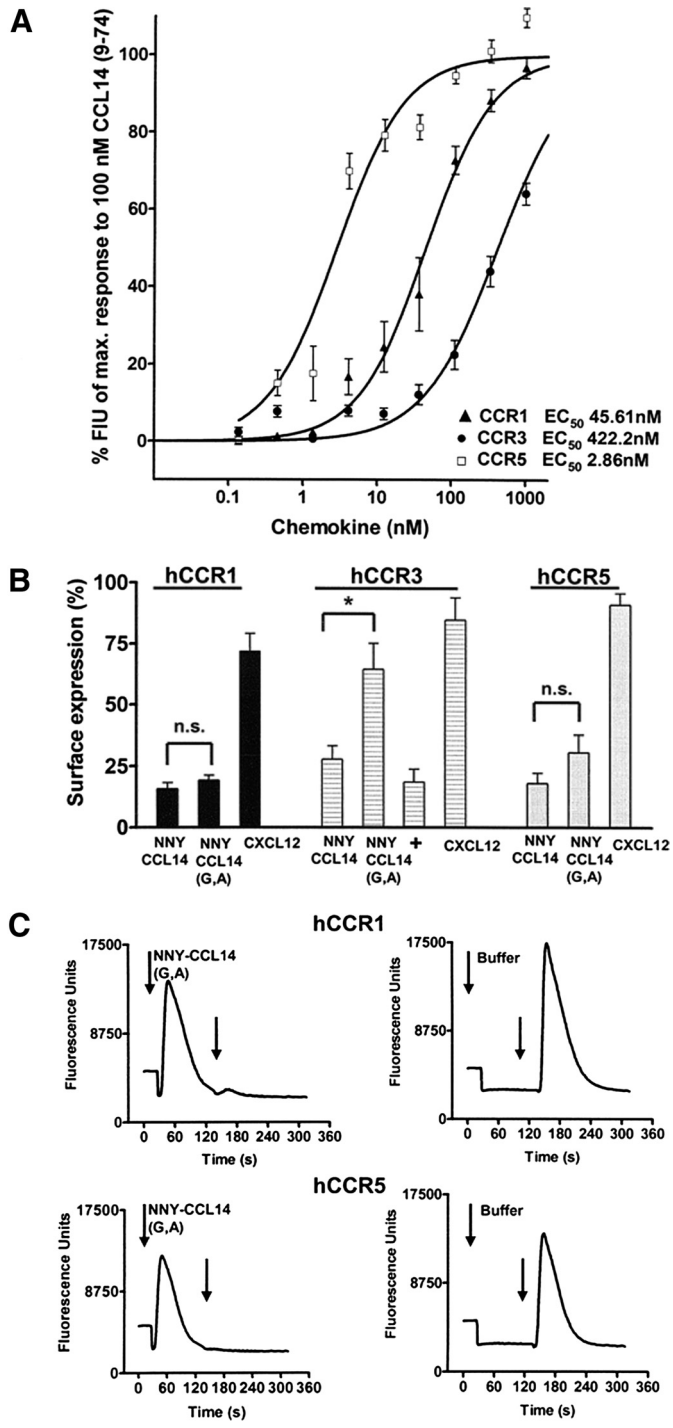

Figure 2. Potency and efficacy of NNY-CCL14(G,A) on hCCR1, hCCR3, and hCCR5. All assays were performed with hCCR1-, hCCR3-, or hCCR5-expressing B300.19 cell lines. (A) The dose-dependent response of NNY-CCL14(G,A) was determined by measuring intracellular calcium mobilization using the FLIPR. EC_{50} values, expressed in nM concentrations, were calculated from the dose-response curves. All values are mean ± SEM ($n \geq 13$). FIU, Fluorescence intensity unit. (B) Surface expression of the indicated cell surface receptor was analyzed by flow cytometry following incubation of the respective cell lines with 100 nM of the indicated chemokines for 30 min at 37°C. CCL11(+) (100 nM) was included as the positive control for CCR3. Data are expressed as the mean ± SEM relative fluorescence intensity as described in Materials and Methods ($n \geq 5$); *, $P < 0.05$; n.s., not significant. (C) For the desensitization experiments, $CCR1^+$ or $CCR5^+$ cells were sequentially stimulated at 90 s intervals with 100 nM NNY-CCL14(G,A) or buffer (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.

GAG-binding motif in the naturally occurring chemokine CCL14(9-74) and its derivative NNY-CCL14.

NNY-CCL14(G,A) is an agonistic inactivator of hCCR1 and hCCR5 but not of hCCR3

To study the efficacy and potency of NNY-CCL14(G,A) on hCCR1, hCCR3, and hCCR5, we analyzed the ligand-induced calcium mobilization by FLIPR on B300.19-$CCR1^+$, -$CCR3^+$, and -$CCR5^+$ cell lines. EC_{50} values for NNY-CCL14(G,A) were calculated from dose-response curves. NNY-CCL14(G,A) is a potent activator of hCCR1 and hCCR5, activating these receptors at nanomolar concentrations. Moreover, the EC_{50} value of these receptors was comparable with the potency of the parent compound NNY-CCL14 described previously [18]. Surprisingly, the activating potential of NNY-CCL14(G,A) on hCCR3 was 20-fold less (**Fig. 2A**) than NNY-CCL14 [21]. In the next set of experiments, B300.19-CCR1, -CCR3, and -CCR5 cell lines were incubated for 30 min at 37°C with 100 nM NNY-CCL14(G,A), alongside previously studied NNY-CCL14 and CCL14(9–74) for comparison [18, 21], CCL11, or CXCL12 as positive and negative controls, respectively; surface expression of the receptor was analyzed by flow cytometry. NNY-CCL14(G,A) retained the ability to internalize hCCR1 and hCCR5 as effectively as its parent compound NNY-CCL14, but there was a significant loss in its ability to internalize CCR3 (Fig. 2B). In addition, the desensitization studies (Fig. 2C) reveal that pretreatment with 100 nM NNY-CCL14(G,A) desensitized hCCR1 and hCCR5 to further stimulation by equimolar concentration of their natural ligand CCL14(9-74) [22]. Hence, these experiments suggest that NNY-CCL14(G,A) is a potent agonist of hCCR1 and hCCR5, activating these receptors, desensitizing them, and internalizing them from the cell surface, making them unavailable for further stimulation with their natural ligands.

Potency and efficacy of NNY-CCL14(G,A) on human primary leukocytes

The potency and efficacy of NNY-CCL14(G,A) were tested on human primary leukocytes. The EC_{50} value was calculated from the dose-response curve (**Fig. 3A**). The potency of NNY-CCL14(G,A) observed on PBMCs was consistent with that on hCCR1 and hCCR5 cell lines described above. Moreover, the EC_{50} = 22.6 nM for NNY-CCL14(G,A) on PBMCs was sixfold lower than its parent compound NNY-CCL14 (EC_{50}=3.5 nM [18]) described previously. The efficacy and potency of NNY-CCL14(G,A) to induce chemotaxis were studied on monocytes that express surface CCR1, CCR2, and CCR5 but not CCR3, which has been described as an eosinophil receptor. The in

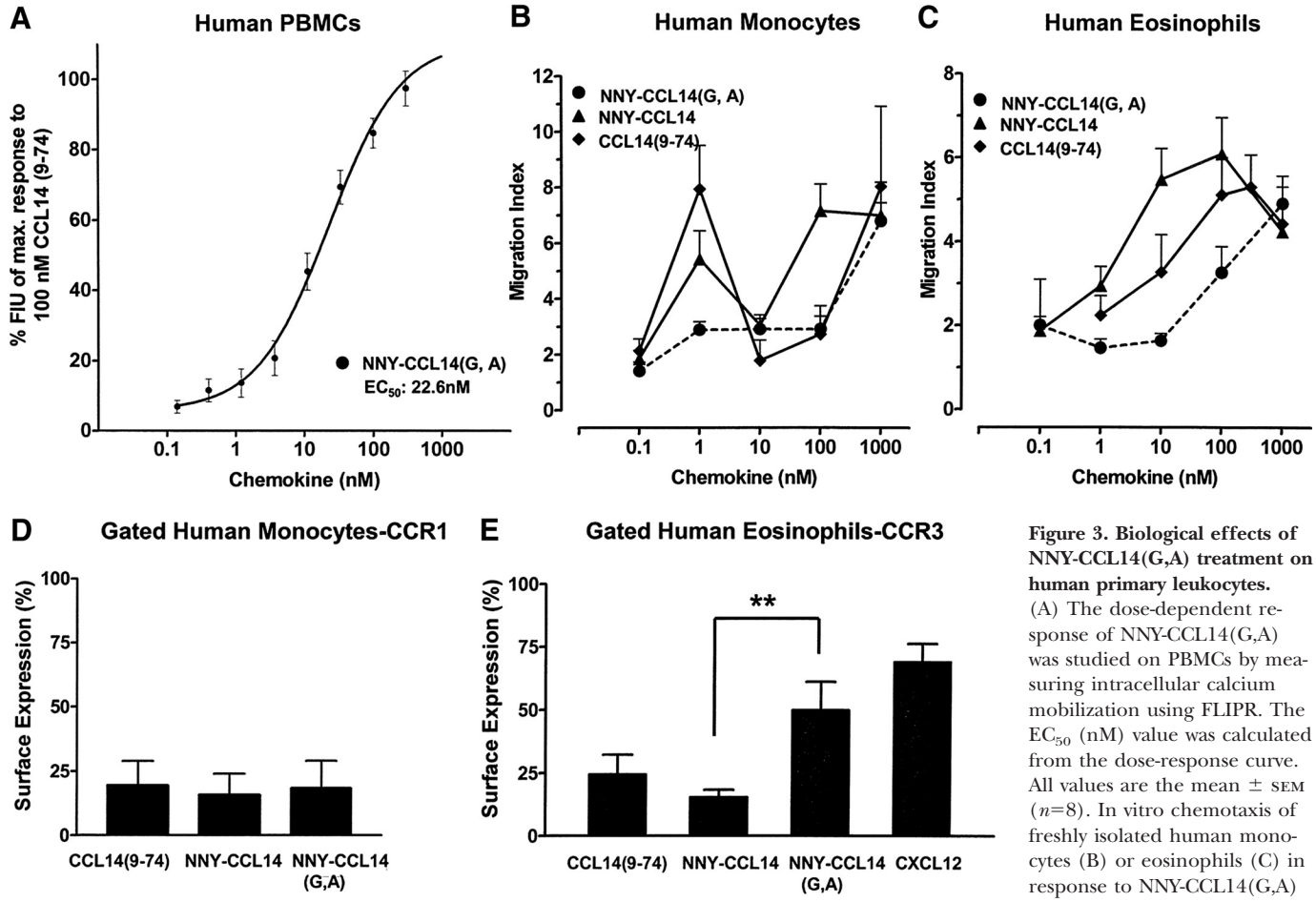

Figure 3. Biological effects of NNY-CCL14(G,A) treatment on human primary leukocytes. (A) The dose-dependent response of NNY-CCL14(G,A) was studied on PBMCs by measuring intracellular calcium mobilization using FLIPR. The EC_{50} (nM) value was calculated from the dose-response curve. All values are the mean ± SEM (n=8). In vitro chemotaxis of freshly isolated human monocytes (B) or eosinophils (C) in response to NNY-CCL14(G,A) was studied in a 48-well microchemotaxis Boyden chamber for 60 min at 37°C in response to 0.1–1000 nM of the indicated ligand. The number of migrating cells/five high-power fields (×1000) is indicated; $n \geq 6$ donors. The spontaneous migration in the absence of chemoattractant was incorporated in the determination of the migration index. Described previously [21], CCL14(9-74) and NNY-CCL14 were included as controls and for comparison. Comparison of the ability of NNY-CCL14(G,A), with its precursors described previously [18, 21], to down-modulate CCR1 on monocytes (D) and CCR3 on eosinophils (E) on treatment for 30 min at 37°C, is depicted. Concentration of all ligands is 100 nM. Data are demonstrated as mean ± SEM relative fluorescence intensity as determined by flow cytometric analysis; **, $P < 0.01$; $n \geq 4$.

vitro migration assays were conducted in 48-well microchemotaxis chambers. Surprisingly, the potency and effectiveness of NNY-CCL14(G,A) to induce chemotaxis of monocytes and eosinophils were reduced significantly (Fig. 3, B and C). The typical bell-shaped curve observed for the reference compound NNY-CCL14 could no longer be observed at concentrations up to 1 μM. The biphasic curve with peaks at 1 nM and 100 nM seen for the control parent compound NNY-CCL14 was not seen for NNY-CCL14(G,A) on monocytes (Fig. 3B), but the migration peak was at a much higher concentration of 1000 nM. Similarly, a reduction in migration of eosinophils was seen for NNY-CCL14(G,A) compared with NNY-CCL14. The peak migration for NNY-CCL14(G,A) was achieved at 1000 nM in comparison with 100 nM for NNY-CCL14 (Fig. 3C). In addition, NNY-CCL14(G,A) internalized CCR1 on monocytes as efficiently as NNY-CCL14 (Fig. 3D), but its ability to internalize CCR3 on eosinophils was reduced significantly (Fig. 3E). Therefore, the properties of NNY-CCL14(G,A) studied on primary leukocytes are in accordance with the study about transfected cell lines above.

The GAG mutated variant of NNY-CCL14 loses its ability to activate and internalize hCCR2

Hereby, the above results demonstrated that the mutation in the GAG-binding site of NNY-CCL14 leads to a loss in its activity on CCR3, but its properties on CCR1 and CCR5 are not perturbed remarkably. Surprisingly, NNY-CCL14(G,A) has a sixfold lower EC_{50} than its parent compound NNY-CCL14 on PBMCs. As CCR2 is a chemokine receptor expressed within the PBMC population alongside CCR1 and CCR5, we further investigated the functional properties of NNY-CCL14(G,A) on hCCR2. Internalization studies on B300.19-CCR2 cells and PBMCs revealed that even at a concentration as high as 1000 nM, NNY-CCL14(G,A) does not internalize CCR2 (**Fig. 4, A** and **B**). Moreover, as CCL2 is the selective ligand for CCR2, we performed desensitization experiments on PBMCs, where the ability of NNY-CCL14(G,A) to desensitize the response of 10 nM CCL2 was studied. Our studies demonstrated (Fig. 4C) that even at a dose of 1000 nM, NNY-CCL14(G,A) (first arrow) does not completely desensitize the response of PBMCs to 10

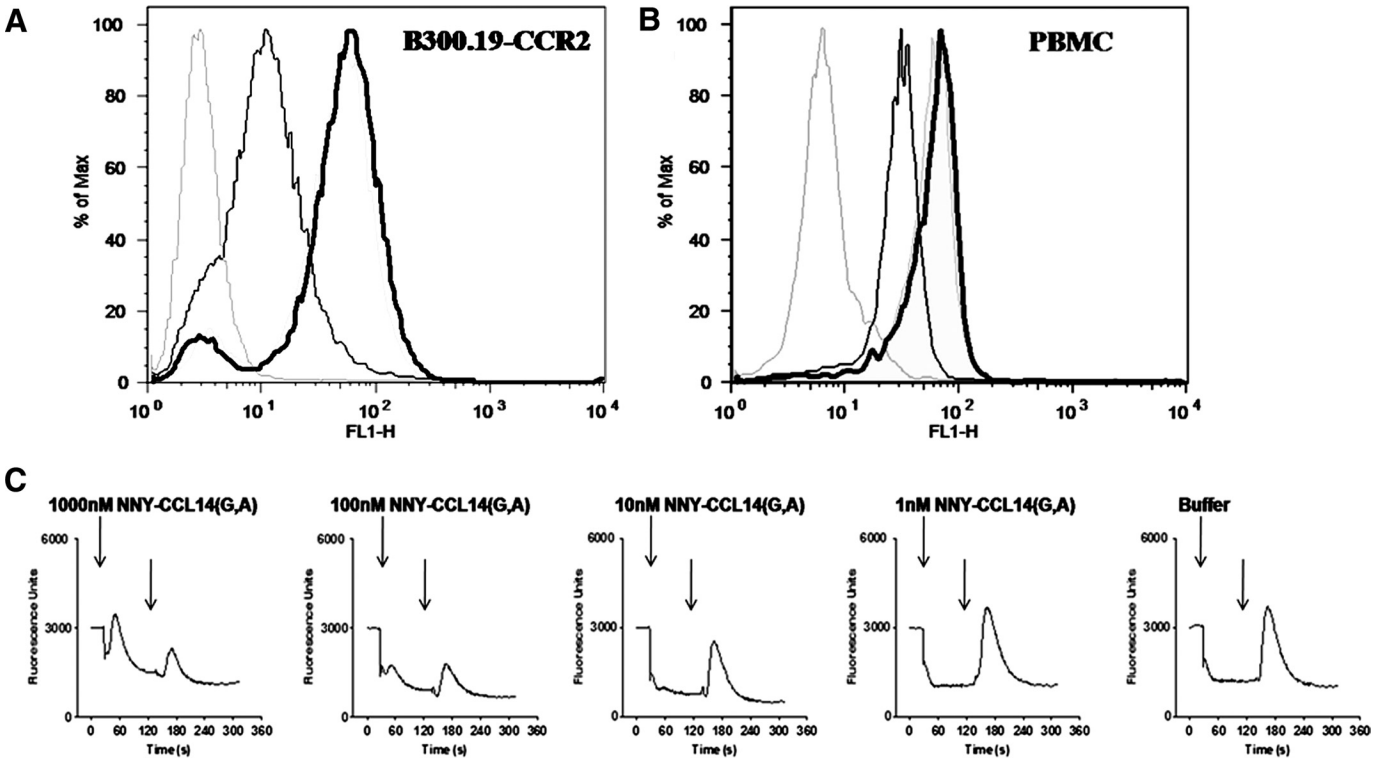

Figure 4. Effects of NNY-CCL14(G,A) treatment on hCCR2. B300.19-CCR2$^+$ cells (A) or purified PBMCs (B) were treated for 30 min at 37°C with 1000 nM NNY-CCL14(G,A) (bold, black line), 100 nM hCCL2 (thin, black line), and medium (shaded, gray region). Thereafter, cells were stained with anti-hCCR2 mAb and were analyzed by flow cytometry. The gray line represents isotype control. (C) Cells were stimulated sequentially at 120 s intervals with varying concentrations of NNY-CCL14(G,A) as indicated (first arrow) and 10 nM CCL2 (second arrow); intracellular calcium fluxes were recorded by FLIPR. The results are representative of three independent experiments.

nM CCL2 (second arrow). Thus, the above studies demonstrated that the altered activity of NNY-CCL14(G,A) on PBMCs can be explained by its loss to activate and ability to internalize CCR2 alongside the observed altered effect on CCR1 and CCR5.

NNY-CCL14(G,A) does not promote the arrest of B300.19-CCR1$^+$ cells on modeled human endothelium

GAGs play a crucial role in arresting leukocytes on vascular endothelium. Chemokines are presented to leukocytes bound to GAGs on the vascular endothelium, which play a crucial role in arresting, rolling, and extravasating leukocytes [33]. The effect of mutating the GAG-binding domain on leukocyte arrest on endothelial cells was studied in flow chamber experiments. Confluent, hTNF-α-activated HUVEC monolayers were preincubated with or without (control) the respective chemokine compounds to allow binding to GAG. After a washout of nonbound chemokines, CCR1- or CCR5-transfected B300.19 cells were subjected to the endothelial monolayer and allowed to adhere to the endothelium for 5 min. Subsequently, the shear stress was set to 2.0 dyn/cm^2. In control experiments, ~10% of CCR1-transfected cells bound to the activated HUVECs. Pretreatment of HUVECs with NNY-CCL14(G,A) did not increase the adhesion of the cells significantly. In contrast, pretreatment of the endothelial monolayer with CCL14(9-74) as well as with NNY-CCL14 induced a significant increase in the adhesion of B300.19-CCR1$^+$ cells to ~30% of the cells subjected initially (**Fig. 5A**).

In comparison with B300.19-CCR1$^+$ cells, CCR5-transfected cells displayed a higher basal adhesion to endothelial monolayers under a shear stress of 2 dyn/cm^2. Pretreatment of HUVEC monolayers with CCL14(9-74), NNY-CCL14, or NNY-CCL14(G,A) did not increase the binding of CCR5-transfected cells significantly (Fig. 5B).

These experiments demonstrate a decreased trapping of B300.19-CCR1$^+$ cells to the modeled endothelium in the NNY-CCL14(G,A)-treated group, validating its decreased binding to GAG compared with its precursor NNY-CCL14.

CCR1 and CCR5 re-expression after removal of NNY-CCL14 or NNY-CCL14(G,A)

The ability of NNY-CCL14 and NNY-CCL14(G,A) to sequester CCR1 and CCR5 after removal of ligand from the extracellular medium was investigated using B300.19-CCR1$^+$ or -CCR5$^+$ cells. To test the ability to inhibit re-expression, the cells were incubated with 100 nM ligand for 30 min at 37°C, washed twice with room temperature PBS, and resuspended in fresh, prewarmed medium at 37°C. The receptor expression was monitored at the indicated time-points by flow cytometry up to

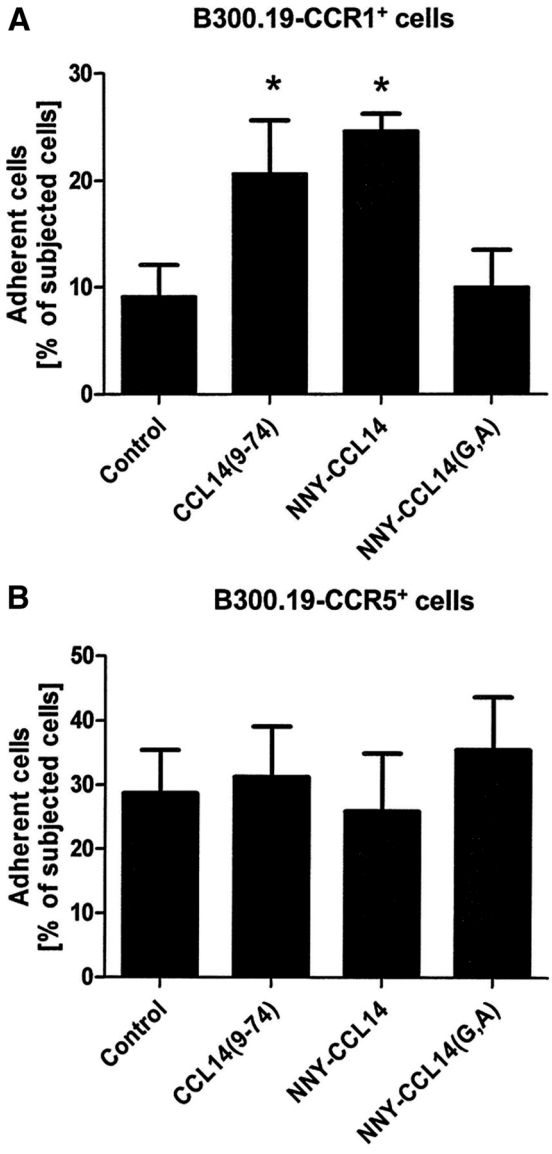

Figure 5. NNY-CCL14(G,A) does not modulate the adhesion of B300.19-CCR1$^+$ and -CCR5$^+$ cells to HUVEC monolayers. In vitro flow chamber experiments with B300.19-CCR1$^+$ (A) and -CCR5$^+$ (B) were performed on hTNF-α-activated HUVEC monolayers. Prior to administration of cells to the endothelium, the monolayer was preincubated with 5 μg/ml CCL14(9-74), NNY-CCL14, or NNY-CCL14(G,A). The yield of cells was documented in high-power fields recorded by photographs, subsequent to the adhesion period of 5 min without flow and subsequent to the shear stress of 2.0 dyn/cm^2. In the figure, the ratio defines yield of cells subsequent to shear stress of 2.0 dyn/cm^2/yield of cells subsequent to the adhesion period. (A) Preincubation of the endothelium with CCL14(9-74) or NNY-CCL14 increased the adhesion of B300.19-CCR1$^+$ cells, which was not induced by pretreatment with NNY-CCL14(G,A). (B) Preincubation of the endothelium with CCL14(9-74), NNY-CCL14, or NNY-CCL14(G,A) did not increase the adhesion of B300.19-CCR5$^+$ cells to hTNF-α-activated HUVEC monolayers; *, $P < 0.05$.

3 h. NNY-CCL14 and NNY-CCL14(G,A) inhibited recycling of hCCR1 during this period (**Fig. 6**). The re-expression rate of CCR5 was faster for NNY-CCL14-treated cells than NNY-CCL14(G,A). For the NNY-CCL14-treated group, surface CCR5 expression increased (t=0, 22.7±2.98; t=180, 52.346±3.33) over 3 h, but NNY-CCL14(G,A) inhibited CCR5 re-expression during this time period (t=0, 44.35±3.86; t=180, 43.167±6.83; Fig. 6).

Therefore, NNY-CCL14(G,A) inhibits recycling of hCCR5 alongside hCCR1 during the time period studied.

NNY-CCL14(G,A) internalizes mCCR5 in vitro and in vivo

Various subsets of murine lymphocytes, namely CD4$^+$ T cells, CD8$^+$ T cells, and DX5$^+$ NK cells, expressing varying levels of CCR5 [24], were analyzed by flow cytometry to study NNY-CCL14(G,A)-mediated in vitro internalization of CCR5 after 30 min at 37°C. Eosinophils were used to study CCR3 in vitro internalization. In comparison with the medium control, 100 nM NNY-CCL14(G,A) internalized surface CCR5 (**Fig. 7A**) from each of these cell types, as visualized by the shift of the cell

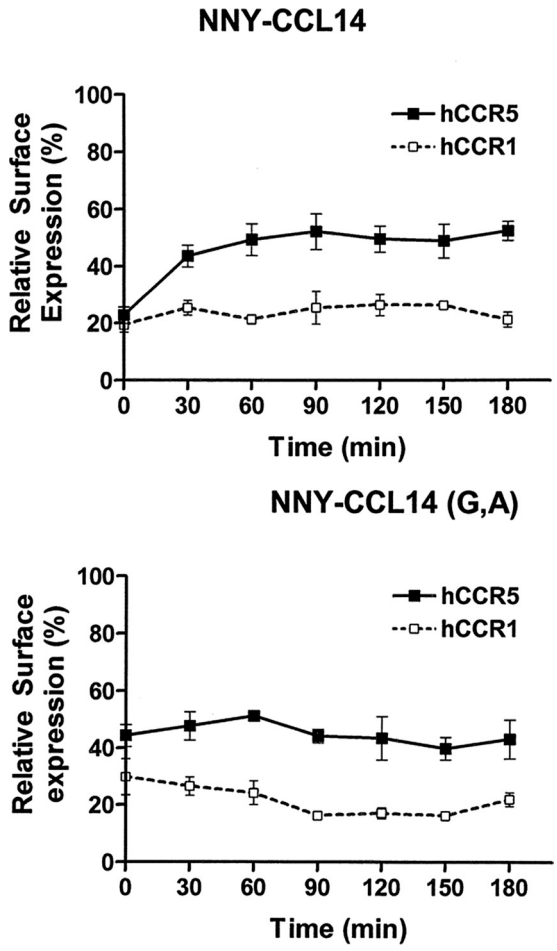

Figure 6. Re-expression kinetics for CCR1 and CCR5. B300.19-CCR1$^+$ or -CCR5$^+$ cells were treated for 30 min at 37°C with 100 nM NNY-CCL14 (A) or NNY-CCL14(G,A) (B). The cells were then washed twice with PBS and resuspended in fresh media at 37°C. Surface expression was analyzed by flow cytometry at the indicated time-points. Data are demonstrated as mean ± SEM relative surface expression; $n = 4$.

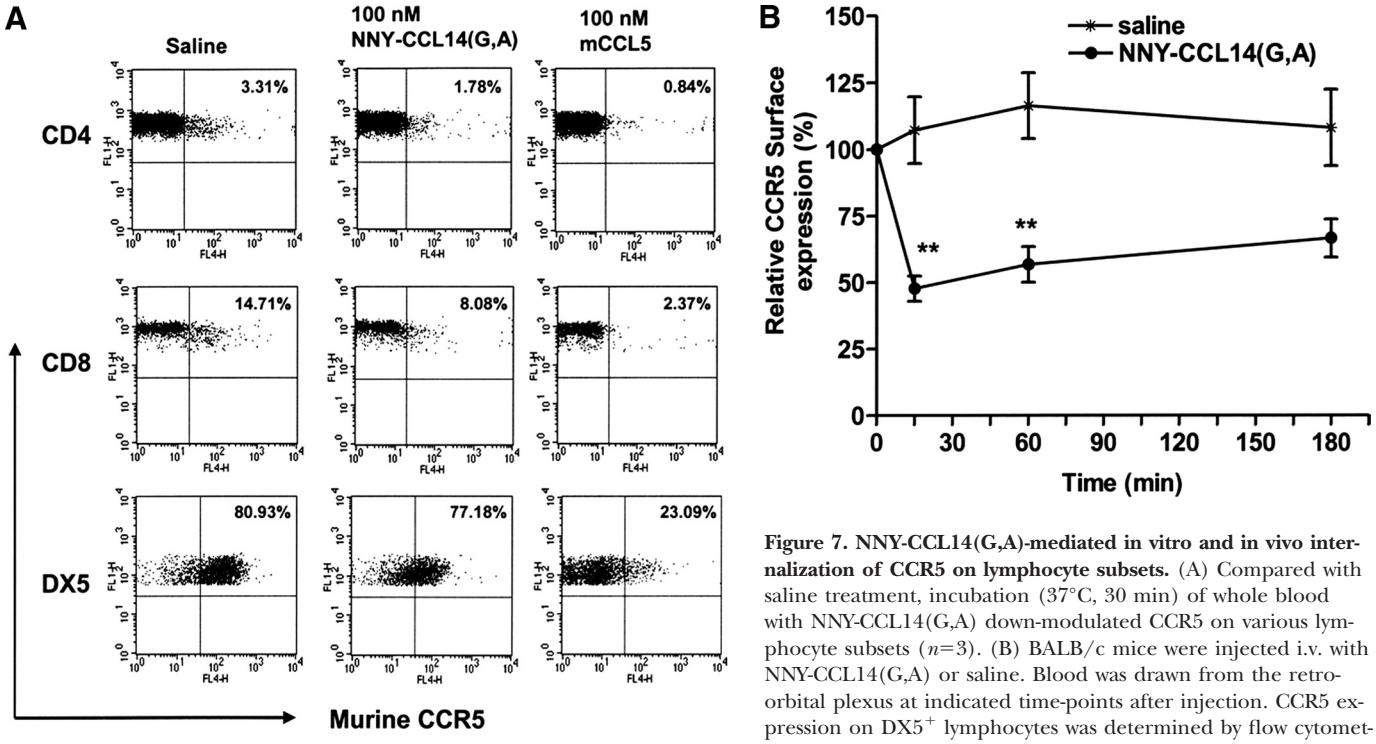

Figure 7. NNY-CCL14(G,A)-mediated in vitro and in vivo internalization of CCR5 on lymphocyte subsets. (A) Compared with saline treatment, incubation (37°C, 30 min) of whole blood with NNY-CCL14(G,A) down-modulated CCR5 on various lymphocyte subsets (n=3). (B) BALB/c mice were injected i.v. with NNY-CCL14(G,A) or saline. Blood was drawn from the retro-orbital plexus at indicated time-points after injection. CCR5 expression on $DX5^+$ lymphocytes was determined by flow cytometric analysis. The graph represents mean ± SEM values for relative percentage expression; $n = 4$; **, $P < 0.01$.

populations. In contrast, NNY-CCL14(G,A), like NNY-CCL14, did not internalize surface CCR3 on murine eosinophils (data not shown). To substantiate the relevance of the effects of NNY-CCL14(G,A) on mCCR5 internalization, we investigated the capacity of NNY-CCL14(G,A) to exert these effects in vivo in the mouse. As it has been demonstrated previously that $DX5^+$ NK cells homogeneously express CCR5 in comparison with other lymphocyte subsets [24], these cells were chosen to demonstrate the ability of NNY-CCL14(G,A) to internalize CCR5 in vivo in mouse blood. The in vivo internalization of mCCR5 and re-expression rate after systemic injection of the inhibitory compound NNY-CCL14(G,A) (100 nmol/kg) were studied. Efficient receptor down-modulation was observed 15 min after systemic treatment, which persisted in the same range for at least 60 min (Fig. 7B). Even after a time period of 180 min, the surface expression of CCR5 detected on $DX5^+$ NK cells was 67% of the level prior to the application of NNY-CCL14(G,A) (Fig. 7B). These experiments suggest the possible in vivo mechanism of receptor inactivation after systemic treatment with NNY-CCL14(G,A).

DISCUSSION

Chemokines play a role in the selective recruitment and activation of leukocytes during routine immunosurveillance and inflammatory conditions. GAGs, on the luminal surface of the blood vessels and the extracellular matrix, sequester chemokines, immobilizing them, and thus, establishing a concentration gradient resulting in a directional signal. Hence, modulation of leukocyte recruitment through intervention with chemokine receptors is an attractive therapeutic strategy [16, 19] for the treatment of inflammatory diseases. The results presented in this report display NNY-CCL14(G,A) as a lead structure for a potential therapy, which exerts its effects via a combination of several mechanisms: Its inability to bind to GAG may allow it to modulate leukocyte functions prior to their interaction with endothelium-bound chemokines expressed under inflammatory conditions; the N-terminal modification decreases its susceptibility to degradation by peptidases, thus increasing the half-life; and it modulates leukocyte functions by desensitizing and internalizing multiple chemokine receptors, making them unavailable for further stimulation with natural chemokines bound to GAG.

The BBXB and BXBXXB motifs have been attributed to GAG-binding in various proteins [34]. In addition, the BBXB motif has been recognized as the principal GAG-binding motif for CXCL12, CCL3, CCL4, CCL5, and CCL7 [6, 7, 30, 31], although for CXCL8 [35] and CCL2 [36], spatially separate residues have been suggested to be responsible for GAG-binding. On scanning the sequence of NNY-CCL14, ^{49}KRGH^{52} was identified as a BBXB motif lying in the β-sheet region of this chemokine derivative. A double mutant of NNY-CCL14 was generated, in which the first two basic amino acids were replaced with glycine and alanine, respectively. These mutations lead to a loss in heparin-binding, and the mutant could be eluted from a heparin column at a much lower salt concentration. The loss of binding affinity to heparin has been shown previously to be dependent on the number of amino acids mu-

tated within the heparin-binding motif, where in the case of CCL7, a triple mutant was the most effective [31].

The potency and efficacy of NNY-CCL14(G,A) were tested on hCCR1, hCCR2, hCCR3, and hCCR5, the principal chemokine receptors, on which the parent compound NNY-CCL14 mediates its effects. The functional studies reveal that NNY-CCL14(G,A) internalizes and desensitizes hCCR1 and hCCR5 as effectively as its parent compound. As suggested by the EC_{50} value, it is twofold less effective in activating hCCR1. A remarkable loss in potency and efficacy was observed for hCCR2 and hCCR3. The ability to internalize hCCR2 and hCCR3 on B300.19-CCR2$^+$/-CCR3$^+$ cells and human PBMCs/eosinophils was lost significantly. Although binding assays were not performed directly, a loss in chemotaxis of eosinophils and monocytes in response to NNY-CCL14(G,A) suggests an altered activation and/or downstream signaling of the receptor.

The perturbations in receptor affinity and activation on mutation in the GAG-binding region have been observed for various chemokine mutants. A profound loss of activity on CCR1 has been depicted for ^{44}AANA^{47}-CCL5, which was most pronounced for this triple mutant, although there was no significant effect on binding and activation of CCR5 [30]. Abrogation of the GAG-binding site in CCL3 also leads to a profound effect on its interaction with CCR1 [37]. Functional studies about the non-GAG-binding CCL7 mutant also showed minor perturbations of its affinity for CCR1, CCR2b, and CCR3 [31]. In contrast to the earlier conventional belief that the receptor-binding and activation regions of chemokines lie in the N-terminal segment, and the GAG-binding region lies in the C-terminal domain, more recent studies mapping these regions have shown possibilities of their overlap. Moreover, mutations in GAG-binding regions have been shown to affect receptor affinity or selectivity [6]. Further investigations to map the binding, activation, and GAG-binding domains of NNY-CCL14(G,A) with respect to CCR1, CCR3, and CCR5 would facilitate a better understanding of the differences in the observed biological properties.

In the flow chamber experiments, a marked decrease in the number of endothelium-bound B300.19-CCR1$^+$ cells was observed when HUVECs were treated with NNY-CCL14(G,A) compared with its precursor compounds. Moreover, the NNY-CCL14(G,A)-treated group demonstrated adhesion properties, such as the controls (untreated group), thus demonstrating the inability of NNY-CCL14(G,A) to bind to GAGs. The arrest of B300.19-CCR5$^+$ cells was not affected by NNY-CCL14(G,A) treatment. Previous studies have shown that shear-resistant arrest of monocytes and T_H1-like T cell clones is mediated by CCR1, and CCR5 participates in the spread and transmigration, thus demonstrating distinct functions of CCR1 and CCR5 during leukocyte recruitment [38, 39]. These findings suggest further that NNY-CCL14(G,A) might inhibit leukocyte migration by inhibiting arrest to the endothelium mediated via CCR1. Moreover, it might counteract further spread and transmigration mediated via CCR5, thus suggesting another of the many properties of NNY-CCL14(G,A).

In the in vitro re-expression studies, unlike the NNY-CCL14 treatment, hCCR5 did not recycle after removal of NNY-CCL14(G,A) over a period of 3 h. Recycling of hCCR1 was similarly inhibited after removal of NNY-CCL14(G,A) or NNY-CCL14. Similar properties have been demonstrated for CCL5 and its derivatives, i.e., inhibition of the recycling of CCR1 after ligand removal [27, 38]. Moreover, NNY-CCL14(G,A) inhibits hCCR5 recycling alongside hCCR1. The nonsignificant difference (P=0.2494), observed in the surface expression of CCR5 at t = 0 on NNY-CCL14(G,A) treatment after washing with room temperature PBS (Fig. 6; 44.353±3.861%), in contrast to the internalization observed after washing with cold PBS (Fig. 2; 30.67±7.149), may be attributed to the re-expression occurring as a result of the washing at room temperature. NNY-CCL14(G,A) internalized cell surface CCR5 on various murine blood lymphocyte subsets as effectively as NNY-CCL14 [18]. In the in vivo re-expression study performed in mice, a gradual re-expression of mCCR5 was observed over a period of 3 h, and the mean surface expression after 3 h was still 67% compared with baseline. Although NNY-CCL14(G,A) is slightly less potent than NNY-CCL14 [18] in effecting the internalization of CCR5 at t = 15 in vivo, the re-expression was faster for NNY-CCL14 compared with NNY-CCL14(G,A). Thus, i.v. injections demonstrate the in vivo ability of NNY-CCL14(G,A) to down-regulate CCR5, exhibiting a part of its complex mechanism of action. NNY-CCL14(G,A), like NNY-CCL14 [18], does not internalize CCR3 (data not shown). In a previous study, the ability of NNY-CCL14 to decrease AHR and inhibit the migration of inflammatory cells to the lung in an OVA-dependent murine model of allergic inflammation has been attributed to its interaction with CCR5 in vivo [18]. Inducing mutations in the GAG-binding region of NNY-CCL14 does not significantly alter its effector properties on CCR5, thus permitting the proposed mechanism of action in vivo.

High specificity and low systemic toxicity suggest peptides as putative, therapeutic candidates; agonistic chemokine derivatives acting via multiple mechanisms of actions have been proposed as potential anti-inflammatory agents [15, 16, 19, 40]. GAG binding is required for the chemokine-driven inflammation. In the present study, we describe NNY-CCL14(G,A), which is a GAG-mutated derivative of NNY-CCL14 described previously. As a result of its loss of binding to GAG, administered NNY-CCL14(G,A) has the potential to disrupt the directional migration of leukocytes to sites of inflammation under the influence of inflammatory chemokines. NNY-CCL14(G,A) is resistant to CD26/dipeptidyl peptidase IV, thus being available to leukocytes in circulation for longer periods and prior to their activation by GAG-bound chemokines expressed under inflammatory conditions. Moreover, NNY-CCL14(G,A) acts as an agonistic inactivator by desensitizing and internalizing CCR1 and CCR5, making them unavailable for further stimulation by natural ligands. Additional studies with experimentation detailed further in the future will help to evaluate the speculation that as a result of the decreased binding to GAGs, this compound can be administered via any other routes to lead to similar therapeutic effects. Thus, NNY-CCL14(G,A) is a potential lead for an anti-inflammatory therapy that exerts its effects on multiple chemokine receptors acting via a combination of several mechanisms.

AUTHORSHIP

S.G., R.R., W.-G.F., J.E., and U.F. contributed to experimental design, project progression, and manuscript writing. S.G., S.R., R.R., S.S.-M., J.M., S.E.S., and A.H. contributed to performing experiments and data analysis. M.M. provided useful reagents for the study.

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