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SMM-Chemokines: A Class of Unnatural Synthetic Molecules as Chemical Probes of Chemokine Receptor Biology and Leads for Therapeutic Development

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Summary

Chemokines and their receptors play important roles in numerous physiological and pathological processes. To develop natural chemokines into receptor probes and inhibitors of pathological processes, the lack of chemokine-receptor selectivity must be overcome. Here, we apply chemical synthesis and the concept of modular modifications to generate unnatural synthetically and modularly modified (SMM)-chemokines that have high receptor selectivity and affinity, and reduced toxicity. A proof of the concept was shown by transforming the nonselective viral macrophage inflammatory protein-II into new analogs with enhanced selectivity and potency for CXCR4 or CCR5, two principal coreceptors for human immunodeficiency virus (HIV)-1 entry. These new analogs provided insights into receptor binding and signaling mechanisms and acted as potent HIV-1 inhibitors. These results support the concept of SMM-chemokines for studying and controlling the function of other chemokine receptors.

Introduction

Chemokine receptors belong to the superfamily of G protein-coupled receptors (GPCRs). As the natural ligands of chemokine receptors, chemokines can be divided into four subfamilies based on the positions of two conserved cysteine residues in their amino (N)-termini: CC, CXC, CX3C, and C. Chemokines act as chemoattractants of various types of leukocytes to sites of inflammation and to secondary lymphoid organs. Chemokines and their receptors are also involved in neurological disorders, cancer, and, most notably, acquired immune deficiency syndrome (AIDS) [1–4]. Two principal coreceptors, CXCR4 and CCR5, are required for human immunodeficiency virus (HIV)-1 entry [5–8]. During the asymptomatic stage of disease, M-tropic strains of HIV-1 primarily use CCR5 as an entry coreceptor [9–11]. However, in 40%–50% of HIV-1-infected individuals, T-tropic strains that predominantly use CXCR4 eventually replace M-tropic strains, leading to rapid disease progression [12–14]. Natural chemokines of CXCR4 or CCR5 can inhibit HIV-1 infection [15, 16] by blocking gp120 binding sites on CXCR4 or CCR5 [17, 18] and/or by inducing receptor internalization [19, 20].

Despite many important roles of chemokines and their receptors in numerous physiological and pathological processes, a challenge to the field of chemokine biology is the search for specific inhibitors of the chemokine system, given that 50 chemokine ligands and 19 functional receptors have been described to date [4]. The lack of selectivity among chemokine ligands is exemplified by viral macrophage inflammatory protein (vMIP)-II, which recognizes a variety of CC and CXC chemokine receptors, including CXCR4, CCR5, and CCR2 [21]. Although the potential benefits of chemokine receptor inhibitors for AIDS and other diseases have been demonstrated, the lack of selectivity in the chemokine system has made it problematic to use natural chemokines or nonspecific, synthetic inhibitors in clinical applications due to their potential side effects. For instance, there is cause for concern regarding undesired side effects of blocking the normal CXCR4 function, since knockout mice lacking either CXCR4 [22, 23] or its only natural ligand, stromal cell-derived factor (SDF)-1α [24], die during embryogenesis, with evidence of hematopoietic, cardiac, vascular, and cerebellar defects. Consequently, the development of new inhibitors engineered with higher selectivity for specific regions of CXCR4 that are selective for HIV-1 coreceptor function only, but not the normal function of SDF-1α, is clearly desirable. In fact, we have recently reported potentially different determinants for CXCR4 interactions with HIV-1 gp120 and SDF-1α, which provided a basis for the development of new inhibitory agents that modulate the functional sites or conformations of CXCR4 for the purpose of reducing or avoiding the limitations and side effects caused by nonselective inhibitors of this important coreceptor [25].

There has been intensive work in the development of new chemokine analogs by other groups in the field. For instance, Clark-Lewis and his colleagues have extensively studied the structure-function relationship of interleukin 8 and other chemokine receptors using chemically synthesized chemokine analogs [26–28]. On the basis of these previous works by others, we have

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been working toward the development of a systematic strategy based on chemokine structures to synthesize a new family of unnatural chemokines that, unlike natural chemokines, have higher receptor binding selectivity. Here, we report our recent progress in developing such a strategy by employing synthetically and modularly modified (SMM)-chemokines as a potential method for the de novo design of novel ligands selective for any chemokine receptor of interest. For this approach, the word "synthetically" refers to the use of total chemical synthesis to incorporate an almost unlimited range of unnatural amino acids and chemical modifications at any specific site(s). The word "modularly" refers to the changes of short, important sequence modules, rather than the entire sequences, to achieve efficiency and be cost-effective in the generation of specificity and diversity. The word "modified" refers to the ability through various chemical modifications to improve biological properties (e.g., low toxicity and high receptor selectivity and affinity) that are more desirable in clinical applications and basic research of receptor biology. We show how this SMM-chemokine approach can be applied to convert the nonselective vMIP-II into highly selective ligands for CXCR4 or CCR5 with desirable binding, signaling, antiviral, internalization, and toxicity profiles. The general implications of such a chemical biology approach for studying chemokine receptor biology and addressing critical medical issues, such as the drug resistance in AIDS treatment, are discussed.

Results

General Design Concept and Chemical Synthesis of SMM-Chemokines Based on vMIP-II

We wanted to demonstrate the concept and feasibility of SMM-chemokine strategy in addressing the issue of lack of selectivity in natural chemokine-receptor interactions and generating unnatural ligands with designed receptor selectivity and improved pharmacological profiles. vMIP-II, designated here as RCP111, was chosen as the target molecule, as it binds a wide range of chemokine receptors including CXC and CC receptor subfamilies [21]. We focused on CXCR4 and CCR5 as two representative receptors in CXC and CC subfamilies, respectively, for the reason that these two receptors are the principal coreceptors in HIV-1 entry. As an additional control, CCR2 was used to test the specificity of our vMIP-II-derived SMM-chemokines. Our goal was to apply the SMM-chemokine approach to overcome the nonselectivity of vMIP-II and generate highly specific analogs of CXCR4 or CCR5.

The structures of several chemokines that bind CXCR4 or CCR5 have been determined by NMR or X-ray techniques, including those of MIP-1β [29], regulated on activation, normal T cell expressed and secreted (RANTES) [30], SDF-1α [27, 31], and vMIP-II [32, 33]. Based on these structures, the structure and function relationship of these chemokines has been investigated mainly by a chemical approach of synthetic chemokine mutants and peptides. For instance, two groups showed, by using synthetic peptides, that the N terminus of SDF-1α is important for CXCR4 binding and signaling [27, 34]. By synthesizing a series of peptides derived from different regions of the full-length sequence, we also found that the N terminus of vMIP-II is the major determinant for CXCR4 binding [35–37]. Finally, the importance of the N terminus was demonstrated by our synthetic N terminus-truncated vMIP-II (RCP112) that had the deletion of residues 1–10 and showed a significant loss in its binding activity to CXCR4, CCR5, and CCR2 (Tables 1 and 2).

Because the N terminus is a key determinant of receptor binding affinity, selectivity, and signaling activity for many chemokines, including vMIP-II [27, 28, 34, 35, 37–40], we modified the N-terminal (1–10) sequence module of vMIP-II by introducing unnatural D-amino acids or specific sequences grafted from other chemokines to achieve our goal of overcoming the nonselectivity of vMIP-II and generating specific analogs of CXCR4 or CCR5 (Figure 1A). We designed a panel of SMM-chemokines containing D-amino acids or other sequence modifications at this module and studied their different affinities and selectivity for CXCR4, CCR5, and CCR2. The chemical preparation yielded these molecules in high purity and correct identity, as shown by the HPLC and MALDI-MS data of vMIP-II as a representative example (Figures 1B and 1C). The CD spectroscopy also indicated that SMM-chemokines display similar patterns on the CD spectra to their parent template, vMIP-II, demonstrating that the modifications at the N terminus have little effect on the overall conformation of the vMIP-II core structure (Figure 1D). The conservation of the core structure among the designed SMM-chemokines described here is further demonstrated by the high-resolution crystal structure of D(1–10)-vMIP-II (RCP168, Table 1), which displays a similar conformation in the core template region as that of RCP111 (unpublished data).

Design of SMM-Chemokines that Bind CXCR4 with High Selectivity and Affinity

To convert the nonselective RCP111 into highly selective SMM-chemokines for CXCR4, we synthesized RCP168 (Table 1) containing D-amino acids at its N terminus. The rationale for introducing D-amino acids at the N-terminal (1–10) sequence module was based on our recent discovery that D-peptides derived from this module of vMIP-II display high binding selectivity for CXCR4, but not for CCR5 [37]. Thus, we reasoned that the incorporation of D-amino acids at the N terminus of the full-length molecule might also endow the new analog with enhanced selectivity and affinity for CXCR4. Indeed, the CXCR4 binding activity of RCP168 (IC_{50} = 5 nM) was over four times greater than that of RCP111 (IC_{50} = 22 nM) (Figure 2A; Table 2). As a negative control, RCP112 in which the N-terminal (1–10) residues of vMIP-II were deleted had a drastic loss in CXCR4 binding, as a plateau of nonspecific binding could not be reached even at 2.7 μM RCP112.

To test whether RCP168 is specific against other chemokine receptors, its binding activity was tested in CCR5 competition binding assays (Figure 2B; Table 2). In these assays, the positive control RCP111 showed very high binding activity for CCR5 (IC_{50} = 4 nM), whereas the negative control RCP112 had a loss in CCR5 binding affinity of almost 40-fold compared with that of RCP111. RCP168 was over 10 times less potent than RCP111 in its binding to CCR5, which was in sharp

Table 1. List of SMM-Chemokines, Their Sequences, and Modifications

Analog Designation	Modification Diagrams	Amino Acid Sequences[a]
RCP111	1-10 NT | 11-71 vMIP-II Core	LGASWHRPDKCCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSKDWVKKLMQQLPVTAR
RCP112	11-71 vMIP-II Core	CCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSKDWVKKLMQQLPVTAR
RCP168	D-Amino Acids 1-10 NT | 11-71 vMIP-II Core	*LGASWHRPDK*CCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSKDWVKKLMQQLPVTAR
RCP169	D-Amino Acids 1-8 NT -GG- 11-71 vMIP-II Core	*LGASWHRP***GG**CCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSKDWVKKLMQQLPVTAR
RCP188	MIP-1β 1-10 NT | 11-71 vMIP-II Core	**APMGSDPPTA**CCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSKDWVKKLMQQLPVTAR
RCP189	D-MIP-1β 1-10 NT | 11-71 vMIP-II Core	***APMGSDPPTA***CCLGYQKRPLPQVLLSSWYPTSQLCSKPGVIFLTKRGRQVCADKSKDWVKKLMQQLPVTAR

[a] D-amino acids are shown in italic, while inserted (GG) or substituted residues (from MIP-1β) are shown in bold.

Table 2. Binding Affinity and Selectivity of SMM-Chemokines for CXCR4, CCR, and CCR2

Analog	CXCR4 Binding (nM)	CCR5 Binding (nM)	CCR2 Binding (nM)
RCP111	22	4	33
RCP112	>2700	146	104
RCP168	5	43	513
RCP169	141	>2700	>2700
RCP188	>2700	6	107
RCP189	>2700	106	>2700

The binding activity of each SMM-chemokine is shown by its IC_{50} value, determined by competition receptor binding assays using either labeled chemokines or receptor-specific antibodies.

contrast to the significant CXCR4 binding enhancement of RCP168. The decrease (10-fold) in CCR5 binding of RCP168 coupled with its increase (4.4-fold) in CXCR4 binding led to a drastically improved selectivity of RCP168 for CXCR4. The selectivity of RCP168 was further demonstrated by CCR2 competition binding assays (Figure 2C; Table 2). Again, the positive control RCP111 bound CCR2 with high affinity (IC_{50} = 33 nM), which is in agreement with the other previous results [41, 42]. However, as observed in the CCR5 binding assays, RCP168 showed about a 16-fold decrease in CCR2 binding (IC_{50} = 513 nM) compared with RCP111. These results show that RCP168 is a selective and high-affinity ligand of CXCR4.

Another CXCR4-selective ligand that differed slightly from RCP168 was D(1–8)-GG-vMIP-II (RCP169). The major difference between RCP169 and RCP168 was that, in RCP169, two glycine residues were incorporated to replace the residues 9 and 10 of RCP168, with the aim of allowing more structural flexibility for the first eight D-amino acid residues. Interestingly, RCP169 completely lost its binding activity to CCR5 and CCR2, and yet retained much of its binding affinity for CXCR4 (Figure 2; Table 2). Residues 9 and 10 may play a more important role in binding CCR5 and CCR2, as the mutations of these residues to glycine residues led to almost complete loss of CCR5 and CCR2 binding. Alternatively, the conformational flexibility presumably introduced by the glycine residues may be more disruptive for the interactions with CCR5 and CCR2 than for those with CXCR4. In either case, the data on RCP169 support our design notion that D-amino acid modifications at the N-terminal module can generate highly selective analogs for CXCR4.

Design of SMM-Chemokines that Bind CCR5 with High Selectivity and Affinity

Following the development of highly selective CXCR4 ligands, we set out to design CCR5-specific SMM-chemokines. However, we did not use the D-amino acid modification strategy because it did not enhance CCR5 binding affinity. Instead, we decided to replace the N terminus of vMIP-II with that from another chemokine known to bind CCR5 but not CXCR4 and/or other receptors, with the aim that such a "foreign N terminus" might import the desired differentiating ability in receptor binding (Table 1). For this reason, the N terminus (residues 1–10) of MIP-1β, a CC subfamily chemokine that is

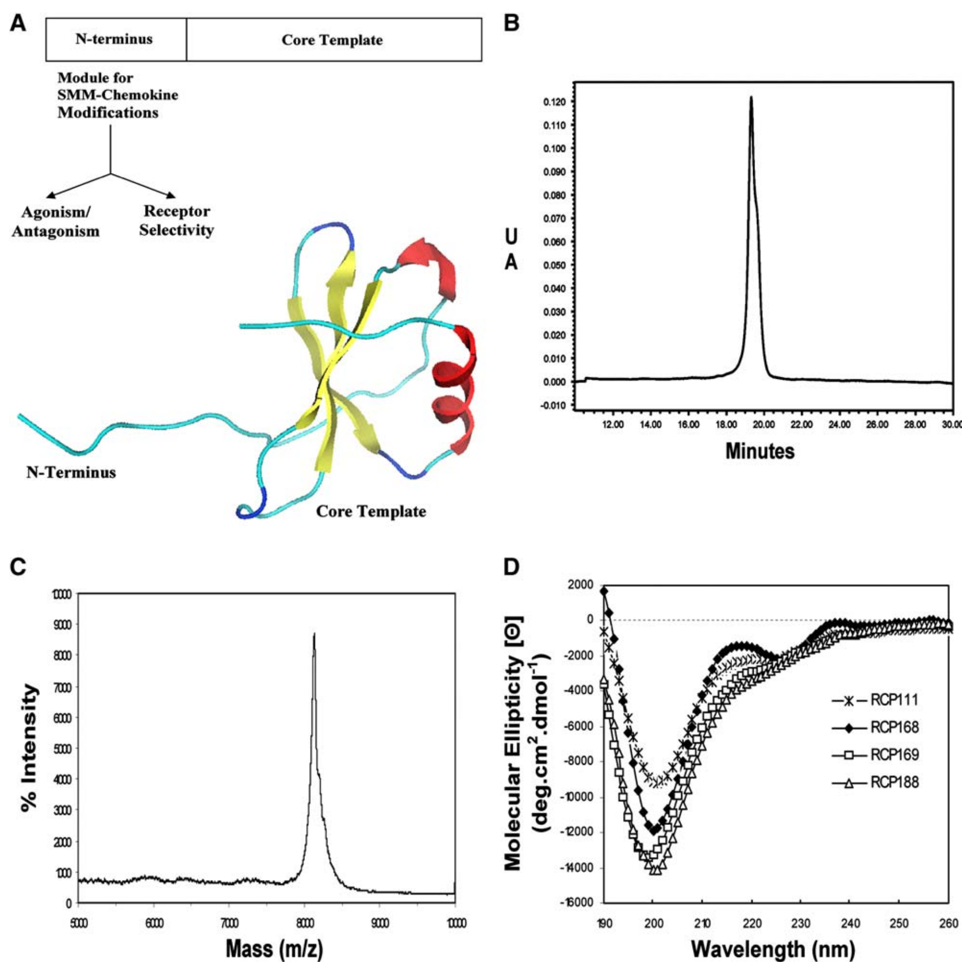

Figure 1. General Design Concept and Chemical Synthesis of SMM-Chemokines

(A) The N-terminal (1–10) sequence module of vMIP-II was modified by introducing unnatural D-amino acids or specific sequences grafted from other chemokines to chemically engineer receptor selectivity and/or signaling property.

(B and C) The HPLC (B) and MALDI-MS (C) of vMIP-II prepared by total chemical synthesis are shown as a representative example.

(D) SMM-chemokines and vMIP-II have similar patterns on the CD spectra.

relatively specific for CCR5 [43], was grafted to replace the N terminus of vMIP-II. The resulting synthetic molecule, (1–10)-MIP-1β-(11–71)-vMIP-II (RCP188), showed very strong binding activity to CCR5 (IC_{50} = 6 nM), but its binding affinity was much weaker in binding CCR2 (by 18-fold) and totally inactive in binding CXCR4 (Figure 2; Table 2). These results demonstrate the selectivity of RCP188 for CCR5.

Signaling Activities of SMM-Chemokines

We conducted biochemical studies on the signaling activities of CXCR4- or CCR5-specific SMM-chemokines.

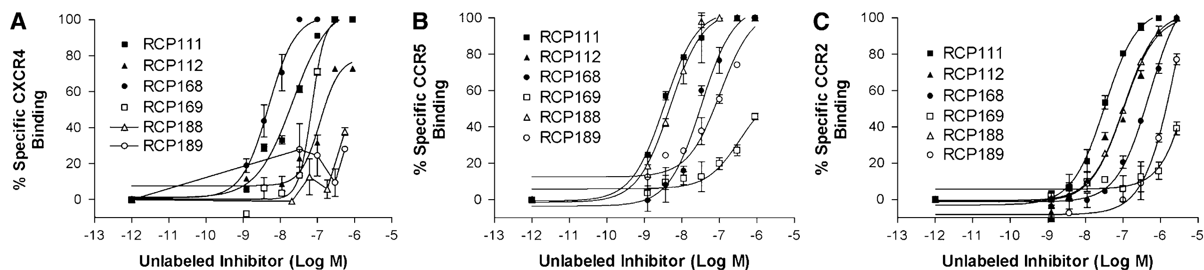

Figure 2. Binding Activities of SMM-Chemokines for CXCR4, CCR5, and CCR2

(A) ^{125}I-SDF-1α competition binding assays.

(B) ^{125}I-MIP-1β competition binding assays.

(C) α-CCR2 competition binding assays.

All data are shown as mean ± SD from at least three independent experiments.

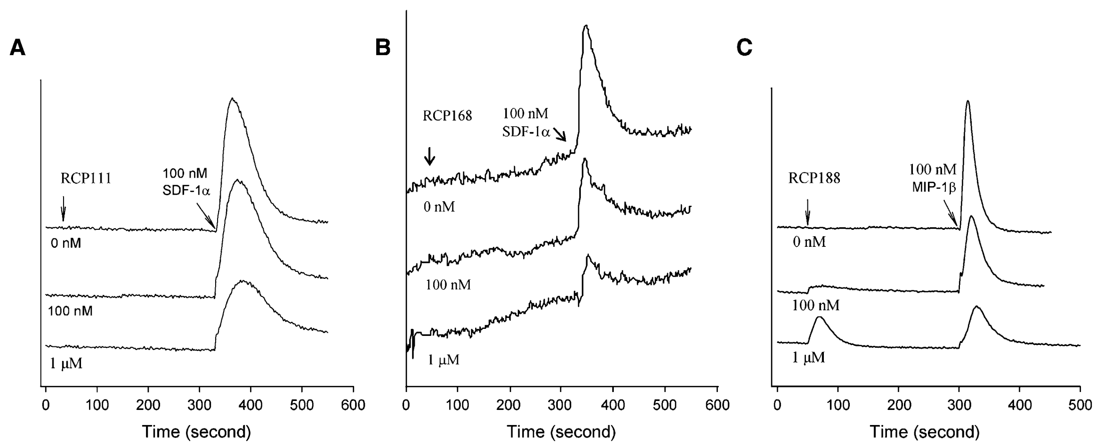

Figure 3. Signaling Activities of SMM-Chemokines

(A) Ca^{2+} influx in Sup T1 cells was measured in response to 0 nM, 100 nM, and 1 μM RCP111, which was followed by adding 100 nM SDF-1α.
(B) The changes in $[Ca^{2+}]_i$ of Sup T1 cells in response to RCP168 were measured. After 5 min of incubation, 100 nM SDF-1α was added.
(C) The Ca^{2+} signals induced by RCP188 in CCR5-transfected 293 cells were followed by 100 nM MIP-1β.
All the data shown are representative of at least three independent experiments.

RCP111 at 100 nM or up to 1 μM did not induce any mobilization of calcium (Ca^{2+}) in Sup T1 cells expressing CXCR4, which is in contrast to the rapid Ca^{2+} mobilization induced by the normal CXCR4 ligand, SDF-1α (Figure 3A). Whether RCP111 may interfere with the normal Ca^{2+} signaling activated by SDF-1α was also examined by adding 100 nM SDF-1α after treating Sup T1 cells with RCP111. RCP111, despite its strong binding to CXCR4, did not interfere with the Ca^{2+} mobilization induced by SDF-1α, even at 100 nM, and only showed a weak inhibitory effect when its concentration was increased to 1 μM. Similarly, RCP168, which binds CXCR4 with very high affinity, did not induce Ca^{2+} mobilization at either 100 nM or even 1 μM. Moreover, it did not interfere with the Ca^{2+} influx induced by SDF-1α until at much higher concentrations, either 100 nM or 1 μM (Figure 3B). As for the CCR5-specific ligand, RCP188, the incorporation of the N terminus from MIP-1β, which is a CCR5 agonist, enabled RCP188 to elicit Ca^{2+} release in CCR5-transfected 293 cells at sufficiently high concentrations, such as 1 μM (Figure 3C). At 1 μM, RCP188 also interfered with the Ca^{2+} mobilization induced by 100 nM MIP-1β. In control experiments, RCP188 neither triggered Ca^{2+} release nor interfered with SDF-1α signaling in Sup T1 cells (data not shown), which is consistent with the binding data showing that RCP188 is a selective CCR5 ligand.

Potent Anti-HIV Activities of SMM-Chemokines

We performed antiviral experiments to investigate whether SMM-chemokines, such as RCP168, with enhanced receptor binding affinity can be used as potential intervening agents for diseases, such as HIV-1 entry and infection mediated by CXCR4. Among the SMM-chemokines listed in Table 1, RCP168 was chosen as the representative compound to be tested for inhibition of HIV-1 infection via CXCR4. As shown in Figure 4A, the antiviral activity of RCP168 was comparable to 3′-azodithymidine or zidovudine (AZT) and nevirapine (NVP), which are nucleoside (NRTI) and nonnucleoside reverse-transcriptase inhibitors (NNRTI), respectively, and commonly used drugs in the clinical treatment of AIDS. Since RCP168 blocks the viral entry pathway that differs from the viral replication step targeted by AZT and many other anti-HIV drugs, it can be expected that molecules like RCP168 may be able to overcome the drug resistance problem encountered by AZT and other anti-HIV drugs in the resistant viral isolates. To test these hypotheses, we assayed RCP168 against several different viral isolates known to be resistant to reverse-transcriptase inhibitors. Whereas RCP168 displayed similar potency to AZT and NVP against a wild-type virus (NL4-3, Figure 4A), reverse-transcriptase inhibitors consistently displayed reduced activity compared with RCP168 against the drug resistant viruses (Figures 4B–4E).

Internalization Activities of CXCR4-Targeting SMM-Chemokines

An important biological activity of SDF-1α is its ability to cause CXCR4 internalization [19, 20], which is suggested to be one mechanism for its anti-HIV activity. In this regard, RCP168 was tested in internalization assays in an effort to examine its mechanism of action. Unlike SDF-1α, which induced 50% receptor loss when its concentration was increased up to 1 μM, RCP168, with concentrations ranging from 0.1 nM to 1 μM, failed to cause CXCR4 downregulation (Figure 5A). The impact of time was also examined. As shown in Figure 5B, neither RCP168 nor RCP111 elicited any significant internalization of CXCR4, even after 90 min of incubation. In contrast, SDF-1α induced rapid receptor downregulation, as 50% of the receptors were internalized after 30 min of incubation. These results suggest that RCP168 inhibits HIV-1 entry through the direct blockade of HIV-1 gp120 binding to CXCR4, rather than causing a decrease in the number of CXCR4 on the cell surface. This feature of RCP168 is desirable, since CXCR4 signal activation and downregulation are considered side effects and should be avoided when using an inhibitor of HIV-1 entry in clinical applications.

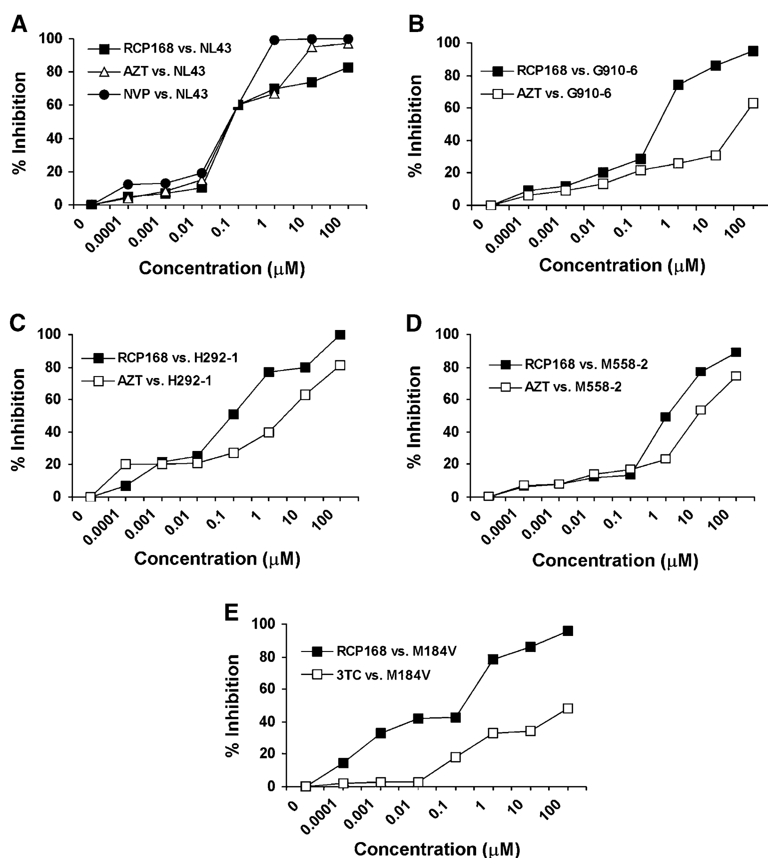

Figure 4. Antiviral Activities of SMM-Chemokines

The antiviral activity of RCP168 was assayed against a panel of HIV-1 strains resistant to approved reverse-transcriptase inhibitors. The strains and their corresponding mutations in reverse transcriptase are: (A) NL 4-3 (wild-type); (B) G910-6 (M41L, D67N, K70R, T215Y, L219G); (C) H292-1 (being sequenced); (D) M558-2 (being sequenced); and (E) S531 (M184).

Toxicities of SMM-Chemokines

In addition to the issue of receptor activation for natural chemokines such as SDF-1α, the cross binding of natural chemokines to receptors other than the targeted one can lead to side effects including toxic effects on uninfected cells. To illustrate this point and demonstrate the advantages of SMM-chemokines over natural chemokines, cytotoxicity assays were performed using CellTiter 96 aqueous nonradioactive cell proliferation assays. The conversion of a tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) into soluble formazan is accomplished by dehydrogenase enzymes found only in metabolically active cells. Thus, the quantity of formazan product (solubilized MTS), as measured by the absorbance at 490 nm, is directly

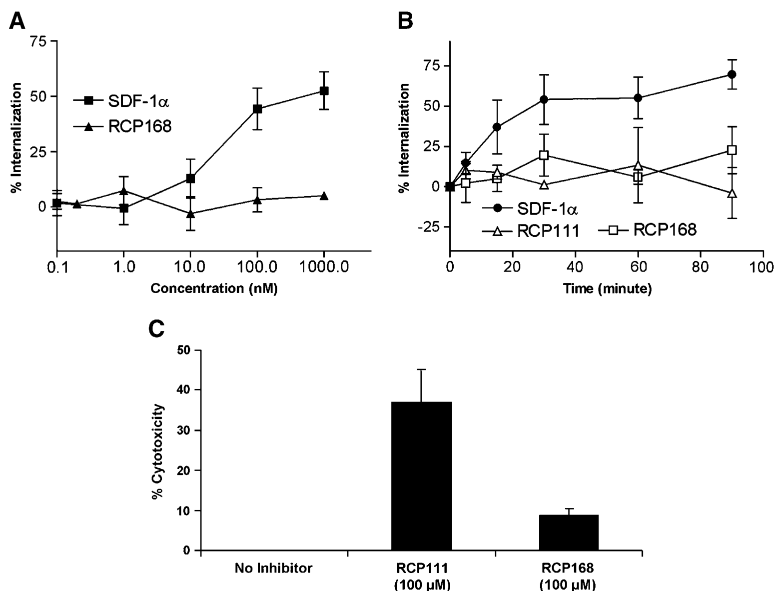

Figure 5. Receptor Internalization Activities and Cytotoxicities of SMM-Chemokines

(A) An increase in the concentration of RCP168 from 0.1 nM to 1 μM failed to cause CXCR4 downregulation, while 1 μM SDF-1α induced 50% receptor loss.

(B) The longer incubation of cells with either RCP168 or RCP111 did not elicit any significant internalization of CXCR4.

(C) The toxicity of 100 μM RCP168 was examined using CellTiter 96 aqueous nonradioactive cell proliferation assays. RCP168 showed no obvious cellular toxicity, whereas the natural chemokine, RCP111, showed a significant cellular toxicity.

All the data shown are representative of at least three independent experiments.

proportional to the number of living cells in culture. The natural chemokine, RCP111, which is known to be nonselective in recognizing many chemokine receptors, showed a significant toxic effect on white blood cells (WBCs), with 40% cell death after 24 hr incubation (Figure 5C). In contrast, RCP168, which is chemically engineered with high receptor selectivity, showed little effect on the cell viability even at 100 μM, a much higher concentration than is required for the inhibition of HIV-1. These data support the notion that SMM-chemokines like RCP168 may have fewer side effects and be more advantageous than the natural chemokines for potential clinical applications.

Discussion

Chemokine ligands and receptors are hotly investigated areas in biomedical research. The dissection of the biological roles of specific ligands and receptors has been challenging because of the lack of selectivity in chemokine ligand-receptor interactions [4]. This also greatly limits the direct applications of natural chemokines in the treatment of various diseases, such as HIV-1 infection. To overcome the limitation of natural chemokines, various approaches have been taken to develop synthetic chemokine analogs that have higher efficacy and improved properties, such as the synthetic modifications of various chemokines conducted in a number of elegant works by Clark-Lewis and colleagues [26–28], and the recent success in the application of medicinal chemistry to the generation of RANTES analogs reported by Offord and colleagues [39]. Here, we demonstrated a chemical biology approach to engineer de novo selective properties into natural chemokines. The effectiveness of this approach was illustrated by the successful conversion of vMIP-II, the most nonspecific and cross-reactive chemokine ligand known to date, into highly specific ligands of CXCR4 or CCR5 through modifying only a small N-terminal module of 10 residues. Two representative SMM-chemokines, RCP168 and RCP188, selective for CXCR4 and CCR5, respectively, showed similar or significantly enhanced binding affinities for their corresponding target receptors but drastically decreased or even completely abolished cross binding activities for other receptors (Table 2).

In addition to high receptor selectivity, another important biological property of these de novo designed ligands is signaling activity. As shown in Figures 3B and 5A–5B, RCP168 did not trigger either Ca^{2+} signaling or receptor internalization, which is distinct from the natural ligand of CXCR4, SDF-1α. More interestingly, RCP168 did not interfere with the Ca^{2+} signaling induced by SDF-1α at its effective CXCR4 binding concentration (5 nM), and only showed its effect at over 20-times higher concentrations, such as 100 nM or 1 μM. We also found that RCP168 potently inhibited HIV-1 entry, in contrast to its much weaker activity in interfering with SDF-1α signaling. These disparate inhibitory activity profiles of RCP168 in differentiating HIV-1 coreceptor function versus the normal function of CXCR4 suggest that this chemically engineered molecule has an interesting and unique receptor binding mechanism, distinct from that of SDF-1α, and may be used to selectively disrupt the coreceptor activity of CXCR4. Furthermore, the disparate activities of RCP168 may prove to be advantageous in clinical applications, as RCP168 may not induce unwanted Ca^{2+} signaling or interfere with SDF-1α signaling important for the normal physiological functions at the concentrations used for inhibiting HIV-1 infection. In fact, the mechanistic basis for the disparate activities of RCP168 was recently investigated and shown by our mutational mapping analysis of binding sites of RCP168 and other D-amino acid-containing SMM-chemokines on CXCR4, revealing that RCP168 binding sites on CXCR4 overlap significantly with HIV-1, but differ from SDF-1α [44].

As for the CCR5-specific SMM-chemokine, RCP188, with its grafting of the N-terminal module of a CCR5 agonist, MIP-1β, was able to mimic the signaling activity of MIP-1β in activating Ca^{2+} release in CCR5-expressing 293 cells. This demonstrates that CCR5-selective binding and signaling activities are encoded in the imported 10 residue sequence from MIP-1β. In subsequent studies, we found that additional modifications on RCP188 or RCP168 can switch their signaling abilities from agonists to antagonists or vice versa (unpublished data). As such, we are able to apply the concept of SMM-chemokines to generate specific agonists and antagonists for either CCR5 or CXCR4, thus demonstrating that both receptor selectivity and signaling property can be designed at will into our synthetic ligands. While antagonists are often used for therapeutic applications, agonists can also be of great interest as the molecular probes to study a particular receptor-mediated signaling pathway.

The results from the first generation of SMM-chemokines reported here seem remarkable and promising, considering that only the N-terminal 10 residue module was modified, yet a significant effect on binding or signaling selectivity was achieved. This illustrates the simplicity and robustness of this method and, at the same time, raises the possibility of its continued refinement and sophistication. We note that, whereas the deletion (as in RCP112) or modifications (as in RCP188 or RCP189) of the N-terminal 10 residue module can completely abolish CXCR4 binding, the same molecules retain residual, although significantly attenuated, binding activity for CCR5 and CCR2. This seems to imply that, unlike CXCR4, other chemokine receptors, or at least some receptors in the CCR subfamily, possess additional, secondary functional modules, such as the N-loop and/or the 30′ loop, for interacting with their receptors [45, 46]. In this regard, we are extending the present work to include these potential functional modules in our design and modification scheme. It may be expected that continued development and optimization of the SMM-chemokine strategy could achieve further improved selectivity and biological profiles.

Specific SMM-chemokines for a designated chemokine receptor can be potentially used in many areas of basic research and therapeutic development. For instance, one can use these SMM-chemokines as highly specific molecular probes to study the biology of chemokine receptors at the cellular level (i.e., to characterize specific biological roles of a receptor in a physiological or pathological process out of the complicated and potentially overlapping receptor network). Also the mechanism of receptor-ligand interactions at the

molecular or atomic level can be investigated by making various artificial changes in ligand probes and testing how the receptor responds. This was demonstrated in another of our studies [44], in which the SMM-chemokines as reported here were used as chemical biology probes to discover new functional sites on CXCR4, important for the selective interactions with HIV-1 but not with the normal physiological ligand of CXCR4, SDF-1α, providing a basis for the development of more selective antiviral therapies than the conventional drugs that interrupt both pathological and physiological pathways. As chemokine receptors belong to the superfamily of GPCRs, which represent the largest class of drug targets and yet are still poorly understood in terms of their structure-function relationship and ligand-receptor interactions, the development of chemical biology probes, such as SMM-chemokines, may have general implications for the study of these important membrane receptors. In addition to being valuable research tools, receptor-selective SMM-chemokines are promising leads for developing highly effective pharmaceutics. For clinical applications, SMM-chemokines can be highly desirable and advantageous for their greater selectivity, higher potency, and reduced toxicity compared with their natural counterparts. Most importantly, not only was RCP168 found, in the study of drug-nonresistant HIV-1 strains, to be comparable in efficacy to some of the most well-known and commonly used HIV-1 drugs, such as AZT, which targets HIV-1 reverse transcriptase, but also RCP168, directed at an HIV-1 coreceptor expressed on the host cells was capable of overcoming the drug resistance problem in drug-resistant viral isolates, including AZT- and 3TC-resistant HIV-1 isolates. This highlights the advantage of targeting a nonviral protein over virus-encoded proteins in terms of tackling the drug resistance issue. Since the drug resistance is a major problem in the treatment of not only AIDS but also other viral infections, the results shown in this study may have broader implications for the development of therapeutic strategies for diseases caused by other viruses.

Significance

Chemokines and their receptors are implicated in a wide range of human diseases, including acute respiratory distress syndrome, allergic asthma, psoriasis, arthritis, multiple sclerosis, cancer, atherosclerosis, and, most notably, AIDS. To enable the applications of chemokines as probes of receptor biology and inhibitors of pathological processes, a major problem with the lack of receptor selectivity of natural chemokines must be overcome. Here, we report the use of a chemical biology approach combining total protein synthesis and modular modifications to generate a class of unnatural chemokines, termed SMM-chemokines, that were chemically engineered with high receptor selectivity and affinity, and reduced toxicity. A proof of the concept is shown by applying this chemical strategy to transform vMIP-II, a very nonselective chemokine, into new analogs with significantly enhanced selectivity and potency for CXCR4 or CCR5, two principal coreceptors for HIV-1 entry. In addition to being valuable chemical probes of receptor biology to study ligand binding and signaling mechanisms, these molecules were shown to be promising leads for the development of anti-HIV therapeutics, as they were more potent in blocking HIV-1 entry and infection and less toxic than natural chemokines. In particular, the seemingly disparate inhibitory activity profiles of RCP168 in blocking HIV-1 entry more potently than CXCR4 normal function suggests a potentially interesting receptor binding mechanism of this compound, which might be exploited as a basis for the development of selective HIV-1 inhibitors. Finally, these inhibitory molecules were able to overcome the resistance of HIV-1 strains against some of the most commonly used anti-HIV drugs, demonstrating a strategy with the advantage of targeting a nonviral protein over virus-encoded proteins for tackling the drug resistance issue, which is a major problem in the treatment of AIDS and other viral infections.

Experimental Procedures

Total Chemical Synthesis of SMM-Chemokines

The automated stepwise incorporation of protected amino acids was performed using a 433A peptide synthesizer (Applied Biosystems, Foster City, CA) with a CLEAR amide resin (Peptides International, Louisville, KY) as the solid support. Fmoc-chemistry was employed for the synthesis. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and N-hydroxybenzotriazole (HOBt) were used as coupling reagents in the presence of diisopropylethylamine (DIEA). In certain coupling steps with potentially slow reaction rates, double coupling followed by capping of the unreacted amino functional groups was performed. After incorporation of the 50th residue, 2% v/v of DMSO was introduced to the solution to enhance the coupling reaction. After removing N-terminal Fmoc protection, the protein was cleaved from the resin support by adding a cleavage cocktail comprised of phenol (4% w/v), thioanisole (5% v/v), water (5% v/v), ethanedithiol (2.5% v/v), triisopropylsilane (1.5% v/v), and trifluoroaceticacid (TFA, 82% v/v). The protein was precipitated by adding ice-cold *tert*-butyl methyl ether and washed repeatedly in cold ether. The crude protein was dissolved in 25% CH_3CN in water containing 0.1% TFA before being lyophilized, and it was then dissolved in water and purified using semipreparative reverse-phase-high performance liquid chromatograph (RP-HPLC). Folding of the purified protein was performed in 1 M guanidinium hydrochloride and 0.1 M trisma base at pH 8.5 (1 mg protein/ml folding buffer), and was monitored by analytical RP-HPLC using a Vydac C-18 column (0.46 $\times$ 15 cm, 5 μm) with a flow rate of 1 ml/min. Solvents used were: A, water with 0.1% TFA; and B, 20% water in CH_3CN with 0.1% TFA; and a linear gradient 30%–70% B over 30 min. Protein desaltation and purification were then performed. The purified protein was characterized by MALDI-TOF MS.

CD Spectroscopy

The spectra were recorded on the JASCO JA-710 spectropolarimeter over the range of 190–300 nm at room temperature with a 1.0 cm pathway length quartz cuvette. The scan speed was 50 nm/s, with the resolution of 1.0 nm. The spectra were signal-averaged four times. The solvent baseline was subtracted from the spectra. The spectra deconvolution was performed using the CDNN program [47].

Materials

The radioiodinated SDF-1α and MIP-1β were purchased from Perkin-Elmer Life Sciences (Boston, MA). Plasmid pcDNA3-CXCR4, plasmid pcDNA3-CCR5, antibodies 12G5 and 2D7, and human kidney cell line 293 were obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, National Institutes of Health, Bethesda, MD). Plasmid pcDNA3-CCR2 was a kind gift from Dr. Israel Charo from the Gladstone Institute (University of California, San Francisco). Anti-human CCR2 antibody (α-CCR2) was

purchased from R&D system (Minneapolis, MN). Sup T1 cell line was obtained through the European Collection of Cell Cultures (ECACC, St. Louis, MO). Cell culture media and G418 were purchased from CAMBREX (Walkersville, MD). While Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS) and 5% penicillin-streptomycin (P/S) was used to maintain 293 cells, RPMI 1640 plus 10% FBS and 5% P/S was used to culture Sup T1 cells.

Transfection of Adherent 293 Cells

Wild-type CXCR4, CCR5, or CCR2 were transfected into 293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The selective medium containing G418 (800 μg/ml) was used to isolate stably transfected cells that were subsequently cloned from a single colony.

Flow Cytometry

Transfected 293 cells (5×10^5 cells/well) were washed with FACS buffer (0.5% bovine serum albumin [BSA], 0.05% sodium azide in PBS) and incubated with monoclonal antibody (MAb) 12G5, 2D7, or α-CCR2 (10 μg/ml) for 30 min at 4°C. After washing with FACS buffer, the cells were incubated with 10 μg of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma, St. Louis, MO) for 30 min at 4°C. The cells were washed with FACS buffer and fixed in the fixing buffer (2% paraformaldehyde in PBS) for 30 min at 4°C before being analyzed on the FACScan flow cytometer.

Competition Receptor Binding Assays Using Labeled Chemokines

Ligand binding experiments were performed using a single concentration (0.2 nM) of ^{125}I-SDF-1α or ^{125}I-MIP-1β in a final volume of 100 μl binding buffer (50 mM Hepes [pH 7.4], 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.1% BSA) containing 5×10^5 cells in 96-well plates in the presence of various concentrations of unlabeled chemokines. Nonspecific binding was determined by adding 150 nM unlabeled SDF-1α or 100 nM unlabeled vMIP-II. Samples were incubated for 60 min at room temperature. The cells were washed with 200 μl binding buffer. Bound ligands were determined by counting γ emissions. The binding data were analyzed using the PRISM program (GraphPad Inc., San Diego, CA).

Competition Receptor Binding Assays with Receptor-Specific Antibodies

Competition binding experiments were performed with a single concentration of 12G5 (50 ng/ml) or α-CCR2 (0.25 μg/ml) in a final volume of 100 μl FACS buffer containing 5×10^5 cells in 96-well plates in the presence of various concentrations of unlabeled chemokines. Samples were incubated on ice for 40 min. The cells were washed with 200 μl FACS buffer and stained with 10 μg FITC-conjugated goat anti-mouse IgG for 30 min at 4°C. As a negative control, cells were stained only with the secondary antibody. The cells were washed with FACS buffer and resuspended in 100 μl FACS buffer before being analyzed on the Wallac Victor^2 1420 Multilabel counter (Turku, Finland).

Intracellular Calcium Measurements

Sup T1 cells (10^7 cells/ml) were loaded with 2 μM fura-2/AM (Molecular Probes, Eugene, OR) and 0.01% Pluronic F-127 (Sigma) in Hank's balanced salt saline (140 mM NaCl, 5 mM KCl, 10 mM Hepes [pH 7.4], 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mg/ml glucose, and 0.025% BSA) for 20 min at room temperature. The cells were washed and resuspended in the same buffer to 10^6 cells/ml. Fura-2 fluorescence was measured on the fluorescence spectrophotometer (ISA SPEX FluoroMax-2; HORIBA Jobin Yvon, Inc., Edison, NJ) using excitation wavelengths of 340 nm and 380 nm, and an emission wavelength of 510 nm.

Antiviral Assays

P4R5 cells were trypsinized, resuspended in complete DMEM (supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 300 μg/ml glutamine) plus 1 μg/ml puromycin, and evenly plated in 48-well plates at the density of 2×10^4 cells/well. The cells were incubated at 37°C, 5% CO_2 overnight. Next day, the cells were infected by adding 100 μl of virus at dilutions yielding 50–100 infectious centers per well. The medium used for infection contained 20 μg/ml DEAE-Dextran without puromycin. The cells were incubated in the CO_2 incubator for 2 hr. After incubation, 400 μl of complete DMEM with different concentrations of SMM-chemokines or control drugs (i.e., AZT, NVP, and 3TC) was added to each well before incubating the cells in the CO_2 incubator for 2 days. Medium from each well was removed before adding to each well 500 μl of 1% formaldehyde plus 0.2% glutaraldehyde in PBS. The cells were fixed for 5 min and washed twice with PBS. Then, 300 μl of staining solution (949 μl PBS, 20 μl of 0.2 M potassium ferrocyanide, 20 μl of 0.2 M potassium ferricyanide, 1 μl of 2 M $MgCl_2$, and 10 μl of 40 mg/ml X-GAL per ml) was added. The cells were incubated at 37°C in a non-CO_2 incubator for longer than 3 hr. The staining process was stopped by removing the stain and washing twice with PBS. The cells were allowed to dry completely. Blue cells in each well were counted and compared with the number of cells in the "no drug" well to calculate the percent reduction.

Internalization Assays

Transfected 293 cells (3×10^5 cells/well) were plated onto 24-well tissue culture plates pretreated with 0.01% poly-L-lysine (Sigma). Nontransfected 293 cells were used as the background. Fixing the cells for 5 min with 4% paraformaldehyde/PBS stopped the reactions. After blocking the nonspecific binding with 1% BSA/PBS and incubating the cells for 45 min, a MAb HA.11 (Covance Inc., Princeton, NJ) was added. The cells were washed with PBS and reblocked with 1% BSA/PBS for 15 min. The cells were incubated with goat anti-mouse-conjugated alkaline phosphate (Bio-Rad, Richmond, CA) for 1 hr. The cells were washed with PBS before colorimetric alkaline phosphate substrate BCIP-NBT (Bio-Rad) was added. The plate was continuously shaken until an adequate color change occurred (~1 hr). The absorbance readings were taken using the Wallac Victor^2 1420 Multilabel counter.

MTS Toxicity Assays

Human blood was mixed with an equal volume of Iscove's modified Dulbecco's medium (IMDM). The mixture was gently added onto the top of Ficoll-Plaque Plus solution (Amersham Biosciences, Piscataway, NJ) and centrifuged at 3000 rpm for 30 min. The band containing the WBCs was collected, mixed with IMDM, and centrifuged at 1500 rpm for 8 min. After aspirating off the media, the WBCs were washed with IMDM and centrifuged at 1200 rpm for another 5 min. A total of 1×10^5 cells/well were plated in the 96-well plates and subsequently incubated with SMM-chemokines for 24 hr. The number of living cells was measured using CellTiter 96 aqueous nonradioactive cell proliferation assays (Promega, Madison, WI), according to the manufacturer's instructions.

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