Staphylococcal Superantigen-like 10 Inhibits CXCL12-Induced Human Tumor Cell Migration[1]

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Abstract

PURPOSE: Tumor cell migration and metastasis share many similarities with leukocyte trafficking, which is critically regulated by chemokines and their receptors. CXCR4 is the most widely expressed chemokine receptor in many different types of cancer and has been linked to tumor dissemination and poor prognosis. Several CXCR4 antagonists have been synthesized. A totally novel approach to discover chemokine receptor antagonists is the use of bacteria. Bacteria produce chemokine receptor inhibitors to prevent neutrophil extravasation and migration toward the infection site to escape clearance by innate immune cells. The aim of the current study was to find and identify the mechanism of a bacterial protein that specifically targets CXCR4, a chemokine receptor shared by neutrophils and cancer cells. *EXPERIMENTAL DESIGN:* Several staphylococcal proteins were screened for their capacity to prevent binding of a function-blocking antibody against CXCR4. *RESULTS:* Staphylococcal superantigen-like 10 was found to bind CXCR4 expressed on human T acute lymphoblastic leukemia, lymphoma, and cervical carcinoma cell lines. It potently inhibited CXCL12-induced calcium mobilization and cell migration. *CONCLUSIONS:* Staphylococcal superantigen-like 10 is a potential lead in the development of new anticancer compounds preventing metastasis by targeting CXCR4.

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Introduction

Metastasis is one of the main hallmarks of cancer and the mechanism responsible for mortality observed for many cancers. The control of metastasis is critical for the control of cancer progression. In addition to cytotoxic and targeted therapies, drugs that target receptors on malignant cells responsible for their metastasizing capacity would be of great value for treatment of most cancers.

In the recent years, striking similarities between leukocyte trafficking and tumor cell migration revealed that they are both critically regulated by chemokines and their receptors [1]. Bacteria are natural producers of chemokine receptor inhibitors that prevent leukocyte migration toward the site of infection. These evolutionary tailored bacterial proteins can be explored for their capacity to antagonize chemokine receptors that play a role in malignant cell behavior as well.

Tumor cells express functional chemokine receptors to sustain proliferation, angiogenesis, and survival and to promote organ-specific localization of distant metastases [2,3]. Increasing evidence suggests the pivotal role of the chemokine stromal cell–derived factor 1 (CXCL12/SDF-1α) and its CXCR4 in the regulation of growth of both primary and metastatic cancers [1,4,5]. CXCR4 is involved in the dissemination of breast cancer, of prostate cancer to the bone marrow [6], of colon cancer to the liver [7], and of undifferentiated thyroid cancer [8]. CXCR4 is highly expressed in human breast cancer cells and metastases. The specific ligand CXCL12/SDF-1α exhibits peak levels of expression in organs representing the first

destination of breast cancer metastasis. *In vivo*, neutralizing the interactions of CXCL12 and CXCR4 significantly impairs metastasis of breast cancer cells to regional lymph nodes and the lungs [9].

CXCR4 expression is associated with cervical adenocarcinoma cell migration and proliferation, and primary cervical adenocarcinoma cells expressing CXCR4 are significantly more likely to metastasize to pelvic lymph nodes [10]. Myeloid and lymphoid leukemia cells express high levels of CXCR4 that plays a critical role in leukemia cell chemotaxis and migration into bone marrow stroma [11–13]. *In vitro*, CXCR4 antagonists strongly inhibit migratory and signaling responses to CXCL12 and partially antagonize the protective effects of marrow stromal cells to spontaneous or drug-induced apoptosis of chronic lymphocytic leukemia, acute lymphocytic leukemia (ALL), and acute myeloid leukemia cells [14–16]. CXCR4 expression levels have a major prognostic impact in acute myeloid leukemia [17]. CXCR4 is expressed by primary T-ALL cells of patients with childhood T-ALL [18], and a high CXCR4 expression predicts extramedullary organ infiltration in childhood [19]. Promising results in preclinical tumor models indicate that CXCR4 antagonists may have additional value to conventional cytotoxic therapy in patients with various malignancies and immune diseases.

For more than a century, bacterial products have been used for the treatment of cancer [20]. Bacteria produce proteins that target chemokine and other chemoattractant receptors to prevent leukocyte migration. We have described chemotaxis inhibitory protein of *Staphylococcus aureus* (CHIPS), an excreted virulence factor of *S. aureus* [21]. CHIPS is known to inhibit formylated peptides and complement factor C5a–induced responses in neutrophils through direct binding to the formyl peptide receptor (FPR) and C5a receptor (C5aR), respectively [22–24]. Thereby, CHIPS inhibits the initial activation and migration of neutrophils to the site of infection, and thus, it hampers the clearance of *S. aureus* by innate immune cells. Recently, the structure of CHIPS was resolved, and it revealed homology to the C-terminal domain of staphylococcal superantigen-like 5 and 7 (SSL5 and SSL7) [25]. SSLs are a family of secreted proteins identified through sequence homology to staphylococcal and streptococcal superantigens, and although structurally related, they do not show superantigenic properties.

The aim of this study was to find a bacterial protein targeting CXCR4 that can prevent malignant cell behavior. Therefore, we screened several staphylococcal proteins for their ability to interfere with a function-blocking antibody directed against CXCR4. We identified SSL10 binding to CXCR4, and SSL10 inhibited the CXCL12-induced migration of a human leukemia (Jurkat) cell line. In addition, migration of the cervical carcinoma cell line HeLa toward CXCL12 was strongly inhibited by SSL10. Inhibition of CXCR4 by SSL10 is a new and attractive prospective into the molecular mechanism of human leukemia, lymphoma, and solid cancer metastases.

Materials and Methods

Reagents

Monoclonal antibodies (mAbs) directed against CXCR4 (clone 12G5), CXCR1 (clone 42705), CXCR7 (clone 11G8), and C5aR were purchased from BD (San Jose, CA), R&D Systems (Minneapolis, MN), and HBT (Uden, the Netherlands), respectively. Fluorescein isothiocynate (FITC)–conjugated mAb directed against CD3 and goat antimouse (Fc-specific)–FITC and goat antimouse (Fc-specific)–PE were from Dako (Carpinteria, CA). Synthetic human CXCL12 and CXCL8 were purchased from Peprotech (Rocky Hill, NJ), and C5a was obtained from Sigma-Aldrich (St. Louis, MO). Anti-HIS antibody was obtained from Novagen (Darmstadt, Germany). Goat antimouse horseradish peroxidase conjugate (GAM-HRP) was from Southern Biotech (Birmingham, AL). Antibodies against phosphoprotein kinase B/Akt and protein kinase B/Akt were purchased from Cell Signaling Technology (Leiden, the Netherlands). AMD3100, a small-molecule CXCR4 antagonist, was purchased from Sigma.

Cells

The human Jurkat T cell ALL, SupT1 T cell lymphoblastic lymphoma (ATCC, Rockville, MD) and A2780 ovarian carcinoma (obtained from Dr. R. Ozols, Philadelphia, PA) cell lines were grown in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 10 μg/ml gentamicin, and L-glutamine. The cervical carcinoma cell line HeLa (ATCC) was grown in DMEM/HAM's F12, 1:1 with 10% FCS. All cell lines were kept at 37°C in a humidified atmosphere consisting of 95% air and 5% CO_2. HEK293EBNA1 cells were maintained in suspension in Freestyle expression medium (Invitrogen, Life Technologies, Paisley, United Kingdom) supplemented with 0.2% FCS and 50 μg/ml G418 (Invitrogen). Twenty-four hours before transfection, cells were seeded to 3×10^5 cells/ml in Freestyle expression medium containing 0.05% FCS. Three hours after transfection, 0.9% of primatone (Kerry Bio-science, Almere, the Netherlands) was added.

For leukocyte isolation, heparinized blood was diluted with an equal volume of PBS and subsequently layered onto a gradient of Ficoll-Paque PLUS (Amersham Biosciences, Piscataway, NJ) and Histopaque 1119 (Sigma-Aldrich). After centrifugation for 20 minutes at 400*g*, neutrophils were collected from the Histopaque layer. After washing with RPMI-1640 containing 25 mM HEPES, L-glutamine (BioWhittaker, Walkersville, MD), and 0.05% human serum albumin (HSA; Sanquin, Amsterdam, the Netherlands) (RPMI/HSA), the neutrophils were subjected to a hypotonic shock with water for 30 seconds to lyse the remaining erythrocytes and were washed. Informed consent was obtained from all subjects and was provided in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht (Utrecht, the Netherlands).

Cloning, Expression, and Purification of SSL10

For the expression of recombinant SSL10 (protein identity YP_498982.1), the SSL10 gene (genomic locus tag SAOUHSC_00395) of *S. aureus* strain NCTC8325, (minus signal sequence coding for the first 30 amino acids) was cloned into the expression vector pRSETB (Invitrogen) directly downstream of the enterokinase (EK) cleavage site (Figure 1*A*). For this purpose, an overhang extension polymerase chain reaction (PCR) was performed. First, we amplified (PCR1) the HIS-tag and EK cleavage site from the pRSETB vector using the *Xba*I recognition sequence (underlined) primer 1: 5′ GCTCTAGAAATAATTTTGTTTAACTTTAAGAAGGAG3′) and introducing the N-terminal first 29-bp sequence of the SSL10 gene through the reverse primer (primer 2: 5′TGTTTATTTACTGACTT-TTGATTTTGTTTCTTGTCGTCATCGTCGTACAG3′). Second, the SSL10 gene was amplified by PCR2 (primer 3: 5′AAACAAAAT-CAAAAGTCAGTAAATAAAC3′, primer 4: 5′GCCGAATTCT-TACTTTAAGTTAACTTCAATATC3′) on chromosomal DNA of

S. aureus strain NCTC8325 introducing an *Eco*RI cleavage site (underlined). Finally, a third PCR (PCR3) was performed on a mixture of PCR1 and PCR2 to anneal the two PCR products together using primers 1 and 4. All PCR products were amplified using *PfuTurbo* DNA polymerase (Stratagene, Cedar Creek, TX). PCR3 was then digested with *Xba*I and *Eco*RI and ligated into the *Xba*I and *Eco*RI sites of the pRSETB vector. After verification of the correct sequence, the pRSET/SSL10 expression vector was transformed in BL21(DE3) *Escherichia coli* according to the manufacturer's protocol (Novagen). Expression of histidine-tagged SSL10 (HIS-SSL10) was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Roche, Basel, Switzerland) for 3 hours. To check for proper *E. coli* expression of HIS-SSL10, 20 μl of *E. coli* culture before and 3 hours after induction by IPTG was treated with sample buffer and run on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Analysis of the presence of HIS-SSL10 was performed by Coomassie brilliant blue staining and enhanced chemiluminescence (Amersham Biosciences) Western blot analysis using 1 μg/ml anti-HIS mAb and an HRP-conjugated secondary antibody (Figure 1*B*). HIS-tagged SSL10 was isolated under denaturing conditions on a HiTrap chelating HP column according to the manufacturer's protocol (Amersham Biosciences). The protein was renatured on the column by gradually exchanging a denaturing buffer (8 M urea, 500 mM NaCl, 500 mM sodium phosphate buffer, pH 5.3) for the native buffer (500 mM NaCl, 500 mM sodium phosphate buffer, pH 5.3). Bound protein was eluted using 50 mM ethylenediaminetetraacetic acid. After dialysis, the HIS-tag was removed from SSL10 by cleavage with EK according to the manufacturer's instructions (Invitrogen Life Technologies). Finally, SSL10 was stored in PBS, and its purity was examined by SDS-PAGE (Figure 1*B*). SSL6, SSL7, SSL8, and SSL11, all from *S. aureus* NTCT8325 (respective protein identities: YP_498978.1, YP_498979.1, YP_498980.1, and YP_498983.1), were also cloned and expressed in the pRSET B vector similarly as described for SSL10. CHIPS, staphylococcal complement inhibitors (SCIN, SCIN-B, and SCIN-C), Orf-D, and FPRL1 inhibitory protein (FLIPr) were expressed as previously described [21,26–28].

SSL10 Binding to CXCR4-Expressing Cells

To determine binding of SSL10 to the CXCR4-expressing cell lines Jurkat and SupT1, SSL10 was labeled with FITC. Therefore, 1 mg/ml SSL10 was incubated with 100 μg/ml FITC in 0.1 M sodium carbonate buffer (pH 9.6) for 1 hour at 37°C. FITC-labeled SSL10 (SSL10-FITC) was separated from unbound FITC using a HiTrap desalting column (Amersham Biosciences). For binding of SSL10-FITC to cells, Jurkat and SupT1 cells (5×10^6 cells/ml) were incubated with increasing concentrations of SSL10-FITC in RPMI/HSA for 30 minutes on ice. After washing, fluorescence was measured on a flow cytometer (FACSCalibur; Becton Dickinson, Franklin Lanes, NJ). To examine the role of sialic acids in the SSL10/CXCR4 interaction, Jurkat and SupT1 cells (5×10^6 cells/ml) were first treated with 0.2 U/ml neuraminidase (from *Clostridium perfringens*; Roche Diagnostics, Almere, the Netherlands) at 37°C for 45 minutes in RPMI/HSA before incubating with SSL10. To study the internalization of SSL10, trypan blue quenching was used as described by Postma et al. [23]. Jurkat cells at 5×10^6 cells/ml were incubated with 10 μg/ml SSL10-FITC in RPMI/HSA at 37°C. At different time points, the SSL10-FITC binding was measured in a flow cytometer, before and immediately after addition of 300 μg/ml trypan blue (Merck, Darmstadt, Germany), to discriminate between cell surface–bound and internalized ligand.

Competition between SSL10 and Antibody Binding to CXCR4 and CXCR7

To determine whether staphylococcal proteins were able to bind CXCR4 and compete with anti-CXCR4 monoclonal antibody (mAb) binding, Jurkat and SupT1 cells (5×10^6 cells/ml) were incubated with 0 to 10 μg/ml SSL10 for 30 minutes on ice in RPMI/HSA. After washing, anti-CXCR4 mAb clone 12G5 was added for
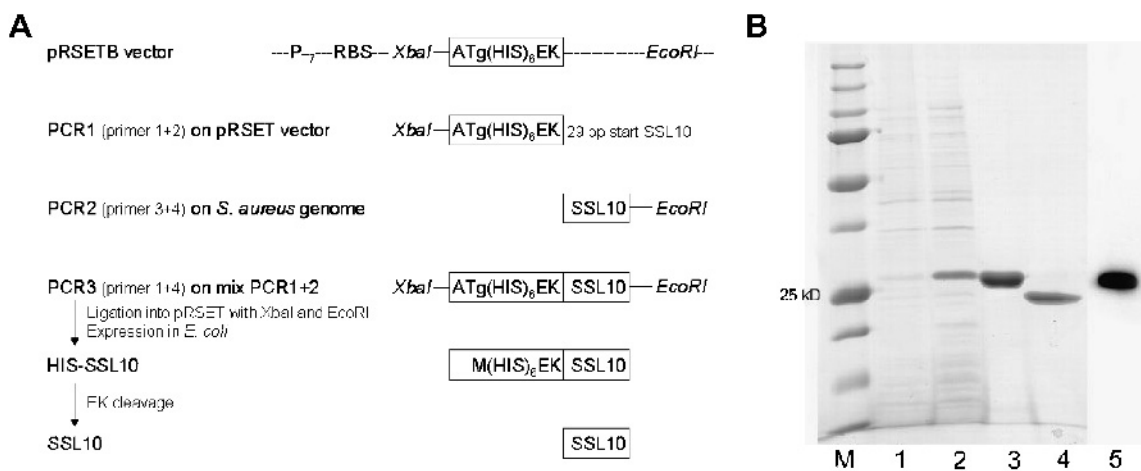
Figure 1. Cloning, expression, and isolation of SSL10. (A) Schematic overview of the cloning and expression procedure of HIS-SSL10. See Materials and Methods for clarification of PCR and primer use. After isolation of HIS-SSL10, the HIS-tag is removed by EK cleavage, resulting in the SSL10 protein of 23.1 kDa as excreted by *S. aureus* strain NCTC8325. (B) Detection of HIS-SSL10 expression in and isolation from BL21(DE3) *E. coli*. Lanes 1 to 4 represent Coomassie brilliant blue staining of HIS-SSL10 expression before (lane 1) and after 3 hours of IPTG induction (lane 2), HIS-SSL10 after isolation by nickel affinity chromatography (lane 3), and the final SSL10 product after EK cleavage (lane 4). Lane 5 represents the expression of HIS-SSL10 detected by Western blot analysis using an anti-HIS mAb and GAM-HRP.

30 minutes on ice. Anti-CD3 mAb was used as a control mAb on Jurkat cells. Anti-CXCR1 and anti-C5aR mAbs were used as control mAbs on human neutrophils. mAbs were detected with FITC-conjugated goat antimouse immunoglobulin G (IgG). After washing, antibody binding was analyzed using flow cytometry. Similar experiments with HeLa cells were performed to test binding of anti-CXCR4 mAb clone 12G5 and anti-CXCR7 mAb clone 11G8; mAbs were detected with PE-conjugated goat antimouse IgG in the presence of 10 µg/ml SSL10.

Transfection of CXCR4 in HEK Cells

CXCR4 was cloned into pABC-intra1-hisN-TEV, a mammalian expression vector that contains the CMV promoter from plasmid pCI (Promega, Madison, WI), OriP from pCEP4 (Invitrogen), the origin of replication and the β-lactamase gene of pcDNA3.1 (Invitrogen), and an expression cassette [29]. The expression cassette consists of a Kozak sequence, followed by an ATG codon, a HIS-tag, and a TEV-protease cleavage site, an in-frame BamHI-NotI cloning site, and an in-frame stop codon. A PCR was performed, which replaced the start methionine of CXCR4 by a BglII restriction site and the stop codon by a NotI restriction site. After confirmation of the sequence, CXCR4 was cloned into BamHI-NotI–digested pABC-intra1-(HIS)N-TEV, generating plasmid pABC-intra1-HIS(N)-TEV-CXCR4. Recombinant CXCR4 was produced in suspension growing HEK293EBNA1 cells by transient transfection. To this end, 2 µg of plasmid pABC-intra-HIS(N)-TEV-CXCR4 was complexed with 4 µg of polyethyleneimine in 100 µl of OptiMEM (Invitrogen).

After 10 minutes of incubation at room temperature, the DNA-polyethyleneimine complex was added to 4 ml of HEK293EBNA1 cells at 0.7×10^6 cells/ml. Cells were harvested by gentle centrifugation 48 hours after transfection, washed with RPMI/HSA, and incubated with the 2 µg/ml anti-HIS mAb (VWR International, Amsterdam, the Netherlands) and 30 µg/ml SSL10-FITC, and subsequently with 2 µg/ml APC-labeled goat antimouse Ig (PharMingen, San Diego, CA). Cells were washed and assayed on a flow cytometer, after addition of propidium iodide, for binding of SSL10-FITC to the CXCR4-expressing (APC-positive) and propidium iodide–negative cells (living) cells. In other experiments, HEK293EBNA1 cells expressing the CXCR4 were used to test whether SSL10 could compete with the binding of PE-labeled anti-CXCR4 mAb 12G5. Therefore, the cells were incubated with 2 µg/ml anti-HIS mAb, 2 µg/ml APC-labeled goat antimouse Ig, and 0 or 30 µg/ml SSL10 with washing steps in between. Finally, the cells were incubated with PE-labeled anti-CXCR4 mAb 12G5. After washing, the binding of PE-labeled anti-CXCR4 mAb was analyzed to CXCR4-expressing (APC-positive) cells.

Calcium Mobilization Assay

The effect of SSL10 on CXCL12-induced intracellular calcium release in Jurkat cells was measured in a flow cytometer, as described before [21]. Briefly, cells (5×10^6 cells/ml) were loaded with 2 µM Fluo-3-AM (Molecular Probes Europe, Leiden, the Netherlands) in RPMI/HSA for 20 minutes at room temperature, protected from light, and kept under constant agitation. Next, cells were washed with buffer and suspended to 1×10^6 cells/ml in RPMI/HSA. Subsequently, Fluo-3–labeled cells were preincubated with buffer or 10 µg/ml SSL10 for 1 minute at room temperature. Cells (1×10^6 cells/ml) were monitored for calcium mobilization over time, first for 10 seconds to determine the basal fluorescence level and then for 40 seconds

after stimulation with CXCL12 (3×10^{-10} to 3×10^{-8} M). Fluorescence was measured using a flow cytometer. The effect of SSL10 on CXCL12, CXCL8, and C5a-induced calcium mobilization in human neutrophils was also tested using the same method. Data are displayed as percentage of maximal calcium mobilization using the following formula: ((X MF – bg MF)/(Maximal MF – bg MF)) × 100%, in which X MF = measured mean fluorescence; bg = background. For the maximal MF, the MF value of cells stimulated with the highest concentration of stimulus was used. For all stimuli, calcium mobilization was determined at 5 seconds after stimulation. To study CXCL12-induced calcium mobilization in SupT1 cells, cells were labeled with 2 µM Fura-2-AM (Molecular Probes Europe) for 45 minutes at room temperature, washed and resuspended in Hank's balanced salt solution containing 25 mM HEPES and 0.1% HSA (HBSS/HEPES/HSA) at 7.5×10^6 cells/ml. Cells were transferred into black clear-bottom microtiter plates and were preincubated with buffer or 10 µg/ml SSL10 for 1 minute at room temperature. Subsequently, the fluorescence was measured every 2 seconds at dual-excitation wavelengths of 340 and 380 nm with 510 nm emission in a fluorescent plate reader (FlexStation; Molecular Devices, Sunnyvale, CA). CXCL12 (final concentration, 10^{-8} M) was automatically added after 30 seconds of baseline reading, and measurement continued for an additional 3 minutes. The ratio of 340:380 was calculated for every reading and plotted versus time.

Chemotaxis and Migration

Chemotaxis of Jurkat cells toward CXCL12 was measured in a 96-well chemotaxis chamber (ChemoTX; NeuroProbe, Gaithersburg, MD) using an 8-µm pore size polycarbonate membrane. Cells (5×10^6/ml) were labeled with 2 µM calcein-AM for 20 minutes at room temperature protected from light. Subsequently, cells were washed with HBSS/HSA, resuspended to 2.5×10^6 cells/ml, and incubated with SSL10 (1-10 µg/ml). Dilutions of CXCL12 (1×10^{-11} to 3×10^{-8} M) were prepared in HBSS/HSA, and 29 µl was placed into each well of the lower compartment of the chamber in triplicate. Wells with the control medium were included to measure the spontaneous cell migration. For total cell fluorescence, wells were filled with 25 µl of labeled cells plus 4 µl of buffer. The membrane holder was assembled, and 25 µl of labeled cells was added as a droplet to each upper well except for the total fluorescence wells. The plate was incubated for 30 minutes at 37°C in a humidified 5% CO_2 atmosphere. The membrane was washed extensively with PBS, and the fluorescence of the wells was measured in a fluorescent plate reader (FlexStation) with excitation at 485 nm and emission at 530 nm. The percentage of chemotaxis was calculated relative to the fluorescence value of cells added directly to the lower well: (fluorescence sample / fluorescence total counts) × 100. SSL10 was also tested on CXCL8- and C5a-induced chemotaxis of human neutrophils using the same method. To test the effect of SSL10 on the migration of adherent HeLa cells, 24-well Transwell plates (Costar 3422, Cambridge, MA) were used. The polyvinylpyrrolidone-free polycarbonate filters with an 8-µm pore size were precoated overnight at 4°C with 1% BSA in PBS. HeLa cells (6.5×10^5 cells/ml) were resuspended in DMEM, containing 1% FCS/0.1% BSA, and preincubated with medium alone or medium with 10 µg/ml SSL10 for 10 minutes at 37°C. Then, 150 µl of cells was added to the upper chamber, whereas 300 µl of medium alone or medium with CXCL12 (1×10^{-9} M) was added to the lower compartment of the Transwell system. Cells were allowed to migrate for 24 hours at 37°C. After incubation, the non-migrated cells remaining on the upper side of the filter were gently

removed using cotton-tipped swabs. Subsequently, cells on the lower surface of the filter were fixed in 75% methanol/25% acetic acid and stained with 0.25% Coomassie brilliant blue in 45% methanol/ 10% acetic acid. Ten high-power fields (×400) were counted under a light microscope, and the results were expressed as the mean number of migrated cells.

Western Blot Analysis

Jurkat or A2780 (10^6) cells were cultured in RPMI-1640 (without FCS) for 24 hours. After the indicated treatments, cell lysates were prepared with a sample buffer (25 mM Tris-HCl, 5% wt/vol glycerol, 1% wt/vol SDS, and 0.05% wt/vol bromophenol blue, pH 6.8). Subsequently, lysates were subjected to SDS-PAGE and transferred to Immobilon-P membranes (Millipore, Bedford, MA). Blots were probed by primary antibodies against protein kinase B/Akt and phospho-Akt and then treated with horseradish peroxidase–conjugated secondary antibodies (Dako). Enhanced chemiluminescence was used for final signal detection.

Results

SSL10 Competes with the Binding of Antibody Directed against CXCR4 on T cells

A screening assay to identify a bacterial inhibitor for surface-expressed CXCR4 was performed with the hematopoietic Jurkat T cell line. For this purpose, 11 excreted staphylococcal proteins, namely, SSL6, SSL7, SSL8, SSL10, SSL11, SCIN-A, -B, -C, Orf-D, FLIPr, and CHIPS, were tested for their ability to block the binding of the function-blocking anti-CXCR4 mAb 12G5. SSL10 clearly blocked the binding of the anti-CXCR4 mAb (Figure 2A), whereas the other *S. aureus* proteins showed no effect (data not shown). In Figure 2B, the dose-dependent inhibition of anti-CXCR4 mAb binding by SSL10 is depicted, showing that 10 µg/ml SSL10 decreased anti-CXCR4 mAb binding by 80%. Sialic acid residues were earlier shown to be critical determinants in the recognition of surface receptors by SSL5 and SSL11 [30,31]. To examine the role of sialic acids in the SSL10/CXCR4 interaction, Jurkat cells were first treated with neuraminidase. Upon treatment, no effect on SSL10-induced inhibition of anti-CXCR4 mAb binding was observed, showing that sialyl Lewis X sugars do not play a role in the binding of SSL10 to CXCR4 (Figure 2B). Same effects of SSL10 on anti-CXCR4 mAb binding with and without neuraminidase treatment were shown for the hematopoietic SupT1 T-cell line (data not shown).

SSL10 Binds to Jurkat and CXCR4-Transfected HEK Cells

The SSL10-mediated blocking of the anti-CXCR4 mAb binding to Jurkat cells could be indicative of binding of SSL10 to CXCR4. We used SSL10-FITC and flow cytometry to confirm binding to CXCR4-expressing Jurkat cells (Figure 2C). To exclude for an aspecific effect of SSL10 on mAb binding to cells, also the binding of other mAbs was studied. Figure 1D shows that there was no inhibition of anti-CD3 mAb binding to Jurkat cells by SSL10 and that there was no effect on the binding of anti-CXCR1 and anti-C5aR mAbs to human neutrophils. To further confirm specific binding of SSL10 to CXCR4, we transiently transfected HEK293EBNA1 cells with CXCR4. SSL10 specifically bound to CXCR4-transfected compared with nontransfected HEK293EBNA1 cells (Figure 2E). Also, binding of anti-CXCR4

mAb to CXCR4-expressing HEK293EBNA1 cells was inhibited by SSL10 (Figure 2F). Thus, SSL10 was identified as a specific CXCR4-binding protein.

SSL10 Inhibits CXCL12-Induced Calcium Mobilization

The hallmark of chemokine receptors is a rapid and transient increase in the free intracellular calcium level on ligand binding. This signaling pathway was used to examine whether SSL10 not only binds CXCR4 but also inhibits the activation by its natural ligand CXCL12. Figure 3A demonstrates that SSL10 inhibited the CXCL12-induced calcium mobilization in Jurkat cells. This effect was not cell type–specific as SSL10 also clearly inhibited the CXCL12-induced calcium mobilization in SupT1 cells (Figure 3B) and neutrophils (Figure 3C). SSL10, used as a stimulus up to 30 µg/ml, did not evoke calcium mobilization itself (data not shown). Calcium mobilization induced by ionomycin and ATP was not affected by SSL10 excluding toxic effects on the cells (data not shown). In control experiments with human neutrophils, SSL10 did not inhibit CXCL8- or C5a-induced calcium responses (Figure 3, D and E). These data show that SSL10 is highly specific toward the CXCR4 receptor.

SSL10 Is Not Internalized after Binding CXCR4

Thus far, we found that SSL10 inhibits binding of anti-CXCR4 mAb at 0°C. This suggests that SSL10 exerts its effect on CXCL12-induced calcium mobilization outside the cells, apparently independent of cell signaling events. To further address this, we determined the possible internalization of SSL10 by Jurkat cells. As a control, FITC-labeled formylated peptide was used, which has been described to be internalized after binding the FPR on neutrophils [23]. Figure 4A shows that SSL10-FITC remains outside the Jurkat cells, whereas in neutrophils, the FITC-labeled formylated peptide is internalized (data not shown). These results strongly indicate that SSL10 affects CXCR4 directly, independently of its signaling events or internalization.

SSL10 Inhibits CXCL12-Induced Phosphorylation of Akt

By using Western blot analysis, we further examined CXCL12/ CXCR4–induced activation of Akt, a pathway associated with cell survival. As shown in Figure 4B, we observed a rapid increase in the phosphorylation of Akt in Jurkat cells on stimulation with CXCL12. Akt phosphorylation was inhibited by AMD3100, a bicyclam antagonist of CXCR4, and, to a lesser extent, by SSL10. The combination of both CXCR4-inhibiting agents showed a synergistic effect of this inhibiting capacity. Neither the CXCR4-negative cell line A2780 nor SSL10 showed CXCL12-induced phosphorylation of Akt (Figure 4C).

SSL10 did not influence the viability of Jurkat cells, as they were still 97% viable after 24 hours of incubation with 10 µg/ml SSL10 at 37°C, as verified by trypan blue exclusion. Furthermore, SSL10 did not influence cell proliferation of Jurkat cells as measured by MTT assay (data not shown).

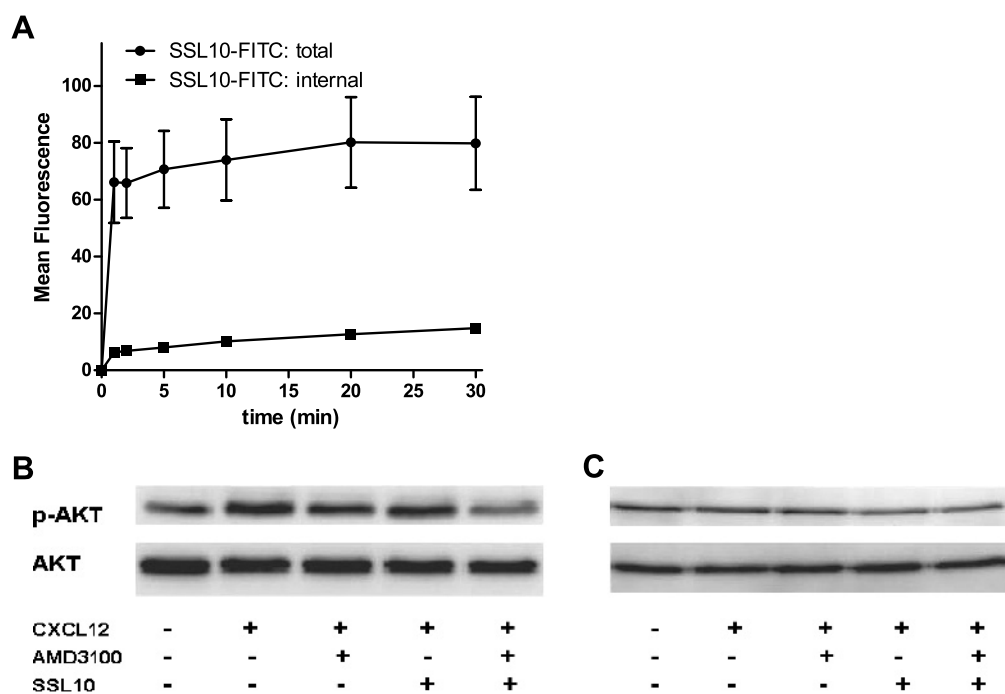### SSL10 Inhibits CXCL12-induced Cell Migration

We examined the ability of SSL10 to inhibit the CXCL12-induced chemotaxis of Jurkat cells. Figure 5A shows that SSL10 clearly inhibits the chemotactic response of Jurkat cells toward CXCL12, whereas background chemotaxis toward buffer was not affected. As a control, CXCL8- and C5a-induced chemotaxis of human neutrophils was not affected by SSL10 (Figure 5, B and C).
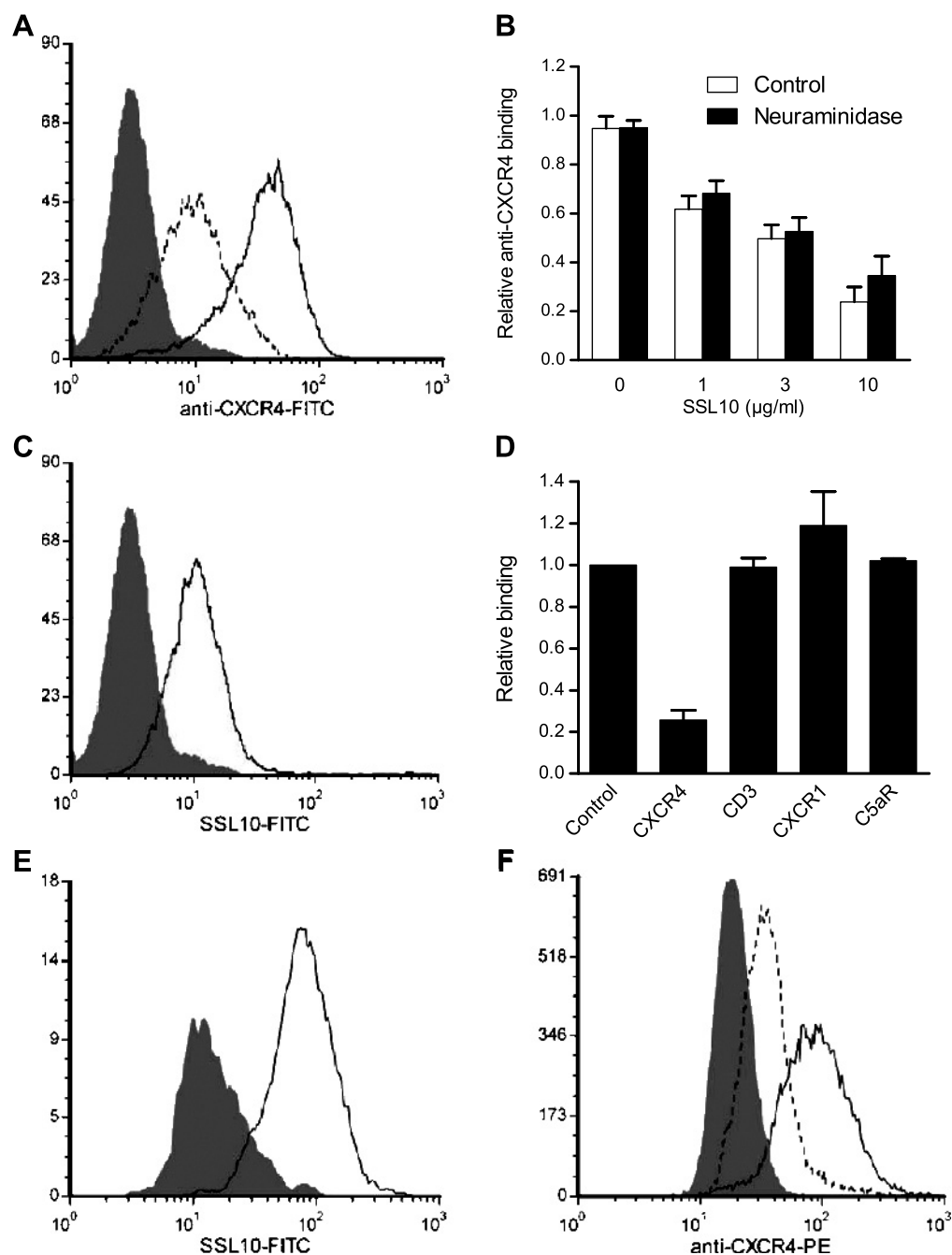
Figure 2. SSL10 binds CXCR4. (A) CXCR4-expressing Jurkat T-ALL cells were incubated with or without 10 μg/ml SSL10 for 30 minutes on ice. After washing, cells were stained with anti-CXCR4 mAb and detected with FITC-conjugated goat antimouse IgG. Continuous and dashed black lines represent untreated and SSL10-treated cells, respectively. Gray histogram represents control-stained cells. Data are one representative example of three independent experiments. (B) Jurkat cells were treated with (black bars) or without (open bars) neur-aminidase before incubation with SSL10 (1-10 μg/ml) for 30 minutes on ice. After washing, cells were stained with anti-CXCR4 mAb that was detected with FITC-conjugated goat antimouse IgG. Data represent mean fluorescence values ± SEM of three independent experi-ments. (C) Jurkat cells were incubated with (black line) or without (gray histogram) 10 μg/ml SSL10-FITC for 30 minutes on ice before analysis on a flow cytometer. Data are one representative example of three independent experiments. (D) Jurkat cells were incubated with 10 μg/ml SSL10 for 30 minutes on ice. After washing, the cells were stained for CXCR4 and CD3 expression. In separate experiments, neutrophils were stained for CXCR1 and C5aR expression. Data represent the relative binding of each antibody compared with untreated cells and are mean values ± SEM of three independent experiments. (E) HEK293EBNA1 cells were transfected with CXCR4. N-terminally HIS-tagged CXCR4 expression was detected by staining with anti-HIS mAb and APC-labeled goat antimouse IgG. Transfected HEK cells were incubated with 30 μg/ml SSL10-FITC for 30 minutes on ice before analysis on a flow cytometer. Data represent SSL10-FITC binding to CXCR4-expressing (APC-positive; black line) and untransfected (APC-negative; gray histogram) HEK cells. Data are one representative ex-ample of three independent experiments. (F) CXCR4-transfected HEK293EBNA1 cells were incubated with anti-HIS mAb and APC-labeled goat antimouse IgG. Then, the effect of 30 μg/ml SSL10 on the binding of PE-labeled anti-CXCR4 mAb was analyzed to CXCR4-expressing (APC-positive) cells. Data represent isotype mAb (gray histogram) and PE-labeled anti-CXCR4 mAb binding with (dotted line) or without (black line) preincubation of SSL10. Data are one representative example of three independent experiments.
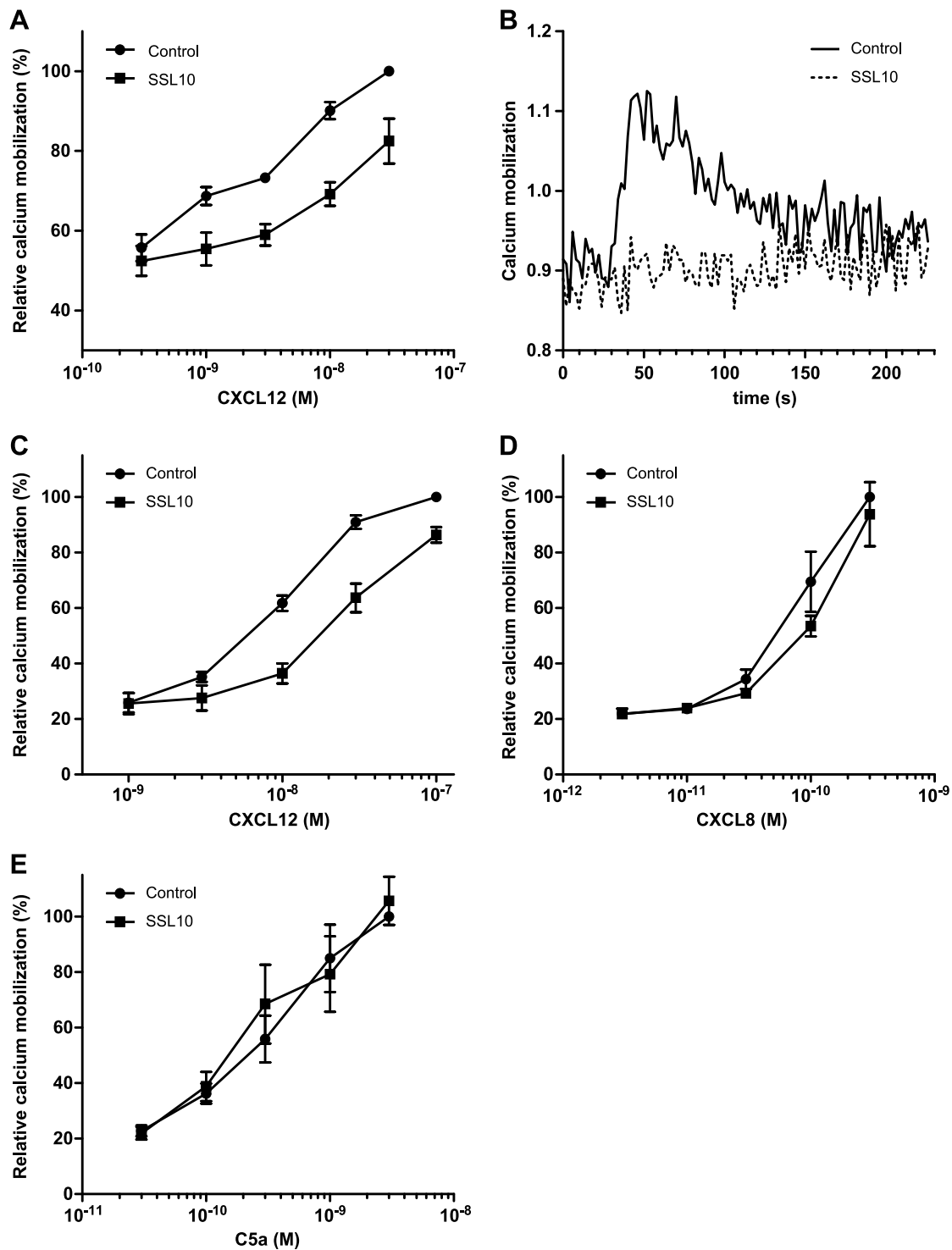
Figure 3. SSL10 specifically inhibits CXCL12-induced calcium mobilization. (A) The effect of SSL10 on intracellular calcium release induced by CXCL12 (10^{-7} to 10^{-10} M) in Fluo-3–loaded Jurkat cells was determined using flow cytometry. After preincubating cells with 10 μg/ml SSL10 (■) or buffer (●), the basal fluorescence level was measured for each sample before CXCL12 was added. Activation of control cells stimulated with the highest concentration of stimulus was set to 100% to display relative calcium mobilization values on the y-axis. Data represent mean values ± SEM of three independent experiments. (B) Representative experiment showing CXCL12 (10^{-8} M) induced calcium mobilization in Fura-2–loaded SupT1 cells treated with buffer or 10 μg/ml SSL10. Results are depicted as the ratio of the fluorescence at 530:590 nm measured in a fluorescent plate reader. (C–E) Effect of SSL10 on the calcium mobilization in neutrophils induced by CXCL12 (10^{-7} to 10^{-9} M) (C), CXCL8 (10^{-9} to 10^{-12} M) (D) and C5a (10^{-8} to 10^{-11} M) (E). Experimental details as described under (A).

Figure 4. SSL10 is not internalized on binding and inhibits CXCL12-induced phosphorylation of Akt. (A) Jurkat cells were incubated with 10 μg/ml SSL10-FITC in RPMI/HSA at 37°C. At different time points, SSL10-FITC binding was measured in a flow cytometer before and immediately after addition of 300 μg/ml trypan blue to discriminate between ligand binding on the outside the cell measured as total SSL10-FITC binding (●) and internalized ligand (■). Data are expressed as fluorescence values and are the mean ± SEM of three separate experiments. (B) Representative experiment of effects of SSL10 on CXCL12-induced phosphorylation of Akt and total Akt in Jurkat cells determined by Western blot analysis. Cells were incubated with buffer, 25 μg/ml AMD3100, 20 μg/ml SSL10, or both for 20 minutes at room temperature before treatment (30 minutes of incubation at 37°C) with buffer or CXCL12 (10^{-7} M). (C) Effects SSL10 on CXCL12-induced phosphorylation of Akt and total Akt in CXCR4-negative A2780 cells as determined by Western blot analysis.

These results are in agreement with our data on receptor expression and calcium mobilization experiments. CXCR7 is a recently discovered deorphanized G-protein–coupled receptor that binds CXCL11 and CXCL12 with high affinity [32]. To investigate whether SSL10 might also affect CXCL12-induced migration through CXCR7 binding, SSL10 was tested for its ability to compete with the binding of the blocking anti-CXCR7 mAb clone 11G8. We could not detect binding of anti-CXCR7 mAb to Jurkat cells, already indicating that CXCR7 plays no role in the CXCL12-induced migration of Jurkat cells. To further exclude SSL10 effects through CXCR7, we tested cervical carcinoma HeLa cells, which are described to express CXCR7 [32]. Figure 6*A* shows clear binding of blocking anti-CXCR7 mAb 11G8 to HeLa cells. This could not be inhibited by SSL10, suggesting that SSL10 does not bind CXCR7 (Figure 6*A*). In contrast, binding of anti-CXCR4 mAb 12G8 to HeLa was clearly inhibited by SSL10 (Figure 6*B*). More importantly, SSL10 completely inhibited the CXCL12-induced migration of HeLa cells (Figure 6*C*).

Discussion

The aim of this study was to identify bacterial antagonists for CXCR4 that prevent malignant cell behavior. We identified SSL10 as a strong inhibitor of CXCR4, as exemplified by CXCR4-expressing human T-ALL, lymphoma, and cervical carcinoma cell lines. SSL10 inhibited CXCL12-induced responses at different levels of the various involved signal transduction pathways, as shown by the inhibition of CXCL12-induced calcium mobilization, Akt phosphorylation, and migration. These results prove our hypothesis that bacterial proteins targeting chemokine receptors on leukocytes can be used to inhibit malignant cell migration.

Beneficial effects of bacterial infections on cancer prognosis are known for a long time. About 100 cases of spontaneous remission of acute leukemia after recovery from sepsis has been described [33,34]. The mechanisms inducing spontaneous remission are thought to be related with an overwhelming immune response leading to raised levels of various cytokines that cause an increased activation of the immune system. An alternative explanation would be that bacteria directly target chemokine receptors on innate immune cells primarily to prevent clearance by these cells, and additionally, they target identical receptors on cancer cells. Thereby, they influence their malignant behavior resulting in spontaneous remission of cancer.

A number of examples of bacterial proteins targeting non–cancer-related chemokine receptors exist. In the past few years, we found CHIPS and FLIPr, *S. aureus* proteins interfering with host cell migration through specific binding to the C5aR, FPR, and FPRL-1 [21,26]. Recently, the structure of CHIPS consisting of residues 31 to 121 (CHIPS$_{31-121}$) was resolved [25]. CHIPS$_{31-121}$ is composed of an α-helix packed onto a four-stranded antiparallel β-sheet and is found highly structural homologous to the C-terminal domain of SSL5 and SSL7. SSLs are a family of secreted proteins identified through sequence homology to staphylococcal and streptococcal superantigens. SSL1 to SSL11 are encoded on staphylococcal pathogenicity island 2 present in all *S. aureus* strains, whereas SSL12 to SSL14 are found on immune evasion cluster [35,36]. Analysis of

the degree of polymorphism among SSL proteins reveals 22% to 65% identity, whereas allelic variations among SSL proteins varies between 54% and 100% [31,35]. Thus far, no relevant sequence homologies with other bacterial proteins are known. Determination of the crystal structures of SSL5 and SSL7 also revealed their high structural homology to superantigens; the N-terminal oligonucleotide/oligosaccharide–binding fold and the C-terminal β-grasp domain characteristic for superantigens are also observed in SSLs. However, residues important for MHC class II and T cell receptor (TCR) binding of superantigens are not conserved in SSLs, which may explain their inability to display superantigenic activities. Three proteins of the SSL family are thus far functionally described: SSL7 binds IgA and complement C5 and inhibits IgA-Fc αRI binding and serum killing of bacteria [37]. SSL5 inhibits P-selectin–mediated neutrophil rolling by binding P-selectin glycoprotein ligand 1 [30]. SSL5 binding to P-selectin glycoprotein ligand 1 was found to be dependent on the presence of sugar moieties. SSL11 was also shown to interact with sLex [31]. Unlike SSL5, SSL10 specifically binds the CXCR4 independent of the presence of sugar moieties. Other bacterial products have been described to target GPCRs, such as cholera and pertussis toxin that covalently modify the α subunits of numerous G proteins by ADP-ribosylating specific amino acid residues [38]. Staphylococcal super-antigens stimulation of human peripheral blood monocytes results in a rapid, dose-dependent, and specific down-regulation of CCR1, CCR2, and CCR5, which correlates with a concomitant hyporesponsiveness of human monocytes to these CC chemokine ligands [39]. Lipopolysaccharide causes a drastic and rapid down-regulation of the expression of CCR2 [40] and is able to down-modulate CXCR4 in neutrophils and monocytes [41]. Like CHIPS, SSL10 probably interferes with its GPCR through specific binding, as it competed with anti-CXCR4 mAb binding at 0°C. Moreover, it remained outside the cells when incubated at 37°C. These results strongly suggest that SSL10 affects the CXCR4 directly, independently of its signaling events or internalization.

For a long time, CXCR4 was described as the sole receptor for CXCL12. Recently, an additional CXCL12-binding chemokine receptor, CXCR7, was identified [32]. Despite its high affinity for CXCL12, the role of CXCR7 in chemotaxis is still a matter of debate. Although one study claims that CXCR7 is involved in chemotaxis [42], more recent data show that CXCR7 lacks intrinsic chemotactic activity toward its ligand CXCL12 [32,43,44]. Our data support the latter because CXCL12-induced migration of HeLa cells could not be inhibited by the blocking anti-CXCR7 mAb 11G8 (data not shown). As SSL10 did not compete with the binding of anti-CXCR7 mAb
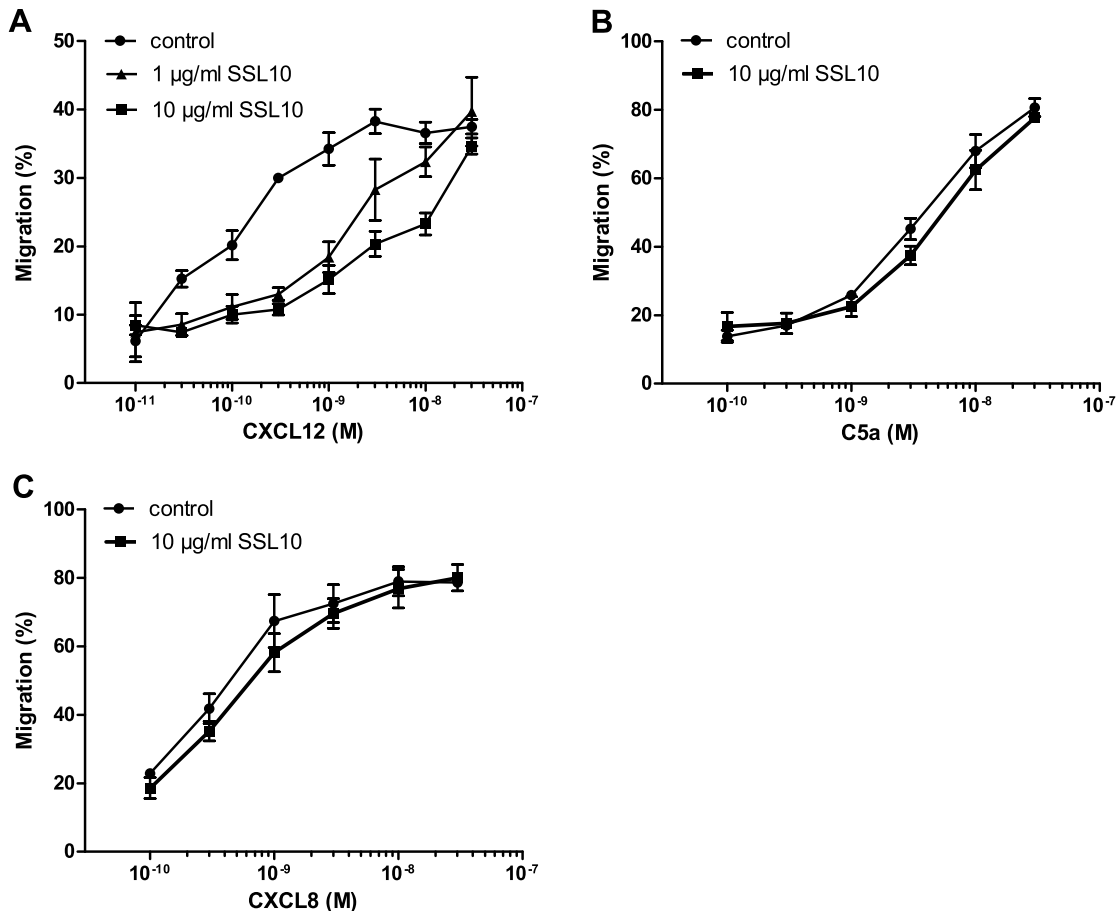
Figure 5. SSL10 inhibits cell migration of Jurkat cells toward CXCL12. (A) Jurkat cells were allowed to migrate toward a concentration range of CXCL12 (10^{-7} to 10^{-11} M) after incubation of cells with buffer (●), 1 μg/ml SSL10 (▲), or 10 μg/ml (■) SSL10. (B and C) Migration of human neutrophils toward a concentration range of C5a (B) and CXCL8 (C), after incubation of the cells with buffer (●) or 10 μg/ml SSL10 (■). Data are expressed as the percentage of migrated cells added to the upper compartment of the 96-well chemotaxis chamber and are mean values ± SEM of four (A) and three (B and C) independent experiments.
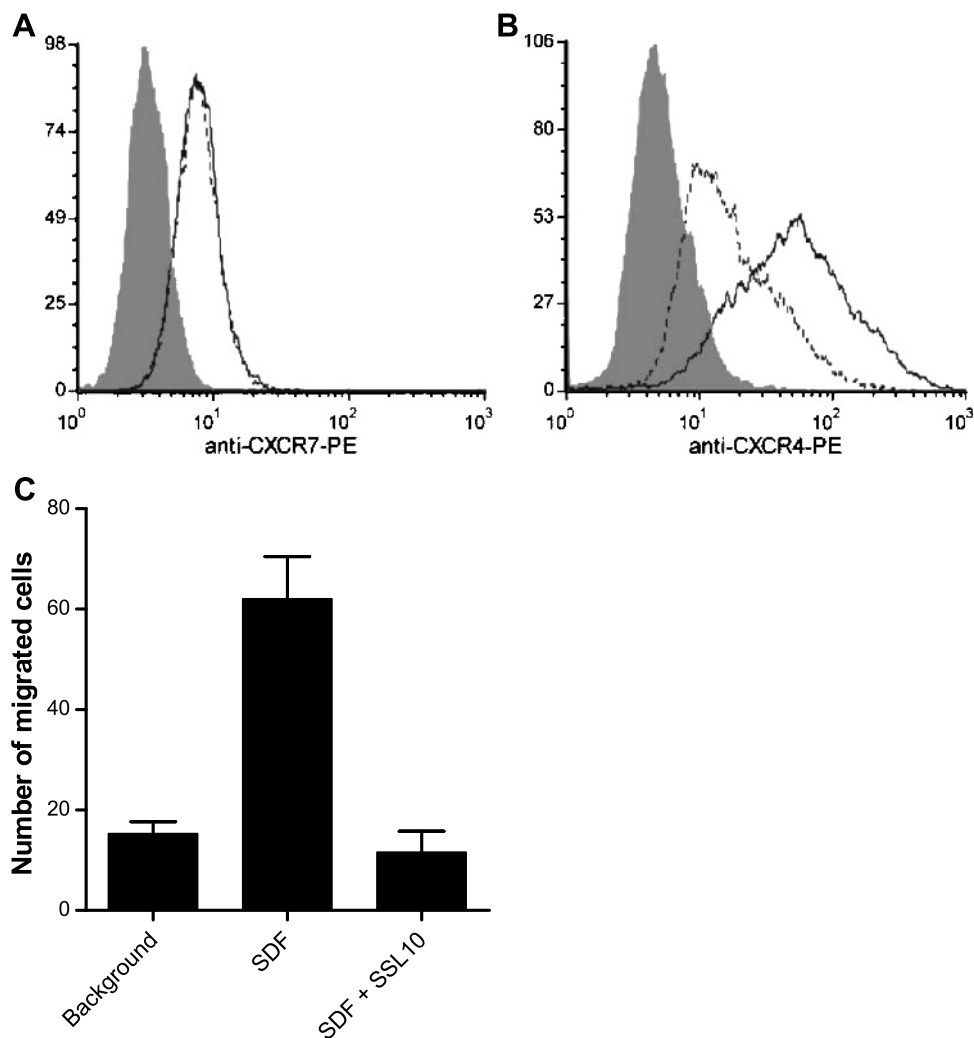
Figure 6. SSL10 does not bind CXCR7 and inhibits CXCL12-induced migration of HeLa cells. (A and B) Binding of blocking anti-CXCR7 mAb 11G8 (A) and blocking anti-CXCR4 mAb 12G5 (B) to HeLa cells in the presence (dotted line) and absence (black line) of SSL10. Gray histograms represent binding of isotype control mAbs. Data are a representative of one of three independent experiments. (C) Migration of HeLa cells preincubated with buffer or 10 μg/ml SSL10 toward 1×10^{-9} M CXCL12 using 24-well Transwell plates. Data are expressed as number of migrated cells counting five high-power field ($\times$400) $\pm$ SEM of four independent experiments.

11G8 to CXCR7, SSL10 only antagonizes CXCR4 for inhibition of CXCL12-induced responses.

The acquired ability of a localized tumor to metastasize is a multistep process involving many pathways, including those involved in angiogenesis, focal adhesion, invasion, and eventually colonization of a distant site [45]. CXCR4 inhibitors may have, like other inhibitors targeting malignant cell migration [46], a role in advanced disease, but even if no activity is observed in this setting, their role in invasion and metastasis might still enable a potential role in the adjuvant setting to reduce the risk of recurrence after definitive therapy. Further studies are necessary to identify potential combinations that will be of benefit including combinations with cytotoxic chemotherapy agents in the frontline setting and after the development of resistance. Recent findings indicate that SDF-1α/CXCR4 interactions contribute to the resistance of leukemic cells to signal transduction inhibitor– and chemotherapy-induced apoptosis in systems mimicking the physiological microenvironment [47]. Disruption of these interactions with CXCR4 inhibitors represents a novel strategy of sensitizing leukemic cells by targeting their protective bone marrow microenvironment.

CXCR4 is the most widely expressed chemokine receptor in many different hematological and solid cancers and has been associated with cancer dissemination and poor prognosis. Interfering with the chemokine system would add possible treatment options for this poor prognosis patient group. Since the disclosure of CXCR4 as a coreceptor for human immunodeficiency virus, various CXCR4 antagonists have been developed, including the horseshoe crab protein polyphemusin II and its analogues [15,48–50], the small-molecule heterocyclic bicyclam AMD3100 [51], and the monocyclam AMD3465 [52]. AMD3100 was originally developed as a CXCR4 inhibitor with anti–human immunodeficiency virus 1 activity but was withdrawn from the phase 2 clinical trial primarily because of a lack of antiviral effect and the occurrence of unexplained cardiotoxicity. AMD3100 was further developed for stem cell mobilization and is now under evaluation in phase 3 clinical studies for mobilization of hematopoietic stem cells (ClinicalTrials.gov) and in preclinical studies as anticancer agent and treatment of autoimmune disease.

Yet, bacteria provide us with evolutionary tailored, highly specific, chemokine receptor inhibitors. SSL10 is the first example of such a

protein interfering with malignant cell migration toward the CXCR4 ligand CXCL12. SSL10 could potentially serve as a supplement to direct cytotoxic therapy to suppress cancer metastasis.

Acknowledgments

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