Loss of New Chemokine CXCL14 in Tumor Tissue Is Associated with Low Infiltration by Dendritic Cells (DC), while Restoration of Human CXCL14 Expression in Tumor Cells Causes Attraction of DC Both In Vitro and In Vivo1

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Breast and kidney-expressed chemokine (BRAK) CXCL14 is a new CXC chemokine with unknown function and receptor selectivity. The majority of head and neck squamous cell carcinoma (HNSCC) and some cervical squamous cell carcinoma do not express CXCL14 mRNA, as opposed to constitutive expression by normal oral squamous epithelium. In this study, we demonstrate that the loss of CXCL14 in HNSCC cells and at HNSCC primary tumor sites was correlated with low or no attraction of dendritic cell (DC) in vitro, and decreased infiltration of HNSCC mass by DC at the tumor site in vivo. Next, we found that recombinant human CXCL14 and CXCL14-positive HNSCC cell lines induced DC attraction in vitro, whereas CXCL14-negative HNSCC cells did not chemoattract DC. Transduction of CXCL14-negative HNSCC cell lines with the human CXCL14 gene resulted in stimulation of DC attraction in vitro and increased tumor infiltration by DC in vivo in chimeric animal models. Furthermore, evaluating the biologic effect of CXCL14 on DC, we demonstrated that the addition of recombinant human CXCL14 to DC cultures resulted in up-regulation of the expression of DC maturation markers, as well as enhanced proliferation of allogeneic T cells in MLR. Activation of DC with recombinant human CXCL14 was accompanied by up-regulation of NF-κB activity. These data suggest that CXCL14 is a potent chemoattractant and activator of DC and might be involved in DC homing in vivo. The Journal of Immunology, 2005, 174: 5490–5498.

The destructive disease head and neck squamous cell carcinoma (HNSCC)3 annually afflicts 40,000 new persons in the United States (1), and 3,000,000 new cases develop worldwide annually (2, 3). Despite improvements in therapy and diagnosis, the overall survival rate of generally 50% for persons diagnosed with HNSCC has remained practically unchanged over the last two decades (1). For this reason, new therapeutic strategies need to be developed to treat HNSCC and the evaluation of alternative treatment strategies for patients with this malignancy is highly justified. Immunotherapy has a long history, but is only rarely considered as the treatment of choice. However, it seems that increasing efficacy of immunotherapy will make it one of the possible therapeutic options.

Specific active immunotherapy is based on the principle that malignant cells contain immunogenic determinants against which an antitumor immune response can be induced. Dendritic cells (DC) that acquire Ags from tumor cells are able to induce and regulate specific antitumor immunity. Several clinical trials have been initiated to evaluate the efficacy of DC-based immunotherapies in cancer, including stimulation of endogenous DC (4–6). However, it is still unclear why endogenous DC do not mediate efficient antitumor immunity in cancer patients. Whereas successful immunotherapy requires a functional immune system, a defect in the immune response may contribute to tumor growth. Such defects include active suppression of immune cells including DC by the tumor causing disturbed longevity and cell dysfunction (7, 8). For instance, it has been shown that many tumor cell lines, including melanoma and colon adenocarcinoma can effectively chemoattract DC in vitro, modulate their phenotype, and eventually, severely damage DC mobility (9). From this point of view, recent reports about loss of certain chemokines in several tumors, including HNSCC, initially sound surprising (10–12). However, it is conceivable to hypothesize a new mechanism of tumor escape: loss of certain chemokines by tumor cells results in a low attraction of DC, decreased number of tumor-infiltrating DC and thus inhibited ability of the immune cells to recognize tumor and initiate specific antitumor immune responses. In fact, analysis of phenotype and distribution of immunocompetent cells in oral leukoplasia with different levels of dysplasia revealed that the levels of immune effector cells varied according to the degree of dysplasia (13). Examining distribution of $^{100}$ DC in the tumor tissues and regional lymph nodes of 60 patients with HNSCC, Deng et al. (14) reported that the $^{100}$ DC density in tumor tissues was correlated with the tumor histologic grade, and the density of $^{100}$ DC was significantly higher in regional lymph nodes without tumor than in those with metastases. A similar conclusion was reported after...
evaluation of 36 cases of primary HNSCC of the lip mucosa or vermilion border for the correlation between tumor-associated DC density and tumor grade, mitotic rate, diameter, ulceration, depth of invasion, muscle invasion, and metastasis (15). Goldman et al. (16) have determined that survival and recurrence rates for patients with squamous cell carcinoma (SCC) of the tongue correlate with the degree of DC infiltration of the primary tumor or adjacent tongue tissue. Patients who had greater numbers of CD1a+ DC adjacent to tumor had better survival and decreased recurrence rates. These suggest that the distribution of DC subsets in HNSCC may reflect the degree of tumor immunity induced in the host-bearing HNSCC. Altogether, these suggest a functional role of DC in the immune response to HNSCC. Localized absence of DC might impair mucosal immunologic protection, allow microbial colonization, and enhance carcinogenesis. However, the mechanisms and chemokines responsible for DC homing and accumulation in HNSCC are unknown.

The various members of chemokine are subdivided into four families known as either the CXC, C-C, C, and CX3C, or the α, β, γ, and δ subfamilies, respectively (17). Approximately 50 human chemokines and 20 receptors are currently known. This large number reflects the highly complex traffic pattern of blood leukocytes, including granulocytes, monocytes, lymphocytes, and DC. Accumulating evidence indicates critical regulatory roles for chemokines during the development of metastatic tumors by stimulating angiogenesis and tumor growth. In addition, by regulating immunity, chemokines critically regulate antitumor immune responses and chronic inflammation such as that associated with various neoplasias (18–20).

Breast and kidney-expressed chemokine (BRACK) CXCL14 is a new CXC chemokine with unknown function and receptor selectivity (11, 12, 21). CXCL14 transcripts are highest in human kidney, small intestine, and liver tissues and expressed constitutively by a variety of epithelia including the basal keratinocytes and dental fibroblasts of skin (21). Importantly, Hromas et al. (12) reported that CXCL14 mRNA was expressed ubiquitously in normal tissues, but absent in a variety of in vitro established tumor cell lines. Moreover, using differential display and in situ mRNA hybridization, Frederick et al. (11) have recently reported that squamous epithelium constitutively express CXCL14, whereas expression in tumors was heterogeneous, with the majority of HNSCC and some cervical SCC showing loss of CXCL14 mRNA. This study demonstrates for the first time up-regulation of CXCL14 mRNA in the inflammatory sites in the tumor microenvironment and loss expression from certain cancers in vivo. The loss of expression in tumors and the presence of CXCL14 in nonmalignant tissues suggest that this chemokine may play a role in host-tumor interactions. It is also possible that down-regulation of the CXCL14 gene expression in tumor cells might be beneficial for tumor growth. However, the role of CXCL14 in the regulation of migration of DC in cancer and their biologic significance has not yet been investigated.

In the present work, we have established new in vitro and in vivo models to address the chemotactic interaction among human DC, CXCL14, and HNSCC tumor cells. We have demonstrated that HNSCC tissues are low in tumor infiltrating DC although DC are present in oral dysplasia lesions. Decreased infiltration of HNSCC by DC was correlated with no or low expression of CXCL14 protein at the tumor site. However, intense CXCL14 staining was observed in oral dysplasia (premalignant) lesions. Furthermore, we showed that CXCL14 is a potent DC chemoattractant in vitro and in vivo and DC are recruited to genetically modified CXCL14-expressing HNSCC cells. In addition to being potent DC chemoattractant, CXCL14 also increased functional activity of DC, which was associated with increased activity of NF-κB.

Materials and Methods

Tumor cell lines and tissues

Human SCC-15, prostate adenocarcinoma LNCaP, and melanoma FemX cell lines were obtained from American Type Culture Collection. The HNSCC PCI-13, PCI-16, and PCI-4B cell lines were prepared from HNSCC tumors (22). Conditioned medium from normal human lymph node cell suspensions served as a positive control. Tumor cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.2 mM l-glutamine, 1 mM sodium pyruvate, and 0.1 mM HEPES (Invitrogen Life Technologies).

The immunohistochemical studies were performed on a variety of formalin-fixed and paraffin-embedded tissue sections, including normal oral tissue (7 blocks), oral epithelial hyperplasia (8 blocks), and oral SCC (8 blocks).

Mice

Male C.B-17 SCID (T−/−, B−/−) mice, 6- to 8-wk-old were obtained from Taconic Farms. Animals were maintained in pathogen-free facility under the controlled temperature, humidity, and a 12-h light to dark cycle.

Immunohistochemistry

Monoclonal Abs recognizing CD83, CD1a (Immunotech), CD11c (DAKO), and S-100 (Sigma-Aldrich) were used for the detection of DC infiltration in formalin-fixed and paraffin-embedded tumor tissue sections. Five-micrometer sections were cut, mounted on positively precharged slides (Superfrost Plus; Fisher Scientific), and allowed to dry overnight at 56°C to ensure optimal adhesion. The sections were deparaffinized and rehydrated. After endogenous peroxidase quenching (0.3% H2O2 in PBS for 30 min), Ags were retrieved by boiling the sections in 1 mM EDTA/NaOH solution, pH 8.0, in a microwave oven for three cycles (5 min each), protease I digestion (5 min), and washed in PBS. A primary Ab was applied to the slides (Superfrost Plus; Fisher Scientific) according to the manufactur-er’s instructions. The positive control was a commercial monoclonal Ab for CD1a. The slides were then covered with a 1:200 dilution of non-immune IgG (Sigma-Aldrich) for 30 min, washed in PBS, and incubated with secondary Ab for 30 min. The sections were then incubated with the avidin-biotin peroxidase technique. Staining was developed with peroxidase and aminoethylcarbazole or diaminobenzidine (Vector Laboratories).

Expression CXCL14 protein in tissue sections and tumor cell lines was determined with anti-CXCL14 mAbs (1/100, overnight incubation; R&D Systems). Staining with normal murine IgG2a was performed as a negative control for CXCL14 stain. Immunohistochemical and immunocytochemical staining was performed using avidin-biotin peroxidase technique described.

Transduction of HNSCC cell lines with the CXCL14 encoding vector

Frederick et al. (11) have demonstrated expression of CXCL14 in PBMC stimulated with LPS. We isolated total RNA from human PBMC activated with Escherichia coli LPS (0.5 μg/ml; Sigma-Aldrich) for 6 h using the TriReagent (Molecular Research Center) and according to the supplier’s instructions. Up to 2 μg of total RNA was reverse-transcribed in a final reaction volume of 25 μl containing 2.5 μl oligonucleotides, 1× reaction buffer (Invitrogen Life Technologies), 0.5 mM each of dNTP (Invitrogen Life Technologies), 10 mM DTT, 1 μl of RNase inhibitor (Boehringer Mannheim), and 200 U of Superscript II reverse transcriptase (Invitrogen Life Technologies). For PCR, 4 μl of cDNA was amplified in a final volume of 50 μl containing 1× Taq buffer, 50 μM each dNTP, and 2.5 U of Taq polymerase enzyme (Invitrogen Life Technologies). Primers for human CXCL14 were: 5′-CAG GTC GAC ATG AGG CTC CTG GCG GCC and 3′-GCG GGA TCC CTA TTC TTT GCA GAC CCT GGC. PCR amplification was performed at 94°C for 10 min, followed by 35 cycles at 94°C for 1 min, 64°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. PCR products were resolved by agarose electrophoresis and visualized with ethidium bromide. To construct a eukaryotic expression vector, the CXCL14 gene was cloned into pCR3.1 plasmid. The PCR products were purified using QIAEX II gel extraction kit (Qiagen) and cloned into pCR3.1 mammalian expression vector using the Eukaryotic TA Expression kit (Invitrogen Life Technologies) according to the manufacturer’s instructions. Highly purified HNSCC primary cell line PCI-16 was transfected with the human CXCL14 gene using Effective Transfection Reagent (Qiagen) according to the supplier’s instructions. Briefly, tumor cells were counted and plated at 70% density in 10-cm petri dish 1 day before the transfection. On the next day, the
medium was removed, the cells were washed in HBSS (Invitrogen Life Technologies), and transfection mixture containing 2 μg of DNA was added to the tumor cells. The cells were incubated with a transfection mixture at 37°C for 6 h. After incubation, fresh medium (RPMI 1640, 10% FCS) was added and tumor cells were incubated at 37°C for additional 48 h. Next, transfected tumor cells were split and fresh medium containing 1 mg/ml geneticin (Invitrogen Life Technologies) was added for selection of transfected cells. Culture medium with geneticin was changed twice a week for 2–3 mo. Expression of CXCL14 protein was confirmed by Western blot (recombinant human CXCL14 served as a positive control; RDI) and immunocytochemistry.

**RT-PCR**

Analysis of mRNA expression of human chemokines in normal oral mucosa and HNSCC tissues was performed using RT-PCR technique. RNA was extracted from five normal mucosae and five oral SCC specimens, transcribed into cDNA using 200 U of superscript reverse transcriptase, and cDNA was amplified with 2.5 U of Taq polymerase using 1.5 pM of the primers specific for MIP-3α, MIP-3β, CXCL8, and GAPDH. RT-PCR was conducted as described earlier (23).

**Generation of human monocyte-derived DC**

CD14-derived DC were generated as described earlier (24). Briefly, PBMC were isolated fromuffy coats by Ficoll gradient centrifugation. The PBMC were further plated at 10^6 cells/well in 2 ml of AIM-V medium (Invitrogen Life Technologies) in six-well plates. After 1-h incubation at 37°C in a humidified 5% CO2 atmosphere, nonadherent cells were removed and adherent monocytes were gently washed with warm AIM-V medium. Adherent monocytes were cultured with recombinant human GM-CSF (100 ng/ml; PeproTech) and IL-4 (1000 U/ml; PeproTech) in complete RPMI 1640 medium for 7 days. Maturation of DC was stimulated by additional supplementation with 20 ng/ml TNF-α (PeproTech) on day 6.

**Analysis of DC migration and chemotaxis in vitro and in vivo**

Spontaneous and chemokine-induced migration of DC in vitro was assessed in the Transwell system with DC placed in the upper chamber (10^6 cells/ml, 100 μl) and chemokines added to the bottom chamber (600 μl) in a 4-h migration assay. Cell migration was measured in 48-well Transwell plates (5-μm pores; Corning Costar). Recombinant human MIP-1α (10–20 ng/ml; PeproTech), synthetic chemotactic peptide N-MLP (0.5–5 μg/ml; Sigma-Aldrich) and recombinant human CXCL14 (5–200 ng/ml; RDI) were diluted in RPMI 1640 medium containing 1% FBS (assay medium), and 600-μl aliquots were placed in the lower chamber of Transwell plates. Assay medium was changed to measure a spontaneous migration of DC. After 4-h incubation at 37°C, the Transwell inserts were removed and cells from the lower chamber were collected. Cells transmigrated through the 5-μm pore size membrane were collected on FACSscan (BD Biosciences) for 60 s. Data are reported as mean number of transmigrated cells from triplicate wells.

To test whether human tumor cell lines produce chemokines that attract DC, cell-free conditioned media were collected from FenX, LNCaP, and different HNSCC cell lines. Tumor cells were seeded at 1 × 10^6 in 4 ml of assay medium. Twenty-four hours later, cell-free supernatant was collected and centrifuged. Conditioned medium from normal human lymph node cell suspensions served as a positive control. Tumor-conditioned or control media were placed in the lower chamber of the Transwell plate and migration of DC was assessed as previously described.

Tumors were harvested 48 h later and tissue sections were analyzed by conventional microscopy and immunohistochemistry with anti-CD1a Abs.

**Flow cytometry**

Expression of DC specific markers was determined as described earlier (25) by flow cytometry on a FACSCalibur (BD Biosciences) using the following Abs: CD1a-PE, HLA-DR-PE (BD Biosciences), CD1a-PE, CD40-PE, CD80-PE, CD83-PE, (Immunotech/Coulter), and CD86-FITC (BD Pharmingen). The analyses were done using the CellQuest software (BD Biosciences).

**MLR assay**

MLR assays were performed to evaluate the effect of CXCL14 on the ability of human DC to stimulate proliferation of allogeneic T cells. Control and CXCL14-treated (200 ng/ml) DC were added in triplicates in graded doses (10^2–10^6 cells per well) to T cells (1 × 10^6 per well) in round-bottom 96-well plates. Proliferation of T cells was measured 72 h later by incorporation of [3H]thymidine (1 μCi/well; DuPont-NEN) added for the last 16 h. Cells were harvested onto GF/C glass fiber filter paper (Whatman) and isotope incorporation was assessed by 1450 MicroBeta TRILUX liquid scintillation counter (Wallac). The counts were expressed as cpm ± SEM.

**NF-κB activity assay**

Monocyte-derived DC were treated with CXCL14 200 ng/ml for 0–30 min. TNF-α (30 ng/ml, 15 min) served as a well-known activator of NF-κB in DC. Nuclear extract from Jurkat cells was used as an internal control. The effect of CXCL14 on NF-κB activation in DC was determined using a method developed by Active Motif. This method was developed in an ELISA format and uses binding of the active form of NF-κB to immobilized oligonucleotides corresponding to NF-κB nuclear consensus site 5'-GGGACCTTCC-3'. The assay was performed according to the manufacturer’s specifications.

We additionally quantitated NF-κB in DC by an activity assay recently developed by Marligen Biosciences using a Luminex technology. The assay is based on a specific binding of transcription factors to cognate sequences on labeled probes. Nuclear extracts were incubated with a mixture of PE-conjugate oligonucleotides containing appropriate cognate DNA binding sequences. This mixture was then incubated with a digestion reagent. In the presence of active transcription factor, label remains associated with the probes, whereas it is removed in the absence of transcription factor binding. Finally, the oligonucleotides were captured onto distinctively colored agarose microspheres that allow each of the reactions to be individually scored, and the quantitative signal generated by the label was detected with a Bio-Plex (Bio-Rad) reader. The amount of label remaining correlates with the amount of active transcription factor derived from the nuclear extract. This format allows better sensitivity and dynamic range compared to EMSA. Furthermore, quantitative results allow comparisons among treatments. The assays were performed according to manufacturer’s protocol.

**Statistical analysis**

For a single comparison of two groups, the Student t test was used after evaluation for normality. If data distribution was not normal, a Mann-Whitney rank sum test was performed. For the comparison of multiple groups, one- or two-way ANOVA was applied. For all statistical analysis, the level of significance was set at a probability of 0.05 to be considered significant. All experiments were repeated at least two or three times. Data are represented as the mean ± SEM.

**Results**

**Immunohistochemical analysis of human HNSCC tissues for infiltration by DC and expression of CXCL14**

First, we confirmed and expanded the published data concerning the reduction of DC numbers in the tumor mass when compared with nonmalignant tissues (14, 15). We analyzed a variety of paraffin specimens of HNSCC and oral dysplastic lesions for the presence of CD1a, S-100, CD83, and CD11c DC by immunohistochemistry. The biopsy specimens were from different patients diagnosed with mild to moderate dysplasia from oral mucosal sites, including buccal mucosa, lateral tongue, and floor of the mouth. For comparison, specimens were also obtained from patients diagnosed with invasive HNSCC from similar oral mucosal sites. The results of the analysis of multiple oral epithelial hyperplasia, and oral SCC specimens suggest that CD83- and CD11c-positive DC were essentially absent in HNSCC tissues, and the numbers of CD1a- and S-100-positive DC were markedly lower in the tumor tissues than in oral dysplasia lesions (Fig. 1A). These data allowed us to hypothesize that DC migration into the HNSCC tissues might be inhibited compared with their migration to the hyperplastic or premalignant lesions. It is likely that chemokines...
A and CXCL14 expression (B) in HNSCC, oral dysplasia, and normal oral tissue specimens. Five-micrometer sections were dried overnight, de-waxed, rehydrated, followed by Ag retrieval with 1 mM EDTA/NAOH solution (see Materials and Methods). A. For evaluating tumor-infiltrating DC, the following DC-specific Abs were used: CD83, CD1a, CD11c, and S-100. Secondary Abs were biotinylated with goat anti-mouse. B. For detection of CXCL14 expression in HNSCC tissues anti-CXCL14 Abs or control murine IgG2a were applied to the tissues overnight. Biotinylated horse anti-mouse secondary Abs were added for 30 min. Staining was developed with amino-9-ethylcarbazole and counterstained with hematoxylin. Positive staining is red-brown. The representative immunohistochemical data from the analysis of 10–12 specimens are shown.

The next series of experiments focused on evaluating the expression of CXCL14 protein in different human tumor cell lines. Tumor cells were cultured on microscopic slides for 48–72 h, and CXCL14 protein was detected by the immunocytochemical procedure. Human PBMC-derived monocytes stimulated with 0.5 µg/ml LPS for 6 h served as a positive control for the expression of CXCL14. Nonstimulated PBMC were used as a negative control. We found that all tested tumor cells, with the exception of PCI4B and FemX, were CXCL14-negative (Fig. 3D). Thus, HNSCC cell line SCC-15 and primary HNSCC cell lines PCI-13, PCI-16, and PCI-38 as well as prostate adenocarcinoma cell line LNCaP express no CXCL14 protein. Interestingly, CXCL14-negative HNSCC cell lines did not attract DC in a chemotaxis assay.

To determine whether DC could be attracted by a CXCL14, we compared its chemotactic activity toward DC with the known DC chemokines. Analysis of DC migration revealed that CXCL14 and two control DC chemokines fMLP, a prototypic bacterial chemotactic stimulus (26) and MIP-1α (27), all dose-dependently chemotactically activated human DC (Fig. 3A). For example, in the presence of 20 ng/ml MIP-1α migration of immature DC reached 6360 ± 650 cells/min vs 3620 ± 380 cells/min spontaneously transmigrated in control wells (p < 0.05). A comparable chemotactic activity of DC (5960 ± 568 cells/min, p < 0.05) was also detected in the presence of 200 ng/ml (20 nM) CXCL14 (Fig. 3A). Thus, CXCL14 is a potent DC chemokine with a chemotactic activity in the nanograms per milliliter range.

Next question was whether CXCL14 chemotacts both immature and mature DC. Fig. 3B demonstrates that only immature, but not mature DC, are chemotactically attracted by CXCL14. These data are in agreement with Shellenberger et al. (28) and the general concept that immature DC are attracted to nonlymphoid tissues where a number of potent DC chemokines, including CXCL14, may be ubiquitously expressed.

In the next set of experiments, we tested whether human tumor cell lines, including different HNSCC, prostate adenocarcinoma, and melanoma, might produce chemokines that could attract human DC in vitro. Cell-free conditioned media were collected from FemX melanoma, LNCaP prostate adenocarcinoma, and HNSCC cell lines SCC-15, PCI-13, PCI-16, PCI-38, and PCI-4B as described in Materials and Methods. Conditioned medium from normal human lymph node cell suspensions served as a positive control. Fig. 3C demonstrates that conditioned media from FemX melanoma cells (5620 ± 483 vs 3630 ± 250 cells in control, p < 0.005), normal lymph node cells (5880 ± 602 cells, p < 0.005), and PCI-4B (6340 ± 436 cells, p < 0.005), but not from LNCaP (3920 ± 286 cells) and HNSCC lines PCI-13 (3720 ± 405), PCI16 (3320 ± 241), and SCC-15 (3390 ± 301) (p > 0.1), displayed chemotactic activity toward human DC. Selective attraction of DC by several tumor cell lines raises the question whether it might correlate with the expression of CXCL14.

Chemoattractive properties of CXCL14 and tumor cell lines toward DC
CXCL14. The results of a representative experiment are shown as mean ± SEM. Three independent experiments have shown similar results. B. Immature (GM-CSF + IL-4, Day 6), but not mature (GM-CSF + IL-4 + TNF-α, Day 8) DC migrate toward CXCL14. The representative results are shown as mean ± SEM (n = 4). *, p < 0.05, one-way ANOVA and t test. C. Differential chemoattraction of human DC to different human tumor cell lines. The results from three independent experiments are shown and presented as the mean ± SEM. *, p < 0.05, Student t test. D. Immunohistochemical evaluation of CXCL14 expression in tumor cell lines was done as described in Materials and Methods. Note the correlation between CXCL14 expression in tumor cell lines (D) and their chemoattractive potential toward DC in a migration assay (C).

whereas CXCL14-expressing cell lines PCI-4B and FenX demonstrated significant chemoattractive potential for DC in the same assay (Fig. 3D).

Migration of DC toward CXCL14-transduced tumors in vitro and in vivo

Our results indirectly support the hypothesis that DC are chemoattracted toward CXCL14-producing cells and do not migrate toward at least certain types of tumors that have lost expression of this chemokine. To test this possibility in direct in vitro and in vivo experiments, we have generated a vector encoding human CXCL14, which was used for a stable transduction of human CXCL14-negative HNSCC cell lines. The HNSCC cell line PCI-16 was transduced with the human CXCL14 gene and, after selection, expression of CXCL14 protein was confirmed by Western blot (Fig. 4A). These data suggest that CXCL14-negative tumor cells could be efficiently engineered to produce high levels of CXCL14 protein. Functional activity of synthesized CXCL14 protein in tumor cells was next tested in vitro and in vivo experiments.

Next we demonstrated that human HNSCC tumor cells transduced with the CXCL14 gene attract significantly higher levels of human DC both in vitro and in vivo. First, cell-free supernatants from CXCL14-negative wild type PCI-16 cultures and PCI-16 cells transduced with CXCL14 were collected and tested for the attraction of human monocyte-derived DC in the chemotaxis assay. Fig. 4B shows that wild type PCI-16 cells did not attract DC (2100 ± 77 vs 2500 ± 105 cells transmigrated in control wells), whereas CXCL14-expressing PCI-16 cells were chemoattractive for DC (6400 ± 135 transmigrated cells, p < 0.05). Together with the results demonstrating no attraction of DC toward control neo-transduced tumor cells, this suggests that CXCL14-transduced tumor cells release biologically active CXCL14 protein. Second, we evaluated trafficking of human DC labeled with a fluorescent dye in SCID mice in vivo. Fluorescent-labeled human DC were i.v. injected in immunodeficient SCID mice (n = 5) bearing both wild type (or control neo-transduced) and CXCL14-transduced PCI-16 cells for 7 days. Two days later, tumors were harvested and fluorescent cells were examined on 6-µm sections immediately by confocal microscopy. The results revealed that infiltration of CXCL14-expressing tumors by labeled DC was significantly higher than in wild type or neo-transduced tumors in all tested mice (Fig. 4C). Similar data were obtained when nonlabeled human DC were i.v. transferred in SCID mice (n = 5) bearing PCI-16/wild type (or neo-transduced) and PCI-16/CXCL14 and infiltration of tumors by injected DC was assessed 48 h later by immunohistochemistry. Fig. 4D demonstrates that the levels of accumulation of CD1a+ human DC in CXCL14-expressing tumors were markedly higher than the number of DC in control tumors. Thus, these results suggest that the recovery of CXCL14 expression in HNSCC cells is associated with increased attraction of DC both in vitro and in vivo.

Regulation of DC function by CXCL14

We next tested whether CXCL14, in addition to being a DC chemoattractant, may also increase functional activity of DC. We first examined whether CXCL14 alters phenotype characteristics of DC. Fig. 5 shows that the addition of 200 ng/ml CXCL14 to DC markedly up-regulated expression of CD83, HLA-DR, CD86, and CD80 molecules when compared with CXCL14-unstimulated DC. For example, the percentage of CD83+ cells increased from 8.4 ± 0.9% in control DC cultures to 38.0 ± 2.3% in DC cultures treated with CXCL14 (p < 0.01). The same pattern was observed for the expression of CD86 and CD80 molecules on DC (Fig. 5). Interestingly, not only the percentage of DC expressing the specific markers was up-regulated after addition of CXCL14, but also the
levels of CD83, CD86, CD80, and HLA-DR expression on DC were significantly up-regulated (Fig. 5). For instance, the mean fluorescence intensity values for CD83 and HLA-DR markers were increased from 4.4 ± 0.5 in control DC to 15.9 ± 1.4 (p < 0.01) on DC treated with CXCL14 and from 123.6 ± 5.8 to 287.7 ± 9.9 (p < 0.01), respectively (Fig. 5). Thus, it is conceivable that CXCL14 stimulates maturation of DC.

Further confirmation of the biologic activity of CXCL14 was obtained in the MLR assay using DC generated from different donors with or without the addition of 200 ng/ml CXCL14 (Fig. 6A). Significantly higher induction of allogeneic T cell proliferation by CXCL14-treated DC (p < 0.01), as compared with untreated DC, was observed. For instance, at DC to T cell ratio 1:30, uptake of [3H]thymidine increased from 17252 ± 897 cpm in control to 30385 ± 689 cpm (p < 0.01) in group treated with CXCL14.

To explore the molecular mechanisms of CXCL14-mediated activation of DC, monocyte-derived DC were treated with CXCL14 (200 ng/ml) and TNF-α (50 ng/ml). The levels of p65 in nuclear extracts were determined using NF-κB Transcription Factor Assay kit. TNF-α served as a well-known activator of NF-κB in DC. Nuclear extract from Jurkat cells was used as internal control. We demonstrated that activation of DC with CXCL14 was accompanied by a significant up-regulation of NF-κB activity in DC up to 200% (p < 0.01) (Fig. 6B). Next, these data were confirmed and further explored by using Luminex-based technique for analyzing NF-κB activation (Fig. 6C). The results also demonstrated that CXCL14 is a strong inducer of NF-κB activation in human DC. Interestingly, the kinetic analysis of transcription factor activity revealed that NF-κB activation induced in DC by CXCL14 was delayed compared with TNF-α-induced activation reaching the maximum at 30 min (Fig. 6C).

In summary, these data suggest that CXCL14, in addition to being DC attractant, also increases functional activity of DC.

**Discussion**

We have demonstrated that expression of a new DC chemokine, CXCL14, is frequently lost in HNSCC tissues, which was accompanied by a low infiltration of the tumor by DC. We speculate that these findings may explain a decreased rate of DC attraction and thus augmented efficacy of tumor escape mechanisms.

CXCL14 was initially named BRAK because it was identified in human breast and kidney derived cells (12). CXCL14 (KS1, Kec, BMAC, NJAC, MIP-2γ) is a chemokine with an as yet unknown function and receptor selectivity (11, 12, 21). The mature sequences of CXCL14 and its murine analog SK1 contain 77 amino acids and are unique with regard to the short N-terminal end of only two amino acids (Ser-Lys), preceding the first of four chemokine-typical Cys residues. The most closely related chemokines, MIP-2α and MIP-2β, share ~30% amino acid identity with CXCL14. Kurth et al. (32) have recently provided evidence that...
CXCL14 is not a chemoattractant for peripheral blood T cells, B cells, and NK cells or neutrophils and is selectively chemotactic for monocytes activated by the cyclic AMP-elevating agents PGE2 and forskolin. The authors proposed that once monocytes enter tissues in response to local inflammation, PGE2 at the site renders them responsive to the high levels of CXCL14 in these tissues, attracting them to the subepithelial locations where they mature into macrophages. In contrast, others have reported that CXCL14 regulates trafficking of B cells (21), is a potent chemoattractant for neutrophils, and weak or inactive for DC, monocytes, NK cells, and T and B lymphocytes (33). Thus, the data on the biologic role of CXCL14 for chemoattraction of immune cells are controversial.

Our results demonstrate that human recombinant CXCL14 and CXCL14-transduced HNSCC cell line PCI-4B are potent inducers of DC migration in vitro and in vivo, whereas CXCL14-negative HNSCC cell lines and prostate adenocarcinoma cell line LNCaP do not attract DC in a chemotaxis assay. Several laboratories demonstrated that CXCL14 mRNA is constitutively expressed in normal tissues, but absent in a number of tumors (11, 12, 34). The majority of HNSCC and some cervical SCC show loss of CXCL14 mRNA. Analysis of the expression of 20,000 genes in human prostate epithelial cells passed to senescence revealed the CXCL14 gene among three genes whose expression was uniformly lost in human prostate cancer cell lines and xenografts (34). The loss of expression in tumors and the presence of CXCL14 in nonmalignant tissues suggest that this chemokine may play a role in host-tumor interactions. It is also possible that down-regulation of the CXCL14 gene expression in tumor cells might be beneficial for tumor growth. In agreement, our new data revealed that the growth of CXCL14-transduced murine HNSCC cell line B7E3/6 in syngeneic BALB/c mice was significantly inhibited in comparison with wild type tumors, which was associated with high infiltration by DC and CD8+ T cells (G. V. Shurin, R. Ferris, I. L. Tourkova, L. Perez, G. S. Chatta, and M. R. Shurin, manuscript in preparation).

Importantly, a leukocyte and chemokine balance in tumors can be manipulated. When murine tumors are engineered to overexpress certain chemokines, the increased intratumoral infiltrate stimulates antitumor responses. For instance, overexpression of CCL19 (MIP-3β) mediated rejection of murine breast tumors in an NK cell and CD4 T cell-dependent mechanism (35). CCL21 (6Ckine) reduced growth of colon adenocarcinoma in mice using a similar pathway (36). Overproduction of CCL20 (MIP-3α) might activate tumor-specific CTLs by attracting DC (37), whereas overproduction of secondary lymphoid tissue chemokine by DC may...

**FIGURE 5.** CXCL14 up-regulated DC maturation. Monocyte-derived DC were coincubated with CXCL14 (200 ng/ml, 72 h) and surface expression of CD83, CD80, CD86, and HLA-DR was assessed by FACScan. Both the percentage and the mean fluorescent intensity (%/MFI) are demonstrated. The results of a representative experiment are shown (n = 3).

**FIGURE 6.** CXCL14 stimulated APC function of human DC and up-regulates activation of NF-κB. A, CXCL14 (200 ng/ml, daily day 3–6) significantly up-regulates Ag-presenting activity of human DC in vitro, as was determined in an allogeneic MLR assay. Data are shown as mean ± SEM. *, p < 0.01, two-way ANOVA (n = 3). B, Monocyte-derived DC were treated with CXCL14 (200 ng/ml, 30 min) and p65 was assessed in nuclear extracts as described in Material and Methods. TNF-α (50 ng/ml, 15 min) served as well-known activator of NF-κB in DC. Nuclear extract from Jurkat cells was used as an internal control. The levels of p65 in nuclear extracts were determined using NF-κB Transcription Factor Assay kit (Active Motif). Data are expressed as mean ± SEM from two independent experiments. *, p < 0.05 (ANOVA). C, NF-κB activity in human DC was determined 0, 15, 30, and 60 min after stimulation with CXCL14 (200 ng/ml) or TNF-α (50 ng/ml) using Luminex-based technique as described in Materials and Methods. The results are shown as mean ± SEM from two independent experiments. *, p < 0.01 (ANOVA).
enhance T cell recruitment and immune priming to tumor-associated Ags (38). In fact, injection of recombinant secondary lymphoid tissue chemokine in the axillary lymph node region in mice with bilateral multilobar pulmonary adenocarcinomas led to a marked reduction in tumor burden with extensive lymphoctic and DC infiltration of the tumors and enhanced survival (39). Together with clinical evidence demonstrating that infiltration of tumor mass by DC is associated with a better patient survival, these results suggest that regulated induction of DC migration into the tumor site might induce efficient antitumor immune responses. However, there are no data on whether CXC cytokines play a role in attraction of immune cells to the tumor site and inducing antitumor immunity. We have shown, that genetic modification of CXCL14-negative PCI-16 HNSCC cell line with the CXCL14 gene results in stimulation of DC attraction in vitro and increased infiltration of the tumor by DC in vivo. In fact, we have shown on a murine HNSCC model that CXCL14-expressing tumors were highly infiltrated by CD11c+ DC suggesting their potential role in developing antitumor immune response at the tumor site (G. V. Shurin, R. Ferris, I. L. Tourkova, L. Perez, A. Lokshin, L. Balkir, B. Collins, G. S. Chatta, and M. R. Shurin, manuscript in preparation).

Next, we evaluated the effect of CXCL14 on DC function. It is known that chemokines may regulate cellular adhesion, proliferation, and cell survival (10, 18, 40). Based on the current knowledge of the life cycle of DC, it has been postulated that chemokines play an important role at several stages of DC development (18). Basal chemokine production and expression at the surface of endothelial cells can mediate DC precursor recruitment into peripheral tissues, which is important for the maintaining DC levels within tissues. Once in the tissue, chemokines, such as MIP-1α, MIP-1β, MIP-3α, MIP-5, MCP-3, MCP-4, RANTES, TECK, and SDF-1 (41), may participate in differentiation of DC precursors into immature DC that are programmed to pick up and process Ag(s). Upon initiation of an inflammatory response, chemokines that recruit immature DC may be up-regulated, resulting in DC accumulation within the tissue. When DC have matured, they enter tissue-draining lymphatic vessels and migrate to the T cell zones in secondary lymphoid organs under the influence of chemokines produced there, such as MIP-3β and 6Ckine. In the T cell zones, DC can produce chemokines that stimulate DC-T cell interaction, thereby enhancing the likelihood of clonal selection (18, 41). Our data show that CXCL14 chemotraffics only immature, but not mature DC, which is in agreement with the concept that nonlymphoid tissue chemokines should attract immature DC. Importantly, we demonstrated that CXCL14 also activated DC through NF-kB-dependent pathways and up-regulated expression of costimulatory molecules on DC as well as enhanced the proliferation of alloresponsive T cells in MLR. Thus our results support the hypothesis that CXCL14 might be a novel DC chemokine regulating their homing and activation in nonlymphoid tissues.

Disclosures

The authors have no financial conflict of interest.

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