Antitumor Effects of the Mouse Chemokine 6Ckine/SLC Through Angiostatic and Immunological Mechanisms

Alain P. Vicari, Smina Ait-Yahia, Karine Chemin, Anja Mueller, Albert Zlotnik and Christophe Caux


http://www.jimmunol.org/content/165/4/1992

**References**

This article cites 57 articles, 35 of which you can access for free at: http://www.jimmunol.org/content/165/4/1992.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Antitumor Effects of the Mouse Chemokine 6Ckine/SLC Through Angiostatic and Immunological Mechanisms

Alain P. Vicari, Smina Ait-Yahia, Karine Chemin, Anja Mueller, Albert Zlotnik, and Christophe Caux

Mouse 6Ckine/SLC (secondary lymphoid tissue chemokine) is a chemotactic factor for dendritic cells, T cells, and NK cells in vitro. In addition, mouse 6Ckine/SLC interacts with the chemokine receptor CXCR3, as do several chemokines with antiangiogenic properties. These dual properties of mouse 6Ckine/SLC were tested for the induction of an antitumor response by transducing the C26 colon carcinoma tumor cell line with a cDNA encoding mouse 6Ckine/SLC. The C26-6CK-transduced cells showed reduced tumorigenicity in immunocompetent or in nude mice. Part of this effect was likely due to angiostatic mechanisms as shown by immunohistochemistry and Matrigel assay. C26-6CK tumors were also heavily infiltrated with leukocytes, including granulocytes, dendritic cells, and CD8+ T cells. In vivo, anti-CD8 treatment increased the tumorigenicity of the C26-6CK tumor cells, and tumor-infiltrating CD8+ T cells had the phenotype of memory effector cells, suggesting the induction of cytotoxic tumor-specific T lymphocytes. On the other hand, anti-asialo-GM1 depletion also increased the tumorigenicity of C26-6CK cells, supporting the participation of NK cells. Finally, tumor-infiltrating dendritic cells had the phenotype and functional features of immature dendritic cells. Overall, these results suggest that mouse 6Ckine/SLC has strong antitumor effects by inducing both angiostatic, CD8+ T cell-mediated, and possibly NK-mediated tumor resistance mechanisms. The Journal of Immunology, 2000, 165: 1992–2000.

Dendritic cells are powerful APCs that possess the unique ability to prime naïve T cells in vivo (1). Several reports (2–6) suggested that manipulating the dendritic cell network could be used to redirect or amplify the immune response against tumors, as an alternative or additional cancer treatment besides chemotherapy and radiotherapy. However, most of these strategies rely on the differentiation of dendritic cells ex vivo and their subsequent reinfusion in vivo after a pulse with tumor Ags expressed as peptides, protein, viruses, or nucleic acids. A possible different strategy would be to favor the interaction between dendritic cells and tumor cells directly in vivo. Introduction of dendritic cell-specific chemokine genes into tumor cells could favor such interaction.

Chemokines belong to a family of small proteins that control the migration of certain leukocyte populations (7). In particular, 6Ckine/SLC3 (secondary lymphoid tissue chemokine) is a chemokine that originally was shown to be strongly expressed in secondary lymphoid organs, in particular in high endothelial venules and in the T cell zone of the lymph nodes (8–11). 6Ckine/SLC has been described as a potent chemotactic factor for dendritic cells (12), a property shared with other chemokines (13–15). This action of 6Ckine/SLC on dendritic cells is likely mediated through the chemokine receptor CCR7 (16, 17), which is strongly up-regulated during dendritic cell maturation (15). More precisely, 6Ckine/SLC has been shown to be involved in the migration of dendritic cells from the skin into the draining lymph nodes (12, 18), suggesting that 6Ckine/SLC would be active in vivo on dendritic cells of an intermediate stage of maturity after Ag capture (1), on their way to secondary lymphoid organs. 6Ckine/SLC is also a chemotactic factor for T and B cells (19), as well as NK cells (20). Collectively, these data suggest that 6Ckine/SLC may play an important role in the early phases of the immune response, when the encounter between dendritic cells and lymphocytes is required. In fact, mice deficient in the expression of 6Ckine/SLC showed defects in lymphocyte homing and dendritic cell localization (21), and mice lacking the CCR7 receptor also had defects in lymph node architecture (22). Furthermore, it has been very recently shown that 6Ckine/SLC could participate itself in lymphoid tissue development and organization, in a transgenic model where the 6Ckine/SLC gene was expressed in pancreatic islets (23).

In addition to CCR7, the mouse 6Ckine/SLC binds to the chemokine receptor CXCR3 (24). Ligands for CXCR3, such as the chemokines IFN-inducible protein 10 (IP-10) and the monokine induced by IFN-γ, Mig, possess angiostatic properties (25–28). Indeed, mouse (m) 6Ckine also was reported to inhibit angiogenesis in a rat corneal micropocket assay (24).

The potential broad spectrum of actions of 6Ckine/SLC, in the recruitment of dendritic cells and effector cells as well as in the inhibition of angiogenesis, prompted us to analyze its role in antitumor activity. To that aim, we inserted into the mouse colon carcinoma cell line C26 a cDNA coding for the mouse chemokine 6Ckine/SLC.

Materials and Methods

Mice

Female BALB/c (H-2b), BALB/c nu/nu, and C57BL/6 (H-2b) mice, 6 to 10 weeks old, were purchased from Charles River (Iffa-Credo, L’Arbresle, France).
France) and maintained in our facilities under standard conditions. Procedures involving animals and their care were conducted in conformity with European Economic Community Council Directive 86/609, OJL 358/1, December 12, 1987.

Tumor cell lines and in vivo procedures

All tumor cell cultures were performed in DMEM (Life Technologies, Paisley Park, U.K.) supplemented with 10% FCS (Life Technologies), 1 mM HEPES (Life Technologies), Gentallin (Schering-Plough, Union, NJ), 2 × 10⁻³ M β-ME (Sigma, St. Louis, MO). All cell cultures were performed at 37°C in a humidified incubator with 5% CO₂. The cDNA encoding m6Ckine/SLC was cloned into the pcDNA3 vector (Invitrogen, Carlsbad, CA) which contains a CMV promoter. C26 colon carcinoma tumor cells (provided by Mario P. Colombo, Instituto Nazionale per lo Studio e la Cura dei Tumori, Milan, Italy) were transfected with this construction using the Fugene reagent (Roche Molecular Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. Single C26 clones expressing m6Ckine/SLC mRNA (C26-6CK) were isolated after neomycin (Sigma) selection at 800 μg/ml. Tumor cells were injected s.c. in the right flank in 100 μl PBS 1 day before tumor inoculation, and mice were sacrificed at the onset of tumors. For Ab depletion or neutralization, 0.5 mg anti-CD8 (clone 2.43), anti-CD4 (GK-1.5), anti-Gr1 (RB6-8C5), anti-asialo GM1 (PharMingen), anti-CD11c (M1/70), anti-IL-2Rb (1D3), or anti-CD54 (3E2), biotinylated secondary Abs were injected i.p. in 200 μl PBS 1 day before tumor inoculation, then 0.2 mg Abs were injected after 3 days and once a week (or twice a week if CD11c expression on purified cells (usually 70 – 80% purity).

Immunohistochemistry

Tumors were removed from animals and embedded in OCT compound (Miles Laboratory, Elkhart, IN) before being snap frozen in liquid nitrogen and stored at −80°C until immunohistochemistry procedures. Cryostat sections (5 μm) applied on glass slides were fixed in acetone and incubated with 1% H₂O₂ for 10 min at room temperature. Slides were then incubated with the Fc-block (anti-human IgG and FcγR) prior to incubating with the primary Ab. As a negative control, sections were treated with PBS before incubation with the primary Ab. Balb/c (anti-asialo GM1 treatment) spleen cell compartments.

RT-PCR analysis of gene expression

Cell or tumor samples were lysed, and total RNA was extracted (31) and stored at −80°C. RT-PCR analysis of gene expression was performed after DNase I treatment (in the presence of RNase inhibitor) of 5 μg total RNA using oligo(dT) primers (Pharmacia, Uppsala, Sweden) and the Superscript kit. RT-PCR was performed using the AmpliTaq enzyme and buffer (Perkin-Elmer, Paris, France), dNTPs at 0.8 mM, and DMSO at a 5% final concentration. Cycle conditions were 92°C for 1 min, 60°C for 2 min, and 72°C for 3 min for 28 –35 cycles. The following primers were used in this study. β₂-microglobulin: TAGTCTTCCTGTTGCTGC (5’); m6Ckine/SLC: TGATGACTGGCTGCTCTAGG (5’); C26-6CK: TCAGTCTTCCTGTTGCTGC (5’); perforin: AGCTGAGAAGCCTATCAGG (5’); GATAAAGTGGCGTG CATAGG (3’); Fas ligand: CTGGTTTGGATGGTAAGT (5’), GAGG GATGTTGGACTGATG (3’); TNF-α: GTGAACTGCGAAGGAGGGA (5’), GGAGGGAGG ATGTAGCAAGAT (3’); CCR6: GTGAACTGCGAAGGAGGGA (5’), GAGGATCTGGATCTGTGAAGC (3’); CCR7: TGAGCTTCAGAGAAGTGGCCG (5’), ACGTATGGTCTAATGCTGATG (3’); CXCR3: TTGTGAGACCTTTCCAGC (5’), AAAC CCACTTGGACGACATT (3’).

Real time quantitative PCR (TaqMan) analysis of CXCR3 and CCR7 mRNA expression

cDNA was diluted to a final concentration of 5 ng/μl. 10 μl cDNA were amplified in the presence of 12.5 μl TaqMan universal master mix (Perkin-Elmer, Foster City, CA), 0.625 μl gene-specific TaqMan probe, 0.5 μl gene-specific forward and reverse primers, and 0.5 μl water. As an internal positive control, 0.125 μl 18S RNA-specific TaqMan probe and 0.125 μl 18S RNA-specific forward and reverse primers were added to each reaction. Specific primers and probes for CXCR3 and CCR7 were obtained from Perkin-Elmer. Gene-specific probes used FAM as reporter, whereas probes for the internal positive control (18S RNA) were associated with the VIC reporter. Samples underwent the following stages: stage 1, 50°C for 2 min; stage 2, 95°C for 10 min; and stage 3, 95°C for 15 s by 60°C for 1 min. Stage 3 was repeated 20 times. Gene-specific RT-PCR products were quantified by means of an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer) continuously during 40 cycles. Specificity of probe/primer combination was confirmed in cross-reactivity studies performed against plasmids of all known chemokine receptors (CCR1–CCR9, CXCR1–CXCR5, CXCR1, CX3CR1). Target gene expression was normalized between different samples based on the values of the expression of the internal positive control.

FACS analysis of tumor-infiltrating leukocytes and lymph node cells

Tumors were surgically removed when reaching an approximate size of 1 cm. The tumor mass was minced into small fragments and incubated in collagenase A (Roche Molecular Biochemicals) solution for 30 min at 37°C under agitation. The suspension was then washed three times in DMEM. In some experiments, an adherence step of 20 h was performed, without any noticeable change in the phenotype of dendritic cells. Single-cell suspensions of draining lymph nodes were also prepared, without the digestion step. Staining of cell suspensions was performed in PBS + 5% FCS. Before incubation with FITC-, biotin- or PE-labeled specific Abs, Fc receptors were blocked using Fc-Block CD16/CD32 Ab (PharMingen). The various Abs (all from PharMingen excepted DEC-205) used in this study were CD4 (clone GK1.5), CD8β (53–58), CD11b (M1/70), CD11c (HL3), CD19 (1D3), CD40 (HM440–3), CD80 (16–10A1), CD86 (GL1), DC-SIGN (3E2), H-2Kd (SF1-1.1), EGF (269), Gr-1 (RB6-8C5), CD3 (145-2C11), CD44 (IM7), CD62L (MEL-14), DEC-205 (NLC-45 hybridoma from American Type Culture Collection, Manassas, VA). Biotinylated Abs were revealed with PE-streptavidin (Becton Dickinson). Phenotypic parameters were acquired on a FACScan (Becton Dickinson, Mountain View, CA) and analyzed using CellQuest software (Becton Dickinson).

Chemotaxis assay

C26 or C26-6CK cells were cultured at 2 × 10⁵ cells/ml in RPMI + 5% FCS for 48 h, and culture supernatants were collected to test their activity in migration assays. Cells used in chemotaxis assays were dendritic cells obtained after culture of bone marrow cells from BALB/c mice in the presence of 10 ng/ml mGM-CSF (Schering-Plough, Kenilworth, NJ) and 100 U/ml TNF-α (R&D Systems) for 5 days. Chemotaxis assays were performed in 5-μm-pore Transwells (Costar, Cambridge, MA) for 2 h at 37°C. Briefly, 600 μl C26 or C26-6CK supernatant in the presence of 10 μg/ml goat IgG or polyclonal anti-m6Ckine neutralizing Ab (R&D Systems) were placed in the lower chamber, and 2 × 10⁵ dendritic cells were placed in the upper chamber. Migrated cell were collected, stained for CD11c and CD86 expression, and analyzed by FACS after resuspension in a defined volume. The number of cells acquired during 2 min was assessed for each condition and used to determine the relative number of migrating cells for CD11c and CD86 defined subsets.

Isolation of tumor-infiltrating dendritic cells

Tumor-infiltrating dendritic cells were purified using CD11c⁺ Microbeads, according to the manufacturer’s instructions (Miltenyi, Bergisch Gladbach, Germany). The Ab used for purification (clone N-418) is different from the one used for FACS analysis and the analysis of CD11c expression on purified cells (usually 70 –80% purity).
Measure of endocytosis using FITC-dextran capture

The procedure for measuring FITC-dextranuptake has been previously described (32). Briefly, purified tumor-infiltrating dendritic cells were re-suspended in 10% FCS medium buffered with 25 mM HEPEs at 37°C in a water bath or at 4°C for negative control. FITC-dextran was added at the final concentration of 0.1 mg/ml. At different time points, the cells were washed four times with cold PBS containing 1% FCS and 0.01% NaN₃. Cells were then stained with CD11c-biotin followed by streptavidin-PE at 4°C and analyzed by flow cytometry.

Mixed leukocyte reaction

For MLR, T lymphocytes were obtained from C57BL/6 (H-2b) mice by depletion of lymph node cell suspensions with anti-CD19, anti-Ia, anti-CD11b, and anti-TER-119 Abs followed by anti-rat magnetic beads. T cells obtained by this method were routinely >98% in purity as assessed by FACS analysis. Control mature bone marrow-derived dendritic cells were obtained after culture of bone marrow cells from BALB/c mice in the presence of 10 ng/ml mGM-CSF (Schering-Plough, Kenilworth, NJ) and 100 U/ml mTNF-α (R&D Systems) for 6 days, with the addition of 10 ng/ml LPS (Sigma) for the last day of culture. Triplicates of 3 × 10⁶ purified T cells were incubated with various numbers of stimulator cells irradiated at 3000 rad in 200 µl 10% FCS culture medium in round-bottom 96-well plates (Nunc, Kamstrup, Denmark) for 5 days at 37°C. The proliferative response was measured by incorporation of [³H]thymidine (Amersham, Les Ulis, France) for the last 18 h of culture. In some experiments, anti-IL-10 Ab (gift from A. O’Garra, DNAX Research Institute, Palo Alto, CA) or control GL113 Ab were added during the culture at 3 × 10³ µg/ml concentration.

Statistical analysis

Statistical analysis of results was performed with Statview software (Abacus Concepts, Berkeley, CA). The χ² test was used to analyze differences between percentages of tumor-bearing mice at a given time, the log rank test was used to analyze curves of tumor development. Student’s t test was used in other analyses when indicated.

Results

Gene transfer of m6Ckine/SLC in the C26 tumor cell line

We transfected the m6Ckine/SLC cDNA into the C26 colon carcinoma cell line and obtained an antibiotic-resistant clone (C26-6CK) that expresses m6Ckine/SLC mRNA as detected by RT-PCR and protein expression (Fig. 1). The supernatant of C26-6CK but not the one of C26 cells was able to induce the chemotaxis of mouse bone marrow-derived dendritic cells (Fig. 1B). This specific chemotactic activity was blocked by a neutralizing anti-m6Ckine polyclonal Ab. There was no difference in cell growth in vitro between the C26 and C26-6CK lines (data not shown). Results presented in this report were obtained with the C26-6CK clone. However, to rule out that the effects were related to a variation of this clone compared with the parental cell line, independent of m6Ckine expression, we recently constructed a C26 cell line expressing m6Ckine/SLC, using a retroviral vector. The tumorigenicity of this C26-6CK cell line was identical with that of the C26-6CK clone.

Tumorigenicity of C26 cells after m6Ckine/SLC gene transfer

C26 and C26-6CK cells were injected s.c. into BALB/c immunocompetent at different doses (Fig. 2A). At the doses of 10⁵, 5 × 10⁵ cells, or 5 × 10⁶ cells, the C26-6CK line showed lower tumorigenicity than the parental cell line (Fig. 2). Moreover, at a 10⁷ cell dose, mice injected with C26-6CK cells did not develop tumors. As a control, a C26 tumor cell line transduced to express β-galactosidase showed the same tumorigenicity than the parental tumor (data not shown). At a 5 × 10⁴ cell dose commonly used with the C26 tumor, that always gives a 100% tumor incidence in our hands, only 55% of mice developed tumors after injection of C26-6CK cells (Fig. 2A). At this 5 × 10⁴ cell dose, the difference between the proportions of mice bearing tumors after C26 or C26-6CK cell injection was statistically significant at 45 days (p < 0.05 by χ² test). Moreover, log rank analysis of the two tumorigenicity curves showed that C26-6CK tumors appeared with a significant delay (p < 0.01) when compared with C26 tumors. In nude mice, all mice injected with 5 × 10⁴ C26 or C26-6CK cells developed tumors (Fig. 2B, left). However, the apparition of tumors was also significantly delayed with C26-6CK cells (p < 0.05 by log rank analysis). In addition, the tumors recovered at 28 days from nude mice injected with C26-6CK cells were significantly smaller than those recovered from mice injected with the parental cell line (p < 0.01 by Student’s t test) (Fig. 2B, right). These data indicate that m6Ckine/SLC gene transfer into C26 tumor cells decreases their tumorigenicity, in immunocompetent mice or in the absence of T cell-mediated immunity.

Angiogenesis induced by C26 and C26-6CK tumor cells

We analyzed the vasculature of C26 and C26-6CK tumors in BALB/c mice using an anti-CD105 Ab in immunohistochemistry. As shown in Fig. 3A, C26-6CK tumors had a much lower density of blood vessels than C26 tumors. Similar results were obtained by staining with an anti-CD31 Ab (data not shown). To quantitatively examine the angiogenesis induced in vivo by C26 or C26-6CK tumor cells, Matrigel pellets containing 2 × 10⁶ tumor cells were injected s.c. into BALB/c mice. After 9 days, the Matrigel pellets were recovered, and their hemoglobin content was measured, as a representation of angiogenesis (Fig. 3B). m6Ckine/SLC gene transfer significantly inhibited angiogenesis induced by the C26 tumor cell line (p < 0.05 by Student’s t test).

FIGURE 1. Expression of m6Ckine/SLC in C26 and C26-6CK tumors. The cDNA-encoding m6Ckine/SLC was transfected into the C26 cell line, and expression of m6Ckine/SLC was analyzed on individual antibiotic-resistant clones. A positive clone for m6Ckine/SLC expression was called C26-6CK and used throughout this study. A. RT-PCR analysis (35 cycles) of β₂-microglobulin (β2-2m) and m6Ckine/SLC mRNA expression in C26 and C26-6CK cell lines or in C26 and C26-6CK tumors 25 days after implantation. B. Secretion of bioactive m6Ckine by C26-6CK cells. Supernatants from C26 or C26-6CK cells were tested for their ability to induce the migration of bone marrow-derived dendritic cells in vitro, in the presence of anti-m6Ckine Ab or control IgG. The number of migrated CD11c⁺,CD86⁺ dendritic cells is indicated for each condition (mean ± SD of triplicates).
C26 (left) or C26-6CK (right) or C26-6CK (right) cells at different doses were injected s.c. in the right flank of female BALB/c mice (6 mice for the 10^4 and 5 x 10^5 doses, 16 mice for the 5 x 10^5 dose). The presence of palpable tumor was checked three times a week. B, 5 x 10^5 C26 or C26-6CK cells were injected s.c. in the right flank of female BALB/c-nu/nu mice (n = 7). The presence of palpable tumor was checked three times a week (left). At 28 days, tumor size was measured for both groups (right). See Results for statistical analysis.

Leukocyte infiltration of C26 and C26-6CK tumors

We analyzed the phenotype of leukocyte population infiltrating C26 and C26-6CK tumors by immunohistochemistry (Fig. 4A) or FACS analysis after collagenase digestion (Fig. 4B). 20–30 days after tumor cell injection. Compared with C26, C26-6CK tumors were characterized by a rich infiltrate of CD11b^+CD11c^- (presumably dendritic cells) and CD11b^+Gr1^- cells as well as a significant increase in CD8^+ cells (Fig. 4A). The tumor-infiltrating leukocytes, including dendritic cells, did not show any particular localization within the tumor in contrast with some human cancers (33, 34), although they were absent from highly necrotic areas and could sometimes form foci at the vicinity of blood vessels (data not shown). After FACS sorting and May-Grunwald-Giemsa (MGG) staining, CD11b^+Gr1^- cells were identified as polymorphonuclear neutrophils (data not shown). Further characterization of the CD11b^+CD11c^- and CD8^+ cells will be shown later in this report. There was no increase of CD4^+ T cells, which represented in both cases <0.5% of the tumor suspension and no significant differences in the percentage of CD11c^-CD11b^- monocyte/macrophages (data not shown). Furthermore, in either C26 or C26-6CK tumors, we did not detect any CD19^- B cells (data not shown).

Relative expression of CXCR3 vs CCR7 mRNA in C26-6CK tumors

Mouse 6Ckine/SLC binds both CCR7 (16, 17) and CXCR3 (24) receptors. To analyze the contribution of these receptors to the cellular infiltration in C26-6CK tumors, we performed real time kinetic RT-PCR on cDNA prepared from C26 and C26-6CK tumors, with 18S mRNA expression as internal control. As shown in Fig. 5, there was no significant change in CXCR3 mRNA expression between C26 and C26-6CK tumors, whereas CCR7 mRNA expression was dramatically increased in C26-6CK tumors when compared with C26 tumors.

Contribution of leukocyte subsets and cytokines in tumor growth delay

To determine the relative contribution of particular leukocyte subsets or cytokines to the tumor growth delay observed in C26-6CK vs C26 tumors, we injected mice with depleting or neutralizing Abs during tumor challenge, as indicated in Materials and Methods. Abs depleting CD4^- or Gr1^- cells, or neutralizing IFN-γ or IL-4 had no significant effect (data not shown). On the contrary, an Ab depleting CD8^- T cells significantly (p < 0.05) increased the tumorigenicity of C26-6CK tumors (Fig. 6A). The same Ab treatment had no significant effect on the growth of C26 tumors. By FACS analysis, CD8^- cells infiltrating C26-6CK tumors expressed CD3 and CD44 but not CD62L, suggesting an effector- or memory-type T cell phenotype (Fig. 6C). These CD8^- T cells, after isolation by FACS sorting to >98% purity, expressed perforin and Fas ligand mRNA, a feature of cytotoxic T cells. They also expressed CCR7, albeit at low levels, but not CXCR3 mRNA (Fig. 6B), suggesting again that interactions with CCR7 were involved in their recruitment. Finally, besides anti-CD8 treatment, anti-asialo-GM1 treatment also significantly (p < 0.05) increased the tumorigenicity of C26-6CK cells (Fig. 7). We did not find, however, a significant infiltration of DX5^-IL-2Rβ^- NK cells at the time we collected C26-6CK tumors. Collectively, these data suggest that both CD8^- T cells and NK cells participate in the reduced tumorigenicity of C26-6CK cells.
Phenotype and chemokine receptor expression of C26-6CK tumor-infiltrating dendritic cells

The high number of dendritic cells infiltrating C26-6CK tumors allowed for their isolation using CD11c-coated magnetic beads (Fig. 8A). Most CD11c+ cells recovered from C26-6CK tumors had a dendritic morphology as seen after MGG staining (Fig. 8B). Moreover, RT-PCR analysis showed that dendritic cells purified from C26-6CK tumors expressed both CCR6 and CCR7 mRNA and relatively low levels of CXCR3 mRNA, resembling immature dendritic cells. Because immature dendritic cells are poor stimulators of naive T cells (1), we analyzed the capacity of dendritic cells from C26-6CK tumors to stimulate T cells in a MLR. We found that dendritic cells from C26-6CK tumors were poor stimulators in MLR, when compared with in vitro bone marrow-derived mature dendritic cells (Fig. 9C). Although the absence of CD86 expression by itself could explain a lower stimulatory capacity, it is possible that factors produced by contaminating macrophages or other cells in our dendritic cell preparation could interfere during the MLR. In particular, we ruled out a contribution of IL-10, because the addition of an anti-IL-10R Ab had no effect on the MLR, compared with a control Ab (data not shown).

Recruitment of dendritic cells in C26-6CK tumors does not affect dendritic cell populations in draining lymph nodes

The high number of dendritic cells in C26-6CK tumors could be due to a direct recruitment via m6Ckine/SLC. Conversely, m6Ckine/SLC expression could prevent dendritic cells from migrating outside the tumor. We injected 10^5 C26 or C26 tumor cells in the rear footpad of mice and analyzed dendritic cells in the popliteal draining lymph node (three determinations from pools of two mice). We found no significant difference between the number of dendritic cells in mice injected with C26 cells (10.24 ± 2.01 × 10^4 CD11c+ cells per lymph node) compared with mice injected with C26-6CK cells (13.44 ± 1.77 × 10^4 CD11c+ cells per lymph node). In addition, the expression of CD40, CD80, CD86, and MHC class II in lymph node dendritic cells was similar in both cases (data not shown).

Discussion

In several studies, cytokine genes have been introduced into tumor cells as an attempt to provide a microenvironment that favors innate and/or acquired immune mechanisms to prevent or reverse tumor development (35). In particular, chemokine gene transfer offers the possibility to trigger the recruitment of initiators or effectors of the immune response within the tumor (36–40). Besides its potential value for the design of anticancer treatments, this method also addresses the biology of chemokines in vivo. The chemokine m6Ckine/SLC has been proposed to play a role in favoring the interactions between dendritic cells and T cells in secondary lymphoid organs, through its interaction with the CCR7 receptor (21, 22). In addition to CCR7, m6Ckine/SLC also interacts with the CXCR3 receptor, which ligands IP-10 and Mig can block angiogenesis in vivo (41) and are chemotactic factors for
activated T cells (42, 43). These potentially dual properties prompted us to express 6Ckine/SLC in tumor cells by gene transfer. The present report shows that 6Ckine/SLC gene transfer decreases the tumorigenicity of the C26 colon carcinoma cell line inducing both angiostatic and CD8+ T cell-mediated tumor resistance mechanisms.

To date, the known angiostatic chemokines structurally belong to the CXC family (26). Thus, 6Ckine/SLC is the first example of a CC chemokine that has angiostatic properties. Moreover, it has not been proved that the angiostatic properties of IP-10 and Mig are mediated through CXCR3. The fact that m6Ckine both binds to CXCR3 and has angiostatic properties is a strong support for a role of CXCR3 in the control of angiogenesis. Of importance, the human 6Ckine/SLC protein does not bind to CXCR3 (44). The introduction of the human 6Ckine/SLC gene into tumors shall define the effect of 6Ckine/SLC on tumor development in the absence of CXCR3-mediated effects.

C26-6CK tumors were characterized by a much richer leukocyte infiltrate than C26 tumors. The strong induction of CCR7 vs CXCR3 mRNA expression in C26-6CK tumors suggests that interactions with CCR7 rather than with CXCR3 were responsible for leukocyte recruitment. It is possible, however, that CXCR3 and CCR7 expression on leukocytes would be differentially regulated within the tumor milieu after recruitment, for example, by m6Ckine/SLC-induced down-regulation, as it has been shown for CCR2 (reduced expression) vs CCR1 and CCR5 (normal expression) in tumor-infiltrating monocytes compared with blood monocytes (45). The CCR7 receptor is particularly resistant to ligand-induced down-regulation (46). C26-6CK tumors were infiltrated by granulocytes. There are no reports of a chemotactic activity of 6Ckine/SLC on granulocytes. It is possible that the pattern of in vivo chemokine responsiveness of granulocyte subpopulations may be different from what is generally observed in vitro (47). Alternatively, m6Ckine/SLC gene transfer may induce secondary mediators that would recruit granulocytes within the tumor. Although in some experimental models granulocyte infiltration was shown to be responsible for decreased tumorigenicity after cytokine gene transfer (48, 49), in our model, similarly to other models (50), granulocyte infiltration was not found to play a significant role.

Although we did not observe mature NK cells within the tumor, we found that anti-asialo-GM1 treatment increased the tumorigenicity of C26-6CK tumor cells, supporting a role for NK cells in this system. NK cells have been shown to respond to CCR7 ligands 6Ckine/SLC and macrophage inflammatory protein-3β/EBI-1 ligand chemokine in vitro (20) and, recently, the introduction of the macrophage inflammatory protein-3β/EBI-1 ligand chemokine in a mouse breast cancer cell line was shown to trigger NK cell-mediated tumor rejection (58). However, we were unable to detect mature NK cells within C26-6CK tumors. Further studies, notably using mice expressing NK1.1- and NK-dependent tumor models, will clarify the role of NK cells in m6Ckine/SLC-transduced tumors.
A significant recruitment of CD8\(^+\) T cells was observed in C266CK tumors, and the decreased tumorigenicity of the C26-6CK cell line compared with the parental cell line was partially reverted by anti-CD8 Ab treatment. These results point to a role for CD8\(^+\) T cells in tumor growth delay. The CD8\(^+\) tumor-infiltrating T lymphocytes expressed perforin and Fas ligand mRNA, suggesting potential cytotoxic functions. However, their cytotoxic activity remains to be tested. These CD8\(^+\) T cells also expressed CCR7 at the mRNA level. Recently, the expression of CCR7 in circulating naive and memory T cell subsets has been analyzed (51, 52). Although naive and effector memory T cells do not express CCR7, a circulating subset of T cells called central memory T cells expresses CCR7 and can differentiate into effector cells (52). According to these observations, m6Ckine/SLC expression in the C26 tumor could promote the recruitment of this intermediate type of T cells, although we do not have clear evidence for that at the present time. CD4\(^+\) T cells were rare in C26-6CK tumors. In vitro, 6Ckine-recruited CD4\(^+\) T cells express CCR7 (17, 19). The condition of m6Ckine/SLC expression in the tumor, the lack of expression of necessary molecules in the tumor blood vessels or a rapid cell death after recruitment may explain this discrepancy between in vitro and in vivo results.

The recruitment of cytotoxic CD8\(^+\) T cells within C26-6CK tumors strongly suggests a priming of the immune system against the tumor. Indeed, C26-6CK tumors were also characterized by a rich infiltrate of dendritic cells, a cell type that plays a major role in triggering antitumor immune responses through the presentation of tumor Ags to naive T cells (1). Dendritic cells from C26-6CK tumors were presumably of myeloid origin (CD11b\(^+\)CD8\(\alpha\)\(^-\)) as opposed to a described CD8\(\alpha\)\(^+\) subset of dendritic cells of lymphoid origin (53). These dendritic cells had an immature phenotype, for example, lacking expression of CD86. They also expressed a chemokine receptor pattern similar to mouse immature dendritic cells, a cell type that plays a major role in Ag capture. Uptake of dextran-FITC by CD11c-enriched C26-6CK tumor-infiltrating dendritic cells (DC) at 37°C (○) or 4°C (□). Representative of three experiments. C, Stimulation of allogeneic T cells by irradiated CD11c-enriched C26-6CK tumor-infiltrating dendritic cells (□) and bone marrow-derived dendritic cells (●) in a MLR reaction. Representative of three experiments.
this hypothesis, it has been proposed that inhibition of dendritic cell function would be a mechanism by which tumors could escape immune surveillance (54–56).

Despite a strong recruitment of dendritic cells and a significant growth delay of C26-6CK tumors compared with parental tumors, we did not observe regression once tumors were established, suggesting that the antitumor response was not efficient enough. The role of CD$^+$ T cells in the delayed tumorigenicity of the C26-6CK tumor suggests that, indeed, the priming of the immune response occurred, although we ignore whether it took place within the tumor or in secondary lymphoid organs. Of note, the introduction of the 6Ckine gene into pancreatic islets has been shown to drive the development of an ectopic lymphoid tissue (23). Thus, it seems that in some conditions, 6Ckine by itself could promote the establishment of the complex environment favorable to T cell priming. To explain our data, one hypothesis would be that the expression of m6Ckine/SLC within the C26 tumor prevents dendritic cells from migrating to the draining lymph node and restricts the immune response to the tumor. Dendritic cell populations from draining lymph nodes were not decreased in mice bearing C26-6CK tumors. This could mean that dendritic cells are still able to migrate from the tumor to the lymph node. On the other hand, one could hypothesize that the strong recruitment of dendritic cells within C26-6CK tumors would lead to a similar increase in the draining lymph population, if the trafficking from the tumor to lymph node is not impaired. However, we still lack information to support one hypothesis or the other: 1) we do not know at the present time whether draining lymph nodes contain dendritic cells that have come from the tumors; 2) we do not know what could be the half-life of dendritic cells migrating from the tumor to the lymph node. If these dendritic cells are short-lived, one would not expect a dramatic increase in lymph node dendritic cell number.

Conversely, another hypothesis for a lack of an optimal immune response is that a strong antitumor response depends on the presence of mature but not immature dendritic cells within the tumor, that could support the activation, recruitment, and/or expansion of effector cells at the vicinity of their targets. Interestingly, the gene transfer of both GM-CSF and CD40 ligand (CD154) in the same C26 tumor cell line led to tumor rejection in a proportion of mice, associated with a rich infiltrate of mature CD8$^+$ dendritic cells as well as CD4$^+$ and CD8$^+$ T cells (57). In humans, mature but immature dendritic cells have been observed in contact with T cells within human breast tumors, suggesting an ongoing immune response (33). Thus, an activation/maturaion signal such as CD154, delivered to dendritic cells within the tumor, may be necessary for an efficient immune response. We will introduce the CD154 gene into the C26-6CK tumor cell line to test this hypothesis.

To conclude, our experimental approach of transducing the m6Ckine/SLC gene into C26 tumor cells has provided information on the biology of this chemokine in vivo, including its angiostatic properties and its ability to induce dendritic cell recruitment. Future experiments will define the value of m6Ckine/SLC gene transfer, as well as other dendritic cell-specific chemokine genes, in other tumor models, as well as the requirement for dendritic cell activation signals to achieve strong antitumor immunity in these models.

Acknowledgments
We thank G. Trinchieri for critically reading the manuscript; M. P. Co- lombo for helpful discussions; I. Durand for expert cell sorting; and M. Vatan, D. Leport, and O. Clear for help in various aspects of this work.

References


