

Antitumor Effect by Interleukin-11 Receptor α -Locus Chemokine/CCL27, Introduced into Tumor Cells through a Recombinant Adenovirus Vector¹

Jian-Qing Gao, Yasuhiro Tsuda, Kazufumi Katayama, Takashi Nakayama, Yutaka Hatanaka, Yoichi Tani, Hiroyuki Mizuguchi, Takao Hayakawa, Osamu Yoshie, Yasuo Tsutsumi, Tadanori Mayumi, and Shinsaku Nakagawa²

Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871 [J.-Q. G., Y. Tsuda, K. K., Y. Tsutsumi, T. M., S. N.]; Department of Microbiology, Kinki University School of Medicine, Osaka-Sayama, Osaka 589-8511 [T. N., O. Y.]; Department of Biomedical Science, DakoCytomation Company Ltd., Nishinotoin-higashiiru, Shijo-dori, Shimogyo, Kyoto 600-8493 [Y. H., Y. Ta.]; and Division of Cellular and Gene Therapy Products, National Institute of Health Sciences, Setagaya-ku, Tokyo 158-8501 [H. M., T. H.], Japan

ABSTRACT

In this study, we examined antitumor activity of a mouse CC chemokine ILC/CCL27 and a mouse CX₃C chemokine fractalkine/CX₃CL1 *in vivo*. We generated recombinant adenovirus vectors with a fiber mutation, encoding mILC (Ad-RGD-mILC) and mFKN (Ad-RGD-mFKN). We confirmed tumor cells infected with Ad-RGD-mILC and Ad-RGD-mFKN to express and release these chemokines. Tumor rejection experiments *in vivo* were carried out by inoculating OV-HM cells infected with Ad-RGD-mILC or Ad-RGD-mFKN into immunocompetent mice. mILC significantly suppressed the tumor growth, whereas no such significant effect was observed by mFKN. The antitumor activity induced by mILC was T cell dependent, involving both CD4⁺ and CD8⁺ T cells. Immunohistochemical analysis revealed accumulation of both CD3⁺ lymphocytes and NK cells in the tumor tissue transduced with mILC and mFKN. However, there was a significant difference in the distribution of infiltrating cells. Furthermore, mFKN appeared to have an angiogenic activity, which might have masked its tumor suppressive activity. Collectively, ILC/CCL27 may be a good candidate molecule for cancer gene therapy.

INTRODUCTION

Considerable attention has been paid recently to the application of chemokines to cancer immunotherapy, because of their chemotactic activity for a variety of immune cells, as well as angiostatic activity of some chemokines, such as IFN-inducible protein-10/CXCL10 and Mig/CXCL9. In addition, it has been known that some tumor cells express a lower level of chemokines than that of normal cells (1). The tumor suppressive activity of several chemokines was also observed after these genes had been transduced into a variety of experimental tumors (2–9). Tumor cells that were transduced with the CC chemokine, macrophage inflammatory protein-1 α /CCL3, had reduced tumorigenicity and significantly increased infiltration of macrophage and neutrophil (7). A strong leukocyte-mediated inflammatory response was observed in tumors expressing macrophage inflammatory protein-1 α , leading to the induction of strong antitumor CTL responses (8). Another CC chemokine, macrophage-derived chemokine/CCL22, also had a strong chemoattractant activity for dendritic, NK,³ and T cells, resulting in tumor regression in a murine lung carcinoma model by its efficient induction of antitumor immunity (9).

More than 40 chemokines have thus far been well characterized (10), but only a few have been demonstrated as candidates for cancer therapy by using as sole agents or with adjuvant. In the present study,

a CC family chemokine, ILC/CCL27, and a CX₃C chemokine, FKN/CX₃CL1, have been studied. ILC is expressed in the skin and selectively chemoattracts CLA⁺ memory T and Langerhans cells (11, 12). FKN shows chemotactic activity for NK cells, T cells, and monocytes (13, 14). FKN is expressed by dendritic cells, and the expression is up-regulated on dendritic cell maturation (15). We hypothesized that if tumor cells could be genetically modified *in vitro* to produce chemokines *in vivo*, the chemokines would accumulate T cells in the tumor. The *in vivo* interaction of T cells with the tumor cells should induce antitumor immunity, resulting in suppression of tumor growth. To test this hypothesis, we used a recombinant adenovirus vector with a fiber mutation (Ad-RGD) containing the Arg-Gly-Asp (RGD) sequence in the fiber knob. This vector has been demonstrated to possess higher transduction and antitumor activities when we used it for cytokine gene therapy against melanoma, compared with conventional adenovirus vectors (16, 17). In this study, the ovarian carcinoma OV-HM cell line (18) was infected with a chemokine-encoding vector, Ad-RGD-mILC or Ad-RGD-mFKN, and inoculated into mice to test its antitumor activity in gene immunotherapy. Ad-RGD-mILC induced the local recruitment of immune cells and suppressed tumor growth. By contrast, Ad-RGD-mFKN did not have such significant antitumor activity against OV-HM cells, although the tumor cells also attracted infiltrating immune cells.

MATERIALS AND METHODS

Cell Lines and Animals. OV-HM ovarian carcinoma cells were kindly provided by Dr. Hiromi Fujiwara (School of Medicine, Osaka University, Japan) and maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS. B16/BL6, A549 lung carcinoma, and human embryonic kidney 293 cells were cultured in DMEM supplemented with 10% FBS. L1.2-mCCR10 and L1.2-mCX₃CR1 cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS and 2-ME (50 μ M; Life Technologies, Inc.). All of the cell lines were cultured at 37°C in a humidified atmosphere with 5% CO₂. Female B6C3F1 and BALB/c nude mice were purchased from SLC, Inc. (Hamamatsu, Japan) and used at 6–8 week of age. All of the experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Adenovirus Vectors. Replication-deficient adenovirus vectors with a fiber mutation used in this study were based on the adenovirus serotype 5 backbone with deletions of E1 and E3 and the expression cassette in the E1 region (19). The RGD sequence was inserted into the HI loop of the fiber knob using a two-step method developed by Mizuguchi *et al.* (20). Murine chemokine genes derived from pFastBac1-mILC and pBlueScriptSK(+)-mFKN were used as sources of their cDNA. Recombinant adenovirus vectors with the RGD fiber mutation, Ad-RGD-mILC and Ad-RGD-mFKN carrying the chemokine cDNA under the control of the cytomegalovirus promoter, were constructed by an improved *in vitro* ligation method as described (19, 21). The Ad-RGD-NUL vector, serving as a negative control, is identical to the Ad-RGD-mILC and Ad-RGD-mFKN vectors without the chemokine gene in the expression cassette. The adenovirus vectors were propagated in human embryonic kidney 293 cells and purified by cesium chloride gradient ultracentrifugation, and their titer was determined by plaque-forming assay using 293 cells (22, 23).

Received 12/4/02; accepted 5/23/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This study was supported by grants from the Ministry of Health and Welfare in Japan and Grants-in-Aid for Scientific Research (C).

² To whom requests for reprints should be addressed, at Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan.

³ The abbreviations used are: NK, natural killer; ILC, interleukin-11 receptor α -locus chemokine; FKN, fractalkine; RT-PCR, reverse transcription-PCR; FBS, fetal bovine serum; MOI, multiplicity of infection.

RT-PCR for Chemokine Gene Detection. To examine the expression of mILC and mFKN mRNA, OV-HM cells were infected with Ad-RGD-mILC, Ad-RGD-mFKN, or Ad-RGD-NULL, as a control vector, at an MOI of 10 for 24 h. After the 24-h cultivation, total RNA of the cells was isolated using TRIzol reagents (Life Technologies, Inc.). SuperScript II reverse transcriptase (Life Technologies, Inc.) was used for the cDNA reverse transcription. cDNA was amplified by PCR in the presence of primers (Pharmacia Biotechnology). Primer sequences for mILC were 5'-AGCAGCCTCCCGTGTACT-GTTG-3' (sense) and 5'-TGCTTTATTAGTTTTGCTGTTGGG-3' (antisense) and for mFKN were 5'-ATGACCTCACGAATCCCAGTGG-3' (sense) and 5'-CCGCCTCAAACCTTCCAATGC-3' (antisense). The following PCR conditions were used: (a) mILC: 30 s at 92°C, 60°C, and 72°C (30 cycles); and (b) mFKN: 30 s at 92°C, 55°C, and 72°C (30 cycles). PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide.

Chemotaxis Assay. A549 cells were infected at an MOI of 10 with Ad-RGD-mILC or Ad-RGD-mFKN for 24 h. The cells were washed and cultured for another 48 h. The resulting conditioned medium was collected, and its chemotactic activity was measured by migration assay across polycarbonate membrane (Chemotax 96; Neuro Probe; Ref. 24) using L1.2-mCCR10 or L1.2-mCX₃CR1 cells expressing the specific receptor for mILC or mFKN, respectively. Migration was allowed for 4 h at 37°C in a 5% CO₂ atmosphere. Migrated cells were lysed and quantitated by using a PicoGreen double-stranded DNA quantitation reagent (Molecular Probe).

Tumor Rejection in Mice and Subsequent Rechallenge by Tumor Re-inoculation. Mice were inoculated intradermally into their flank with 1×10^6 OV-HM cells that had been infected with Ad-RGD-mILC or Ad-RGD-mFKN (at an MOI of 10 each in a volume of 100 μ l diluted in RPMI 1640) for 24 h. Tumor volume was calculated by measuring the length and width of the tumor twice a week. Animals were euthanized when one of the two measurements was >15 mm. Three months after complete regression of primary tumors, mice were rechallenged with freshly isolated OV-HM tumor or B16/BL6 melanoma cells by intradermal injection of 1×10^6 cells into the flank. mILC gene-transduced OV-HM cells, made by transfection of Ad-RGD-mILC, were also inoculated into BALB/c nude mice to observe its antitumor activity.

T- or NK-cell Infiltration into OV-HM Tumors Expressing Chemokine. Immunohistochemical analysis was used to determine lymphocytes infiltrated into tumors. Tumor-bearing mice were sacrificed in 2 days after the administration of OV-HM cells transfected with Ad-RGD-mILC or Ad-RGD-mFKN. The tumor nodules were harvested, embedded in OCT compound (Sakura, Torrance, CA), and stored at -80°C. Frozen thin sections (6 μ m in thickness) of the nodules were fixed in acetone, washed with PBS, and then incubated in PBS containing 0.3% hydrogen peroxide for 10 min at room temperature to block endogenous peroxidase activity. The sections were preincubated with 5% BSA in PBS for 10 min and sequentially incubated with optimal dilution of primary antibody, rabbit antisialo GM1 (WAKO), rabbit antihuman CD3 antibody (DakoCytomation), or normal rabbit IgG (Santa Cruz Biotechnology). Each primary antibody bound was detected with biotinylated goat anti-rabbit immunoglobulins (DakoCytomation) and streptavidin-horseradish peroxidase (Vector Laboratories). Each of the incubations lasted for 30 min and was followed by a 15-min wash in Tris-buffered saline. The sections were stained

with 3,3'-diaminobenzidine (WAKO) and finally counterstained with hematoxylin. The number of immunostained cells was counted under a light microscope with $\times 400$ magnification.

Depletion of Lymphocytes and Tumor Growth. Injection (i.p.) of anti-mouse CD4 or CD8 ascitic fluid, 100 μ l each, into mice was carried out seven times on days -3, -2, -1, 0, 5, 10, and 15. For NK cell depletion, mice were treated with asialo GM1 antiserum (40 μ l/dose; WAKO) by i.p. injection six times on days -2, -1, 0, 5, 10, and 15. OV-HM cells infected with Ad-RGD-mILC at an MOI of 10 were intradermally inoculated on day 0. Tumor growth was examined twice a week.

RESULTS

Expression of mILC and mFKN by Infection with Adenovirus Vectors. We infected OV-HM, a mouse ovary carcinoma line, with recombinant adenovirus vectors encoding mILC or mFKN and examined their expression by RT-PCR at 24 h. As shown in Fig. 1, OV-HM cells infected with Ad-RGD-mILC and Ad-RGD-mFKN indeed expressed mILC and mFKN, respectively. To verify that mILC and mFKN produced by cells infected with the adenovirus vectors were biologically active, A549 cells were infected with the vectors for 24 h, and the culture supernatants were harvested after further cultivation for 48 h. The chemotactic activity in the culture supernatants was examined by a migration assay using L1.2-mCCR10 or L1.2-mCX₃CR1 cells. In this experiment, we used A549 cells instead of OV-HM cells because of a strong background chemotactic activity in the culture supernatant of the latter. As shown in Fig. 2, the culture supernatant of A549 cells infected with Ad-RGD-mILC and Ad-RGD-mFKN efficiently induced migration of L1.2-mCCR10 or L1.2-mCX₃CR1 cells, respectively. Because mFKN encoded by Ad-RGD-mFKN was the full-length transmembrane type, the results also suggested efficient generation of soluble mFKN through cleavage at the membrane-proximal site (13).

In Vivo Antitumor Effect by Infection with Ad-RGD-mILC. OV-HM cells infected with Ad-RGD-mILC, Ad-RGD-mFKN, or Ad-RGD-NULL were intradermally inoculated into B6C3F1 mice. As shown in Fig. 3, OV-HM infected with Ad-RGD-mILC showed significant retardation in tumor growth *in vivo*. On the other hand, OV-HM infected with Ad-RGD-mFKN did not show any difference in tumor growth from that infected with Ad-RGD-NULL. In fact, 9 of 12 mice inoculated with OV-HM infected with Ad-RGD-mILC were tumor free (data not shown). In rechallenge experiment, mice that had complete regression were intradermally injected with OV-HM or B16/BL6 cells 90 days after the initial challenge. Results demonstrated that >50% of mice rechallenged with OV-HM (5 of 9 mice) remained tumor free for ≥ 2 months, whereas other mice showed

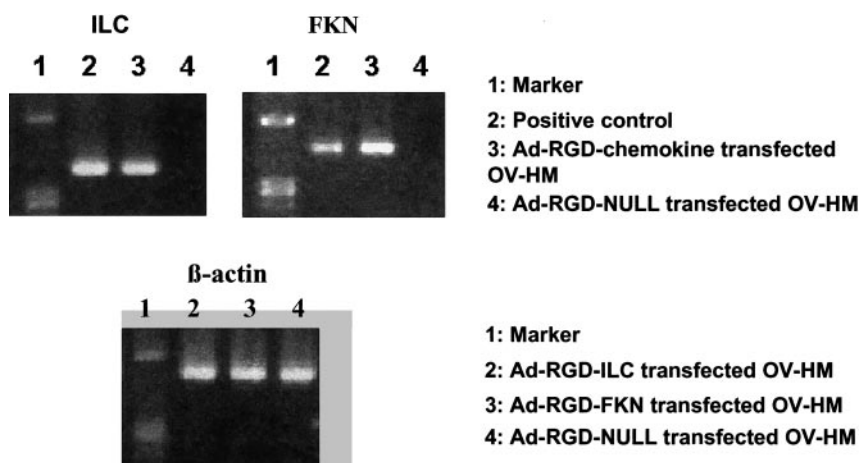


Fig. 1. RT-PCR analysis of chemokine mRNA expression in OV-HM cells infected with Ad-RGD-mILC or Ad-RGD-mFKN. Total RNA was isolated by TRIzol reagents from OV-HM cells infected with indicated chemokine-encoding adenovirus vectors at an MOI of 10 for 24 h, and SuperScript II reverse transcriptase was used for cDNA transcription. cDNA was amplified by PCR with indicated mouse chemokine-specific primers. Simultaneously, amplification of cDNA coding for β -actin was performed as an internal control. Amplified products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide.

Fig. 2. Directed migration of cells, expressing chemokine receptors, induced by culture supernatants of A549 cells infected with Ad-RGD-ILC or Ad-RGD-FKN. A549 cells were infected at an MOI of 10 with: (a) Ad-RGD-mILC or (b) Ad-RGD-mFKN. After 24 h, the cells were washed and cultured for an additional 48 h. The culture supernatants were collected and used for the migration assay by adding to the bottom chamber. L1.2-mCCR10 or L1.2-mCX₃CR1 cells, expressing specific receptors for mILC or mFKN, respectively, were suspended at a concentration of 1×10^7 cells/ml in migration buffer containing 1.04% RPMI 1640 without phenol red, 0.476% HEPES, and 0.5% BSA. After installing a filter on the bottom chamber, the cell suspensions, 20 μ l each, were placed in the top chamber. Cell migration was allowed for 4 h at 37°C in a 5% CO₂ atmosphere. Migrated cells to the bottom chamber were lysed and quantitated by using a PicoGreen double-stranded DNA quantitation reagent. Data are expressed as the mean \pm SD of triplicate results. Statistical analysis was carried out by Student's *t* test.

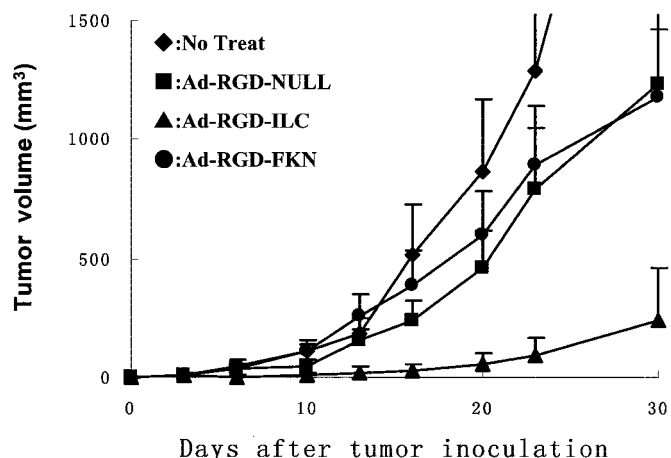
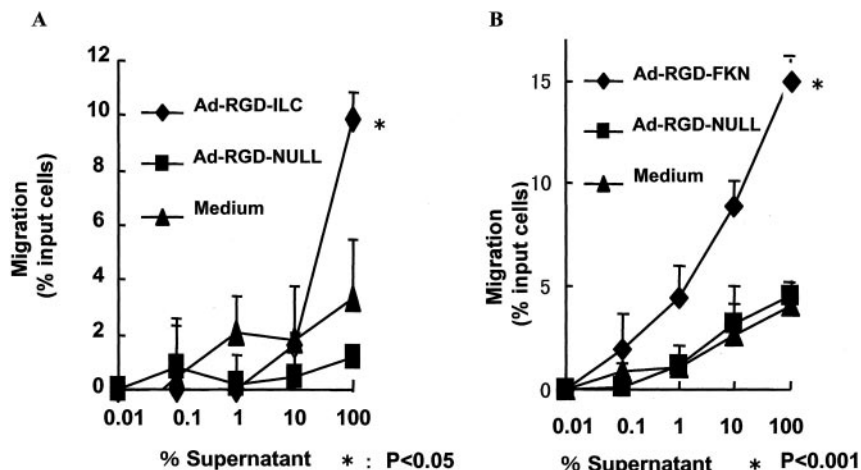


Fig. 3. Growth of OV-HM tumor cells, infected with chemokine-encoding adenovirus vectors, in B6C3F1 mice. Mice were inoculated intradermally in the flank with 1×10^6 OV-HM cells (100μ l in RPMI 1640) infected at an MOI of 10 with Ad-RGD-mILC or Ad-RGD-mFKN for 24 h. Tumor volume was calculated after measuring the length and width of the tumor at indicated periods of time, and data are expressed as the mean \pm SE of results obtained from at least five mice. Animals were euthanized when one of the two values measured was >15 mm.

retarded growth of tumor (data not shown). In contrast, 100% of the mice rechallenge with B16/BL6 developed palpable tumors within 2 weeks. These results indicated the generation of specific immunity against OV-HM in mice that rejected OV-HM-expressing mILC. We also confirmed that OV-HM infected with Ad-RGD-mILC did not show such marked growth suppression in nude mice deficient in T cells (Fig. 4). These results suggested that the importance of T cells in the antitumor effect against OV-HM-expressing mILC.

Infiltration of T and NK Cells into Tumor Infection with Ad-RGD-mILC and Ad-RGD-mFKN. To examine the antitumor mechanism of Ad-RGD-mILC, tumor tissues were immunohistochemically stained for CD3 (a T-cell marker) and asialoGM1 (an NK-cell marker; Refs. 25 and 26) 2 days after the inoculation of OV-HM cells infected with Ad-RGD-mILC, Ad-RGD-mFKN, or Ad-RGD-NULL. As shown in Figs. 5 and 6, both OV-HM cells infected with Ad-RGD-mILC and Ad-RGD-mFKN demonstrated the accumulation of CD3⁺ T and NK cells in tumor tissues. CD3⁺ T and NK cells were evenly distributed in the tumor tissue of OV-HM infected with Ad-RGD-mILC. However, these cells were mostly accumulated around the tumor blood vessel in the tumor tissues of OV-HM infected with Ad-RGD-mFKN. Furthermore, promoted growth of tumor blood vessels appeared to be present in OV-HM-expressing mFKN (Figs. 5 and 6).

Involvement of CD4⁺ and CD8⁺ T Cells in the Antitumor Effect of mILC. To investigate the role of T and NK cells in the antitumor effect by Ad-RGD-mILC, B6C3F1 mice selectively depleted with CD4⁺ T, CD8⁺ T, or NK cell were inoculated with OV-HM cells infected with Ad-RGD-mILC. As shown in Fig. 7, no tumor grew in the NK cell-depleted group, whereas tumors efficiently developed in both of the CD4⁺ or CD8⁺ T cell-depleted mice. These results indicated that the inhibition of tumor growth by mILC was mostly dependent on CD4⁺ and CD8⁺ T cells but not on NK cells.

DISCUSSION

Cytokine or chemokine encoded by a viral vector is currently regarded as a promising way of cancer gene immunotherapy. Researchers have paid attention to chemotactic activity of chemokines for immune cells and expected that they may be able to play an important role in cancer treatment, because the basis and premise of immunotherapy is the accumulation of immune cells in tumor tissues.

The CC chemokine ILC, also called cutaneous T cell-attracting

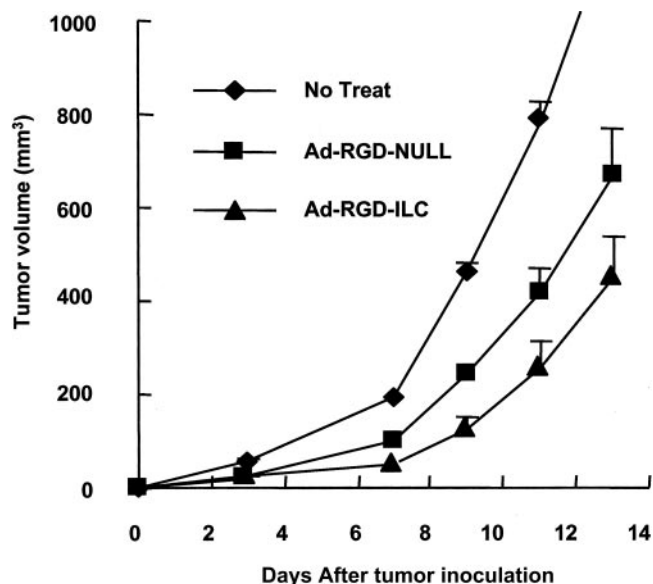


Fig. 4. Growth of OV-HM tumor cells infected with Ad-RGD-mILC in BALB/c nude mice. Mice were inoculated intradermally in the flank with 1×10^6 OV-HM cells (100μ l in RPMI 1640) infected at an MOI of 10 with Ad-RGD-mILC or Ad-RGD-mFKN for 24 h. Tumor volume was calculated after measuring the length and width of the tumor at indicated periods of time, and data are expressed as the mean \pm SE of results from at least five mice.

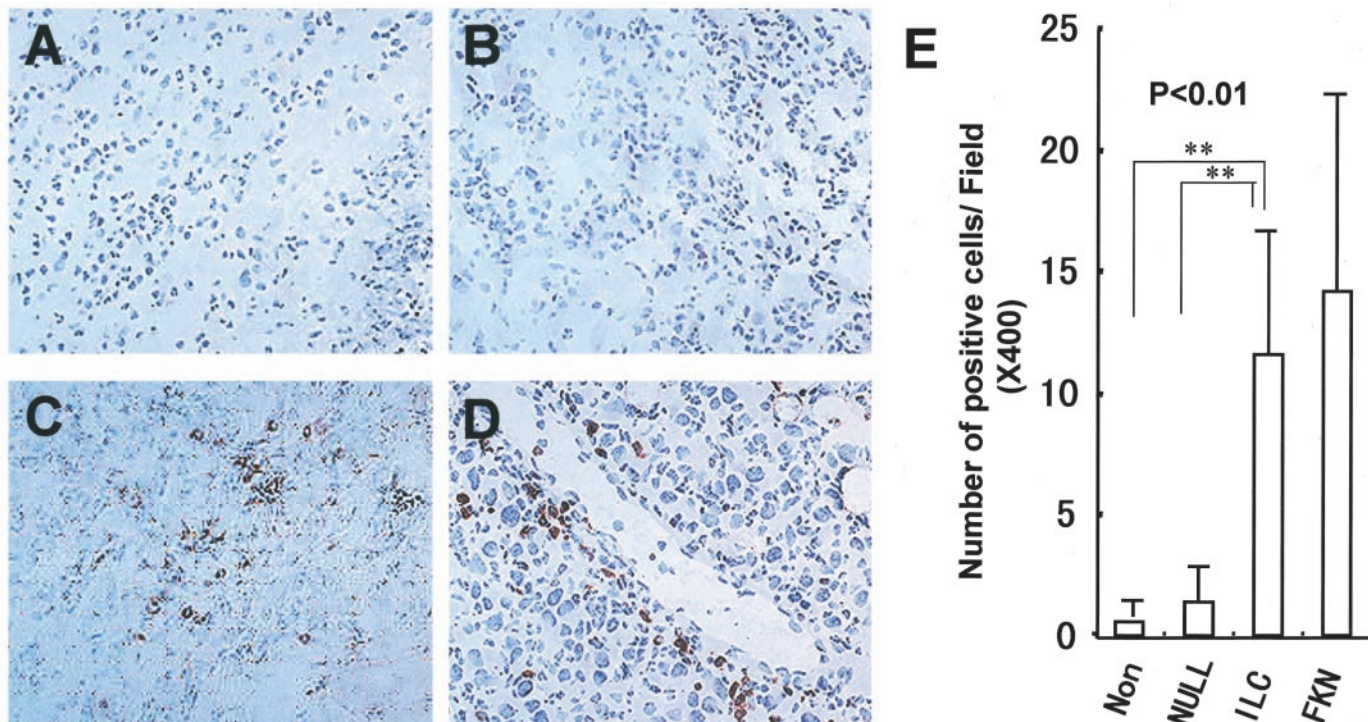


Fig. 5. CD3-positive lymphocytes infiltrate into OV-HM tumors infected with Ad-RGD-mILC and Ad-RGD-mFKN. A–D, representative immunohistochemical appearances of tumor nodules from mice inoculated intradermally with 1×10^6 OV-HM cells infected with none (A), Ad-RGD-NULL (B), Ad-RGD-mILC (C), or Ad-RGD-mFKN (D). Statistical analysis was carried out by Welch's t test.

chemokine or CCL27, was reported to recruit T cells to the site of its injection (27). The CX₃C family chemokine FKN (also called CX₃CL1) could also attract a variety of cytotoxic lymphocytes (13, 14, 28) and enhance the cytotoxicity of NK cells (29). In the present

study, we hypothesized that the transfer of the mILC or mFKN gene to tumor cells, by using recombinant adenovirus *in vitro*, could render the tumor to express the chemokine *in vivo*. The chemokine would consequently induce the accumulation of immune cells in the tumor

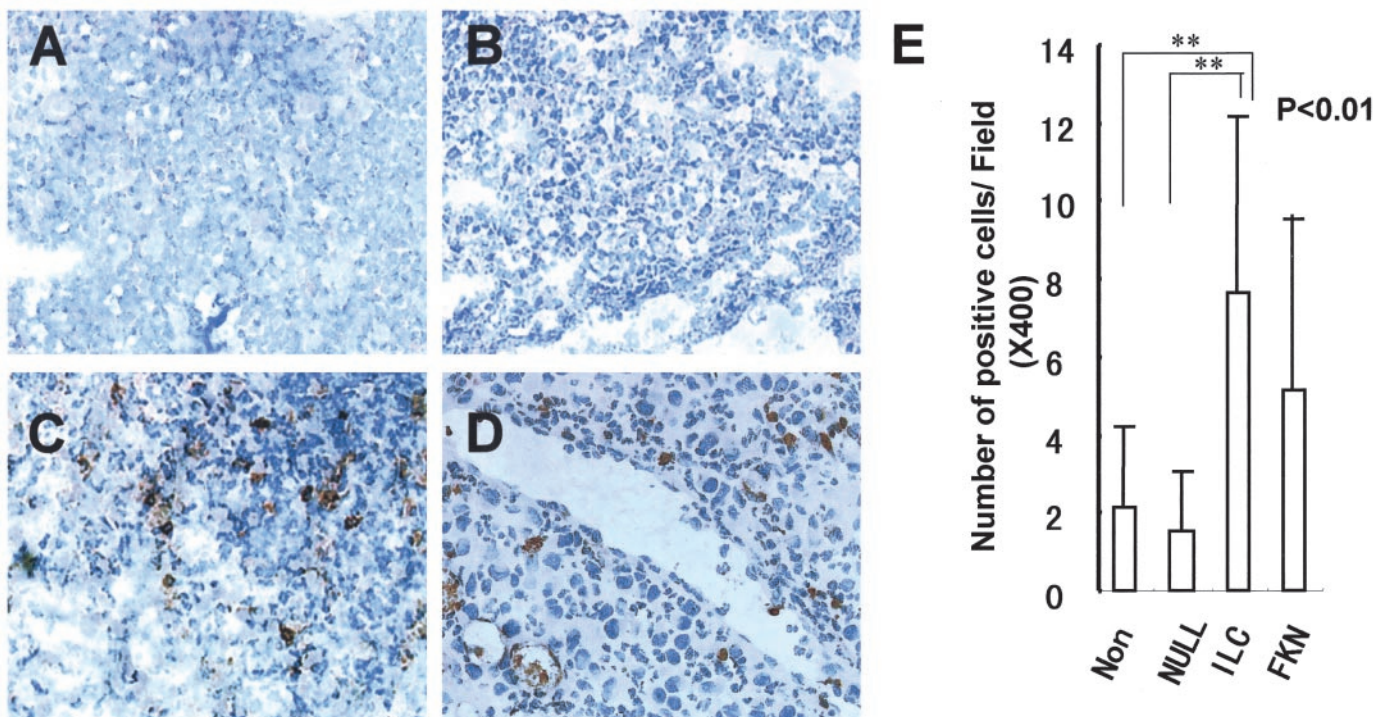


Fig. 6. NK cells infiltrate into OV-HM tumors infected with Ad-RGD-mILC and Ad-RGD-mFKN. A–D, representative immunohistochemical appearances of tumor nodules from mice inoculated intradermally with 1×10^6 OV-HM cells infected with none (A), Ad-RGD-NULL (B), Ad-RGD-mILC (C), or Ad-RGD-mFKN (D). Statistical analysis was carried out by Welch's t test.

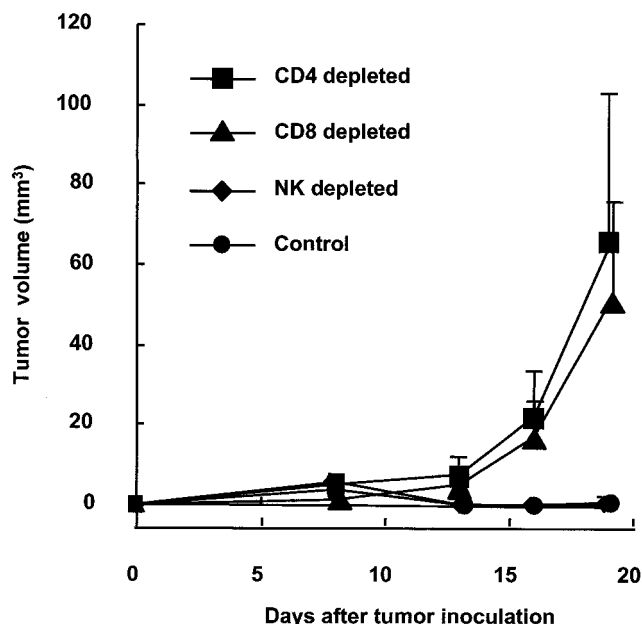


Fig. 7. $CD4^+$ and $CD8^+$ T cells contribute to lowering tumorigenicity of mILC-expressing OV-HM. $CD4^+$ or $CD8^+$ T cells were depleted in B6C3F1 mice by i.p. injection of respective anti- $CD4$ or - $CD8$ ascitic fluid, 100 μ l each, seven times on days -3, -2, -1, 0, 5, 10, and 15. NK cell depletion was carried out by i.p. injection of asialo GM1 antiserum, 40 μ l/dose, six times on days -2, -1, 0, 5, 10, and 15. OV-HM cells infected at an MOI of 10 with Ad-RGD-mILC were intradermally inoculated on day 0. Tumor sizes were measured at indicated time intervals, and data are expressed as the mean \pm SE of results from at least five mice.

tissue and initiate the antitumor immune response. To test this hypothesis, we developed chemokine-encoding fiber mutant adenovirus vectors, Ad-RGD-mILC and Ad-RGD-mFKN. Efficient production of biologically active mILC and mFKN could be detected in the culture supernatants of cells infected with Ad-RGD-mILC or Ad-RGD-mFKN. The *in vivo* tumor growth experiment further showed that mILC markedly suppressed the growth of the tumor. By contrast, antitumor effect by mFKN was not observed. To exclude the possibility that the growth suppression of the tumor cells by Ad-RGD-mILC was attributable to the cytotoxicity of adenovirus or chemokine, OV-HM cells transfected with Ad-RGD-mILC, Ad-RGD-mFKN, or Ad-RGD-NULL were cultured for 48 h, and the cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The *in vitro* growth of the cells infected with these vectors was essentially identical to that of control cells (data not shown). The rechallenge experiment also demonstrated that specific immunity was developed, although only at a limited level, after the initial challenge with mILC-expressing OV-HM cells.

Using immunohistochemical staining, we studied how mILC but not mFKN had antitumor activity. Tumor cells infected with Ad-RGD-mILC or Ad-RGD-mFKN attracted a significantly elevated number of $CD3^+$ lymphocytes and NK cells. Interestingly, however, lymphocytes accumulated only around the blood vessel in tumors expressing mFKN, whereas lymphocytes infiltrated deep within tissues in mILC-expressing tumors. We also observed abundant blood vessels in the tumor tissue expressing mFKN (Figs. 5 and 6). This is in good agreement with the previous results clearly demonstrating that FKN was angiogenic and enhanced the growth of blood vessels *in vivo*, a necessary step for the development of tumors (30). In that paper, the authors demonstrated that FKN significantly induced migration of human dermal microvascular endothelial cells *in vitro*. *In vivo*, FKN also elicited 2.3-fold more blood vessel growth than control in the Matrigel plug assay, which could be inhibited by immunodepletion of FKN. Some of the CXC family chemokines, such

as IFN-inducible protein-10/CXCL10, monokine induced by IFN γ /CXCL9, or platelet factor-4/CXCL4, have potent angiostatic activity because of the lack of the Glu-Leu-Arg (ELR) motif (10). These have stronger antitumor activity than the others attributable not only to their efficient immune cell attraction but also to suppression of blood vessel growth (31–33). Moreover, IL-8/CXCL8, which is an angiogenic CXC chemokine, facilitated tumor growth by suppressing the antitumor activity of IL-2 (34). These results also suggest that the angiogenic or angiostatic activity of the chemokines may influence their antitumor activity and can be an important parameter for cancer immunotherapy using the chemokines. Thus, in the present study, mFKN might not have efficient antitumor activity, despite the recruitment of lymphocytes and NK cells, partly because of its angiogenic activity. Furthermore, to our knowledge, no other reports described a potential relationship between antitumor activity of a chemokine and distribution of recruited lymphocytes within tumor tissues. However, the present observations need to be verified by additional studies. Another factor that may induce the difference of antitumor effect with mILC or mFKN is the difference of CX_3CR1 -expressing immune cells and $CCR10$ -expressing ones. It has been reported that CX_3CR1 only defines peripheral blood cytotoxic effector lymphocytes commonly armed with intracellular perforin and granzyme B, which include NK, $\gamma\delta T$, and terminally differentiated $CD8^+$ T cells (28), whereas $CCR10$ is preferentially expressed among blood leukocytes by both the subset of memory $CD4$ and $CD8$ T cells. $CLA^+/CCR10^+$ memory $CD4$ T cells from normal donor can secrete tumor necrosis factor and IFN- γ (35). Our results showed both the $CD4$ and $CD8$ T cells played important roles in the tumor suppressive effect induced by the infection of ILC. These results can be helpful to explain the different antitumor effect with ILC and FKN.

We found the absolute requirement of T lymphocytes for the antitumor effect of mILC. Using BALB/c nude mice and T or NK cell-depleted mice, the tumor suppressive activity of mILC was found to depend on T cells but not on NK cells. Both $CD4^+$ and $CD8^+$ T cells play roles in the antitumor effect by mILC. It is known that in many cases, only $CD8^+$ T cells were required for antitumor effect by chemokines and/or cytokines against malignant cells lacking MHC class II expression (36). Furthermore, $CD4^+$ T cells have also been found to: (a) enhance tumor cell killing by macrophages; (b) stimulate dendritic cells to prime CTLs; (c) activate NK cells via cytokines secreted by $CD4^+$ cells; and (d) have direct antitumor activity (37, 38). In the present study, we have failed to detect a role for NK cells in the tumor suppression by ILC but cannot completely rule out the contribution of NK cells. Therapy with mILC failed in BALB/c nude mice with normal NK activity, and NK cell-depleted mice did not show tumor development activity after the inoculation of mILC-expressing OV-HM cells.

Taken together, the transduction of OV-HM cells with the mILC-encoding recombinant adenovirus vector induced the recruitment of lymphocytes and NK cells to the tumor, resulting in T cell-dependent antitumor effect. By contrast, Ad-RGD-mFKN did not show any therapeutic effect despite its chemoattractant activity for T and NK cells. Our present study suggests that ILC, a CC family chemokine, may be a good candidate for cancer gene immunotherapy. Using recombinant adenovirus vectors, we are currently trying combination therapy with ILC and cytokines for cancer.

REFERENCES

- Paillard, F. Cytokine and chemokine: a stimulating couple. *Hum. Gene Ther.*, 10: 695–696, 1999.
- Mule, J. J., Custer, M., Averbook, B., Yang, J. C., Weber, J. S., Goeddel, D. V., Rosenberg, S. A., and Schall, T. J. RANTES secretion by gene-modified tumor cells results in loss of tumorigenicity *in vivo*: role of immune cell subpopulations. *Hum. Gene Ther.*, 7: 1545–1553, 1996.

3. Strieter, R. M., Polverini, P. J., Arenberg, D. A., Walz, A., Opendakker, G., Van Damme, J., and Kunkel, S. L. Role of C-X-C chemokines as regulators of angiogenesis in lung cancer. *J. Leukoc. Biol.*, *57*: 752–762, 1995.
4. Hirose, K., Hakozaiki, M., Nyunoya, Y., Kobayashi, Y., Matsushita, K., Takenouchi, T., Mikata, A., Mukaida, N., and Matsushima, K. Chemokine gene transfection into tumor cells reduced tumorigenicity in nude mice association with neutrophilic infiltration. *Br. J. Cancer*, *72*: 708–714, 1995.
5. Huang, S., Xie, K., Singh, R. K., Gutman, M., and Bar-eli, M. Suppression of tumor growth and metastasis of murine renal adenocarcinoma by syngeneic fibroblasts genetically engineered to secrete the JE/MCP-1 cytokine. *J. Interferon Cytokine Res.*, *15*: 655–665, 1996.
6. Lanning, J., Kawasaki, H., Tanaka, E., Luo, Y., and Dorf, M. E. Inhibition of *in vivo* tumor growth by the beta chemokine, TCA3. *J. Immunol.*, *153*: 4625–4635, 1994.
7. Maric, M., and Liu, Y. Strong cytotoxic T lymphocyte responses to a macrophage inflammatory protein 1 α -expressing tumor: linkage between inflammation and specific immunity. *Cancer Res.*, *59*: 5549–5553, 1999.
8. Nakashima, E., Oya, A., Kubota, Y., Kanada, N., Matsushita, R., Takeda, K., Ichimura, F., Kuno, K., Mukaida, N., Hirose, K., Nakanishi, I., Ujiiie, T., and Matsushima, K. A candidate for cancer gene therapy: MIP-1 alpha gene transfer to an adenocarcinoma cell line reduced tumorigenicity and induced protective immunity in immunocompetent mice. *Pharm. Res.*, *13*: 1896–1901, 1996.
9. Guo, J., Wang, B., Zhang, M., Chen, T., Yu, Y., Regulier, E., Homann, H., Qin, Z., and Ju, D. W. Macrophage-derived chemokine gene transfer results in tumor regression in murine lung carcinoma model through efficient induction of antitumor immunity. *Gene Ther.*, *9*: 793–803, 2002.
10. Yoshie, O., Imai, T., and Nomiya, H. Chemokines in immunity. *Adv. Immunol.*, *78*: 57–110, 2001.
11. Reiss, Y., Proudfoot, A. E., Power, C. A., Campbell, J. J., and Butcher, E. C. CC chemokine receptor (CCR) 4 and the CCR 10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. *J. Exp. Med.*, *194*: 1541–1547, 2001.
12. Morales, J., Homey, B., Vicari, A. P., Hudak, S., Oldham, E., Hedrick, J., Orozco, R., Copeland, N. G., Jenkins, N. A., McEvoy, L. M., and Zlotnik, A. CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc. Natl. Acad. Sci. USA*, *96*: 14470–14475, 1999.
13. Bazan, J. F., Bacon, K. B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D. R., Zlotnik, A., and Schall, T. J. A new class of membrane-bound chemokine with a CX₃C motif. *Nature*, *385*: 640–644, 1997.
14. Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiya, H., Schall, T. J., and Yoshie, O. Identification and molecular characterization of fractalkine receptor CX₃CR1, which mediates both leukocyte migration and adhesion. *Cell*, *91*: 521–530, 1997.
15. Papadopoulos, E. J., Sasseti, C., Saeki, H., Yamada, N., Kawamura, T., Fitzhugh, D. J., Saraf, M. A., Schall, T., Blauvelt, A., Rosen, S. D., and Hwang, S. T. Fractalkine, a CX₃C chemokine, is expressed by dendritic cells and is up-regulated upon dendritic cell maturation. *Eur. J. Immunol.*, *29*: 2551–2559, 1999.
16. Okada, N., Saito, T., Masunaga, Y., Tsukada, Y., Nakagawa, S., Mizuguchi, H., Mori, K., Okada, Y., Fujita, T., Hayakawa, T., Mayumi, T., and Yamamoto, A. Efficient antigen gene transduction using Arg-Gly-Asp fiber-mutant adenovirus vectors can potentiate antitumor vaccine efficacy and maturation of murine dendritic cells. *Cancer Res.*, *61*: 7913–7919, 2001.
17. Okada, Y., Okada, N., Nakagawa, S., Mizuguchi, H., Kanehira, M., Nishino, N., Takahashi, K., Mizuno, N., Hayakawa, T., and Mayumi, T. Fiber-mutant technique can augment gene transduction efficacy and antitumor effects against established murine melanoma by cytokine-gene therapy using adenovirus vectors. *Cancer Lett.*, *177*: 57–63, 2002.
18. Hashimoto, M., Niwa, O., Nitta, Y., Takeichi, M., and Yokoro, K. Unstable expression of E-Cadherin adhesion molecules in metastatic ovarian tumor cells. *Jpn. J. Cancer Res.*, *80*: 459–463, 1989.
19. Mizuguchi, H., Kay, M. A., and Hayakawa, T. *In vitro* ligation-based cloning of foreign DNAs into the E3 and E1 deletion regions for generation of recombinant adenovirus vectors. *Biotechniques*, *30*: 1112–1114, 2001.
20. Mizuguchi, H., Koizumi, N., Hosono, T., Utoguchi, N., Watanabe, Y., Kay, M. A., and Hayakawa, T. A simplified system for constructing recombinant adenoviral vectors containing heterologous peptides in the HI loop of their fiber knob. *Gene Ther.*, *8*: 730–735, 2001.
21. Mizuguchi, H., and Kay, M. A. Efficient construction of a recombinant adenovirus vector by an improved *in vitro* ligation method. *Hum. Gene Ther.*, *9*: 2577–2583, 1998.
22. Rosenfeld, M. A., Siegfried, W., Yoshimura, K., Yoneyama, K., Fukayama, M., Stier, L. E., Paakko, P. K., Gilardi, P., Stratford-Perricaudet, L. D., and Perricaudet, M. Adenovirus-mediated transfer of a recombinant alpha 1-antitrypsin gene to the lung epithelium *in vivo*. *Science*, *252*: 431–434, 1991.
23. Crystal, R. G., McElvaney, N. G., Rosenfeld, M. A., Chu, C. S., Mastrangeli, A., Hay, J. G., Brody, S. L., Jaffe, H. A., Eissa, N. T., and Danel, C. Administration of an adenovirus containing the human CFTR cDNA to the respiratory tract of individuals with cystic fibrosis. *Nat. Genet.*, *8*: 41–51, 1994.
24. Vicente-Manzanares, M., Montoya, M. C., Mellado, M., Frade, J. M., del Pozo, M. A., Nieto, M., de Landazuri, M. O., Martinez, A. C., and Sanchez-Madrid, F. The chemokine SDF-1 α triggers a chemotactic response and induces cell polarization in human B lymphocytes. *Eur. J. Immunol.*, *28*: 2197–2207, 1998.
25. Kitching, A. R., Topping, P. G., Kurimoto, M., and Holdsworth, S. R. IL-18 has IL-12-independent effects in delayed-type hypersensitivity: studied in cell-mediated crescentic glomerulonephritis. *J. Immunol.*, *165*: 4649–4657, 2000.
26. Di Carlo, E., Comes, A., Basso, S., De Ambrosio, A., Meazza, R., Musiani, P., Moelling, K., Albin, A., and Ferrini, S. The combined action of IL-15 and IL-12 gene transfer can induce tumor cell rejection without T and NK cell involvement. *J. Immunol.*, *165*: 3111–3118, 2000.
27. Homey, B., Alenius, H., Müller, A., Soto, H., Bowman, E. P., Yuan, W., McEvoy, L., Lauerma, A. I., Assmann, T., Bünemann, E., Lehto, M., Wolff, H., Yen, D., Marxhausen, H., To, W., Sedgwick, J., Ruzicka, T., Lehmann, P., and Zlotnik, J. ILX-CCR10 interactions regulate T cell-mediated skin inflammatory. *Nat. Med.*, *8*: 157–165, 2002.
28. Nishimura, M., Umehara, H., Nakayama, T., Yoneda, O., Hieshima, K., Kakizaki, M., Dohmae, N., Yoshie, O., and Imai, T. Dual functions of fractalkine/CX₃C ligand 1 in trafficking of perforin⁺/granzyme B⁺ cytotoxic effector lymphocytes that are defined by CX₃CR1 expression. *J. Immunol.*, *168*: 6173–6180, 2002.
29. Yoneda, O., Imai, T., Goda, S., Inoue, H., Yamauchi, A., Okazaki, T., Imai, H., Yoshie, O., Bloom, E. T., Domae, N., and Umehara, H. Fractalkine-mediated endothelial cells injury by NK cells. *J. Immunol.*, *164*: 4055–4062, 2000.
30. Volin, M. V., Woods, J. M., Amin, M. A., Connors, M. A., Harlow, L. A., and Koch, A. E. Fractalkine: a novel angiogenic chemokine in rheumatoid arthritis. *Am. J. Pathol.*, *159*: 1521–1530, 2001.
31. Kolber, D. J., Knisely, T. L., and Maione, T. E. Inhibition of development of murine melanoma lung metastases by systemic administration of recombinant platelet factor 4. *J. Natl. Cancer Inst.* (Bethesda), *87*: 304–309, 1995.
32. Tannenbaum, C. S., Tubbs, R., Armstrong, D., Finke, J. H., Bukowski, R. M., and Hamilton, T. A. The CX₃C chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor. *J. Immunol.*, *161*: 927–932, 1998.
33. Sgadari, C., Farber, J. M., Angiolillo, A. L., Liao, F., Teruya-Feldstein, J., Burd, P. R., Yao, L., Gupta, G., Kanegane, C., and Tosato, G. Mig, the monokine induced by interferon-gamma, promotes tumor necrosis *in vivo*. *Blood*, *89*: 2635–2643, 1997.
34. Arenberg, D. A., Kunkel, S. L., Polverini, P. J., Glass, M., Burdick, M. D., and Strieter, R. M. Inhibition of interleukin-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice. *J. Clin. Investig.*, *97*: 2792–2802, 1996.
35. Hudak, S., Hagen, M., Liu, Y., Catron, D., Oldham, E., McEvoy, L. M., and Bowman, E. P. Immune surveillance and effector function of CCR10⁺ skin homing T cells. *J. Immunol.*, *169*: 1189–1196, 2002.
36. Mazzolini, G., Qian, C., Xie, X., Sun, Y., Lasarte, J. J., Drozdziak, M., and Prieto, J. Regression of colon cancer and induction of antitumor immunity by intratumoral injection of adenovirus expressing interleukin-12. *Cancer Gene Ther.*, *6*: 514–522, 2000.
37. Hung, K., Hayashi, R., Lafond-Walker, A., Lowenstein, C., Pardoll, D., and Levitsky, H. The central role of CD 4⁺ T cells in the antitumor immune response. *J. Exp. Med.*, *188*: 2357–2368, 1998.
38. Lanzavecchia, A. Immunology: license to kill. *Nature*, *393*: 413–414, 1998.