

Expression of Inflammatory Chemokines Combined with Local Tumor Destruction Enhances Tumor Regression and Long-term Immunity¹

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ABSTRACT

Expression of chemokines within tumors can be used to recruit immature dendritic cells (DCs) for the initiation of antitumor T-cell responses. Here, we describe the chemokine receptor expression on murine bone marrow-derived immature DCs. On the basis of these receptor studies, we chose to express the chemokines CCL3 (Mip-1 α) or CCL20 (Mip-3 α) in tumors. We show that expression of these chemokines in the colorectal tumor model CMT93 significantly decreases tumorigenesis. This decrease is associated with an increase in CD8 T cells, natural killer cells, and Class II DCs in the tumor within the first 24 h. Furthermore, studies in immunodeficient mice show that both natural killer cells and T cells are required for this decrease in immunogenicity. CCL3 and CCL20 expression alone did not significantly inhibit the development of the B16 melanoma tumor. However, coexpression of the Herpes Simplex Virus thymidine kinase gene (HSVtk) and CCL20, cured large established tumors where HSVtk expression alone was not sufficient. Finally, coexpression of HSVtk with either CCL3 or CCL20 was able to significantly increase protection against subsequent tumor rechallenge.

Overview Summary

On the basis of the pattern of expression of chemokine receptors that we observed on murine bone marrow-derived immature DCs, we expressed the chemokines CCL3 (Mip-1 α) or CCL20 (Mip-3 α) alone, or in tandem, with the Herpes Simplex Virus thymidine kinase gene, HSVtk, in tumors. Coexpression of HSVtk and CCL20 cured large established B16 tumors where chemokine or HSVtk expression alone was not sufficient. In addition, coexpression of HSVtk with either CCL3 or CCL20 was able to significantly increase protection against subsequent tumor rechallenge. Our data demonstrate that as well as provision of signals to recruit and activate DCs, a source of antigen release is also required to generate optimal antitumor responses.

INTRODUCTION

DCs⁴ function as APCs, priming naïve T cells and initiating immune responses. IDCs circulate and reside in the periphery where they can pick up antigen released from cells at sites of infection, undergo maturation following signals from resident macrophages or inflamed and stressed tissue, and migrate to the draining lymph node where they prime naïve T cells (1, 2). An understanding of the DC's pivotal role in priming immune responses has led to their use in various approaches to develop antitumor immune responses. These include use of *in vitro* manipulated DCs for direct intratumoral injection or

vaccination with DCs cocultured with syngeneic or allogeneic tumors, purified tumor-associated antigen protein, or purified tumor-associated antigen peptides, as well as DCs fused to tumor cells (3–6).

Expression of the cytokine GM-CSF in irradiated tumor vaccines is an effective way to generate an antitumor immune response (7). One observation in GM-CSF therapy is the observed infiltrate of DCs into the tumor site, and it is this influx of DCs that has been postulated to be responsible for the long-term T-cell mediated immunity (8–10). However, GM-CSF is not a chemotactic factor for DCs, and GM-CSF has multiple effects on other immune cells, including macrophages and endothelial cells, which may be the intermediate effectors in the recruitment of DCs (11). Vaccination with irradiated GM-CSF-expressing tumors results in an increased expression of the chemokine Mip-1 α at the tumor site that may be the cause of DC recruitment (12). Additionally, GM-CSF may have confounding effects attributable to its role in hematopoiesis, which includes profound leukocytosis and fatality in mice receiving tumors expressing GM-CSF (7).

The evolving field of chemokine biology has identified a surprising specificity in the matching of chemokine expression patterns in immune processes, with patterns of receptor expression by cells and cell subsets, that appear to play key roles in orchestrating immune responsiveness through location (13–17). Chemokines may, therefore, represent an ideal option to recruit a particular repertoire of immune cells characteristic of a specific T cell-mediated response, including DC, macrophages, NK cells, CTL, and Th1 cells, while excluding the broader effects of cytokine secretion alone.

To determine the role of DC recruitment to tumors in activation of antigen-specific antitumor immune responses, we hypothesized that we could use chemokines to selectively recruit immature DCs to the tumor site. Although DC recruitment is an important step in the generation of an immune response, this is only part of the story. The release of antigen at appropriate levels and in appropriate form is also required for T-cell priming (9, 18–20). Therefore, we have used a model that distinguishes between the roles of immune cell recruitment and/or antigen release in the generation of an antitumor immune response. On the basis of *in vitro* data, we show that murine bone marrow-derived DCs express the chemokine receptors CCR1, CCR5, and CCR6. Therefore, we chose mCCL3 and mCCL20, the ligands for these receptors, to recruit immune cells into tumors. We also coexpressed these chemokines with the HSVtk, a viral enzyme that converts ganciclovir from a prodrug to a toxic agent that kills the cells and is associated with a bystander effect (21). Using an IRES, we coexpressed HSVtk with a chemokine in order to induce cytotoxicity with concomitant release of tumor antigen, thus combining recruitment of DCs and inflammatory cells with antigen release. Our data demonstrate that coexpression of HSVtk and chemokine is able to increase tumor immunity in animal models where chemokine or HSVtk expression alone is not sufficient.

MATERIALS AND METHODS

Construction of Expression Plasmids. Total RNA from LPS-activated IC-21 murine macrophage cells was reverse transcribed then PCR amplified using specific primers for murine CCL3 (5' CCATGGAGGTCTCCACCACTG; 3' TCTAGATCCTCGTGCCTCC) and CCL20 (5' CCATGGCCTCGGGTGGCAAGC; 3' GCTCTAGAGCATCAGTTTTTTACATC). Each 5'

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⁴ The abbreviations used are: DC, dendritic cell; IDC, immature dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; APC, antigen presenting cell; IRES, internal ribosomal entry site; HSVtk, herpes simplex virus thymidine kinase; IL, interleukin; GCV, ganciclovir; RPA, ribonuclease protection assay; LPS, lipopolysaccharide; NK, natural killer; MDC, mature dendritic cell; FACS, fluorescence-activated cell sorter.

primer contained a Nco-1 site, and each 3' primer contained a Xba-1 site for further subcloning, described below. Specific chemokine bands were cloned into pCR3.1 (Invitrogen) and confirmed by restriction digest and sequencing. Bicistronic vectors were constructed with chemokine genes inserted 3' of the IRES in the pCITE-2 vector (Novagen) between Nco-1 and Xba-1 sites to ensure optimal internal transcription from the IRES element. The IRES-chemokine fragment was PCR amplified using a new 5' primer upstream of the IRES that incorporated a Xho-1 site and the appropriate 3' primer described above, and the specific band cloned into pCR3.1. Finally, this IRES chemokine element was excised by Xho-1-Xba-1 digest and subcloned into the pCR3.1HSVtk plasmid 3' of the HSVtk coding sequence to create HSVtk-IRES-CCL3 and HSVtk-IRES-CCL20.

Cell Culture. The murine melanoma B16 and B16tk lines and the colorectal tumor CMT93 used in this study have been described previously (20). The murine macrophage cell line IC-21 was obtained from the American Type Culture Collection and grown according to American Type Culture Collection guidelines. All cell lines were monitored routinely and found to be free of *Mycoplasma* infection. For cell killing *in vitro*, medium was supplemented with GCV (Cytovene; Roche, Indianapolis, IN) to a final concentration of 5 $\mu\text{g}/\text{ml}$. B16neo, B16CCL3, B16CCL20, B16tkIRES-CCL3, and B16tkIRES-CCL20 are stable clones generated by transfection of B16 cells with pCR3.1 alone or the appropriate expression vector followed by selection in 5 mg/ml G418 (Life Technologies, Inc.). CMT93neo, CMT93CCL3, CMT93CCL20, CMT93tkIRES-CCL3, and CMT93tkIRES-CCL20 are stable clones generated by transfection of CMT93 cells with pCR3.1 alone or the appropriate expression vector followed by selection in 1 mg/ml G418.

DC Culture. DCs were cultured from the bone marrow of C57BL/6 mice as described previously (22). Briefly, bone marrow was isolated from the tibia and femur, and after RBC lysis, the resulting cell suspension was incubated in 1 ml of RPMI 1640 containing 10 $\mu\text{g}/\text{ml}$ antibodies to MHC class II (I-Ab), Mac 3, CD8a (HO2.2), B220, CD3e, and Gr-1 (all from PharMingen, San Diego, CA) on ice for 20 min. The cells were washed and suspended in baby rabbit complement (Accurate Chemical and Scientific Corp., Westbury, NY) at a concentration of 10^7 cells/ml (1:15, complement: medium) at 37°C for 60 min. Cells were washed and plated at a concentration of 10^6 cells/ml in RPMI 1640 supplemented with 10 ng/ml GM-CSF for IDCs and 20 ng/ml GM-CSF plus 1 ng/ml IL-4 for MDCs. Cultures were washed at 3 days to remove nonadherent cells. IDCs were collected on day 7, whereas MDCs were supplemented with LPS on day 7 and cells collected on day 9.

Detection of Chemokine Production from Cell Lines. Cell line chemokine production was detected by specific ELISA. Antibodies and recombinant protein were purchased from R&D Systems (Minneapolis, MN). Five $\mu\text{g}/\text{ml}$ antimouse CCL3 (AF-450-NA) or CCL20 (AF-760) capture antibody were coated on treated 96-well ELISA plates (Rainin). Recombinant murine CCL3 (450-MA) or CCL20 (760-M3) were used as standards alongside cell supernatants. One $\mu\text{g}/\text{ml}$ biotinylated antimouse CCL3 (BAF450) or CCL20 (BAF760) was used for detection followed by 0.2 $\mu\text{g}/\text{ml}$ peroxidase-conjugated Streptavidin (016-030-084; Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, TMB substrate reagent (PharMingen) was added and stopped after 20 min using 100 μl of 2N H_2SO_4 .

RPA. For analysis of DC chemokine receptor expression, a custom multi-probe template set was assembled to order by PharMingen, with templates including mCCR1, mCCR5, mCCR6, mCCR7, and the housekeeping control genes L32 and GAPDH. RNA probes were synthesized from the template mix using a T7 MAXIScript *in vitro* transcription kit (Amnion, Austin, TX) incorporating radiolabeled [α - ^{32}P] UTP (NEN) according to manufacturer's instructions. DNA template sequences were digested for 30 min with DNaseI, and RNA templates were purified from unincorporated nucleotides using a Chromaspin-30 column (Clontech, Palo Alto, CA). RNA hybridization was performed using an RPAIII kit (Ambion). Briefly, 5 μg of total RNA prepared using RNeasy kit (Qiagen) was combined with 4×10^4 cpm labeled probe, denatured at 95°C for 3 min, and then incubated overnight at 56°C. Single-stranded RNA was digested with an RNaseA/T1 mix for 45 min at 45°C; then samples were precipitated and run on a 5% acrylamide sequencing gel until resolved. For analysis, gels were transferred to filter paper and exposed to X-ray film overnight at -70°C. For quantitation, films were scanned, and band intensities were calculated using the public domain NIH Image program.

Calcium Mobilization Assay. Changes in levels of intracellular Ca^{2+} in response to chemokine were analyzed using calcium-responsive dye fluores-

cence by flow cytometry. IDCs were washed and resuspended at a concentration of $10^7/\text{ml}$ in HBSS containing 10 mM HEPES and combined with an equal volume of 20 μM Indo-1AM (Molecular Probes, Eugene, OR) in HBSS for 30 min at 37°C. The cells were washed and resuspended in HBSS containing 10 mM HEPES 0.05% BSA. The sample was analyzed with a FACStar+ flow cytometer (Becton Dickinson, San Jose, CA) using a UV laser for excitation with violet (390 nm) and blue (500 nm) fluorescence emissions recorded. Recombinant chemokine (R&D Systems) or cell supernatant was added after a 60-s baseline reading. Data plots were generated using the FlowJo software program (Tree Star, Palo Alto, CA).

In Vivo Injection of Chemokine-expressing Tumor Cells. C57BL/6, beige, or SCID mice were age and sex matched for individual experiments. For tumorigenicity studies, 1×10^6 CMT93 or 5×10^5 B16 cells or clones were injected s.c. (100 μl) into the flank region. Animals were examined daily until the tumor became palpable; thereafter, the diameter, in two dimensions, was measured three times weekly using calipers. For tumor free data, animals were scored as tumor free if they failed to develop tumors > 0.3 cm in diameter. For survival data, animals were euthanized when tumor size reached 1 cm in any one perpendicular direction. Statistical analysis of survival curves was done using the Log-rank test. A *P* of <0.05 was considered significant.

In Vivo Injection of HSVtk-expressing Tumor Cells. Tumors were established by injection of 5×10^5 s.c. (100 μl). Once tumors reached a size of 0.3 cm in one diameter, appropriate groups received daily i.p. injection of GCV (70 mg/kg) in 100- μl volumes for seven consecutive days. If primary GCV treatment failed, when tumors reached a size of 0.5×0.5 cm, mice received surgery to remove tumors. For rechallenge experiments, mice received an s.c. injection of 1×10^5 parental B16 cells on the opposite flank 10 days after the last surgical removal of tumor in any of the cohorts, along with a control group of naive mice. Animals were euthanized when tumor size reached 1×1 cm in two perpendicular directions. Statistical analysis of survival curves was done using the Log-rank test. A *P* of <0.05 was considered significant.

Analysis of Immune Cell Infiltrate in Vivo. To study immune infiltrate, randomly selected untreated tumors were removed when they reached a size of $\sim 0.5 \times 0.5$ cm. Half of the tumors were fixed, and sections were H&E stained. The remaining half was FACS analyzed for immune infiltrate. Tumors were dissociated and three color stained with directly conjugated antibodies as follows: CD45 PerCP; CD8 PE; CD4 PerCP; CD3e FITC; NK1.1 PE; CD11c PE; Mac3 PE; I-a^b PE; CD11c FITC; Ly-6G PE; I-a^b FITC; and CD14 FITC (BD PharMingen). To study immune infiltrate in CMT93 tumors where chemokine expression prevented tumor development, we used a gelfoam matrix to isolate cells infiltrating the inoculation site based on a modification of a protocol described previously (38). Briefly, sterile gelfoam sponges (Pharmacia and Upjohn, Kalamazoo, MI) were cut to a size of $1.5 \times 1.5 \times 1$ cm and rehydrated in sterile HBSS. The sponge was implanted s.c. on the back of the mice, and the incision site was closed with wound clips. Three days after implantation, 1×10^6 CMT93 cells (100 μl) were injected into the sponge, and 24 h later, the sponge was removed. Sponges were finely chopped and then incubated in 15 ml of collagenase enzyme cocktail (20 mg/ml BSA and 400 units/ml collagenase in Saline G, 1.1 grams/liter glucose, 8 grams/liter NaCl, 0.4 gram/liter KCl, 0.29 gram/liter $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.15 gram/liter KH_2PO_4 , 0.15 gram/liter $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.016 gram/liter $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ in endotoxin free water) for 3 h at 37°C with agitation. The suspension was passed through a 100- μm filter, washed with HBSS, and analyzed for specific antibody staining by FACS as above. Infiltrating cells from three mice per group were pooled, and total cells were counted by trypan blue exclusion on a hemacytometer and phenotypically characterized by FACScan analysis.

RESULTS

IDCs Express Receptors for the Inflammatory Chemokines CCL3 and CCL20 and Mobilize Calcium on Stimulation.

To determine the most rational chemokine to express in tumor cells for the recruitment of immature DCs, the pattern of chemokine receptor expression on bone marrow-derived DCs was characterized by RPA analysis. RNA expression of mCCR1 and mCCR5, the receptors for mCCL3; mCCR6, the receptor for mCCL20; and mCCR7, the receptor for mCCL19, in bone marrow-derived IDCs and MDCs was analyzed by RPA (Fig. 1A). Immature DCs expressed RNA for all

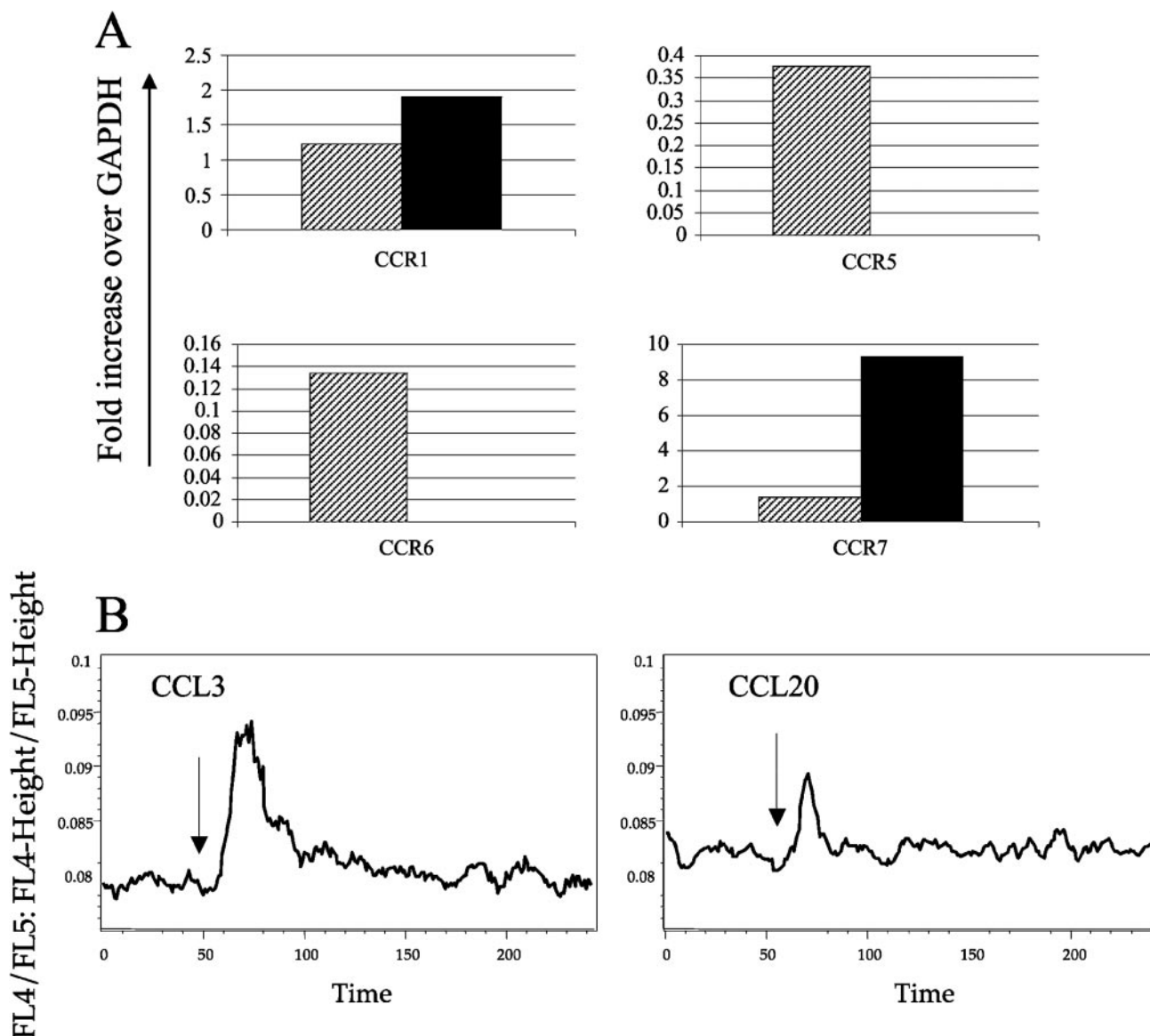


Fig. 1. DC chemokine receptor expression. *A*, densitometry analysis of band intensity after RPA of RNA from *in vitro* cultured bone marrow-derived DC. Values are fold increase in intensity over GAPDH. *Hatched bars*, IDC; *Solid bars*, MDC. *B*, calcium mobilization in Indo-1-labeled DC in response to recombinant CCL3 and CCL20. *Arrows*, the time recombinant chemokine was added to the cells.

four receptors, but on maturation with LPS, RNA expression was lost for mCCR6 and mCCR5, remained constant for mCCR1, and up-regulated for mCCR7 (Fig. 1A). The response of IDC to mCCL3 and mCCL20 was analyzed using a calcium mobilization assay. Bone marrow-derived IDCs mobilized calcium in response to both CCL3 (40 ng/ml) and CCL20 (200 ng/ml; Fig. 1B) but not to a similarly formulated CCL24/eotaxin 2 negative control (40 ng/ml; data not shown). Thus, these chemokines were shown to be able to activate specific chemokine receptors expressed by bone marrow-derived immature DCs in mice. On the basis of these data, we decided to express CCL3 and CCL20 in tumor cells to recruit immature DCs into the tumor.

Construction of Stable Clones Expressing CCL3, CCL20, HSVtk IRES CCL3, HSVtk IRES CCL20. Plasmids were generated that incorporated a neomycin resistance cassette along with the murine CCL3 or CCL20 cDNA expressed from a cytomegalovirus promoter. Bicistronic plasmids were generated that expressed both HSVtk and CCL3 or HSVtk and CCL20 from a cytomegalovirus promoter through the use of an IRES sequence. The murine colorectal

tumor line CMT93 and murine melanoma line B16 were transfected with these plasmids or the parental plasmid expressing the neomycin resistant gene, and stable clones were selected. Secretion of chemokines from appropriate clones was confirmed by ELISA (Table 1). To confirm the biological activity of the secreted CCL3 or CCL20, calcium mobilization assays were performed on IDCs using supernatants from B16 clones expressing either CCL3 or CCL20. Supernatants from both B16 CCL3 and B16 CCL20 induced calcium mobilization in IDCs, but no calcium flux was seen using supernatants from parental B16 cells (Fig. 2). All HSVtk-coexpressing cell clones showed 100% cytotoxicity when grown in 5 μ g/ml GCV (data not shown). Additionally, the *in vitro* bystander cytotoxicity for clones expressing HSVtk alone or HSVtk plus chemokines to non-HSVtk-expressing parental cells was similar (data not shown), indicating similar levels of gene expression.

CCL3 and CCL20 Modification of CMT93 Tumor Development *in Vivo* Is NK Cell and T Cell Dependent. We hypothesized that chemo-attraction of immune cells by tumor expression of the chemokines CCL3 and CCL20 would influence tumor development.

C57BL/6 mice were s.c. challenged with CMT93 tumors expressing CCL3, CCL20, or the *neo^r* gene alone. Development of tumors was followed for 60 days. Only mice who failed to develop tumors were scored as tumor free. Expression of CCL3 or CCL20 was sufficient to significantly inhibit tumor development within these mice when compared with control tumor (Table 2) in a chemokine dose-dependent fashion. Thus, for chemokine tumors mixed with CMT93 neo tumors an increasing number of mice developed tumors (Table 2).

To better understand the mechanisms by which chemokine expression abrogated tumor growth, we wished to characterize the immune infiltrate in tumors expressing chemokines to complement the *in vitro* studies of receptor expression (Fig. 1). However, the failure of CMT93 expressing CCL3 and CCL20 to develop tumors made it difficult to analyze *in vivo* immune infiltrate by histology. For this reason, we used a gelfoam matrix to isolate cells from the site of tumor inoculation. Both CCL3- and CCL20-transfected CMT93 tumors showed an overall increase in leukocytes (CD45⁺) and, more specifically, an increase in CD8⁺, NK1.1, and Class II⁺/CD11c⁺

Table 1 Chemokine production from B16 and CMT93 clones (ng/ml/5 × 10⁵ tumor cells/48 h)

Cell line	Concentration (ng/ml/5 × 10 ⁵ /48 h)	SD
CMT93 neo	0.0005	0.008
CMT93 CCL3	4.8	0.91
CMT93 CCL20	2.218	0.071
B16 neo	0.007	0
B16 CCL3	12	0.9
B16 CCL20	3.07	0.085
B16 HSVtk IRES CCL3	3.074	0.86
B16 HSVtk IRES CCL20	2.7	0.058

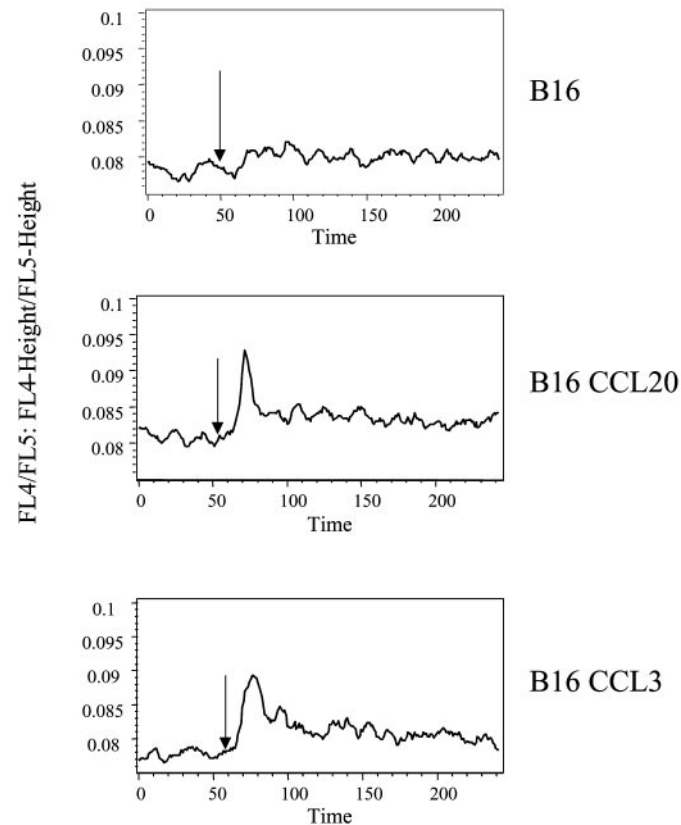


Fig. 2. Functional analysis of chemokines secreted from B16 clones. Calcium mobilization in Indo-1-labeled, bone marrow-derived DC in response to cell free supernatants from B16, B16 CCL3, and B16 CCL20 tumors. B16 cells were cultured at a concentration of 5 × 10⁵ cells/ml/48 h. Arrows, the time 100 μl of supernatant was added to the cells.

Table 2 Effect of chemokine expression on CMT93 tumorigenicity

Tumor	Percentage of mice tumor free
CMT93	20% (30 mice)
CMT93 CCL3	73% (30 mice)
CMT93 CCL20	80% (10 mice)
CMT93 CCL3 50%	80% (10 mice)
CMT93 CCL3 25%	40% (10 mice)
CMT93 CCL20 50%	80% (10 mice)
CMT93 CCL20 25%	40% (10 mice)

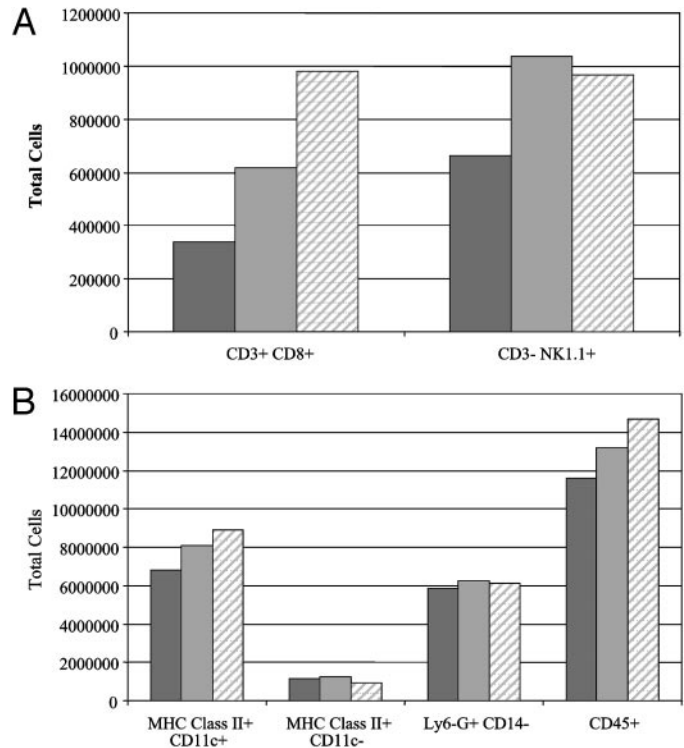


Fig. 3. CCL3 and CCL20 expression in CMT93 tumors increases immune cell infiltrate into tumors. Gelfoam sponges were surgically placed s.c. on the flanks of C57BL/6 mice. Forty-eight h later, 10⁶ tumor cells were injected into the sponge. Twenty-four h later, the sponges were harvested and pooled from each group, and single cell suspensions were prepared. FACS was done for cell surface molecules. Here, three animals per group were pooled and analyzed. Absolute cell numbers recovered from each sponge were assessed using the total cell counts from each group and multiplied by the percentage determined by FACS. Black bars, CMT93 neo; gray bars, CMT93 CCL3; hatched bars, CMT93 CCL20. This is representative of two experiments done.

cells but no difference in Ly6G⁺/CD14⁻ or Class II⁺/CD11c⁻ cells (Fig. 3, A and B). Thus, within 24 h of implantation, chemokine expression attracted cells that phenotypically resembled DCs consistent with our earlier *in vitro* studies on bone marrow-derived DCs (Fig. 1B). In addition, we observed infiltrates of NK and T cells.

Because subsets of NK and T cells express receptors for both CCL3 and CCL20, we investigated whether either of these cells play a role in the observed effects (16, 23, 24). Beige mice that lack functional NK cells (Fig. 4A) and SCID mice that lack functional T and B cells (Fig. 4B) were challenged with CMT93 tumors expressing CCL3, CCL20, or *neo^r*. All tumors developed at a similar rate in beige mice. Thus, no significant difference in survival between chemokine-expressing and parental tumors was observed in mice lacking NK cells. These data suggest that NK cells are necessary for the inhibition of tumor development in chemokine-expressing CMT93 cells. Similar results were observed in SCID mice (Fig. 4B), indicating a similar requirement for functional T and B cells.

Thus, we observed that in the immunogenic CMT93 model, chemokine expression alone is sufficient to stimulate an immune response against the tumor.

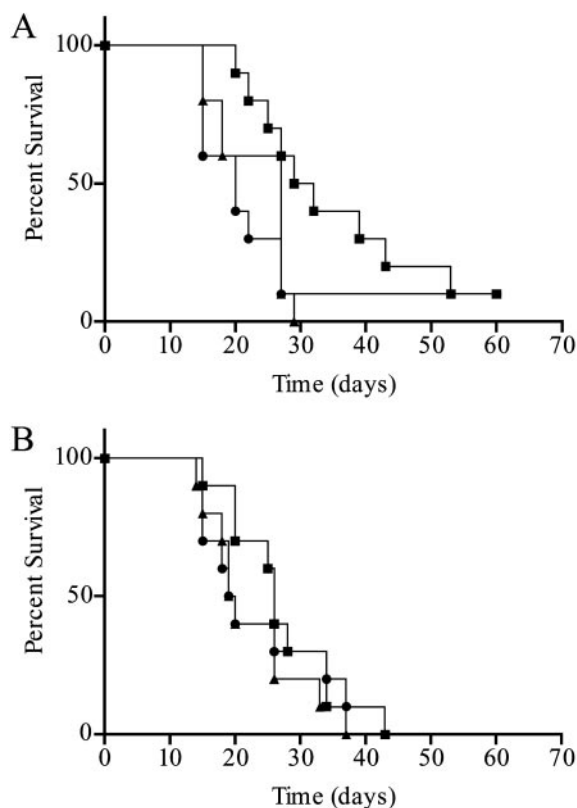


Fig. 4. CCL3 and CCL20 expression in CMT93 tumors decrease tumor development *in vivo* in NK- and T cell-dependent fashion. 10^6 CMT93 neo (10 mice), CMT93 CCL3 (10 mice), or CMT93 CCL20 (10 mice) cells were injected s.c. into beige (A) or SCID (B) mice. Tumor growth was measured, and animals were sacrificed when tumors reached a size of 1 cm in any direction. Survival data are shown. For all curves, CMT93 neo (■), CMT93 CCL3 (●), and CMT93 CCL20 (▲).

Neither Expression of CCL3 nor CCL20 from B16 Tumors Results in a Decrease in Tumorigenicity Despite Modifying the Immune Infiltrate into Tumors. CMT93 tumors have a higher level of immunogenicity when compared with the murine melanoma B16. Specifically, vaccination with irradiated CMT93 tumors protects against subsequent rechallenge in the majority of cases. By contrast, vaccination with irradiated B16 does not lead to protection (data not shown). We therefore wanted to see whether chemokine expression in a less immunogenic tumor model would lead to a similar decrease in tumorigenicity. However, expression of CCL3 and CCL20 in poorly immunogenic B16 tumors showed no decrease in tumorigenicity when compared with tumors expressing *neo*' alone (Fig. 5A). It was possible that B16 was secreting a factor that blocked the effects of the chemokines *in vivo*. However, based on our earlier data, we had shown *in vitro* that supernatants from chemokine-expressing B16 cells resulted in calcium mobilization within IDCs (Fig. 2). The outgrowth of tumors was not attributable to loss of chemokine expression. Reverse transcription-PCR of RNA prepared from growing tumors demonstrated the presence of sustained chemokine gene expression in chemokine-transfected tumors but not in controls (data not shown). Furthermore, we went on to look at the immune infiltrate in chemokine-expressing tumors by FACS. An overall increase in leukocytes (CD45+) was observed in both CCL3- and CCL20-expressing tumors (Fig. 6A). Specifically, increased numbers of CD4+, CD8+, and NK1.1+ (Fig. 6B) lymphocytes were detected, along with increased numbers of Class II+, Mac3+, and CD11c+ APCs (Fig. 6C). These data are consistent with those seen with gelfoam sponges in CMT93 tumors described above (Fig. 4, A and B). Examination of H&E-stained histological sections of tumors expressing chemokine indi-

cated an increase in necrosis in tumors where chemokine was expressed (Fig. 7, A–C). Additionally, a perivascular cuff of mononuclear cells (lymphocytes plus macrophages) was observed within the CCL20 tumor, indicating recruitment of immune cells into the tumor from the vasculature (Fig. 7D). This perivascular cuffing was never observed in B16 tumors. Thus, chemokine expression alone causes an increased infiltrate of specific immune cells into B16 tumors that was associated with an increase in necrosis but was unable to affect tumor growth.

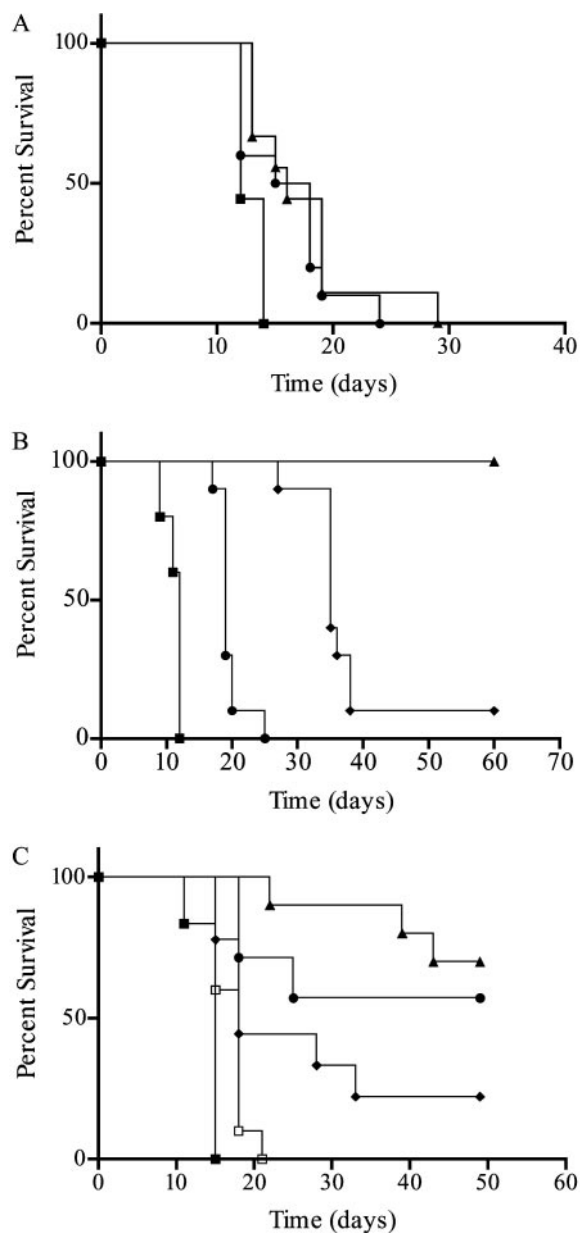


Fig. 5. CCL3 and CCL20 expression alone or with HSVtk in B16 tumors *in vivo*. In A, 5×10^5 cells were injected s.c. in C57BL/6 mice. Tumor growth was measured, and animals were sacrificed when tumors reached a size of 1 cm in any direction. B16 neo (10 mice, ■), B16 CCL3 (10 mice, ●), and B16 CCL20 (10 mice, ▲). In B, 10^5 tumor cells were injected s.c. into C57BL/6 mice. Tumors were allowed to reach a size of 0.3 cm in any one direction. Then GCV treatment was given i.p. at a concentration of 70 mg/kg for 7 days. Tumor growth was monitored, and survival data are shown. B16 (10 mice, ■), B16 HSVtk (10 mice, ◆), B16 HSVtk IRES CCL3 (10 mice, ●), and B16 HSVtk CCL20 (10 mice, ▲). In C, after treatment with GCV as described in B and "Materials and Methods," mice received a second tumor challenge with parental B16 tumor on the opposite flank. Tumor growth was monitored, and survival data are shown. B16 (6 mice, ■), B16 HSVtk (9 mice, ◆), B16 HSVtk IRES CCL3 (7 mice, ●), B16 HSVtk CCL20 (10 mice, ▲).

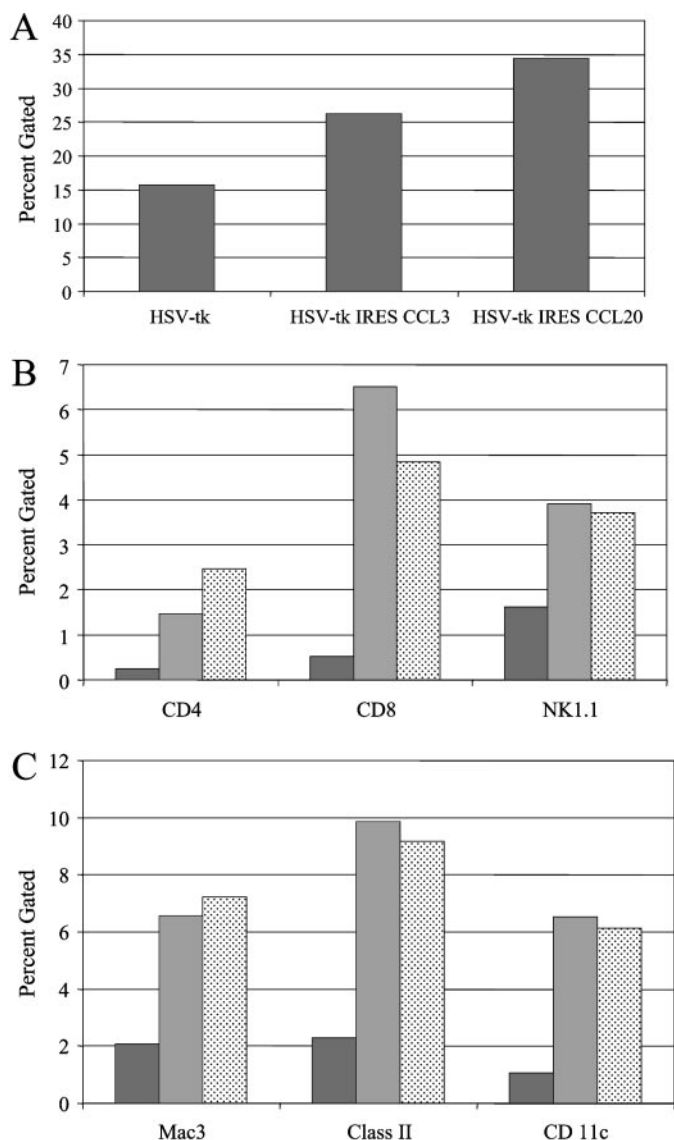


Fig. 6. FACS analysis on immune cells infiltrating B16 tumors. 10^5 tumor cells were injected s.c. into C57BL/6 mice. Tumors were allowed to reach a size of 0.3 cm in any one direction and then surgically removed. Half was used to prepare a single cell suspension for FACS analysis. In A, the live population was gated on, and the percentage of cells expressing CD45 is shown. In B and C, the live population was gated on, and the percentage of cells coexpressing CD45 and indicated cell surface molecule are shown. Black bars, B16 HSVtk; gray bars, B16 HSVtk IRES CCL3; spotted bars, HSVtk IRES CCL20.

Coexpression of CCL20 with HSVtk Enhances the Antitumor Immune Effects of HSVtk or CCL20 Alone in Established B16 Tumors.

We hypothesized that where attraction of APCs alone was insufficient to generate an effective immune response to tumor, additional provision of tumor antigen in the appropriate context to these APC at the tumor site would ensure effective stimulation of effector immune cells. On the basis of our previous work with B16 and HSVtk, the cell death associated with a growing B16 tumor is itself not immunogenic. However, HSVtk can be used to generate death and antigen release in an immunogenic context associated with an increase in proinflammatory factors secreted from the tumor, including HSP-70 (20, 25). Furthermore, by coculturing macrophages with HSVtk lysates and HSP-70, we have shown an increase in inflammatory cytokine release (26). Therefore, we attempted to enhance the effects of chemokines by generating B16 clones that coexpressed HSVtk and CCL3 or HSVtk and CCL20. All HSVtk-coexpressing cell

clones show 100% cytotoxicity when grown in 5 μ g/ml GCV (data not shown). Additionally, the *in vitro* bystander cytotoxicity for clones expressing HSVtk alone or HSVtk plus chemokines to non-HSVtk-expressing parental cells was similar (data not shown), indicating similar levels of gene expression. C57BL/6 mice were challenged s.c. with parental B16, B16 HSVtk, B16 HSVtk IRES CCL3, or B16 HSVtk IRES CCL20, and tumors were treated for 7 days with 70 mg/kg GCV i.p. GCV therapy was initiated either 3 days after inoculation when tumor size is still small or at a later time point when tumors had reached a size of 0.3 \times 0.3 cm. When treatment was given 3 days after tumor challenge, all of the tumors expressing HSVtk regressed but not the parental B16 tumors (data not shown). When mice received GCV treatment for larger (0.3 \times 0.3 cm) tumors, despite the fact that similar levels of HSVtk killing and bystander effect were observed *in vitro*, only the mice expressing HSVtk IRES CCL20 showed complete regression with no regrowth of tumor after GCV treatment ($P < 0.0001$; Fig. 5B). Mice bearing tumors expressing HSVtk IRES CCL3 or HSVtk alone showed significant delay in tumor growth when compared with parental B16 tumors ($P < 0.0001$; Fig. 5B).

To test for the development of effective immune responses not observable during primary tumor development, mice in all groups receiving late GCV therapy underwent surgery to remove any remaining tumors. Ten days after the last surgery, all four groups plus a naive group were challenged with parental B16 tumors on the opposite flank. In both the naive group and that in which mice were initially challenged with B16, all mice succumbed to tumor rechallenge. In mice initially challenged with HSVtk-expressing B16 cells, the majority of mice developed tumor on rechallenge, whereas both CCL3- and CCL20-expressing tumors showed a significant increase in protection against subsequent tumor rechallenge ($P < 0.0001$; Fig. 5C). So despite the fact that chemokine expression from B16 cells is sufficient to modify the infiltrate, additional provision of tumor antigen in the appropriate context, through HSVtk/GCV-mediated cell death, was needed to generate a sufficient immune response to clear residual tumor cells. This was true for CCL20-expressing tumors but not for CCL3 tumors. However, coexpression of either CCL20 or CCL3 with HSVtk was able to generate protection against subsequent tumor rechallenge.

DISCUSSION

We have shown that chemokines can be used to modify the immune cell infiltrate in tumors. Specifically, inflammatory chemokines can be used to generate a profile of infiltrating leukocytes resembling that generated during an ongoing antiviral immune response. Moreover, the recruitment of these cells, including DCs, NK cells, and T cells, is sufficient in the moderately immunogenic colorectal tumor model CMT93 to decrease tumorigenicity significantly. Interestingly, both NK and T cells were required for this response. In the poorly immunogenic melanoma tumor model B16, chemokine expression alone was not sufficient to inhibit tumor development. This could be overcome by combining the cytotoxic gene HSVtk with chemokine expression. In large B16 tumors, HSVtk and HSVtk IRES CCL3 expression significantly inhibited tumor progression when compared with parental B16, but they were unable to eradicate the tumors completely. In contrast, HSVtk IRES CCL20 mediated complete tumor inhibition and regression. Furthermore, we have shown that on subsequent rechallenge with parental B16 tumors, coexpression of a chemokine with HSVtk showed a significant increase in protection when compared with HSVtk expression alone. So although CCL3 coexpressed with HSVtk did not show any effect on primary tumor regression, expression of the chemokine did improve the generation of

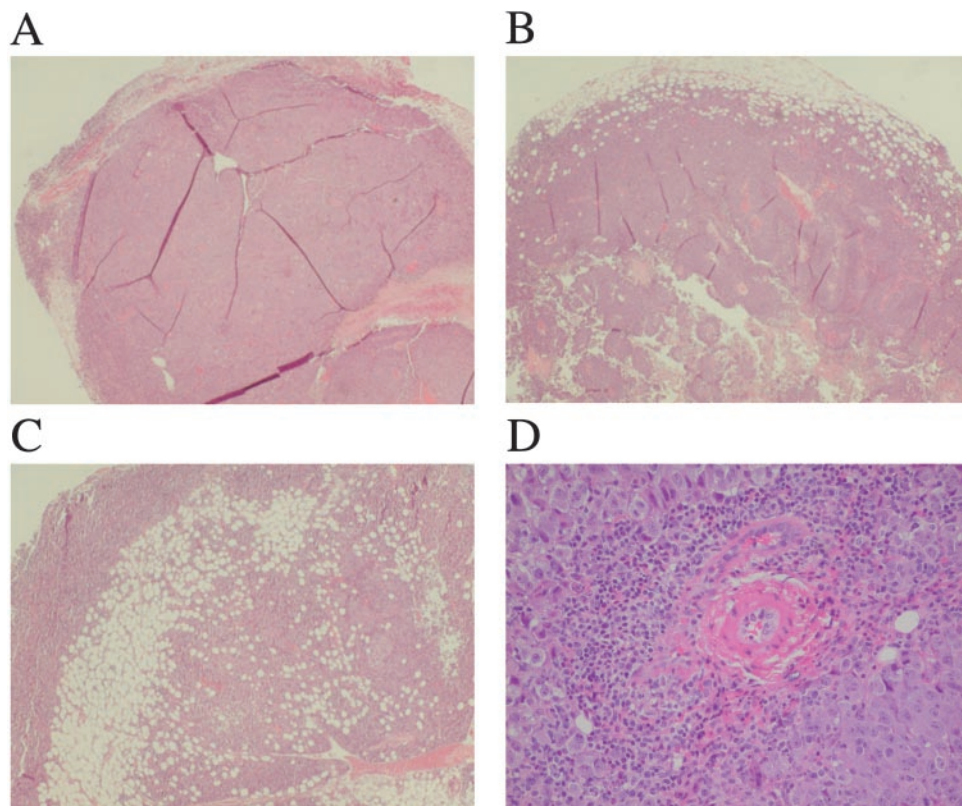


Fig. 7. H&E staining of B16 tumors. 10^5 tumor cells were injected s.c. into C57BL/6 mice. No GCV was given to these mice. Tumors were allowed to reach a size of 0.3 cm in any one direction and then surgically removed. Half of the tumor was fixed for histological analysis. Sections were stained with H&E. A, B16 tumor; B, B16 HSVtk IRES CCL3; C, B16 HSVtk IRES CCL20; D, perivascular cuff in B16 HSVtk IRES CCL20 tumor.

an immune response to B16. Impressively, CCL20 coexpressed with HSVtk was both able to eliminate large B16 tumors and generate a robust immune response to subsequent tumor rechallenge.

We have shown that, although in some models, chemokine expression alone is sufficient to prevent tumor development, this is not the case for all tumors. There may be several reasons why this is true. Although chemokine expression will attract a repertoire of immune cells into tumors, other environmental factors within the tumor may influence the ability of these cells to initiate or perpetuate an immune response, *e.g.*, certain tumors aggressively suppress immune cell activation through the release of anti-inflammatory molecules, such as IL-10, transforming growth factor- β , and vascular endothelial growth factor (27–29). Other factors that may influence the immune response within tumors include the relative susceptibility of tumor cells to innate immune recognition by cells such as macrophages and NK cells.

Another consideration is that tumors may release varying amounts of antigen as they develop. The context in which this antigen is presented to the immune cells is important, in some cases leading to tolerance and in other instances leading to activation (30). Therefore, although DCs may be recruited into tumors, without antigen release in the appropriate context, they will be either unable to cross-prime naïve T cells in draining lymph nodes, or this cross-priming will lead to T-cell suppression. Expression of the cytotoxic gene HSVtk with the chemokine CCL20 was able to eliminate large B16 tumors. We have described previously that B16 tumors killed by HSVtk undergo a nonapoptotic cell death (20). Furthermore, we have shown that nonapoptotic cell death is associated with increased immunogenicity *in vivo* (26). The enhanced effect of HSVtk when coexpressed with chemokines that recruit DCs may in part be caused by this release of antigen in the appropriate context for DCs to phagocytose and cross-present. Certainly, both CCL3 and CCL20 were able to enhance the immunogenicity of B16 HSVtk tumors.

Other studies have examined the combinations of cytokines with cytotoxic gene expression. Although some cytokines, such as GM-CSF, in combination with HSVtk have been shown to enhance protection against subsequent tumor rechallenge, they showed no enhancement in the regression of established primary tumors. In contrast, although IL-2 coexpression with HSVtk has been shown to enhance the regression of primary tumor, this did not extend to protection against subsequent tumor rechallenge (31). Significantly, here we have shown that CCL20 was capable of doing both. This may reflect the importance in choosing an appropriate chemokine that will recruit DCs to prime a naïve T cell as well as effector cells, such as NK and T cells.

The different results of different chemokines, despite some overlap in responsive cells, emphasize additional influences within the immune response. It will be necessary to further understand the cell types that contribute to the success or failure of protective immunity. In addition, it will be necessary to determine the relative contribution of chemokine expression in initiation of *de novo* immune responses and in attracting effector cells for tumor clearance once immune responses have begun.

Studies investigating the expression effects of chemokines have shown inhibition of tumor development to varying degrees. HCCL3 delayed tumor progression in a murine adenocarcinoma model after foot pad inoculation but was unable to completely eliminate tumors in the majority of cases (32). Adenoviral delivery of hCCL20 into murine tumors inhibited tumor progression in a CD8 T cell-dependent fashion. In these studies, the effectiveness of the chemokine may have been enhanced as a result of damage and antigen release during delivery (33). In our B16 model, we were unable to show that chemokine expression alone was sufficient to prevent tumor growth if it was expressed over the course of the development of tumor. Therefore, we conclude that cell death along with antigen release in an immunogenic context is required in the B16 model.

The present studies indicate that chemokine expression and immune cell recruitment alone are sufficient in some tumor models to inhibit tumor development, but that in other tumors, additional factors may be required. Consistent with this, chemokine expression, including CCL20, has been shown within human tumors and is clearly not sufficient to inhibit tumor development (34–36). Furthermore, some chemokines, including RANTES, have been shown to enhance formation of human tumors in nude mice (37). We show here that cytotoxic gene expression resulting in release of antigen from tumor cells for uptake and cross-presentation by infiltrating DCs significantly enhances the generation of tumor immunity when compared with chemokine expression alone. We would propose that future approaches to tumor therapy focus on both recruitment of inflammatory cells into tumors and provision of antigen through appropriate cell death that allows DC to pick up antigen, mature, and migrate to draining lymph nodes. We have shown in previous studies that the mode of cell death associated with HSVtk expression in B16 tumors is proinflammatory. Signals released from B16 tumors as they die, such as Hsp-70, activate macrophages that can provide signals for DC maturation (26). Therefore, provision of signals to recruit and activate DCs will all be required to generate optimal antitumor responses.

These data emphasize local expression of chemotactic factors within established tumors to generate immune-mediated tumor clearance. Clinical translation of these data would be most compatible with gene therapy approaches targeting accessible tumor deposits, *e.g.*, in an adjuvant setting. Additional work is underway to generate suitable gene therapy vectors to test this hypothesis.

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