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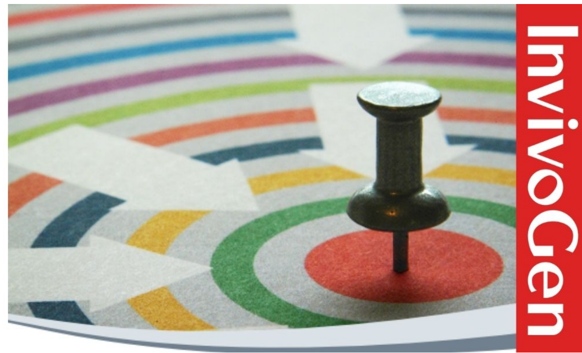

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CXCR3/CXCR3 Ligand Biological Axis Impairs RENCA Tumor Growth by a Mechanism of Immunoangiostasis[1]

Judong Pan,* Marie D. Burdick,* John A. Belperio,* Ying Ying Xue,* Craig Gerard,§ Sherven Sharma,* Steven M. Dubinett,* and Robert M. Strieter[2]*†‡

Metastatic renal cell carcinoma (RCC) responds poorly to chemo- or radiation therapy but appears to respond to systemic immunotherapy (i.e., IL-2 and/or IFN-α), albeit with only 5–10% durable response. The CXCR3/CXCR3 ligand biological axis plays an important role in mediating type 1 cytokine-dependent cell-mediated immunity, which could be beneficial for attenuating RCC if optimized. We found that systemic IL-2 induced the expression of CXCR3 on circulating mononuclear cells but impaired the CXCR3 ligand chemotactic gradient from plasma to tumor by increasing circulating CXCR3 ligand levels in a murine model of RCC. Moreover, the antitumor effect of systemic IL-2 was CXCR3-dependent, as IL-2 failed to inhibit tumor growth and angiogenesis in CXCR3$^{-/-}$ mice. We hypothesized that the immunotherapeutic effect of the CXCR3/CXCR3 ligand biological axis could be optimized by first priming with systemic IL-2 to induce CXCR3 expression on circulating mononuclear cells followed by enhancing the intratumor CXCR3 ligand levels to establish optimal CXCR3-dependent chemotactic gradient. We found that combined systemic IL-2 with an intratumor CXCR3 ligand (CXCL9) lead to significantly greater reduction in tumor growth and angiogenesis, increased tumor necrosis, and increased intratumor infiltration of CXCR3$^+$ mononuclear cells, as compared with either IL-2 or CXCL9 alone. The enhanced antitumor effect of the combined strategy was associated with a more optimized CXCR3-dependent chemotactic gradient and increased tumor-specific immune response. These data suggest that the combined strategy of systemic IL-2 with intratumor CXCR3 ligand is more efficacious than either strategy alone for reducing tumor-associated angiogenesis and augmenting tumor-associated immunity, the concept of immunoangiostasis. ***The Journal of Immunology,*** **2006, 176: 1456–1464.**

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Renal cell carcinoma (RCC)[3] accounts for ~3% of new cancer incidence and mortality in the United States (1). In general, approximately one-third of patients at the time of presentation have metastatic RCC (mRCC), and another third that present with local disease will eventually experience recurrence and metastases with a median survival of less than 1 year (2). Although treatment of local disease is surgical resection, the therapeutic dilemma of mRCC is that it does not respond to conventional chemo- or radiation therapy (3). As a potential alternative therapeutic approach, immunotherapy with IL-2 and/or IFN-α has led to only objective responses in 10–25% of patients with mRCC, and evidence of ~5–10% durable responders (4–6). Given the potential immunogenic nature of RCC, the response of mRCC to systemic immunotherapy is relatively low. Further insights into the underlying mechanisms of this phenomenon will be necessary for the development of more effective immunotherapy strategies.

IFN-inducible CXC chemokines such as CXCL9, CXCL10, and CXCL11 are multifunctional chemokines that are potent inhibitors of angiogenesis (7–9). All the IFN-inducible CXC chemokines act through the G-protein coupled receptor CXCR3 (10–16). CXCR3/CXCR3 ligands play a critical role in orchestrating type 1 cytokine-induced cell-mediated immunity via the recruitment of CXCR3-expressing mononuclear cells (11, 13–16). CXCR3 has been reproducibly found on type 1 cells, such as memory and activated CD4 and CD8 lymphocytes, B cells, and NK cells (11, 12). The recruitment of these specific cells is necessary to develop a type 1 cell-mediated immune phenotype in the local environment. IL-2 has been found to be a major agonist for induction of the expression of CXCR3 on mononuclear cells (10–12). Increasing evidence suggests that the CXCR3/CXCR3 ligand (i.e., CXCL9, CXCL10, and CXCL11) biological axis may be important in the development of type 1 cytokine-induced cell-mediated antitumor immunity (17–23), and at the same time inhibit tumor-associated angiogenesis leading to suppression of tumor growth (24–28).

We postulated that the low response rate of RCC to systemic immunotherapy with IL-2 may be related to increased CXCR3 on mononuclear cells without the establishment of an effective CXCR3 ligand chemotactic gradient within the tumor microenvironment, the concept of activated systemic circulating cells without an effective chemotactic gradient in which to extravasate. In support of this notion, we found that systemic IL-2 therapy induced expression of CXCR3 on circulating mononuclear cells in CXCR3$^{+/+}$ mice, but impaired the CXCR3 ligand chemotactic gradient within a murine model of RCC (RENCA) by increasing

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[3] Abbreviations used in this paper: RCC, renal cell carcinoma; mRCC, metastatic RCC; MIG, monokine induced by IFN-γ; IP-10, IFN-γ-inducible protein 10; mCXCL, murine CXCL; MMC, mitomycin C; BC, buffy coat cell.

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systemic levels of CXCL9 and CXCL10 without markedly increasing the intratumor levels of these CXC chemokines. Moreover, in the absence of CXCR3, IL-2 therapeutic benefit was essentially lost. From these studies, we hypothesized that "systemic priming" with IL-2 to induce the expression of CXCR3 on circulating mononuclear cells, combined with a strategy to increase intratumor levels of a CXCR3 ligand, could maximize the CXCR3/CXCR3 ligand biological axis, and concomitantly reduce tumor-associated angiogenesis and augment the host immune response to the tumor. Our results indicated that systemic IL-2 and concomitant intratumor CXCR3 ligand (i.e., CXCL9) lead to significantly greater suppression of tumor growth, enhanced tumor necrosis, reduced tumor-associated angiogenesis, and increased infiltration of $CXCR3^+$ mononuclear cells into murine RCC (RENCA) tumors, as compared with treatment with either IL-2 or CXCL9 alone. Moreover, the augmented antitumor effect of combined systemic IL-2 and intratumor CXCL9 therapy was associated with an improved CXCR3/CXCR3 ligand chemotactic gradient, as compared with systemic IL-2 therapy alone. Furthermore, mononuclear cells isolated from the spleen of the $CXCR3^{+/+}$ mice bearing RENCA tumors that had been treated with combined systemic IL-2 and intratumor CXCL9 were found to have enhanced proliferative capacity when rechallenged with RENCA tumor cells, but not with syngeneic control tumor cells. These findings support the notion that the CXCR3/CXCR3 ligand biological axis is critical in mediating the antitumor effect of systemic IL-2 therapy and illustrates a strategy to optimize immunotherapy by combining systemic activation of mononuclear cells to express CXCR3 and at the same time enhance the spatial CXCR3 ligand chemotactic gradient to promote greater mononuclear cell extravasation within the tumor, inhibit tumor-associated angiogenesis, and enhance the host immune response to the tumor.

Materials and Methods

Reagents

Polyclonal goat anti-murine monokine induced by IFN-γ (MIG)/CXCL9 and anti-murine IFN-γ-inducible protein 10 (IP-10)/CXCL10-specific antiserum were produced by the immunization of goats with the appropriate recombinant murine chemokines (MIG/CXCL9 or IP-10/CXCL10; R&D Systems) (21, 24, 26, 29, 30). The specificity of anti-murine CXCL9 (mCXCL9) and anti-mCXCL10 Abs was assessed by Western blot analysis against a panel of human and murine recombinant cytokines. The anti-mCXCL9 and anti-mCXCL10 Abs were specific in our sandwich ELISA without cross-reactivity to a panel of cytokines including human and murine IL-1 receptor antagonist, IL-1, IL-2, IL-6, IL-4, TNF-α, IFN-γ, and members of the CXC and CC chemokine families (21, 24, 26, 29, 30). The anti-mCXCL9 and anti-mCXCL10 Abs have been previously used in vivo in mouse models to neutralize CXCL9 and CXCL10, respectively (21, 24, 26, 29, 30). The recombinant murine chemokine CXCL9 (R&D Systems) was used for intratumor injection in a mouse RENCA model.

Mouse RENCA model

The murine RCC tumor cell line (RENCA; a gift from Dr. A. Belldegrum, David Geffen School of Medicine, University of California, Los Angeles, CA) was originally isolated from a renal carcinoma that developed spontaneously in BALB/c mice (31). BALB/c $CXCR3^{+/+}$ mice (6–8 wk old) were obtained from The Jackson Laboratory. BALB/c $CXCR3^{-/-}$ mice were provided by one of the coauthors (C. Gerard) (32). To determine the effects of systemic IL-2 therapy on RENCA tumor growth and CXCR3 chemotactic gradient, BALB/c $CXCR3^{+/+}$ mice were injected s.c. with RENCA cells (s.c., 10^6 cells/100 μl) into one flank using a modification as previously described (24–26). The tumors were allowed to establish for 3 days. Four days after tumor inoculation, mice were subjected to systemic administration of IL-2 (30,000 IU or vehicle control i.p. bid per mouse for 5 days). Mice were subsequently sacrificed at 4 wk after initiation of IL-2 therapy for the measurement of tumor size and CXCR3 ligand levels in tumors and plasma. To test the effect of systemic IL-2 therapy on the induction of CXCR3 expression on circulating mononuclear cells, a separate experiment was performed in a similar manner except that systemic IL-2 was administered for 7 days. Buffy coat cells (BC) from peripheral blood were collected daily for 7 days for FACS analysis of CXCR3 expression. In separate experiments, BALB/c $CXCR3^{+/+}$ and $CXCR3^{-/-}$ mice were injected s.c. with RENCA cells, subjected to systemic administration of IL-2 for 5 days, and subsequently sacrificed at 4 wk after initiation of IL-2 therapy as described above for determination of the role of CXCR3 in mediating the antitumor effect of systemic IL-2 therapy. In addition, to test the therapeutic efficacy of different immunotherapy strategies, one cohort of mice were subjected to systemic IL-2 for 5 days as described above. Two other cohorts of mice were injected with intratumor mCXCL9 or murine serum albumin 1 μg/20 μl on Monday, Wednesday, and Friday for up to 4 or 7 wk starting on day 4 after tumor cell inoculation. Another cohort received combined systemic IL-2 and intratumor CXCL9 as described above for each injection. The animals were maintained under specific pathogen-free conditions and sacrificed in groups of 6–10 mice at specified time points. Heterotopically placed RENCA tumors were dissected from the mice at specified times and measured with a Thorpe caliper (Biomedical Research Instruments). Tumor volume was calculated using the formula: volume = $(d_1 \times d_2 \times d_3) \times 0.5236$, where d_n represents the three orthogonal diameter measurements. Tumor and tissue specimens were then processed. All studies were approved by the University of California (Los Angeles, CA) institutional animal care and use committee.

MIG/CXCL9 and IP-10/CXCL10 ELISAs

Intact murine CXCL9 and CXCL10 protein levels in plasma and tumor tissue were quantitated using a modification of a double ligand method as previously described (24, 26, 33). The amount of intact CXC chemokine present was determined by interpolation of a standard curve generated by known amounts of recombinant CXC chemokine protein. The sensitivity for the specific CXC chemokine ELISAs was ≥50 pg/ml, and these assays failed to cross-react with a panel of known cytokines and other chemokines as previously described (24, 26, 33).

FACS analysis of leukocytes, endothelial cells, and CXCR3 expression

For FACS analysis of a subpopulation of leukocytes and endothelial cells, buffy coats from peripheral blood or single-cell suspensions of the tumors were made using a modification as previously described (29, 30, 33–35). Briefly, BC isolated from peripheral circulation were stained with primary FITC-conjugated CXCR3 (Santa Cruz Biotechnology) Ab. Tumors were harvested from animals and minced with scissors to a fine slurry in 15 ml of digestion buffer (RPMI 1640, 5% FCS, 1 mg/ml collagenase (Boehringer Mannheim Biochemical), 30 μg/ml DNase (Sigma-Aldrich)). Tissue slurry was enzymatically digested for 45 min at 37°C. Drawing the solution up and down through the bore of a 10-ml syringe further dispersed any undigested fragments. The total cell suspension was pelleted, and resuspended in FACS analysis buffer. Cell counts and viability were determined using trypan blue exclusion on a hemocytometer. Single-cell suspensions from tumors were stained with the primary biotinylated pan-endothelial cell marker MECA-32 (BD Pharmingen) or isotype controls. The primary Abs were detected with streptavidin-FITC. Tumor and buffy coat samples were also stained with Tricolor-conjugated anti-murine CD45 (Caltag Laboratories), PE-conjugated CD3, CD4, CD8A, NK DX5, Ly6 (BD Biosciences), MAC519 (Serotec), or FITC-conjugated CXCR3 (Santa Cruz Biotechnology). Cells were analyzed on a FACScan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences) as previously described (29, 30, 33–35).

Tumor-specific lymphocyte proliferation assay

The spleens from RENCA tumor-bearing mice from each of the treatment groups (control, systemic IL-2, intratumor CXCL9, or combined systemic IL-2 and intratumor CXCL9) were removed from mice and placed in cold RPMI 1640. Single-cell suspensions of spleen cells were prepared by gently pressing spleen cells through a sterile stainless steel 60-gauge mesh screen into cold RPMI 1640 supplemented with 10% heat-inactivated FBS and L-glutamine. RENCA and L1C2 (i.e., syngeneic tumor control) BALB/c tumor cells were used as stimulator cells, and were pretreated with mitomycin C (MMC) using a modification as previously described (36). Briefly, trypsinized RENCA and L1C2 tumor cells were incubated with 50 μg/ml MMC at 37°C for 30 min followed by washing to remove MMC. MMC-treated RENCA (10^5) or L1C2 (10^5) cells/well were seeded in 96-well culture plates and mixed with 10^5 isolated spleen mononuclear cells/well and incubated for 72 h. After 72 h, 1 μCi/well [^{3}H]thymidine was added to each well, and the plates were incubated for another 18 h. The cells were harvested using a cell harvester, and [^{3}H]thymidine incorporation was quantitated by scintillation counting as previously described (24).

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Morphometric analysis of tumor necrosis

For analysis of mean tumor necrotic areas, morphometric analysis was performed on a minimum of 16 separate H&E-stained sections taken 60 μm apart from different tumors in each group at ×200 magnification. An Olympus BH-2 microscope coupled to a Sony 3CCD camera was used to capture images that were then analyzed using the NIH Image 1.55 software. For determination of tumor necrosis, the average percentage of necrotic area per high power field (×200) for each tumor was determined by morphometric analysis, and this number was then multiplied by the tumor area (square pixels) yielding the average necrotic area for each tumor.

Statistical analysis

The animal studies involved 6–10 mice bearing RENCA tumors for each treatment group. Data were analyzed on a personal computer using the Statview 5.0 statistical package (Abacus Concepts). Comparisons were evaluated by the ANOVA test with the post hoc analysis Bonferroni/Dunn. Data were expressed as mean ± SEM. Data were considered statistically significant if p values were 0.05 or less, designated by an asterisk (*).

Results

Systemic IL-2 therapy alone impairs tumor growth, yet reduces the CXCR3 ligand chemotactic gradient within the RENCA tumors

IL-2 has been shown to have antitumor activity against RCC in various clinical studies (4–6). To begin to assess strategies to improve immunotherapy in RCC, we used a heterotopic murine model of RCC (RENCA), in which we found that systemic IL-2 therapy, as compared with control vehicle, led to significant reduction in RENCA tumor growth at 4 wk (Table I), which was consistent with previous findings for IL-2 therapy in this common model (37–39). In conventional immunotherapy against RCC, however, IL-2 is given systemically with the potential of both systemic immune activation leading to increased expression of CXCR3 on circulating immune cells and elevated levels of plasma CXCR3 ligands that could impact on the CXCR3/CXCR3 ligand-dependent chemotactic gradient for extravasation into the tumor. To test this notion, we assessed the effect of systemic IL-2 therapy on the potential chemotactic gradient of CXCR3 ligands (CXCL9 and CXCL10) between plasma and the intratumor compartments. BALB/c mice were inoculated with RENCA cells into one flank, subjected to systemic murine IL-2 30,000 IU or vehicle control. The mice were sacrificed at week 4 after RENCA cell inoculation and the plasma and tumors were isolated for measurement of protein levels of CXCL9 and CXCL10. Systemic IL-2 therapy resulted in markedly increased levels of both CXCL9 and CXCL10 in the plasma (Fig. 1*A*). In the tumor compartment, systemic IL-2 therapy predominantly increased the levels of CXCL9, but not CXCL10 (Fig. 1*B*). However, the CXCR3-dependent chemotactic gradient between the plasma and tumor compartments in response to systemic IL-2 therapy was neglectable. In fact, the sum of the levels of both CXCR3 ligands under conditions of IL-2 therapy was higher in the plasma, as compared with the tumor.

Table I. *Reduction in RENCA tumor growth by systemic IL-2 treatment*

	RENCA Tumor Size (mm³)		
Weeks	Control Treatment	Systemic IL-2 Treatment	p Value
1	179 ± 7	151 ± 6	$p < 0.05$
2	610 ± 19	367 ± 15	$p < 0.05$
3	1190 ± 41	347 ± 19	$p < 0.05$
4	1551 ± 45	955 ± 60	$p < 0.05$

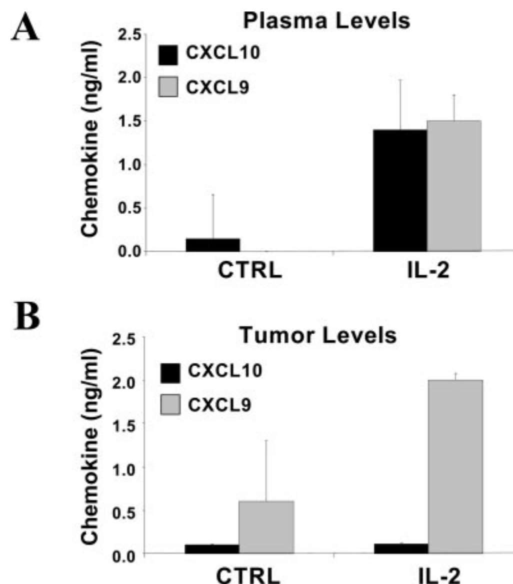

FIGURE 1. Systemic IL-2 therapy impairs the establishment of a CXCR3 ligand chemotactic gradient. Plasma (*A*) and intratumor (*B*) CXCL9/CXCL10 levels in response to systemic IL-2. Plasma and intratumor CXCL9 and CXCL10 protein levels were quantitated at 4 wk using ELISA from mice inoculated with heterotopic RENCA tumors and subjected to systemic IL-2 or control therapy.

Systemic IL-2 therapy induced the expression of CXCR3 on circulating cells

Based on previous studies that have shown that IL-2 is a major agonist in vitro for the expression of CXCR3 on immune cells (10–16), we wanted to determine whether systemic IL-2 therapy could induce in vivo the expression of CXCR3 on circulating cells in BALB/c mice. BALB/c mice were treated with systemic IL-2 and sacrificed in a kinetic manner. At time of sacrifice, BC were harvested for FACS analysis of the percent of CD45 cells expressing CXCR3. On day 1 of IL-2 treatment, we observed that 19 ± 2% of CD45 cells expressed CXCR3 in BC, as compared with 14 ± 2% in the control treated mice (Table II). In contrast, by treatment days 3–7, we detected a 3- to 4-fold increase in the presence of CXCR3$^+$ cells in the BC of IL-2-treated, as compared with control, mice (Table II). The expression of CXCR3 in the BC peaked at day 3, and remained significantly greater than controls throughout the 7-day time course.

Systemic IL-2 therapy is CXCR3-dependent to impair RENCA tumor growth

Because CXCR3 ligands suppress tumor growth by type 1 cytokine-induced cell-mediated immunity and by inhibition of angiogenesis

Table II. *Percentage of CXCR3$^+$ cells in circulation in response to IL-2 therapy*

	% CXCR3$^+$ Circulating Cells[a]		
Day	Control Treatment	IL-2 Treatment	p Value
1	14 ± 2	19 ± 2	NS
2	19 ± 1	21 ± 3	NS
3	15 ± 3	49 ± 6	<0.05
4	12 ± 2	39 ± 7	<0.05
5	10 ± 3	41 ± 4	<0.05
6	12 ± 2	35 ± 6	<0.05
7	14 ± 4	42 ± 5	<0.05

[a] Measured by FACS analysis of single cell suspensions of buffy coat cells from peripheral blood.

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(18–26, 28, 40), and IL-2 induces the expression of CXCR3 on mononuclear cells, we next examined whether the therapeutic effect of systemic IL-2 immunotherapy in BALB/c mice bearing RENCA tumors was CXCR3-dependent. To perform these studies, we used $CXCR3^{+/+}$ and $CXCR3^{-/-}$ mice on a BALB/c background. BALB/c mice bearing RENCA tumors were subjected to systemic IL-2 or control treatment as described above. As shown in Fig. 2*A*, we found that systemic IL-2 therapy reduced tumor growth in only the $CXCR3^{+/+}$ mice, however, there was no significant difference in tumor size between the $CXCR3^{+/+}$ control group, the $CXCR3^{-/-}$ control group, or the $CXCR3^{-/-}$ mice treated with systemic IL-2. Moreover, morphometric analysis of mean tumor necrotic area and FACS analysis of the pan-endothelial cell marker (MECA-32) for angiogenesis demonstrated that systemic IL-2 induced tumor necrosis and reduced angiogenesis only in RENCA tumors grown in $CXCR3^{+/+}$, but not $CXCR3^{-/-}$, mice (Fig. 2*B* and data not shown).

Combined systemic IL-2 and intratumor CXCL9 immunotherapy is more effective in reducing RENCA tumor growth

On the basis that systemic IL-2 therapy induced the expression of CXCR3 on mononuclear cells and IL-2 therapy was CXCR3-dependent, we hypothesized that for optimal extravasation of $CXCR3^{+}$ mononuclear cells within the tumor together with promotion of CXCR3 ligand-mediated inhibition of tumor-associated angiogenesis, a combination of systemic IL-2 and intratumor CXCR3 ligand (i.e., CXCL9) would be more effective for reducing tumor size, as compared with either strategy alone. To test this postulate, we inoculated 10^6 RENCA cells into the flanks of 6- to 8-wk-old BALB/c mice (10 animals in each group). Three days after the establishment of the tumor, tumor-bearing mice were treated in the following manner: 1) systemic IL-2 (30,000 IU) for the first 5 days; 2) intratumor CXCL9 (1 μg/20 μl) on Monday, Wednesday, and Friday for 7 wk; 3) a combination of systemic IL-2 and intratumor CXCL9; or 4) control treatment with systemic vehicle control for IL-2 and intratumor murine serum albumin as a specific control for CXCL9. Tumor-bearing animals were assessed weekly for tumor size, and were sacrificed at 7 wk. We used a 1-μg dose of CXCL9 for intratumor injection on the basis of our previous work for injecting CXCL9 or CXCL10 in tumor models of non-small cell lung cancer (24, 26), and our unpublished data that demonstrated that 5 and 10 μg of either intratumor injected CXCL10 or CXCL9 did not increase the efficacy over the intratumor dose of either 100 ng or 1 μg for attenuating tumor growth. We found that systemic IL-2, intratumor CXCL9, or the combination of both systemic IL-2 and intratumor CXCL9 therapy resulted in marked reduction of RENCA tumor growth, as compared with the control-treated group (Fig. 3). The reduction in tumor growth was most apparent in mice treated with the combined strategy of systemic IL-2 and intratumor CXCL9, as compared with either systemic IL-2 or intratumor CXCL9 alone. At weeks 4–7, tumor growth in the combined systemic IL-2 and intratumor CXCL9 treatment group was virtually flat, as compared with either systemic IL-2 or intratumor CXCL9 alone (Fig. 3). In addition, we found that three animals in the control-treated group, two animals in the systemic IL-2-treated group, and none in the intratumor CXCL9 alone or combined systemic IL-2 and intratumor CXCL9-treated group died related to tumor burden before the end of the experiment between weeks 4 and 7. Moreover, two tumors in the combined systemic IL-2 and intratumor CXCL9 treatment group were barely detectable for measurement from weeks 5 to 7, whereas all other tumors were easily detectable in the control and other treatment groups.

Combined systemic IL-2 and intratumor CXCL9 therapy of RENCA tumors leads to increased tumor necrosis and reduced tumor-associated angiogenesis

Based on the findings of significant differences in the size and growth pattern of RENCA tumors in BALB/c mice between the

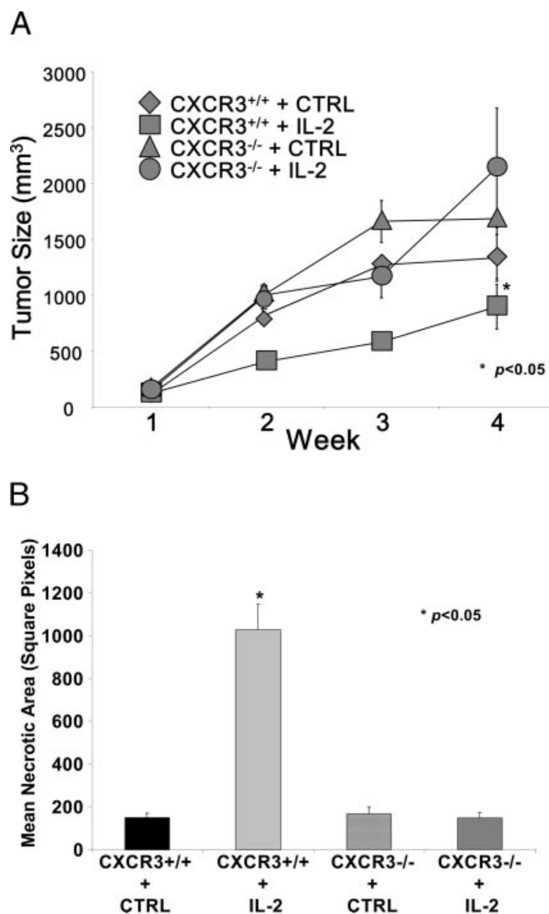

FIGURE 2. IL-2 immunotherapy is CXCR3-dependent. *A*, RENCA tumor growth in $CXCR3^{-/-}$, as compared with $CXCR3^{+/+}$, mice in response to systemic IL-2 therapy or control (CTRL) vehicle. Tumor volume was calculated using the formula: volume $= (d_1 \times d_2 \times d_3) \times 0.5236$, where d_n represents the three orthogonal diameter measurements. *B*, Mean necrotic area in $CXCR3^{-/-}$, as compared with $CXCR3^{+/+}$, mice in response to systemic IL-2 therapy. Mean necrotic area (square pixels at $\times 200$ magnification) was assessed at 4 wk by morphometric analysis. $*$, $p < 0.05$.

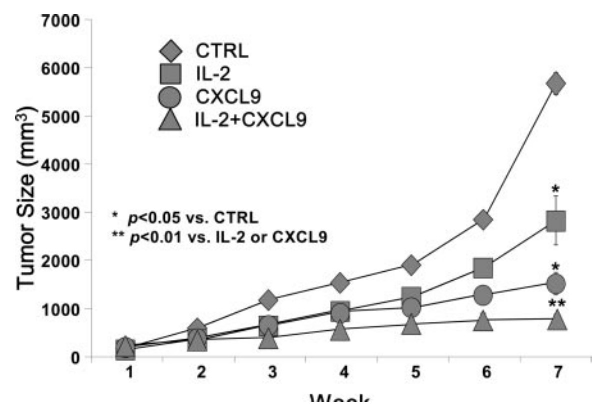

FIGURE 3. Combined therapy with systemic IL-2 and intratumor CXCL9 therapy markedly attenuates RENCA tumor growth, as compared with either treatment strategy alone. Tumor volume was calculated using the formula: volume $= (d_1 \times d_2 \times d_3) \times 0.5236$, where d_n represents the three orthogonal diameter measurements.

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various treatment groups, we next assessed the effect of different treatment strategies on the biology of the tumors. For this purpose, we examined tumor necrosis and the change in angiogenesis at 4 wk of tumor growth. Our results demonstrated that concomitant systemic IL-2 and intratumor CXCL9 treatment lead to the most significant increase in RENCA tumor necrosis (Fig. 4*A*). Treatment with systemic IL-2 or intratumor CXCL9 alone resulted in less marked, but still significant, increase in tumor necrosis (Fig. 4*A*). In addition, the magnitude of tumor necrosis paralleled the marked attenuation of angiogenesis in the tumors as measured by FACS analysis of the pan-endothelial cell marker (MECA-32) from single-cell suspensions of the RENCA tumors (Fig. 4*B*). Concomitant systemic IL-2 and intratumor CXCR9 > intratumor CXCL9 alone > systemic IL-2 alone resulted in significant inhibition of angiogenesis in the RENCA tumors (Fig. 4*B*).

Combined systemic IL-2 and intratumor CXCL9 therapy resulted in marked increase in infiltration of CXCR3$^+$ mononuclear cells within the RENCA tumors

To determine whether there were differences in the magnitude of infiltrating mononuclear cells expressing CXCR3 in the RENCA tumors under the different treatment conditions, we assessed H&E staining and dual-color FACS analysis of subpopulations of mononuclear cells expressing CXCR3 from single-cell suspensions of

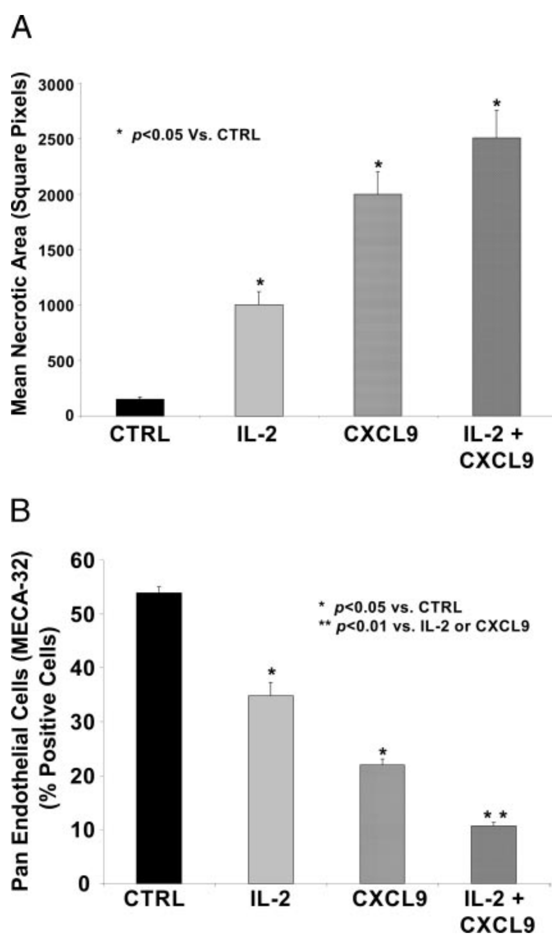

FIGURE 4. Combined therapy with systemic IL-2 and intratumor CXCL9 markedly increases tumor necrosis and decreases tumor-associated angiogenesis as compared with either treatment strategy alone. *A*, Mean necrotic area was assessed at 4 wk by morphometric analysis and expressed as square pixels at ×200 magnification. *B*, Reduction in angiogenesis in RENCA tumors was determined by FACS analysis of MECA-32-positive cells from a single-cell suspension of heterotopic RENCA tumors.

the RENCA tumors at 4 wk. As shown in Fig. 5*D* (×400), we found more mononuclear cell infiltration in the tumors from animals that had been treated with concomitant systemic IL-2 and intratumor CXCL9, as compared with IL-2 alone (Fig. 5*B*, ×400), intratumor CXCL9 alone (Fig. 5*C*, ×400), or control treatment (Fig. 5*A*, ×400). These findings were confirmed using FACS analysis of dual staining of tumor-infiltrating leukocytes expressing CXCR3 (Fig. 6). We found greater tumor infiltration of leukocytes expressing CXCR3 under conditions of concomitant systemic IL-2 and intratumor CXCR9 > intratumor CXCR9 alone > systemic IL-2 alone (Fig. 6*A*). In addition, we determined that the expression of CXCR3 on tumor-infiltrating leukocytes was significantly related to the increased infiltration of the following subset of mononuclear cells: CD4$^+$CXCR3$^+$ (Fig. 6*B*), CD8$^+$CXCR3$^+$ (Fig. 6*C*), and NK$^+$CXCR3$^+$ (Fig. 6*D*).

To determine whether the enhanced recruitment of CXCR3$^+$ cells in RENCA tumors of BALB/c mice treated with the combined systemic IL-2 and intratumor CXCL9, as compared with either strategy alone, was associated with an optimized plasma to tumor CXCR3 ligand chemotactic gradient, we measured plasma and tumor protein levels of CXCL9 and CXCL10 at week 4. We found that plasma to tumor gradients of CXCR3 ligands, as represented by tumor minus plasma levels of the sum of CXCL9 and CXCL10, were increased in the CXCL9 alone and combined systemic IL-2 and intratumor CXCL9 treatment groups (Table III). The tumor levels of CXCL9 in the intratumor CXCL9 group (6.9 ± 2.7 ng/ml) were not statistically different from the CXCL9 levels in the combined systemic IL-2 and intratumor CXCL9 group (4.7 ± 1.3 ng/ml). In contrast, systemic IL-2 treatment alone impaired the chemotactic gradient. These findings support the notion that the tumor to plasma chemotactic gradient of CXCR3 ligands was preserved in response to concomitant systemic IL-2 and intratumor CXCL9 treatment, as compared with IL-2 treatment alone.

Combined systemic IL-2 and intratumor CXCL9 therapy induced specific tumor immunity to RENCA cells

Because we had found that combined systemic IL-2 and intratumor CXCL9 treatment resulted in reduced tumor growth with increased tumor necrosis, decreased tumor-associated angiogenesis, and increased tumor infiltration of CXCR3$^+$ mononuclear cells, we next determined whether the treated animals developed a tumor-specific immune response as assessed by splenocyte proliferation in response to rechallenge of RENCA cells or control syngeneic tumor cells. To perform this study, we isolated mononuclear cells from the spleen of the RENCA tumor-bearing animals that were treated with systemic IL-2 alone, intratumor CXCL9 alone, a combination of systemic IL-2 and CXCL9, or control-treated groups and performed mixing experiments with MMC-pretreated RENCA cells or syngeneic BALB/c L1C2 lung cancer cells pretreated with MMC using a modification as previously described (36). MMC has been shown to be able to completely block the proliferation of RENCA cells and L1C2 cells (36). Subsequent to pulsation with [^{3}H]thymidine, we found that MMC-pretreated RENCA cells stimulated active [^{3}H]thymidine uptake in mononuclear cells isolated from the spleens of animals bearing RENCA tumors treated with either systemic IL-2, intratumor CXCL9, or the combined systemic IL-2/intratumor CXCL9, normalized to the control treatment group (Fig. 7*A*). However, we detected the most robust mononuclear proliferative response to rechallenge with RENCA cells from animals bearing RENCA tumors that had been treated with combined systemic IL-2 and intratumor CXCL9 (Fig. 7*A*). To determine whether this proliferative response was specific to RENCA tumor cells, we performed the same experiment in response to

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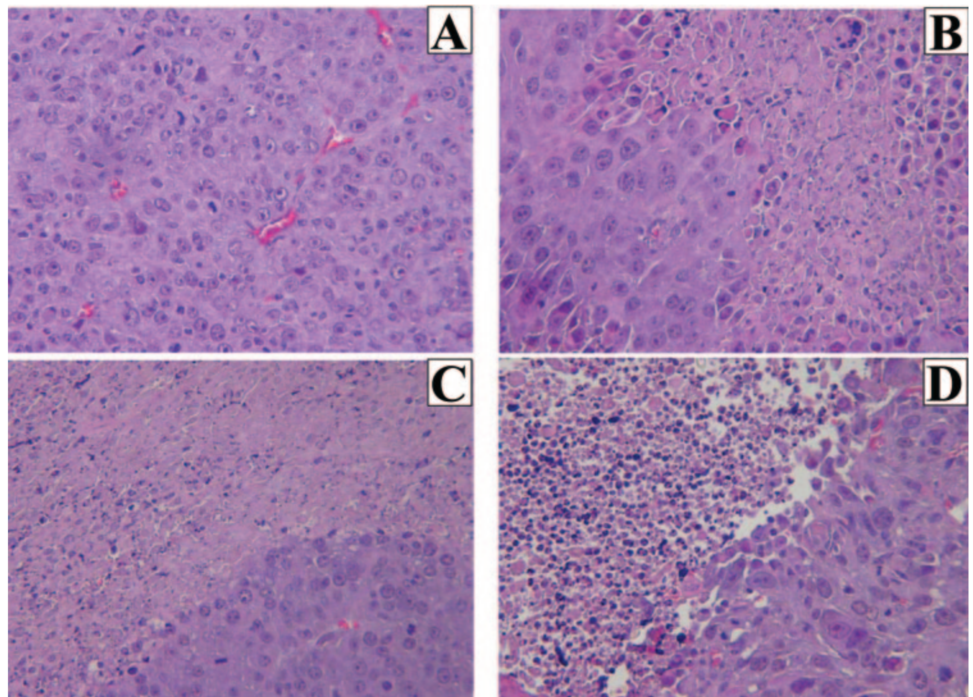

FIGURE 5. Combined therapy with systemic IL-2 and intratumor CXCL9 increases the infiltration of RENCA tumors with mononuclear cells, as compared with either treatment strategy alone. *A*, Representative photomicrograph (×200) from control-treated mice. *B*, Representative photomicrograph (×200) from systemic IL-2-treated mice. *C*, Representative photomicrograph (×200) from intratumor CXCL9-treated mice. *D*, Representative photomicrograph (×200) from combined systemic IL-2 and intratumor CXCL9-treated mice.

challenge with the syngeneic L1C2 cells. We found that challenge of mononuclear cells with L1C2 did not induce a mononuclear proliferative response that was any different between the treatment groups normalized to the control treatment group (Fig. 7*B*).

Discussion

RCC is characterized by a significant propensity for metastases, and patients with mRCC do not respond to conventional chemotherapy or radiation therapy (3). Therefore, alternative therapeutic approaches, such as immunotherapy, remain attractive to optimize more effective anti-RCC strategies. However, immunotherapy with IL-2 and/or IFN-α has led to disappointing response rates in patients with mRCC (4–6). In this study, we examined the role of CXCR3/CXCR3 ligand immunotherapy in targeting RCC in an effort to address the relatively low response rate of mRCC to systemic IL-2 therapy. Our results suggested that while systemic IL-2 administration alone led to marked increases in the cell surface expression of CXCR3 on mononuclear cells, it also led to a marked increase in plasma levels of CXCR3 ligands, ultimately inhibiting the chemotactic gradient that favors recruitment of CXCR3$^+$ mononuclear cells to tumor sites. The failure to effectively establish a CXCR3 ligand chemotactic gradient (i.e., the concept of "hit or miss") may explain why only a minority of patients with mRCC respond to immunotherapy. The mechanism underlying the ability of IL-2 to induce CXCR3 ligand expression needs to be further studied, but it is most likely indirect and related to local or systemic generation of type I and II IFNs or other agonists. In this study, we found that while systemic IL-2 induced both CXCL9 and CXCL10 in the circulation, it induced predominately CXCL9 in the local tumor microenvironment. It is our speculation that the differential induction of these CXCR3 ligands may be due to different cellular sources (systemic vs local tumor microenvironment) of these ligands.

We found that in CXCR3$^{+/+}$ animals, systemic IL-2 significantly reduced RENCA tumor growth. In CXCR3$^{-/-}$ animals, however, systemic IL-2 therapy alone failed to suppress tumor growth, induce tumor necrosis, or impair angiogenesis in RENCA tumors, supporting the notion that systemic IL-2 therapy was CXCR3-dependent. The importance of the CXCR3/CXCR3 ligand biological axis for inhibiting tumor growth has been substantiated by the recent studies demonstrating that intratumoral injection of recombinant CC chemokine CCL21 in murine tumors induced potent antitumor responses; and depletion of CXCR3 ligands or IFN-γ attenuated the antitumor effects of CCL21 (20, 21). Similarly, IL-12-mediated regression of murine RENCA tumors was markedly attenuated when either CXCL9 or CXCL10 was depleted by specific neutralizing Abs (22). In addition, the antitumor effect of imiquidod, a TLR7 agonist, was associated with strong induction of IFN-α production and enhanced CXCR3$^+$ lymphocyte infiltration in cutaneous malignant lesions, which are characteristic of type 1 cytokine-based cellular immunity (23). Moreover, it was

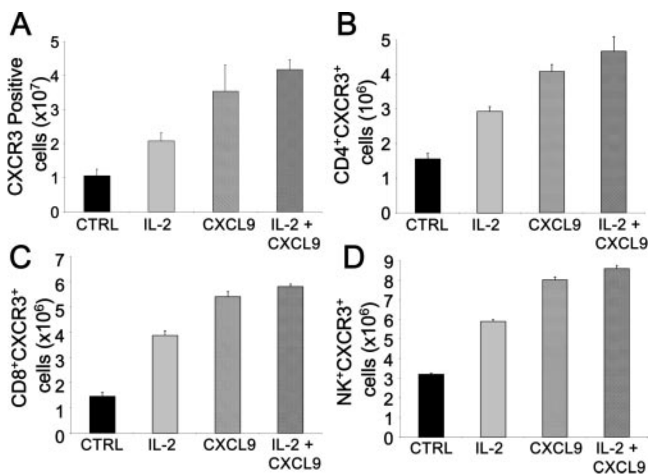

FIGURE 6. Combined therapy with systemic IL-2 and intratumor CXCL9 results in an increase in the infiltration of RENCA tumors with CXCR3$^+$ mononuclear cells, as compared with either treatment strategy alone. *A*, FACS analysis of infiltration of CD45$^+$CXCR3$^+$ cells in RENCA tumors. *B*, FACS analysis of infiltration of CD4$^+$CXCR3$^+$ cells in RENCA tumors. *C*, FACS analysis of infiltration of CD8$^+$CXCR3$^+$ cells in RENCA tumors. *D*, FACS analysis of infiltration of NK$^+$CXCR3$^+$ cells in RENCA tumors.

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Table III. *Plasma and tumor levels of CXCL9 and CXCL10 in response to systemic IL-2 and/or intratumor CXCL9 therapy*[a]

Treatment	Plasma CXCL10 (ng/ml)	Plasma CXCL9 (ng/ml)	Sum of Plasma CXCR3 Ligands (ng/ml)	Tumor CXCL10 (ng/ml)	Tumor CXCL9 (ng/ml)	Sum of Tumor CXCR3 Ligands (ng/ml)	Chemotactic Gradient (Tumor-plasma, ng/ml)
Control	0.15 ± 0.05	0.00	0.15	0.10 ± 0.014	0.6 ± 0.5	0.70	+0.55
IL-2	1.40 ± 0.57	1.50 ± 0.3	2.90	0.11 ± 0.006	2.0 ± 0.08	2.11	−0.79
CXCL9	0.10 ± 0.1	0.90 ± 0.07	1.00	0.13 ± 0.009	6.9 ± 2.7	7.03	+6.03
IL-2 + CXCL9	1.00 ± 0.1	1.08 ± 0.15	2.08	0.084 ± 0.023	4.7 ± 1.3	4.78	+2.70

[a] Gradient of higher concentration in tumor compared to plasma.

recently demonstrated that CXCL11 is able to recruit CXCR3$^+$ macrophages and CXCR3$^+$/CD8$^+$ T cells to tumor sites and establish a systemic protective immune response (17). Taken together, these findings suggest that the CXCR3/CXCR3 ligand biological axis plays a pivotal role in the suppression of tumor growth.

On the basis of the dependence of the CXCR3/CXCR3 ligand biological axis in mediating the antitumor effects of systemic IL-2 in our murine RENCA model, we anticipate that the temporal magnitude of expression of CXCR3 will serve as a biomarker for mRCC patients who respond to systemic IL-2 therapy. In support of this notion, a recent pilot study of our patients with mRCC suggested a positive correlation between CXCR3 expression on circulating mononuclear cells and the responsiveness to high-dose systemic IL-2 immunotherapy (our unpublished findings).

To enhance the CXCR3 ligand chemotactic gradient attenuated by systemic IL-2 therapy alone while continuing to promote the effect of IL-2 effect for inducing the expression of CXCR3 on circulating mononuclear cells, we developed a two-step strategy to achieve optimal recruitment of CXCR3$^+$ mononuclear cells and at the same time inhibit angiogenesis. The first step was "systemic priming" with IL-2 to induce the expression of CXCR3 on circulating mononuclear cells. The second step was to spatially induce higher intratumor levels of a CXCR3 ligand (CXCL9) to enhance a chemotactic gradient from the plasma to within the tumor, as well as inhibit angiogenesis. This paradigm would enhance selective and specific extravasation of type 1 cells into the tumor, enhance type 1-mediated immunity "in situ," increase the expression of local IFNs, further augment expression of CXCR3 ligands in the local tumor microenvironment, amplify further in situ type 1-mediated immunity, and at the same time promote CXCR3 ligand-mediated angiostasis. Our findings support the notion that the combined systemic IL-2 and intratumor CXCL9 strategy was more efficacious than either intervention alone for reducing tumor growth, enhancing tumor necrosis, promoting inhibition of tumor-associated angiogenesis, and enhancing the recruitment of CXCR3$^+$ mononuclear cells in the tumors.

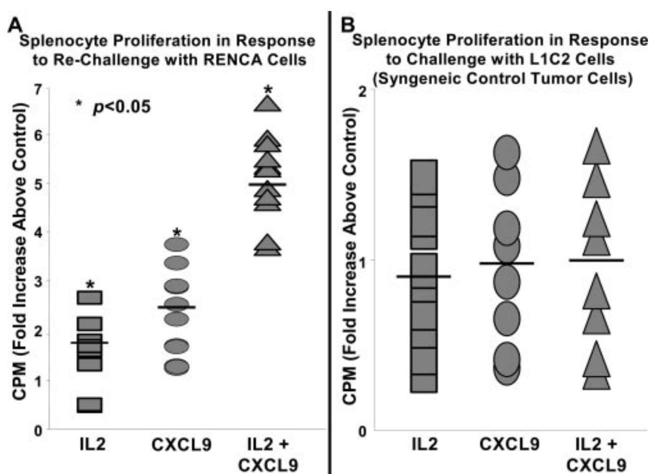

FIGURE 7. Combined therapy with systemic IL-2 and intratumor CXCL9 leads to enhanced host-derived specific immunity to RENCA tumor cells, as compared with either treatment strategy alone. Mononuclear cells were isolated from the spleens of RENCA bearing mice, mixed with MMC-pretreated RENCA (*A*) or syngeneic L1C2 cells (*B*) and incubated for 72 h. Proliferation of mononuclear cells was measured by [^{3}H]thymidine incorporation using scintillation counting. Fold induction in cpm represents increase in [^{3}H]thymidine incorporation by splenocytes from mice treated with either systemic IL-2, intratumor CXCL9, or combined systemic IL-2 and intratumor CXCL9, normalized to the control-treated group.

In this study, intact CXCR3 ligand levels were measured with ELISA. Although we did not test the bioavailability (stability) and clearance of these chemokines, the biological effects of intratumor CXCL9 on tumor growth, tumor necrosis, and tumor-associated angiogenesis were demonstrated. Once exogenous CXCL9 was injected into a murine tumor, we were unable to distinguish exogenous from the endogenous intact murine CXC chemokine, which may be induced by IFN-γ secreted by CXCR3$^+$ cells recruited to tumor by the CXCR3-dependent chemotactic gradient. Therefore, the levels of CXC chemokine measured by ELISA will reflect both exogenous and endogenous intact CXC chemokine in the case of CXCL9 in our studies. In addition, the clearance of chemokines in a local tumor microenvironment is complex and may be related to both degradation by extracellular proteinases, as well as by intracellular mechanisms associated with activation of its putative receptor by binding followed by internalization and targeting of the ligand to the endosomes for degradation. Thus, any measured degradation product of a CXC chemokine in the local tumor microenvironment would be a reflection of both extracellular and intracellular mechanisms for degradation.

In this study, we demonstrated that concomitant systemic IL-2 and intratumor CXCL9 therapy increased infiltration of CXCR3$^+$ mononuclear cells to the tumor, and increased specific immune cell proliferation in response to rechallenge with RENCA cells. The role of CXCR3/CXCR3 ligands in promoting antitumor immunity, especially type 1 cytokine-mediated cellular immunity, has also been supported by several recent studies (18, 19, 41–44). One study showed that intratumor injection of adenoviral vectors expressing CXCL10 increased recruitment of tumor-specific CD8$^+$ T cells to the tumor site and led to enhanced eradiation of tumor mass (18). In addition, expression of CXCR3 on activated CD8$^+$ T cells was associated with prolonged survival in stage III melanoma patients (19). Furthermore, CXCR3 ligands were shown to have stimulatory effects on T lymphocyte proliferation (44), and NK cells were shown to be recruited to lymph nodes in a CXCR3-dependent manner and produce IFN-γ for type 1 priming (42).

We further examined the role of CXCR3/CXCR3 ligand biology in mediating inhibition of angiogenesis. Our data support the notion that IL-2 loses its ability to inhibit angiogenesis in the absence

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of CXCR3, and that concomitant systemic IL-2 and intratumor CXCL9 therapy inhibited more tumor-associated angiogenesis as compared with either strategy alone. Although our previous studies showed that the suppressive effects of CXCR3 ligands, CXCL9 and CXCL10, on human non-small cell lung cancer was mediated by their angiostatic functions (24, 26), the specific receptor which these CXC chemokines use to mediate their angiostatic effects is CXCR3. An alternatively spliced form of CXCR3, CXCR3B, is expressed on human microvascular endothelial cells and mediates the angiostatic effects of CXCR3 ligand (41). Although further studies may be necessary to further clarify whether CXCR3 mediates the angiostatic activity of IFN-inducible CXC chemokines (CXCL9, CXCL10, and CXCL11) in mice, we (27) and others (28) have recently demonstrated that the angiostatic activity of CXCR3 ligands in mice is mediated by CXCR3.

In summary, our results suggested that the CXCR3/CXCR3 ligand biological axis plays a critical role in mediating the antitumor effect of systemic IL-2 therapy. Moreover, our results support the notion that the systemic immunotherapy regimen can be further optimized by combining systemic activation of mononuclear cells to express CXCR3 and at the same time enhance the CXCR3 ligand chemotactic gradient to promote greater mononuclear cell extravasation within the tumor, induce enhanced type 1 cytokine-dependent cell-mediated immunity, and concurrently inhibit tumor-associated angiogenesis, the concept of immunoangiostasis. The findings of these studies could have a significant impact on how we provide immunotherapy to patients with mRCC and lead to improved positive and durable responses in these patients.

Disclosures

The authors have no financial conflict of interest.

References

1. American Cancer Society. 2004. *Cancer Facts and Figures 2004: Selected Cancers*. American Cancer Society, Atlanta, GA.
2. Pantuck, A. J., A. Zisman, F. Dorey, D. H. Chao, K. R. Han, J. Said, B. Gitlitz, A. S. Belldegrun, and R. A. Figlin. 2003. Renal cell carcinoma with retroperitoneal lymph nodes: impact on survival and benefits of immunotherapy. *Cancer* 97: 2995–3002.
3. Figlin, R. A. 1999. Renal cell carcinoma: management of advanced disease. *J. Urol.* 161: 381–386; discussion 386–387.
4. Negrier, S., B. Escudier, C. Lasset, J. Y. Douillard, J. Savary, C. Chevreau, A. Ravaud, A. Mercatello, J. Peny, M. Mousseau, et al. 1998. Recombinant human interleukin-2, recombinant human interferon α-2a, or both in metastatic renal-cell carcinoma. Groupe Francais d'Immunotherapie. *N. Engl. J. Med.* 338: 1272–1278.
5. Ravaud, A., N. Trufflandier, J. M. Ferriere, M. Debled, J. Palussiere, L. Cany, R. Gaston, S. Mathoulin-Pelissier, and B. N. Bui. 2003. Subcutaneous interleukin-2, interferon α-2b and 5-fluorouracil in metastatic renal cell carcinoma as second-line treatment after failure of previous immunotherapy: a phase II trial. *Br. J. Cancer* 89: 2213–2218.
6. Verra, N., R. Jansen, G. Groenewegen, H. Mallo, M. J. Kersten, A. Bex, F. A. Vyth-Dreese, J. Sein, W. van de Kasteele, W. J. Nooijen, et al. 2003. Immunotherapy with concurrent subcutaneous GM-CSF, low-dose IL-2 and IFN-α in patients with progressive metastatic renal cell carcinoma. *Br. J. Cancer* 88: 1346–1351.
7. Belperio, J. A., M. P. Keane, D. A. Arenberg, C. L. Addison, J. E. Ehlert, M. D. Burdick, and R. M. Strieter. 2000. CXC chemokines in angiogenesis. *J. Leukocyte Biol.* 68: 1–8.
8. Strieter, R. M., J. A. Belperio, D. A. Arenberg, M. I. Smith, M. D. Burdick, and M. P. Keane. 2002. *CXC chemokine in angiogenesis.* Elsevier Science, Amsterdam.
9. Strieter, R. M., P. J. Polverini, S. L. Kunkel, D. A. Arenberg, M. D. Burdick, J. Kasper, J. Dzuiba, J. Van Damme, A. Walz, D. Marriott, et al. 1995. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J. Biol. Chem.* 270: 27348–27357.
10. Beider, K., A. Nagler, O. Wald, S. Franitza, M. Dagan-Berger, H. Wald, H. Giladi, S. Brocke, J. Hanna, O. Mandelboim, et al. 2003. Involvement of CXCR4 and IL-2 in the homing and retention of human NK and NK T cells to the bone marrow and spleen of NOD/SCID mice. *Blood* 102: 1951–1958.
11. Loetscher, M., B. Gerber, P. Loetscher, S. A. Jones, L. Piali, I. Clark-Lewis, M. Baggiolini, and B. Moser. 1996. Chemokine receptor specific for IP10 and Mig: structure, function, and expression in activated T-lymphocytes. *J. Exp. Med.* 184: 963–969.
12. Loetscher, M., P. Loetscher, N. Brass, E. Meese, and B. Moser. 1998. Lymphocyte-specific chemokine receptor CXCR3: regulation, chemokine binding and gene localization. *Eur. J. Immunol.* 28: 3696–3705.
13. Luster, A. D. 1998. Chemokines–chemotactic cytokines that mediate inflammation. *N. Engl. J. Med.* 338: 436–445.
14. Moser, B., and P. Loetscher. 2001. Lymphocyte traffic control by chemokines. *Nat. Immunol.* 2: 123–128.
15. Qin, S., J. B. Rottman, P. Myers, N. Kassam, M. Weinblatt, M. Loetscher, A. E. Koch, B. Moser, and C. R. Mackay. 1998. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J. Clin. Invest.* 101: 746–754.
16. Rabin, R. L., M. K. Park, F. Liao, R. Swofford, D. Stephany, and J. M. Farber. 1999. Chemokine receptor responses on T cells are achieved through regulation of both receptor expression and signaling. *J. Immunol.* 162: 3840–3850.
17. Hensbergen, P. J., P. G. Wijnands, M. W. Schreurs, R. J. Scheper, R. Willemze, and C. P. Tensen. 2005. The CXCR3 targeting chemokine CXCL11 has potent antitumor activity in vivo involving attraction of $CD8^+$ T lymphocytes but not inhibition of angiogenesis. *J. Immunother.* 28: 343–351.
18. Huang, H., and J. Xiang. 2004. Synergistic effect of lymphotactin and interferon γ-inducible protein-10 transgene expression in T-cell localization and adoptive T-cell therapy of tumors. *Int. J. Cancer.* 109: 817–825.
19. Mullins, I. M., C. L. Slingluff, J. K. Lee, C. F. Garbee, J. Shu, S. G. Anderson, M. E. Mayer, W. A. Knaus, and D. W. Mullins. 2004. CXC chemokine receptor 3 expression by activated $CD8^+$ T cells is associated with survival in melanoma patients with stage III disease. *Cancer Res.* 64: 7697–7701.
20. Sharma, S., M. Stolina, J. Luo, R. M. Strieter, M. Burdick, L. X. Zhu, R. K. Batra, and S. M. Dubinett. 2000. Secondary lymphoid tissue chemokine mediates T cell-dependent antitumor responses in vivo. *J. Immunol.* 164: 4558–4563.
21. Sharma, S., S. C. Yang, S. Hillinger, L. X. Zhu, M. Huang, R. K. Batra, J. F. Lin, M. D. Burdick, R. M. Strieter, and S. M. Dubinett. 2003. SLC/CCL21-mediated anti-tumor responses require IFNγ, MIG/CXCL9 and IP-10/CXCL10. *Mol. Cancer* 2: 22.
22. Tannenbaum, C. S., R. Tubbs, D. Armstrong, J. H. Finke, R. M. Bukowski, and T. A. Hamilton. 1998. The CXC chemokines IP-10 and Mig are necessary for IL-12-mediated regression of the mouse RENCA tumor. *J. Immunol.* 161: 927–932.
23. Wenzel, J., M. Uerlich, O. Haller, T. Bieber, and T. Tueting. 2005. Enhanced type I interferon signaling and recruitment of chemokine receptor CXCR3-expressing lymphocytes into the skin following treatment with the TLR7-agonist imiquimod. *J. Cutan. Pathol.* 32: 257–262.
24. Addison, C. L., D. A. Arenberg, S. B. Morris, Y. Y. Xue, M. D. Burdick, M. S. Mulligan, M. D. Iannettoni, and R. M. Strieter. 2000. The CXC chemokine, monokine induced by interferon-γ, inhibits non-small cell lung carcinoma tumor growth and metastasis. *Hum. Gene Ther.* 11: 247–261.
25. Arenberg, D. A., M. P. Keane, B. DiGiovine, S. L. Kunkel, S. B. Morris, Y. Y. Xue, M. D. Burdick, M. C. Glass, M. D. Iannettoni, and R. M. Strieter. 1998. Epithelial-neutrophil activating peptide (ENA-78) is an important angiogenic factor in non-small cell lung cancer. *J. Clin. Invest.* 102: 465–472.
26. Arenberg, D. A., S. L. Kunkel, P. J. Polverini, S. B. Morris, M. D. Burdick, M. C. Glass, D. T. Taub, M. D. Iannettoni, R. I. Whyte, and R. M. Strieter. 1996. Interferon-γ-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. *J. Exp. Med.* 184: 981–992.
27. Burdick, M. D., L. A. Murray, M. P. Keane, Y. Y. Xue, D. A. Zisman, J. A. Belperio, and R. M. Strieter. 2005. CXCL11 attenuates bleomycin-induced pulmonary fibrosis via inhibition of vascular remodeling. *Am. J. Respir. Crit. Care Med.* 171: 261–268.
28. Yang, J., and A. Richmond. 2004. The angiostatic activity of interferon-inducible protein-10/CXCL10 in human melanoma depends on binding to CXCR3 but not to glycosaminoglycan. *Mol. Ther.* 9: 846–855.
29. Belperio, J. A., M. P. Keane, M. D. Burdick, J. P. Lynch III, Y. Y. Xue, K. Li, D. J. Ross, and R. M. Strieter. 2002. Critical role for CXCR3 chemokine biology in the pathogenesis of bronchiolitis obliterans syndrome. *J. Immunol.* 169: 1037–1049.
30. Belperio, J. A., M. P. Keane, M. D. Burdick, J. P. Lynch III, D. A. Zisman, Y. Y. Xue, K. Li, A. Ardehali, D. J. Ross, and R. M. Strieter. 2003. Role of CXCL9/CXCR3 chemokine biology during pathogenesis of acute lung allograft rejection. *J. Immunol.* 171: 4844–4852.
31. Murphy, G. P., and W. J. Hrushesky. 1973. A murine renal cell carcinoma. *J. Natl. Cancer Inst.* 50: 1013–1025.
32. Hancock, W. W., B. Lu, W. Gao, V. Csizmadia, K. Faia, J. A. King, S. T. Smiley, M. Ling, N. P. Gerard, and C. Gerard. 2000. Requirement of the chemokine receptor CXCR3 for acute allograft rejection. *J. Exp. Med.* 192: 1515–1520.
33. Keane, M. P., J. A. Belperio, D. A. Arenberg, M. D. Burdick, Z. J. Xu, Y. Y. Xue, and R. M. Strieter. 1999. IFN-γ-inducible protein-10 attenuates bleomycin-induced pulmonary fibrosis via inhibition of angiogenesis. *J. Immunol.* 163: 5686–5692.
34. Belperio, J. A., M. P. Keane, M. D. Burdick, V. Londhe, Y. Y. Xue, K. Li, R. J. Phillips, and R. M. Strieter. 2002. Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. *J. Clin. Invest.* 110: 1703–1716.
35. Keane, M. P., J. A. Belperio, Y. Y. Xue, M. D. Burdick, and R. M. Strieter. 2004. Depletion of CXCR2 inhibits tumor growth and angiogenesis in a murine model of lung cancer. *J. Immunol.* 172: 2853–2860.
36. Hara, I., H. Nagai, H. Miyake, K. Yamanaka, S. Hara, M. J. Micallef, M. Kurimoto, K. Gohji, S. Arakawa, M. Ichihashi, and S. Kamidono. 2000.

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Effectiveness of cancer vaccine therapy using cells transduced with the interleukin-12 gene combined with systemic interleukin-18 administration. *Cancer Gene Ther.* 7: 83–90.

37. Dybal, E. J., G. P. Haas, R. L. Maughan, S. Sud, J. E. Pontes, and G. G. Hillman. 1992. Synergy of radiation therapy and immunotherapy in murine renal cell carcinoma. *J. Urol.* 148: 1331–1337.
38. Futami, H., L. Eader, T. T. Back, E. Gruys, H. A. Young, R. H. Wiltrout, and B. C. Baguley. 1992. Cytokine induction and therapeutic synergy with interleukin-2 against murine renal and colon cancers by xanthenone-4-acetic acid derivatives. *J. Immunother.* 12: 247–255.
39. Sonouchi, K., T. A. Hamilton, C. S. Tannenbaum, R. R. Tubbs, R. Bukowski, and J. H. Finke. 1994. Chemokine gene expression in the murine renal cell carcinoma, RENCA, following treatment in vivo with interferon-α and interleukin-2. *Am. J. Pathol.* 144: 747–755.
40. Strieter, R. M., M. D. Burdick, B. N. Gomperts, J. A. Belperio, and M. P. Keane. 2005. CXC chemokines in angiogenesis. *Cytokine. Growth Factor Rev.* 16: 593–609.
41. Lasagni, L., M. Francalanci, F. Annunziato, E. Lazzeri, S. Giannini, L. Cosmi, C. Sagrinati, B. Mazzinghi, C. Orlando, E. Maggi, et al. 2003. An alternatively spliced variant of CXCR3 mediates the inhibition of endothelial cell growth induced by IP-10, Mig, and I-TAC, and acts as functional receptor for platelet factor 4. *J. Exp. Med.* 197: 1537–1549.
42. Martin-Fontecha, A., L. L. Thomsen, S. Brett, C. Gerard, M. Lipp, A. Lanzavecchia, and F. Sallusto. 2004. Induced recruitment of NK cells to lymph nodes provides IFN-γ for T_H1 priming. *Nat. Immunol.* 5: 1260–1265.
43. Taub, D. D., A. R. Lloyd, K. Conlon, J. M. Wang, J. R. Ortaldo, A. Harada, K. Matsushima, D. J. Kelvin, and J. J. Oppenheim. 1993. Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J. Exp. Med.* 177: 1809–1814.
44. Whiting, D., G. Hsieh, J. J. Yun, A. Banerji, W. Yao, M. C. Fishbein, J. Belperio, R. M. Strieter, B. Bonavida, and A. Ardehali. 2004. Chemokine monokine induced by IFN-γ/CXC chemokine ligand 9 stimulates T lymphocyte proliferation and effector cytokine production. *J. Immunol.* 172: 7417–7424.

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