CXCL12/CXCR4 Blockade Induces Multimodal Antitumor Effects That Prolong Survival in an Immunocompetent Mouse Model of Ovarian Cancer

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Abstract

The chemokine CXCL12 and its receptor CXCR4 are expressed widely in human cancers, including ovarian cancer, in which they are associated with disease progression at the levels of tumor cell proliferation, invasion, and angiogenesis. Here, we used an immunocompetent mouse model of intraperitoneal papillary epithelial ovarian cancer to show that modulation of the CXCL12/CXCR4 axis in ovarian cancer has multimodal effects on tumor pathogenesis associated with induction of antitumor immunity. siRNA-mediated knockdown of CXCL12 in BR5-1 cells that constitutively express CXCL12 and CXCR4 reduced cell proliferation in vitro, and tumor growth in vivo. Similarly, treatment of BR5-1–derived tumors with AMD3100, a selective CXCR4 antagonist, resulted in increased tumor apoptosis and necrosis, reduction in intraperitoneal dissemination, and selective reduction of intratumoral FoxP3+ regulatory T cells (Treg). Compared with controls, CXCR4 blockade greatly increased T-cell–mediated antitumor immune responses, conferring a significant survival advantage to AMD3100-treated mice. In addition, the selective effect of CXCR4 antagonism on intratumoral Tregs was associated with both higher CXCR4 expression and increased chemotactic responses to CXCL12, a finding that was also confirmed in a melanoma model. Together, our findings reinforce the concept of a critical role for the CXCL12/CXCR4 axis in ovarian cancer pathogenesis, and they offer a definitive preclinical validation of CXCR4 as a therapeutic target in this disease. Cancer Res; 71(16); 5522–34. ©2011 AACR.

Introduction

Chemokines and their cognate receptors have been shown to play a role in favoring tumor growth, progression, and immune escape through multiple mechanisms (1–6). CXCL12 and its receptor CXCR4 constitute a chemokine–receptor axis that is known to be expressed in various tumors and to correlate with poor clinical outcome (7–14). CXCL12 is expressed by primary human ovarian cancer and has been detected in tumor tissue and malignant ascites (2, 15, 16), whereas CXCR4 is the only chemokine receptor shown to be constitutively expressed in epithelial ovarian cancer (15, 16). CXCL12 can stimulate ovarian cancer cell proliferation by direct activation of kinases such as protein kinase B and mitogen-activated protein kinase and by indirect protection attracting CXCR4+ cancer cells, and increasing matrix metalloproteinase expression (8, 15, 16, 21). CXCL12 has also been shown to repel tumor-specific effector T cells (Teff) and to recruit suppressive cell populations at tumor sites, including interleukin-10–producing plasmacytoid dendritic cells, regulatory T cells (Treg), and myeloid-derived suppressor cells (22–25).

We proposed that modulation of the CXCL12/CXCR4 axis in ovarian cancer would impact multiple aspects of tumor pathogenesis including immune dysregulation. Therefore, this
ligand–receptor axis would provide an attractive therapeutic target. The availability of an immunocompetent murine model of intraperitoneal papillary epithelial ovarian cancer derived from a syngeneic cell line that constitutively expresses CXCL12 and CXCR4 and the existence of a clinically validated, highly specific CXCR4 antagonist, AMD3100, facilitated this approach (26, 27). To date, AMD3100 has been tested in vivo in ovarian cancer in the context of its impact on tumor growth and dissemination in immunodefective mice (21). Knocking down CXCL12 expression or CXCR4 blockade with AMD3100 led to increased tumor cell apoptosis and reduction in tumor growth, peritoneal dissemination, and angiogenesis. CXCR4/CXCL12 blockade also led to a selective reduction in intratumoral Tregs as compared with CD8+ T cells and increased antitumor immunity. These antitumor effects were associated with significantly prolonged survival. The selective effect of CXCR4/CXCL12 blockade on intratumoral Tregs was associated with significantly greater expression of CXCR4 and chemotactic responses to CXCL12 of this T-cell subpopulation compared with CD8+ T cells. These findings support the critical role of the CXCL12/CXCR4 axis in ovarian cancer pathogenesis and immune evasion and provide validation for a new therapeutic target in this disease.

Materials and Methods

Animal models and cell lines

Tumor generation involved a single intraperitoneal injection of the syngeneic cancer cell line BR5-1 (1 × 10^7 cells per mouse) into 5-week-old FVB/NJ mice (28) and was consistently first evident after 3 to 5 weeks via abdominal distension secondary to malignant ascites. Tumor-bearing mice were euthanized at the endpoint when there were signs of distress, including fur ruffling, rapid respiratory rate, hunched posture, reduced activity, and progressive ascites formation. At necropsy, tumor weight, volume of ascites, and peritoneal dissemination were measured and lungs, brain, and liver were examined for metastasis by visual inspection. A second tumor model, B16F10 murine melanoma, was used, as described previously (see Supplementary Methods; ref. 29). All measurements were conducted in a blinded manner (to control or experimental group), and all animal experiments were executed according to Public Health Service Policy on Humane Care of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

CXCL12 knockdown using RNA interference vector

CXCL12 expression knockdown in BR5-1 cells was achieved by transducing the cells with MISSION lentiviral transduction particles (Sigma-Aldrich) through the use of 5 different short hairpin RNA sequences targeting CXCL12 (see Supplementary Methods).

Cell proliferation and CXCL12 quantitation

Cell proliferation was measured by manual counting and by using the Premix WST-1 Cell Proliferation Assay System (Clontech Laboratories). In certain experiments, 100 ng/mL of exogenous recombinant murine CXCL12 (Peprotech) was added to the culture of CXCL12 knocked down BR5-1 cells (kdBR5-1) in fresh media at 0, 24, 48, and 72 hours. CXCL12 was measured in cancer cell culture supernatants using a CXCL12-ELISA kit (R&D Systems).

AMD3100 treatment in vivo

Administration of AMD3100 into mice was achieved using osmotic minipumps (DURECT Co.) implanted dorsolaterally under the skin. For the murine ovarian cancer model, animals were treated with AMD3100 or vehicle (PBS) by 2 different schedules; schedule A—treatment began upon the development of ascites either for 3 days (for time-matched study) or until evident signs of distress as described earlier (for survival study); schedule B—treatment began 2 weeks after intraperitoneal injection of tumor cells either for 14 days (for time-matched study) or until evident signs of distress as described earlier (for survival study). For the murine B16F10 melanoma model, AMD3100 treatment was initiated when tumors reached 4 mm in mean diameter either for 4 days (for time-matched study) or until tumors reached a size of more than 12 mm in diameter or the mice showed signs of distress (for survival study). The details of the treatment are described in the Supplementary Methods. All monitoring of animal survival and tumor progression was carried out in a manner that was blinded to the observer.

Quantitation of tumor vessel density by immunofluorescence

Mice were euthanized at day 3 after tumor development, at which point the sizes of the tumors analyzed were comparable in the treatment group versus controls. Tumor vessel density was determined and scored in a blinded manner using fluorescein-labeled lectin (Vector Laboratories) and image analysis (Carl Zeiss LSM5 Pascal; Carl Zeiss, Inc.) and Image J 1.43 freeware (NIH) as previously described (30).

Quantitation of tumor-infiltrating lymphocytes by immunofluorescence

Immunofluorescence analysis of tumor-infiltrating CD8- and FoxP3-positive cells was carried out. T cells were visualized using confocal microscopy and scored in a blinded manner using Image J 1.43 freeware (Supplementary Methods).

Quantitation of apoptosis using terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining

Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining was carried out on 10-μm thick tumor sections prepared, as described in the previous section, using the In situ Cell Death Detection Kit (TMR Red; Roche). Slides were imaged and analyzed using Image J and were blinded to the analyzer.

Flow cytometry

For subpopulation analysis of tumor-infiltrating lymphocytes (TIL), mononuclear cells, including tumor cells, stromal
cells, and immune cells derived from disaggregated tumor tissue, were separated by centrifugation on Ficoll Hypaque and directly stained with fluorophore-conjugated anti-CD3, anti-CD8, anti-CD4, anti-CD25, anti-FoxP3, and anti-CXCR4 antibodies (BD Bioscience). Cell subpopulations, including CD3⁺CD4⁺ T-helper cells, CD3⁺CD8⁺ CTLs, and CD4⁺CD25⁺FoxP3⁺ Tregs, were quantified as a percentage of cell per gram of tumor tissue through examination of both positive control and fluorescence-minus-one (FMO) gating strategy (31). For in vitro assessment of tumor-specific T-cell function, splenocytes and TILs isolated and prepared as single-cell suspensions were plated and pulsed with tumor lysates, Her2/neu peptide (0.1 μg/mL, PDSLRDLSVF; EZBiolab; ref. 32), or medium alone for 18 hours when Golgi Plug (BD Bioscience) was added (0.75 μL/well) for 6 hours, stained with anti-Granzyme B (R&D Systems), and analyzed on a LSRII 3 laser, BD Biosciences (see Supplementary Methods).

Treg depletion and CD8⁻ T-cell isolation
Magnetic beads were used to separate Tregs and CD8⁻ T cells (MACS; Miltenyi Biotech) yielding a population with greater than 95% purity. Treg–depleted cells (CD4⁺CD25⁻) were plated, stimulated, and stained as described earlier.

Transmigration assay
Treg and CD8⁻ T-cell migration was measured using Transwells (96-well format, 3-μm pore size; ChemoTx System, Neuro Probe Inc.) as previously described (33) in CXCL12 concentrations (5, 50, 500 ng/mL, and 5 μg/mL) and in the presence or absence of AMD3100 (1 μg/mL) or preincubation with 100 ng/mL pertussis toxin (PTX; Sigma) for 1 hour at 37°C.

Statistical analysis
All of the results are expressed as mean ± SEM. Differences between treatment groups were determined by the Mann–Whitney test and other numerical variables by unpaired t test. A P value of less than 0.05 was considered significant. All statistical analyses were independently conducted on data sets by Dr. Gebremichael at the Harvard School of Public Health.

Results

CXCL12 knockdown impacts BR5-1 proliferation in vitro and tumor growth in vivo
CXCL12 has been shown to stimulate ovarian cancer cell proliferation in vitro (16). We used the murine ovarian cancer cell line BR5-1, which constitutively expresses CXCL12 and CXCR4, to observe the effect of RNA interference (RNAi)-mediated CXCL12 silencing on tumor growth. CXCL12 levels from wild-type BR5-1 cells (wtBR5-1), RNAi-transfected, CXCL12 kdBR5-1, and scrambled RNAi–transfected BR5-1 cells (scBR5-1) were quantitated using an ELISA (Fig. 1A). Similar levels of CXCL12 were released by wtBR5-1 and scBR5-1 (1.05 ± 0.19 and 0.85 ± 0.09 ng/mL), whereas CXCL12 from kdBR5-1 was negligible (<0.02 ng/mL, P < 0.0001). CXCR4 expression was not significantly different among wtBR5-1, kdBR5-1, and scBR5-1 (Fig. 1B). kdBR5-1 proliferated at a significantly slower rate than wtBR5-1 and scBR5-1 (P < 0.0001; Fig. 1C). The results were confirmed using a WST-1 proliferation assay, showing a significantly higher absorbance in wtBR5-1 than in kdBR5-1 at 48 hours (absorbance ratio 12.5 ± 0.23 vs. 3.37 ± 0.30, respectively; P < 0.001; data not shown). Addition of exogenous CXCL12 significantly increased kdBR5-1 proliferation to a level comparable with wild-type (Fig. 1D). Apoptosis was excluded as a mechanism responsible for the reduced kdBR5-1 cell proliferation (Fig. 1E), thus confirming a direct effect of CXCL12 on ovarian cancer cell proliferation (16).

To test whether defective CXCL12 production was maintained in vivo, cancer cells derived from wtBR5-1 (WT) and kdBR5-1 tumors (RNAi) were grown for 48 hours in media. Quantitation of CXCL12 in supernatants showed results comparable with in vitro data, with CXCL12 less than 0.02 ng/mL in tumor cells extracted from kdBR5-1–injected mice and CXCL12 levels of 1.2 ± 0.10 ng/mL in tumor cells extracted from wtBR5-1 tumors. Furthermore, RNAi tumors displayed a marked reduction of CXCL12 (stained red in Fig. 1F) compared with WT tumors via immunofluorescence. Small numbers of cells expressing CXCL12 were detected and thought to be due to the infiltration of CXCL12-expressing stromal and endothelial cells in the tumor microenvironment (Fig. 1F, arrowheads).

FVB/NJ mice injected with wtBR5-1 normally generate intraperitoneal tumors in 3 to 5 weeks. The time interval between tumor implantation and appearance of ascites was found to be significantly higher in kdBR5-1–injected mice than in wtBR5-1- and scBR5-1–injected mice (53 ± 5.5 days vs. 33 ± 2.1 and 32 ± 3.3 days, respectively, P < 0.05, Supplementary Fig. S1A). A reproducible “survival window” was defined between the first clinical sign of disease (abdominal swelling secondary to ascites formation) and euthanasia required by advanced clinical condition. The overall survival was significantly longer in kdBR5-1–injected mice than in wtBR5-1–injected mice (8.7 ± 1.8 days vs. 3.4 ± 0.4 days, respectively, P < 0.005; Fig. 1G). Mice injected with scBR5-1 had similar survival windows (3.3 ± 0.5 days) compared with wtBR5-1–injected mice.

AMD3100 treatment prolongs mouse survival and reduces dissemination of tumors
Mice were treated with AMD3100 either at the onset of ascites until the end-stage time point (schedule A) to simulate the treatment in human disease (34) or from day 15 after the implantation of tumor cells (schedule B). AMD3100 treatment schedules were based on previous pharmacokinetic evaluations to maintain steady serum levels (35). AMD3100 treatment on schedule A resulted in a 2-fold increase in survival compared with PBS-treated mice (6 days vs. 3 days, P < 0.05; Fig. 2A), and a higher overall survival was reported in the AMD3100-treated mice compared to controls (38 days ± 1.1 vs. 32 days ± 1.5, P < 0.05; Fig. 2B). In a separate experiment, AMD3100-treated and PBS-treated mice were euthanized at day 3 from the onset of clinical signs to compare total tumor mass, volume of ascites, and tumor dissemination (Fig. 2C–E). Mean tumor weight and amount of ascites were lower in
AMD-treated than in PBS-treated mice (1.3 ± 0.23 g and 4.4 ± 0.4 mL vs. 0.8 ± 0.17 g and 3.6 ± 0.4 mL, respectively; Fig. 2D and E), although these differences were not significant.

BR5-1 injection robustly resulted in multiple tumor nodules growing on organ surfaces without significant invasion. Sites of tumor spread included the mesothelial lining of the peritoneum, intestines, spleen, and diaphragm but not the lungs (28). Tumor dissemination was compared between AMD3100-treated and PBS-treated mice (Fig. 2F and G) and classified as localized (localizations ≤3), limited (3 < localizations ≤6, with no visible masses on mesentery and diaphragm), or disseminated (localizations ≥6, including mesentery and diaphragm).
Figure 2. Effect of AMD3100 treatment on tumor progression and mice survival. A–G, AMD3100 or PBS was delivered into FVB/NJ mice with wtBR5-1 tumors at the onset of ascites (schedule A). A and B, survival curves from the onset of ascites (A) and the time of tumor cell injection (B); n = 10 per group (*, P < 0.05). C–E, effect of AMD3100 treatment on tumor weight and ascites formation. Tumor-bearing mice were treated with AMD3100 or PBS under schedule A (n = 8 from 3 experiments) and euthanized at day 3 post-ascites formation. C, representative mice from AMD3100 and PBS groups are shown. Total tumor weight (D) and ascites volumes (E) were measured. F, bar graph representing the percentage of mice with visible tumor deposits on organs at necropsy (n = 10 per group; *, P < 0.01). G, tumor dissemination. Classification: localized (localizations/C20/C3, limited (3<Clocalizations<C6, with no visible masses on mesentery and diaphragm), and disseminated (localizations>C6, including mesentery and diaphragm localization); *, P < 0.01. H and I, AMD3100 or control PBS delivered 15 days after tumor cell injection (schedule B). H, survival curves of the mice treated under schedule B from the onset of ascites showing that AMD3100-treated mice increased survival window (n = 6; *, P < 0.01) and (I) reduced tumor dissemination (n = 6; *, P < 0.05).
The percentage of mice with tumor deposits on organ surfaces at necropsy was lower in AMD3100-treated than in PBS-treated mice. Although all AMD3100-treated mice had localized or limited tumors, 70% of the tumors in PBS-treated mice fell under the disseminated category.

In schedule B, when the treatment was started at day 15 after tumor implantation, a significant delay in the onset of ascites was recorded in the AMD3100-treated mice versus controls (21 ± 0.5 days vs. 24 ± 1.3 days, P < 0.05). An increase in survival in AMD3100-treated versus control-treated animals was observed (3.6 days vs. 7 days; Fig. 2H) as well as higher overall survival times (41 ± 2.1 days vs. 36 ± 0.5 days, P < 0.05; Supplementary Fig. S1B). Tumor dissemination in schedule B examined 5 weeks after the tumor injection was comparable with that in schedule A (Fig. 2I).

**CXCL12/CXCR4 manipulation markedly reduces tumor angiogenesis**

CXCL12 is a proangiogenic factor in ovarian cancer via upregulation of VEGF, which, in turn, increases CXCR4 expression by endothelial cells (19, 36). Vessel density was significantly lower in AMD3100 tumors and RNAI tumors than in PBS tumors (Fig. 3A and B; 91.23 ± 17.57 and 71.11 ± 28.73 vessels/mm² vs. 167.84 ± 37.92 vessels/mm², respectively; AMD3100 vs. PBS, P < 0.05; RNAI vs. PBS, P < 0.05). scrBR5-1-injected tumors displayed similar vessel density (111.11 ± 11.07 vessels/mm²) to PBS tumors.

**AMD3100 treatment increases tumor apoptosis and necrosis**

It has been previously reported that CXCL12-mediated inhibition of the NF-kB/TNFα apoptotic pathway (17, 18) affords in vivo protection from tumor apoptosis and that this can be impaired via CXCR4 inhibition (37, 38). Here, significantly higher numbers of apoptotic cells were found in AMD3100 tumors than in PBS tumors (90.86 ± 17.99 cells/mm² vs. 37.18 ± 11.60 cells/mm², P < 0.05; Fig. 4A and B). RNAI tumors showed a nonsignificant increase in apoptotic cells compared with controls (97.35 ± 46.5 cells/mm²), suggesting a compensatory role for stromal-derived CXCL12 in these tumors. Hematoxylin and eosin (H&E)-stained selected tumor tissues from AMD3100-treated and PBS-treated mice showed areas of necrosis in 50.0% of the AMD3100 tumor sections, whereas PBS tumor sections appeared microscopically negative for necrotic tissue (Fig. 4C).

**CXCR4 antagonism selectively reduces intratumoral FoxP3+ T-cell infiltration in comparison with CD8+ T cells**

CXCL12 can act as both a chemoattractant and a chemorepellent for T-cell subpopulations in a concentration- and CXCR4-dependent manner in tumor models (25). Therefore, we examined the effect of the manipulation of the CXCL12/CXCR4 axis on intratumoral T-cell trafficking in this model of ovarian cancer. CD8+ T-lymphocyte infiltration was not significantly different in PBS-treated, kdBR5-1–injected, and AMD3100-treated mice via immunofluorescence [Fig. 5A (top) and B] and flow cytometry (CD3+CD8+; 11.09% ± 0.20%, 10.44% ± 1.18%, 9.54% ± 0.16% and CD3+CD4+: 42.1% ± 2.39%, 40.53% ± 0.15%, 40.33% ± 2.30%, respectively). In contrast, RNAI tumors and AMD3100 treatment resulted in a significant 4- to 5-fold reduction in FoxP3+ T cells compared with PBS tumors, as determined by immunohistochemistry [18.77 ± 7.29 and 14.46 ± 4.48 cells/mm² vs. 76.0 ± 25.02 cells/mm², P < 0.05; Fig. 5A (bottom) and C] and flow cytometry (Fig. 5D). No differences were detected in the spleen for CD3+CD8+, CD3+CD4+, and CD4+CD25FoxP3+ cells among PBS-treated, kdBR5-1–injected, and AMD3100-treated mice (data not shown). To determine whether this was the result of selective FoxP3+ T-cell redistribution (39) or an alteration in TIL trafficking (25), thymus and spleens were obtained from tumor-bearing mice and stained for FoxP3 expression. No significant differences were detected in PBS-treated (1,088.23 ± 196.15 and 69.54 ± 21.77 cells/mm²) versus AMD3100-treated mice with regard to the number of
FoxP3+ T cells in these tissues (948.15 ± 156.03 and 153.40 ± 86.65 cells/mm²; Fig. 5E). AMD3100 treatment resulted in a 6-fold increase in the CD8+ /FoxP3+ ratio, a parameter correlated with increased survival in ovarian cancer (40), compared with control PBS–treated animals (3.12 and 3.50 vs. 0.58, respectively, $P < 0.01$; Fig. 5F).

**AMD3100 treatment selectively increases tumor-specific T-cell responses and impairs Treg migration in vitro**

To test whether the differential distribution of Tregs was associated with impaired tumor-specific T-cell function, we analyzed the in vitro production of Granzyme B in T lymphocytes extracted from spleen and tumors in AMD3100-treated and PBS-treated mice as described earlier. Splenocytes and TILs were stimulated with tumor lysates and Her2/neu-specific peptide for 24 hours and then analyzed for the intracellular expression of Granzyme B by flow cytometry. Her2/neu was shown to be expressed by BR5-1 by flow cytometry (data not shown). Granzyme B production was significantly higher in TILs from AMD3100 tumors showed higher Granzyme B production than PBS tumors, whereas Treg–depleted TILs from PBS tumors showed increased Granzyme B production compared with nondepleted TILs from PBS-treated tumors (Fig. 6C and D). No differences in Granzyme B production were observed in the spleen-derived T-cell populations for either AMD3100- or PBS-treated tumors (Fig. 6D and data not shown).

We observed that intratumoral Tregs consistently expressed higher CXCR4 levels than CD4+CD25− and nondepleted CD4+CD25+ cells were stimulated as before, and Granzyme B expression was determined using flow cytometry. Nondepleted CD4+ TILs from AMD3100 tumors showed higher Granzyme B production than PBS tumors, whereas Treg–depleted TILs from PBS tumors showed increased Granzyme B production compared with nondepleted TILs from PBS-treated tumors (Fig. 6C and D). No differences in Granzyme B production were observed in the spleen-derived T-cell populations for either AMD3100- or PBS-treated tumors (Fig. 6D and data not shown).

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These data support the view that differential expression of CXCR4 may selectively affect the intratumoral trafficking of regulatory versus effector cells toward intratumoral CXCL12 gradients.
CXCL12/CXCR4 antagonism using AMD3100 selectively impacts Treg infiltration in a second murine tumor model

To test the impact of CXCL12/CXCR4 axis blockade on Treg infiltration in a second solid tumor, we employed the B16F10 murine melanoma model. Previous reports reveal that B16 melanoma cells express both CXCR4 and CXCL12 (41) and have assessed the effect of CXCR4 antagonists on tumor spread (10). To test the effect of CXCL12/CXCR4 manipulation on T-cell infiltration into melanomas, osmotic pumps delivering AMD3100 were implanted as described above. We observed a significant decrease in CD4+CD25+ and CD4+CD25+FoxP3+ T cells in the AMD3100-treated tumors compared with controls (1,277.28 ± 36.73 and 202.56 ± 29.14 T cells/g vs. 3,494.27 ± 831.46 and 573.55 ± 111.40 T cells/g, P < 0.05; Fig. 7A), whereas the CD8+ TIL count was not significantly different between the experimental group and controls (1,569.28 ± 599.99 T cells/g vs. 3,337.51 ± 687.12 T cells/g, P = 0.89; Fig. 7A). Intratumoral Tregs were shown to express higher levels of CXCR4 than CD8+ cells (Fig. 7B). These data are consistent with those generated in the ovarian cancer model and support the view that the antagonism of the CXCR4/
CXCL12 axis results in selective Treg depletion compared with CD8+ T cells potentially as a result of the expression of higher levels of CXCR4 on the Treg subpopulation. Tumor growth delay at the levels of 4 and 8 times the initial volume was 2.9 ± 0.6 and 4.7 ± 1.5 days in control tumors (n = 11) and 2.7 ± 0.7 and 4.3 ± 1.5 days in AMD-treated tumors (n = 12), respectively. There was no significant difference in the rate of tumor growth or in overall survival between the groups, which is consistent with reports that the CXCL12/CXCR4 axis does not impact B16F10 melanoma cell proliferation (42) and that depletion of Tregs alone is not sufficient to promote tumor eradication in the B16 melanoma model.
Discussion

This first-of-its-kind study confirms that blockade of the CXCR4/CXCL12 axis alone can reduce ovarian tumor growth and peritoneal dissemination, resulting in a significant survival advantage in immunocompetent mice. The murine model that was used reproduces a clinical sequence of events from the detection of ascites to the appearance of disseminated intraperitoneal disease that resembles the progression of the most common form of papillary epithelial ovarian cancer in humans (28). In this context, we investigated the ability of the CXCL12/CXCR4 axis blockade to target multiple aspects of tumor progression, including cancer growth, angiogenesis, and tumor-specific immune dysregulation.

This study confirmed that CXCL12 directly impacts cancer cell proliferation in this model (13, 14, 16, 43). When CXCL12 was knocked down in BR5-1 cells, which natively express both CXCL12 and CXCR4, we observed a deceleration of tumor growth \textit{in vivo} and a significant reduction in tumor cell proliferation \textit{in vitro} that was reversed by adding exogenous CXCL12. Furthermore, AMD3100-treated tumors had increased level of tumor cell apoptosis and tumor necrosis compared with controls, which is consistent with published data showing that AMD3100 treatment can abrogate CXCL12-mediated protection from apoptosis in cancer (44, 45) and reduced the number of intraperitoneal masses in nude mice (21).

Tumor-derived CXCL12 plays a part in the trafficking of proangiogenic cells (i.e., plasmacytoid dendritic cell) and the induction of proangiogenic factors (i.e., CCL8) in the tumor microenvironment (46). VEGF and CXCL12 have been shown to act synergistically to induce CXCR4 expression and migration of vascular endothelial cells (19). In this study, impaired vascularization in the RNAi and AMD3100 treatment groups was observed. Tumor angiogenesis is also known to contribute to tumor immune escape through multiple mechanisms, including suppression of adhesion molecule expression, reduction of tumor-specific CTL function and adenomatous polyposis coli (APC) activation, and induction of intratumoral Treg localization (47, 48). Therefore, the antiangiogenic effect that AMD3100 elicited in this study may be associated with a reduction in tumor growth and dissemination, as well as a partial correction of tumor immunity.

This model of ovarian cancer in an immunocompetent mouse, allowed us to show for the first time that AMD3100 resulted in a significant and selective 4- to 5-fold reduction in FoxP3\(^+\) cells in ovarian tumors that was not associated with either a change in the distribution of Tregs in other organs (i.e., spleen and thymus) or a diminution of intratumoral CD8\(^+\) T cells. CXCL12-mediated mechanisms hypothesized to be involved in immunosuppression are either the repulsion of antitumor effector T cells or the active recruitment of immunosuppressive cells (25, 39). The preferential accumulation of tumor-specific, T-cell–suppressive Tregs in ovarian cancer tissue and ascites was previously correlated with poor survival (49).
Our results show that AMD3100 differentially blocks intratumoral Treg infiltration and migration in comparison with CD8+ T cells in vivo and in vitro, respectively. We also found that this was associated with the finding that intratumoral Tregs express significantly higher levels of CXCR4 than CD8+ T cells. These findings were recapitulated in a second tumor model. Furthermore, we showed that depletion of Tregs from TILs reversed an apparent immunosuppressive effect on tumor antigen-specific Granzyme B production in vitro, supporting the concept that exclusion of Tregs from the intratumoral milieu may augment local antitumor immunity (50).

These findings reveal a new mechanism for modulating intratumoral T-cell subsets that compares favorably with other therapeutic strategies. In this study, AMD3100 treatment resulted in a 6-fold increase in the intratumoral Teff/Treg ratio. This highly significant change was associated with significantly improved cytotoxic T-cell function in tumor tissue, prolonged survival in this model, and is consistent with recent approaches to depleting intratumoral Tregs (51, 52). Tuve and colleagues reported that the CD8/Treg ratio in TC-1/anti-CTLA-4-52). Tuve and colleagues reported that the CD8/Treg ratio in TC-1/anti-CTLA-4-52). Tuve and colleagues reported that the CD8/Treg ratio in TC-1/anti-CTLA-4-52). Tuve and colleagues reported that administration of an engineered hepatic tissue-targeting Listeria monocytogenes with cyclophosphamide led to a 5-fold increase in the T eff/Treg ratio in a murine model of colorectal cancer metastasis to the liver (51).

Importantly, we clearly showed that CXCR4 antagonism induced selective reduction of intratumoral Tregs without affecting systemic Tregs and intratumoral CD8+ effector T cells. Conventional strategies to reduce the number of intratumoral Tregs, including the systemic application of anti-CD25 antibody and cyclophosphamide do not specifically target Tregs and therefore also deplete effector T cells. Furthermore, such strategies have significant drawbacks, including toxicity and induction of autoimmunity due to the systemic elimination of Tregs (53). In our study, we have successfully identified an effective strategy to selectively deplete intratumoral Tregs, resulting in prolonged survival in a murine model of ovarian cancer.

In summary, these data generated in an immunocompetent syngeneic and orthotopic murine model lend important support to the view that the CXCL12/CXCR4 axis plays multiple roles in the pathogenesis of ovarian cancer. This study shows a new role for this axis in the selective trafficking of Tregs into the tumor in addition to reaffirming its established roles in tumor apoptosis, growth, and angiogenesis. We also show that these multimodal effects of specific single-agent CXCR4/CXCL12 axis blockade in this model of ovarian cancer are associated with a significantly prolonged survival and reductions in tumor progression. The availability of a safe, effective, and clinically applicable CXCR4 antagonist in AMD3100 adds both to the direct clinical translatability of this study and to the enthusiasm amongst clinicians to explore the use of this agent as an adjunct to conventional chemotherapy, including taxol in platinum-resistant disease, or in combination with other immunotherapies aimed at enhancing CD8+ T-cell responses in human ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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