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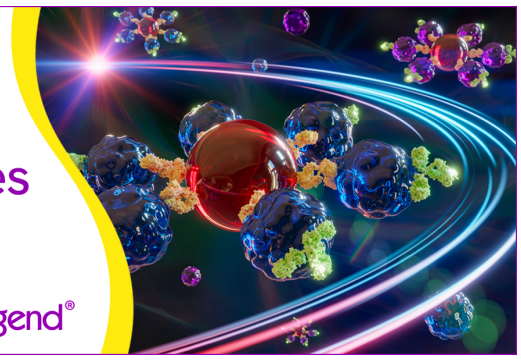

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Murine B16 Melanomas Expressing High Levels of the Chemokine Stromal-Derived Factor-1/CXCL12 Induce Tumor-Specific T Cell Chemorepulsion and Escape from Immune Control[1]

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The chemokine, stromal-derived factor-1/CXCL12, is expressed by normal and neoplastic tissues and is involved in tumor growth, metastasis, and modulation of tumor immunity. T cell-mediated tumor immunity depends on the migration and colocalization of CTL with tumor cells, a process regulated by chemokines and adhesion molecules. It has been demonstrated that T cells are repelled by high concentrations of the chemokine CXCL12 via a concentration-dependent and CXCR4 receptor-mediated mechanism, termed chemorepulsion or fugetaxis. We proposed that repulsion of tumor Ag-specific T cells from a tumor expressing high levels of CXCL12 allows the tumor to evade immune control. Murine B16/OVA melanoma cells ($H2^b$) were engineered to constitutively express CXCL12. Immunization of C57BL/6 mice with B16/OVA cells lead to destruction of B16/OVA tumors expressing no or low levels of CXCL12 but not tumors expressing high levels of the chemokine. Early recruitment of adoptively transferred OVA-specific CTL into B16/OVA tumors expressing high levels of CXCL12 was significantly reduced in comparison to B16/OVA tumors, and this reduction was reversed when tumor-specific CTLs were pretreated with the specific CXCR4 antagonist, AMD3100. Memory OVA-specific $CD8^+$ T cells demonstrated antitumor activity against B16/OVA tumors but not B16/OVA.CXCL12-high tumors. Expression of high levels of CXCL12 by B16/OVA cells significantly reduced CTL colocalization with and killing of target cells in vitro in a CXCR4-dependent manner. The repulsion of tumor Ag-specific T cells away from melanomas expressing CXCL12 confirms the chemorepellent activity of high concentrations of CXCL12 and may represent a novel mechanism by which certain tumors evade the immune system. *The Journal of Immunology,* **2006, 176: 2902–2914.**

Chemokines are a family of structurally related proteins that regulate leukocyte migration through interactions with a subset of seven-transmembrane G protein-coupled receptors (1). The chemokine, stromal-derived factor-1 (SDF-1)[4]/CXCL12, is expressed by stromal cells, including fibroblasts and endothelial cells, and interacts specifically with the receptor CXCR4. CXCR4 is expressed by various cells including T lymphocytes, monocytes, neutrophils, and endothelial cells (2). We recently demonstrated that whereas CXCL12 at low concentration (<10 nM) serves as a T cell chemoattractant, higher concentrations of the chemokine can repel T cells in vitro and in vivo via a CXCR4 receptor-mediated, pertussis toxin and wortmannin-sensitive mechanism, termed chemorepulsion or fugetaxis (3). This novel mechanism was shown to contribute to the physiological process of T cell emigration from the thymus potentially in combination with the activity of sphingosine-1-phosphate and its receptor (4, 5). High concentrations of CXCL12 were also shown to abrogate T cell infiltration into an anatomic site of Ag challenge (3). Recently, it has been demonstrated that monocytes, dendritic cells, and neutrophils are repelled by the chemokines, eotaxin-3, CXCL10, and IL-8 (6–8). In addition, Ag-specific T cells have also been shown to migrate away from the HIV-1 envelope protein, gp120, in vitro and in vivo in a concentration-dependent and CXCR4-mediated manner, and it has been suggested that this may contribute to a novel mechanism by which the retrovirus evades the host immune system (9–11).

It is known that chemokines play a role in both tumor biology and the migration and localization of T cells that precede the initiation of an antitumor immune response (2, 12). CXCL12 has been shown to be highly expressed by primary brain tumors, melanomas, and ovarian carcinomas, and is thought to exert a proliferative effect on tumors, and to determine the destination of metastatic tumor cells through its interaction with CXCR4 (13–16). CXCL12, as a potent chemoattractant for T cells, is also thought to modulate the immune response to tumors (17–19). Paradoxically, it has been observed that both normal tissues such as the bone

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[4] Abbreviations used in this paper: SDF-1, stromal-derived factor-1; LN, lymph node; CM, conditioned medium; MFI, mean fluorescent intensity; CLIO-HD, tat peptide-derivatized CLIO; MR, magnetic resonance; MRI, MR imaging; TR, repetition time; TE, echo time; MTX, matrix; FOV, field of view; TIL, tumor-infiltrating lymphocyte.

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marrow and thymus and dysplastic tissues producing abundant CXCL12 are rarely infiltrated by significant numbers of T cells (3, 11, 20).

Tumors are thought to be able to evade immune recognition using a number of different mechanisms (21–24). We proposed that tumor expression of high levels of CXCL12 induces tumor-specific CTL chemorepulsion or fugetaxis and thereby favors tumor growth by limiting the efficacy of effector T cells in infiltrating the tumor and killing neoplastic cells. We investigated the effects of the secretion of high levels of CXCL12 by the B16 melanoma tumor on the antitumor response in vaccinated mice and after adoptive transfer of tumor-specific CTL. We demonstrated that T cell infiltration is significantly reduced and immune control of tumor growth is impaired in tumors expressing high levels of CXCL12 compared with control tumors that expressed low levels of the chemokine or no CXCL12. T cell infiltration into tumors expressing high levels of CXCL12 could be restored by CXCR4 blockade. By using a modified ^{51}Cr release assay in which T cell migration is critical to killing efficacy, we demonstrated that the killing activity of tumor-specific CTL against tumor cells expressing a high level of CXCL12 is significantly reduced in comparison to control tumor cells and that this effect can be abrogated by blocking chemokine binding to its cognate receptor, CXCR4. We demonstrated that the constitutive expression of high levels of CXCL12 within the tumor microenvironment abrogates localization toward and infiltration of tumors by Ag-specific T cells, which, in turn, allows the neoplasm to escape destruction by the immune system.

Materials and Methods

C57BL/6 and OT-1 mice

C57BL/6 mice (6- to 10-wk-old) were used in all experiments (The Jackson Laboratory). OT-1 TCR transgenic mice were kindly provided by W. R. Heath and F. Carbone (Walter and Eliza Hall Institute, Melbourne, Australia). The OT-1 TCR is expressed on CD8$^+$ T cells and is specific for the peptide $OVA_{257–264}$ (SIINFEKL) bound to the class I MHC molecule H2-K^b (25).

Cell lines and preparation of OT-1 CTLs

B16 melanoma cells (H2^b) stably expressing chicken OVA (B16/OVA.pc) were provided by Drs. E. Lord and J. Frelinger (University of Rochester Medical Center, Rochester, NY) (26). OT-1 CD8$^+$ T cells were isolated from the spleens and lymph nodes (LN) of OT-1 mice using the MACS system (Miltenyi Biotec) and cultured and expanded (27). For CXCR4 expression, B16 cells and naive or effector OT-1 CD8$^+$ T cells were immunostained using anti-CXCR4 Ab (clone 2B11; BD Pharmingen) and analyzed on a FACS (BD Biosciences) using FlowJo software (Tree Star). The effect of CXCL12 on OT-1 CD8$^+$ T cell apoptosis, activation and proliferation, was evaluated by annexin V/propidium iodide staining (Detection Kit I; BD Pharmingen), by quantitation of CD69 (clone H1-2F3) and CD25 (clone PC61; all obtained from BD Pharmingen) expression, and by CFSE staining (Molecular Probes) (28–30).

Generation of genetically modified B16/OVA cells expressing CXCL12

The coding region of murine CXCL12α (82–232 bp) was amplified by PCR and cloned into the enhanced green fluorescent protein-encoding bicistronic murine stem cell virus-derived retroviral transfer vector MSCV2.2 (provided by Dr. N. Carlesso, Massachusetts General Hospital, Boston, MA), using *Xho*I and *Eco*RI cloning sites. Cloning results were confirmed by DNA sequencing. 293T HEK packaging cells were cotransfected using the calcium-phosphate method and the following vectors: enhanced green fluorescent protein encoding vectors MSCV2.2-CXCL12 or MSCV2.2, a packaging vector pKat and pCMV-VSV-G (encoding the vesicular stomatitis virus G-glycoprotein). VSV-G pseudotyped retroviral vectors encoding CXCL12 and GFP or GFP alone were collected at 48 and 72 h posttransfection and used for transduction of B16/OVA melanoma cells (31). Cell sorting was performed using a FACSVantage cells sorter (BD Biosciences) to select the brightest MSCV cells (B16/OVA.MSCV) and two cell populations with bright and low levels of fluorescence termed B16/OVA.CXCL12-high and B16/OVA.CXCL12-low cells.

Quantitation of CXCL12 production and bioactivity

B16/OVA.MSCV and B16/OVA.CXCL12 cells were grown until they were 80–90% confluent. Cells were incubated for a further 24 h in medium containing 0.5% FCS. Conditioned medium (CM) was harvested, concentrated, and analyzed by Western blotting, ELISA, and transmigration assay. Western blotting was performed using mouse monoclonal anti-CXCL12 Ab (R&D Systems), HRP-labeled sheep anti-mouse Ab (Amersham Biosciences), and ECL detection kit (Amersham). The ELISA was performed using sandwich ELISA (Quantikine; R&D Systems). Levels of OVA secreted in CM from B16/OVA.MSCV and B16/OVA.CXCL12 cells cultured for 24 h were also measured by ELISA (Alpha Diagnostic).

For quantitative transmigration assays, purified murine CD8$^+$ T cells (6×10^4 cells) were added to the upper chamber of each well in a total volume of 150 μl of Iscove's modified medium. CM, 10- or 20-fold concentrated, undiluted or diluted 1/10 in DMEM containing 0.5% FCS, was added in the lower, upper, or both lower and upper chambers of the Transwell to generate a standard "checkerboard" analysis of cell migration including measurements of chemotaxis, fugetaxis, and chemokinesis (3, 5, 9, 10). Further control wells assessed the chemotactic, fugetactic, and chemokinetic activity of rCXCL12 at concentrations from 10 ng/ml to 1 μg/ml. Cells were harvested from the lower chamber after 3 h, and cell counts were performed using a hemocytometer, as described previously (3).

To test for heterologous receptor desensitization of CCR5 induced by high concentrations of CXCL12, freshly prepared murine CD8$^+$ T cells were exposed to CXCL12 at concentrations of 100 ng/ml and 1 μg/ml for 15 min. Cells were then fixed in paraformaldehyde, and CCR5 expression was quantitated on a FACS using anti-CXCR4 (clone 2B11), anti-CCR5 Abs (clone C34-3448). The mean fluorescent intensity (MFI) of cells was determined for CCR5 before treatment and immediately after treatment with CXCL12. For each marker, the threshold of positivity was found beyond the nonspecific binding observed in the presence of irrelevant isotype-matched control Ab. MFI values were obtained by subtracting the MFI of the isotype control from the MFI of the positively stained sample. The Kolmogorov-Smirnov test was used to evaluate whether the differences between the distributions of MFI were statistically significant with respect to controls.

To determine whether exposure of cells to high concentrations of CXCL12 might result in the inhibition of chemotactic responses of CD8$^+$ T cells to the CCR5 binding chemokine, CCL3, murine CD8$^+$ T cells were or were not exposed to CXCL12 at a concentration of 100 ng/ml and 1 μg/ml and then subjected to positive and negative gradients of the CCL3 at peak concentrations of 10 ng/ml and 100 ng/ml as well as uniform concentrations of this chemokine. Transmigration of T cells under these conditions was then quantitated as described above.

Tumorigenicity of B16/OVA cells in nonimmune and immunized mice

Tumor cells (5×10^5) were seeded in T25 flasks (BD Biosciences) and cell yield determined for three consecutive days. Tumorigenicity was determined by s.c. injection of 2×10^5 viable cells into the flank of mice in a total volume of 200 μl of PBS. Tumor growth was also evaluated in naive mice challenged with B16/OVA.MSCV or B16/OVA.CXCL12-high cells and injected twice a day s.c. with the CXCR4 antagonist, AMD3100 (1.25 mg/kg; Sigma-Aldrich) (32). Tumor growth was measured every 3 or 4 days using a caliper. Tumor volume was recorded as the product of two orthogonal diameters (a $\times$ b; a = longest diameter; b = orthogonal width) (33). In immunization studies, mice received injections s.c. (at multiple sites) with 1×10^6-irradiated (8568 cGy) B16/OVA.pc cells in a volume of 200 μl of PBS at day 0 and day 10. Fourteen days after immunization, mice were challenged s.c. in both flanks with 2×10^5 B16/OVA.MSCV and B16/OVA.CXCL12-high cells, and tumor growth was measured as described above (34). In some experiments, mice were immunized with B16/OVA.CXCL12-high cells and then challenged with B16/OVA.CXCL12-high or B16/OVA.CXCL12-low cells. Mice were sacrificed when one or both tumors reached 200 mm^2 in volume.

Adoptive immunotherapy, transfer of nanoparticle-labeled CTL and survival analysis

Tat peptide-derivatized CLIO (CLIO-HD) was synthesized as described previously (35). For each experiment, mice received injections s.c. in the right and left flank with 5×10^5 B16/OVA.MSCV and B16/OVA.CXCL12-high cells. When tumors reached 10–15 mm in at least one diameter, OT-1 CD8$^+$ T cells expanded in vitro were harvested, incubated

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with CLIO-HD (300 μg/ml/1 $\times$ 10^7 cells), and 3 $\times$ 10^7-labeled cells were injected i.p. into mice bearing bilateral tumors of similar size (35). Distribution of CLIO-HD-labeled cells over time was assessed via magnetic resonance (MR) imaging at 24 and 48 h after adoptive transfer. MR imaging (MRI) experiments were performed on a 4.7 T 16-cm bore MRI system (Bruker Pharmascan). Mice were anesthetized throughout imaging with 1–2% isoflurane at 1.5 l/min. For the analysis of T cell recruitment, an axial T2-weighted gradient echo sequence (repetition time (TR) = 600 ms, echo time (TE) = 6.0, field of view (FOV) 4.24 $\times$ 2.12 cm, matrix (MTX) 256 $\times$ 128, 4 averages) and an axial T2-weighted fast spin echo sequence (TR = 2000 ms, TE = 48.6 ms, FOV 4.24 $\times$ 2.12 cm, MTX 256 $\times$ 128, 8 averages) were used. T2 fit values were derived from a multislice multiecho sequence (TR = 2000 ms, TE = 6.5 $\times$ 16 ms, FOV 4.24 $\times$ 2.12 cm, MTX 128 $\times$ 128, 2 averages). Regions of interest for the tumors were drawn by hand, and T2 fit values were calculated using an in-house program, CMIR-Image (35).

In some experiments, activated OT-1 $CD8^+$ T cells were also preincubated with the CXCR4 antagonist, AMD3100, at concentrations of 0.08 and 0.25 μg/ml (15 min at RT) in a 12-well plate at a density of 1 $\times$ 10^7 cells/well before adoptive transfer (36).

To study the effect of low-level secretion of CXCL12 by tumors on T cell infiltration, mice also received injections into the right and left flank with B16/OVA.MSCV and B16/OVA.CXCL12-low cells.

Survival analysis was evaluated in mice after in vivo generation of a tumor-specific memory T cell compartment (37). Twenty-one days after adoptive transfer of 1 $\times$ 10^7 OT-1 $CD8^+$ T cells (in 500 μl of HBSS via tail vein injection), two groups of mice (4 or 6 per group) were challenged s.c. in the right flank with 2 $\times$ 10^5 B16/OVA.CXCL12-high cells or B16/OVA.MSCV control cells in 200 μl of HBSS using a 27-gauge needle (37). As control, two groups of mice that did not receive adoptive transfer were challenged with tumors. Detectable tumor was considered to be >5 mm^2. Mice were sacrificed when tumors reached 400 mm^2 in size.

Tumor immunohistochemistry and FACS analysis of tumor-infiltrating lymphocytes (TIL)

Formalin-fixed tumors were embedded in paraffin. Serial 5-μm sections of tumors were stained with H&E or with polyclonal anti-CD3 Ab or isotype control Ab, followed by incubation with secondary HRP-labeled anti-rabbit Dako EnVision Ab (DakoCytomation). T cell infiltration was quantified by counting four random 200$\times$ field powers of the tumor sections, each 0.5 mm^2 in area. Quantitation of TIL by FACS was performed in mice that received OT-1 $CD8^+$ T cells for survival analysis (38). The total number of CD8/TCR-Vβ-5-1/Vα-2-positive cells was calculated from the percentage of the total number of cells recovered from 100 mg of tumor.

Quantitation of CTL efficacy in vitro

Cytotoxicity of OT-1 $CD8^+$ cells was measured in a standard ^{51}Cr-release assay in round-bottom wells as described previously (9). The assay was also performed in flat-bottom wells to determine the effects of CTL migration on killing efficacy in the context of target cells expressing low or high levels of CXCL12 (9). Dilutions of effector cells were added to ^{51}Cr-labeled target cells to give the indicated E:T cell ratios. Following a 5-h incubation, 30 μl of supernatant were harvested, and the radioactivity was counted in a gamma radiation counter. Cytotoxicity was expressed either as percentage of lysis or as Lytic Unit 30 as described previously (9, 39). ^{51}Cr release assays were also performed in flat-bottom wells after preincubation of OT-1 $CD8^+$ T cells with the CXCR4 antagonist AMD3100 at concentrations of 0.25 and 1 μg/ml as described above (36).

The effect of CXCL12 on CTL killing efficacy was measured after proliferating OT-I $CD8^+$ T cells were cocultured with B16/OVA.CXCL12-high or B16/OVA.MSCV cells. In this assay, 5 $\times$ 10^6 OT-I $CD8^+$ T cells were allowed to proliferate for 5 days in 12 well plates in the presence of 10 μg/ml anti-CD3, 2 μg/ml anti-CD28, and 50 U/ml IL-2. A total of 5 $\times$ 10^5 B16/OVA.MSCV or B16/OVA.CXCL12-high cells was seeded onto a 0.4-μm pore polycarbonate membrane (Corning Life Sciences) and placed in the upper chamber from the first day of culture. In experiments performed in parallel, OT-I $CD8^+$ T cells were also allowed to proliferate for 5 days in the presence of rCXCL12 at concentrations of 500 or 1000 ng/ml, which was added daily to each well.

Analysis of activation, proliferation, and apoptosis of OT-1 T cells exposed to CXCL12

Activated OT-1 $CD8^+$ T cells were exposed to murine rCXCL12α (PeproTech) for 24 or 72 h at final concentrations of 10, 100, and 1000 ng/ml. Cells were incubated with FITC-conjugated annexin V and propidium iodide (Detection Kit I; BD Pharmingen). The percentage of apoptotic cells was analyzed by FACS using FlowJo software (28). To analyze the effect of CXCL12 on T cell proliferation, 2 $\times$ 10^5 OT-1 total splenocytes or purified $CD8^+$ T cells were allowed to proliferate for 3 days in 96 round-bottom well plates precoated with 10 μg/ml anti-CD3 mAb (BD Pharmingen) in the presence of 2 μg/ml soluble anti-CD28 mAb and 50 U/ml murine rIL-2. OT-1 T cells were preincubated for 3 h with murine rCXCL12 at final concentrations of 500 or 1000 ng/ml. CXCL12 at each concentration was also added every 24 h to proliferating T cells.

For CFSE analysis, T cells were preincubated with 1 μM CFSE (Molecular Probes) in PBS for 10 min. At the time of harvest, cells were washed twice in cold PBS buffer and incubated for 15 min at 4°C with anti-CD69 and anti-CD25 mAb, and flow cytometry was performed on a FACSCalibur (BD Biosciences). Data analysis was performed with the FlowJo software (Tree Star). For each marker, the threshold of positivity was found beyond the nonspecific binding observed in the presence of irrelevant isotype-matched control Ab. MFI values were obtained by subtracting the MFI of the isotype control from the MFI of the positively stained sample. The Kolmogorov-Smirnov test was used to evaluate whether the differences between the distributions of MFI were statistically significant with respect to controls.

Results

Engineered B16/OVA.CXCL12 cells expressing high or low levels of CXCL12 induce T cell chemorepulsion and chemoattraction, respectively

Cell sorting of transduced B16/OVA.pc cells was performed to select the brightest and dimmest B16/OVA.CXCL12 cells, termed B16/OVA.CXCL12-high and B16/OVA.CXCL12-low. The cells transduced with the MSCV vector encoding GFP alone were used as the control cell line in all experiments. Concentrations of soluble CXCL12 in the CM from B16/OVA.CXCL12-high and B16/OVA.CXCL12-low cultures (from 1 $\times$ 10^6 cells cultured for 24 h) as measured by Western blot and ELISA ranged from 35 to 110 ng/ml (mean, 58 $\pm$ 28.5) and 2.4 to 13 ng/ml (mean, 7.6 $\pm$ 4.1), respectively. No CXCL12 was detected in CM from untransduced B16/OVA cells or B16/OVA cells transduced with MSCV-GFP by Western blotting or ELISA. No significant difference in class I MHC expression and OVA production between B16/OVA.CXCL12 transductants and B16/OVA.MSCV cells (Table I) was detected. B16/OVA.MSCV and B16/OVA.CXCL12 cells expressed very low levels of CXCR4 by flow cytometry (Table I).

The effect of the CXCL12/CXCR4 axis on tumor growth was evaluated in mice untreated and treated with the specific CXCR4 antagonist, AMD3100, in vivo from the first day after challenge with tumor cells until the appearance of tumors. The average time

Table I. *MHC class I and CXCR4 expression determined by FACS analysis and OVA secretion in B16/OVA.MSCV and B16/OVA.CXCL 12-high and -low cells determined by Western blotting and ELISA*

	MHC-I (MFI $\pm$ SD)	OVA (pg/ml $\pm$ SD)	CXCL12 (ng/ml $\pm$ SD)	CXCR4 (MFI $\pm$ SD)
B16.OVA/MSCV	16.2 $\pm$ 2.1	950 $\pm$ 70		3.06 $\pm$ 0.65
B16.OVA/CXCL12-high	17.1 $\pm$ 1.9	1200 $\pm$ 282	58 $\pm$ 28.5	2.45 $\pm$ 0.67
B16.OVA/CXCL12-low	17.9 $\pm$ 1.3	994 $\pm$ 34	7.6 $\pm$ 4.1	2.88 $\pm$ 0.96

[a] Results are shown as MFI. Levels of CXCL12 and OVA secretion in the supernatant from 1$\times$$10^6$ B16/OVA.MSCV and B16/OVA.CXCL12-high cells cultured for 24 h are shown (mean $\pm$ SD).

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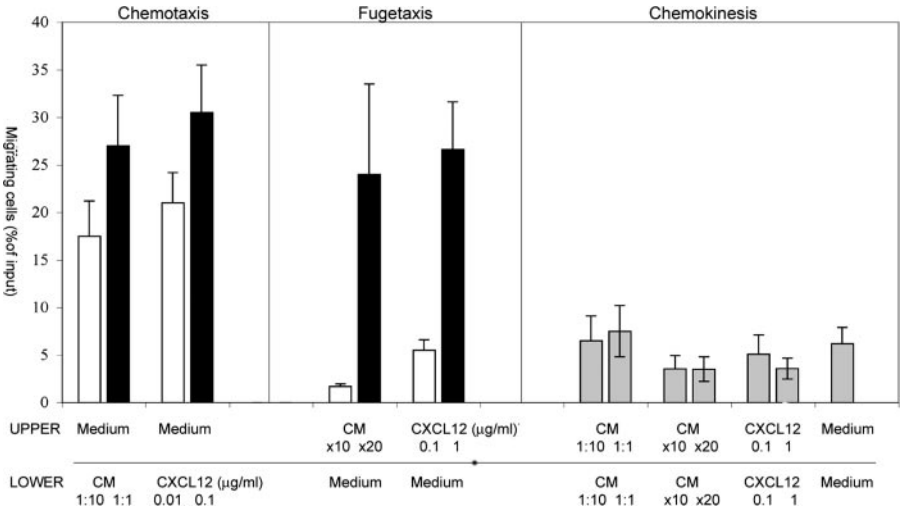

FIGURE 1. CXCL12 expressed by engineered B16/OVA tumor cells acts as a bidirectional cue for murine T cells in vitro. B16/OVA.CXCL12-high cells were incubated for 24 h in DMEM containing 0.5% FCS. CM was then collected and used undiluted, 1/10 diluted, or 20× concentrated in transmigration assays. rCXCL12 at concentrations of 10-100 ng/ml and 1 μg/ml were used as controls. To evaluate chemotaxis and fugetaxis, CM was placed in the lower or upper chamber, respectively, and purified murine $CD8^+$ T cells (6×10^4 cells) were added to the upper chamber of each well in a total volume of 150 μl. Chemokinesis was assessed by added rCXCL12 or diluted or concentrated CM in both the upper and lower chambers of the transwell. The percentage of input cells in the upper chamber migrating into the low chamber under each condition was assessed in three independent experiments, and the mean ± SD is shown for each setting of the assay.

to tumor growth to a size of 200 mm^2 size was 22 ± 2 (average ± SD) and 24 ± 1.3 days, respectively, for B16/OVA.MSCV treated or untreated with AMD3100 ($p = 0.32$; data not shown). The time for tumor growth to 200 mm^2 for AMD3100 untreated and treated B16/OVA.CXCL12-high tumors was 23 ± 1.5 and 23.6 ± 2.08 days, respectively ($p = 0.72$; data not shown).

CM from B16/OVA.CXCL12-high cells and B16/OVA.MSCV cells was tested for functional activity in transmigration assays (Fig. 1). Undiluted and 1/10 diluted CM from B16/OVA.CXCL12-high cells resulted in the chemotactic migration of 27 ± 5.3 and 17.5 ± 3.7% of input cells, respectively (Fig. 1). Recombinant CXCL12 at concentrations of 0.01 and 0.1 μg/ml resulted in the chemotactic migration of 21 ± 3.2 and 30.5 ± 5.0% of input cells, respectively. CM was concentrated 20-fold by ultrafiltration to generate chemorepellent concentrations of CXCL12. Ten- or 20-fold concentrated CM resulted in the fugetactic migration of 1.7 ± 2.6 and 24 ± 9.5% of input cells, respectively. Murine rCXCL12 at concentrations of 0.1 and 1 μg/ml resulted in the fugetactic migration of 5.5 ± 1.1 and 26.6 ± 5.0% of input cells, respectively. Chemokinetic responses (random movement in the absence of a gradient of a chemokinetic agent) of T cells were also measured in transmigration assays in which recombinant CXCL12,

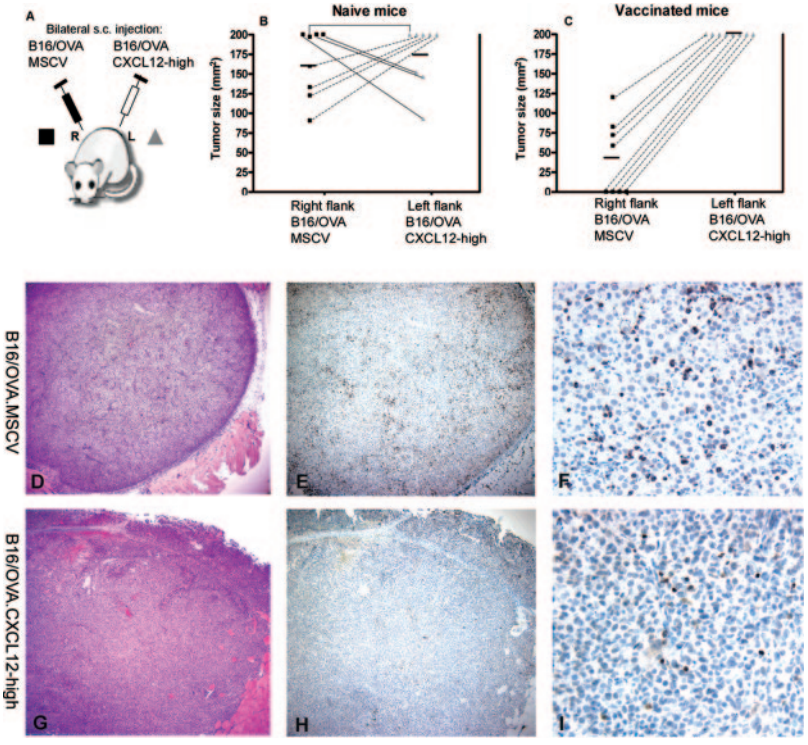

FIGURE 2. Prophylactic immunization of mice is effective against control but not B16/OVA tumors secreting high levels of CXCL12. Nonimmunized mice were simultaneously inoculated with B16/OVA.MSCV (■, right flank) and B16/OVA.CXCL12-high cells (▲, left flank) (*A*). Mice ($n = 8$) were sacrificed when at least one tumor reached 200 mm^2 in size. Tumor sizes for each mouse at time of sacrifice are shown (*B*). No significant trend in tumor development was observed in naive mice (B16/OVA.MSCV vs B16/OVA.CXCL12-high tumors) ($p = 0.7$). Tumor growth was also recorded in mice that were immunized with irradiated B16/OVA.pc ($n = 8$) before inoculation with B16/OVA.MSCV (■, right flank) and B16/OVA.CXCL12-high cells (▲, left flank) (*C*). The growth of B16/OVA.MSCV was absent in 50% of immunized animals and delayed in 4 of 8 mice. All (8 of 8) B16/OVA.CXCL12-high tumors implanted in the opposite flank of each immunized mouse rapidly progressed to 200 mm^2 in size ($p = 0.0001$). Paraffin-embedded sections of tumors from immunized mice were stained with H&E (*D* and *G*) or with polyclonal anti-CD3 Ab (*E*, *F*, *H*, and *I*). Prominent infiltrates of CD3-positive cells were observed in B16/OVA.MSCV tumors (*E* and *F*), but not in tumors expressing high levels of CXCL12 (*H* and *I*). Magnification, ×40 or ×200.

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diluted or concentrated CM from B16/OVA.CXCL12-high cells was applied to both the upper and lower chambers (Fig. 1). Chemokinetic responses under all these conditions varied between 3.5 ± 1.1% of input cells for 1 μg/ml CXCL12 and 7.5 ± 2.7% for 2 undiluted CM for example, and were always significantly less than peak chemotactic or fugetactic responses to CM or recombinant CXCL12 ($p = 0.008$ and $p = 0.032$, respectively).

Immunized mice fail to control the growth of B16/OVA.CXCL12-high tumors

To study the local effect of CXCL12 secretion by a tumor, nonimmunized or immunized mice were challenged with both B16/OVA.MSCV and B16/OVA.CXCL12-high tumor cells into the right and left flank, respectively (Fig. 2*A*). Subsequent tumor growth was measured every 3 or 4 days, and mice were sacrificed when at least one tumor reached 200 mm^2. When naive nonimmunized mice received injections bilaterally with B16/OVA.MSCV and B16/OVA.CXCL12-high cells, tumors in all cases reached a size of 200 mm^2, and all mice were sacrificed by day 25 after challenge. No mouse died of causes directly or indirectly related to the growth of the tumor itself. When comparing the bilateral tumor size in each mouse, a random pattern of growth was observed (Fig. 2*B*). In 60% of animals, B16/OVA.CXCL12-high tumors grew more rapidly than the contralateral B16/OVA.MSCV tumor, and there was no significant growth advantage of CXCL12-expressing tumors over control B16/OVA.MSCV tumors (Fig. 2*B*; $p = 0.7$). In contrast, mice immunized with irradiated B16.OVA.pc cells demonstrated a strikingly different pattern of tumor growth (Fig. 2*C*). Fifty percent of immunized mice showed no evidence of B16/OVA.MSCV tumor development in the right flank at the end of follow-up, whereas all mice developed a B16/OVA.CXCL12-high tumor in the left flank. When comparing the tumor size in mice that developed bilateral tumors, all mice developed a >200 mm^2 B16/OVA.CXCL12-high tumor when the contralateral B16/OVA.MSCV tumor was significantly smaller in size (Fig. 2*C*; $p = 0.0001$). It should also be noted that B16/OVA.CXCL12-high tumor growth in immunized mice was significantly delayed in comparison to tumor growth in nonimmunized mice (29.4 ± 4.9 vs 12.8 ± 2 days (mean ± SD), respectively; $p = 0.0002$), supporting the view that the immune system could play a role in the control of tumor growth in this model.

Immunohistochemistry was performed to quantitate T cell infiltration into tumors that were not rejected. In nonimmunized mice, there was minimal $CD3^+$ T cell infiltration into both B16/OVA.MSCV and B16/OVA.CXCL12-high tumors (5.5 ± 1.5 and 4.2 ± 1.4 cells/mm^2, respectively). In contrast, there was consistent $CD3^+$ T cell infiltration into B16/OVA.MSCV tumors in immunized mice (Fig. 2, *D–F*). T cell infiltration into B16/OVA.CXCL12-high tumors in immunized mice was significantly reduced (Fig. 2, *G–I*). The number of $CD3^+$ T cells (in four 200× fields from three mice) infiltrating B16/OVA.CXCL12-high tumors was significantly lower (34.5 ± 10.6 cells/mm^2; mean ± SD) compared with B16/OVA.MSCV tumors (92.2 ± 28.8 cells/mm^2; $p = 0.016$). These data support the view that intratumoral expression of high concentrations of CXCL12 leads to a reduction in TIL.

We also evaluated the effect of vaccination of mice with irradiated B16/OVA cells expressing high levels of CXCL12 to protect mice from bilateral challenge with B16/OVA.SDF.1-high and -low cells. As shown in Fig. 3*A*, naive mice challenged with bilateral B16/OVA.CXCL12-low and -high cells showed a random pattern of growth ($p = 0.088$). In contrast, vaccination with irradiated B16/OVA cells expressing high levels of CXCL12 resulted in rejection of 40% of B16/OVA.CXCL12-low tumors, whereas all mice developed B16/OVA.CXCL12-high tumors in the left flank (Fig. 3*B*; $p = 0.021$). Furthermore, mice that developed bilateral tumors showed a slower rate of growth of B16/OVA.CXCL12-low compared with B16/OVA.CXCL12-high tumors (data not shown). Quantitation of T cell infiltration by immunohistochemistry revealed a significantly greater infiltration of $CD3^+$ cells into B16/OVA.CXCL12-low tumors (101 ± 10 cells/mm^2) than into B16/OVA.CXCL12-high tumors (31 ± 5.1 cells/mm^2; $p = 0.007$; Fig. 3*C*). These data support the concept that although CXCL12 does not affect the immunogenicity of B16/

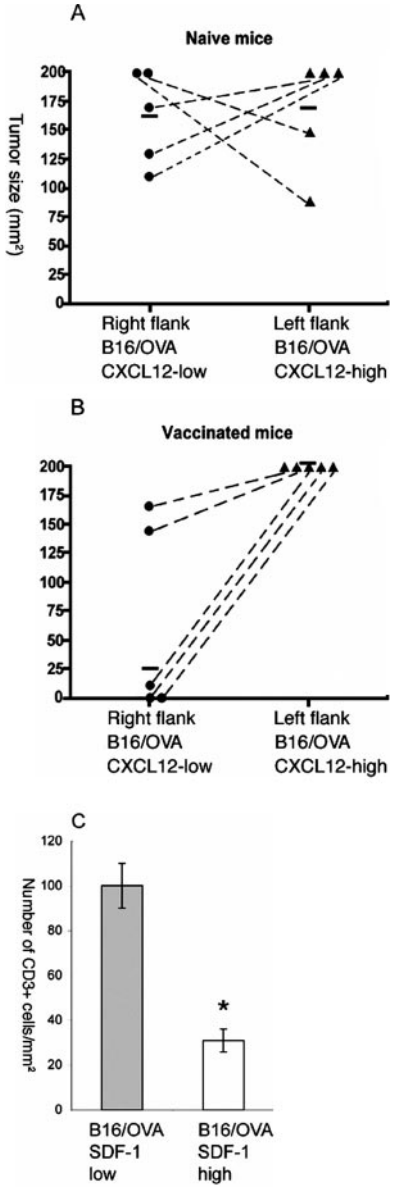

FIGURE 3. B16/OVA.CXCL12-high cells are immunogenic. Mice (groups of 5) were prophylactically immunized with irradiated B16/OVA.CXCL12-high cells before inoculation with B16/OVA.CXCL12-low (●, right flank) and B16/OVA.CXCL12-high cells (▲, left flank) (*B*). Nonimmunized mice were challenged bilaterally with both tumors as control (*A*). Forty percent of mice did not develop the B16/OVA.CXCL12-low tumors implanted in the right flank (*B*). All mice showed growth of the B16/OVA.CXCL12-high tumors in the left flank ($p = 0.021$) (*B*). No significant difference in tumor development was observed in naive mice (*A*; $p = 0.88$). Anti-CD3 staining of tumor sections showed T cell infiltrates in B16/OVA.CXCL12-low tumors but not in tumors expressing high levels of CXCL12 (*C*; ∗, $p = 0.007$).

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OVA cells it can serve as a bidirectional cue for T cells, attracting at low concentration and repelling at high concentration.

High but not low levels of CXCL12 reduce recruitment of adoptively transferred OVA-specific CTL into tumors

It has been shown previously that OT-1 $CD8^+$ cells can be efficiently and specifically recruited into tumors as early as 12 h after adoptive transfer into C57BL/6 mice bearing OVA-expressing B16 tumors (35). In this study, mice were implanted s.c. with control B16OVA.MSCV (right flank) and B16OVA.CXCL12-high cells (left flank) so that each animal served as its own control. Serial MR images were taken of the animals when tumors reached 10–15 mm in one diameter. Animals bearing tumors similar in size were imaged at 24 and 48 h after adoptive transfer of CLIO-tat-labeled OT-1 cells.

Lymphocyte infiltration of tumors, as evidenced by quantitation of signal reduction, was consistently reduced in B16/OVA.CXCL12-high as compared with B16/OVA.MSCV tumors. In a representative axial slice (Fig. 4*A*) a clear, although heterogeneous, signal reduction could be visualized in the B16/OVA.MSCV tumor 24 h after injection of OT-1 $CD8^+$ T cells, whereas a small change in signal intensity was observed in the contralateral B16/OVA.CXCL12-high tumor, indicating that few T cells were recruited (T2 fit value ratio between the right and left tumor: 0.58). MRI at 48 h after CTL injection did not differ significantly from 24-h analysis for both B16/OVA.MSCV and B16/OVA.CXCL12-high tumors (data not shown). Tumors were harvested 48 h after adoptive transfer of labeled T cells, and sections were stained with H&E and anti-CD3 Ab (Fig. 4). $CD3^+$ T cell infiltration was clearly observed in B16/OVA.MSCV tumors (Fig. 4, *C* and *D*), whereas only rare $CD3^+$ T cells were found infiltrating the B16/OVA.CXCL12-high tumors in the same mouse (Fig. 4, *F* and *G*). Quantitative analysis of $CD3^+$ cells in immunohistochemical samples (Fig. 4*H*) showed a 3-fold difference in T cell infiltration in B16/OVA.MSCV (115.2 cells/$mm^2 \pm$ 35.5; mean ± SD) compared with B16/OVA.CXCL12-high tumors (38 cells/$mm^2 \pm$ 17; $p = 0.018$). $CD3^+$ T cells were very rarely seen infiltrating B16/OVA.MSCV and B16/OVA.CXCL12-high tumors from control nonimmunized mice that did not receive OT-1 T cells (5.9 ± 1.1 and 6.8 ± 0.9 cells/mm^2, respectively), and these levels of T cell infiltration were significantly different compared with those seen in mice adoptively transferred with OT-1 T cells ($p = 0.024$ and $p = 0.033$, respectively).

No evidence of intravascular accumulation of T cells was observed in B16/OVA.CXCL12-high or B16/OVA.MSCV tumors (data not shown).

To confirm that CXCL12 impairs the recruitment of OT-I $CD8^+$ T cells when expressed at high but not low levels, we evaluated the level of T cell infiltration in tumors expressing low levels of CXCL12. Mice bearing bilateral B16/OVA.MSCV and B16/

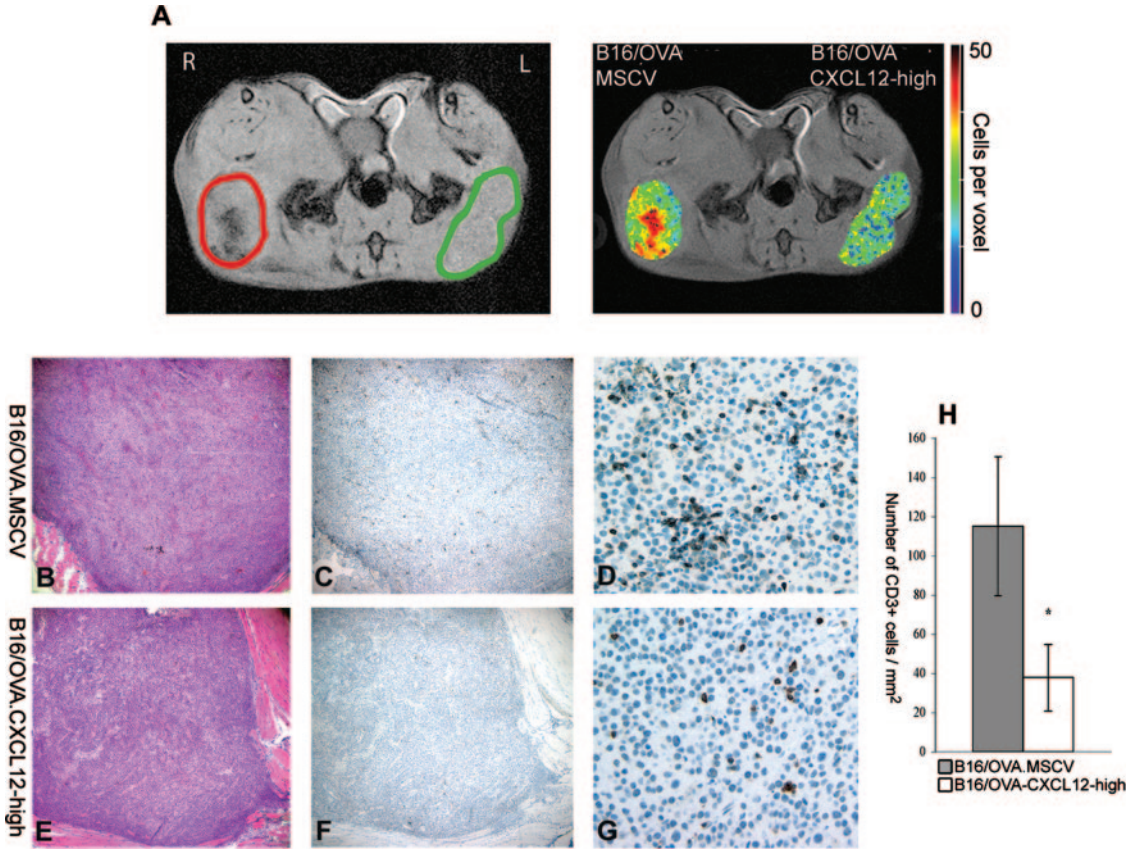

FIGURE 4. Early recruitment of CLIO-tat-labeled OT-1 $CD8^+$ T cells is impaired in B16/OVA. CXCL12-high tumors. Axial MRI slices through mouse thighs showed a significant and consistent signal reduction in B16/OVA.MSCV tumors compared with B16/OVA.CXCL12-high tumors, indicating that more labeled OT-1 T cells had been recruited into the B16/OVA.MSCV tumor. The intensity of T cell recruitment corresponding to dark areas (*A*, *left panel*) is also shown as a T2 spectral color map (*A*, *right panel*). Number of cells/voxel is indicated in the color scale. Immunohistochemical studies of T cell infiltration in B16/OVA tumors correlates with MR imaging. Tissues were collected 48 h after adoptive transfer and sections stained with H&E (*B* and *E*) or with polyclonal anti-CD3 Ab (*C*, *D*, *F*, and *G*). Numerous $CD3^+$ T cells were present within B16/OVA.MSCV tumors (*C* and *D*). In contrast, very few $CD3^+$ T cells could be detected in the B16/OVA.CXCL12 tumors (*F* and *G*). Results are representative of four independent experiments. Quantitation of $CD3^+$ cell infiltration into tumors was performed. The mean number (±SD) of $CD3^+$ cells per mm^2 of tumor from six animals is shown (*H*). Magnification, ×40 or ×200. ∗, $p = 0.018$.

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OVA.CXCL12-low or B16/OVA.MSCV and B16/OVA.CXCL12-high tumors were adoptively transferred with OT-I $CD8^+$ T cells, and T cell infiltration was quantitated 48 h later by immunohistochemistry. Quantitation of T cell infiltration showed that the expression of low levels of CXCL12 was associated with a higher number (164 ± 14 cells/mm^2) of T cells infiltrating B16/OVA.CXCL12-low tumors compared with control B16/OVA.MSCV tumors (101 ± 8.5 cells/mm^2) growing in the contralateral thigh (Fig. 5; $p = 0.032$). Therefore, recruitment of adoptively transferred activated tumor Ag-specific CTLs into B16/OVA tumors is significantly increased when CXCL12 is expressed at low level and reduced when CXCL12 is expressed at high level by the tumor.

Failure of T cells to infiltrate CXCL12-secreting tumors is CXCR4-mediated

In view of the fact that we proposed that the expression of high levels of CXCL12 had a direct chemorepellent effect on tumor-specific T cells, we studied the effect of CXCR4 blockade by AMD3100 on the early recruitment of OT-1 T cells (Fig. 6) (36). CLIO-tat-labeled OT-1 $CD8^+$ T cells were incubated with AMD3100 at 0.08 and 0.25 μg/ml and adoptively transferred into mice bearing bilateral tumors. T cell recruitment was evaluated by MRI and by immunohistochemistry. A clear MRI signal reduction was seen in the B16/OVA.MSCV tumor and not in the B16/OVA.CXCL12-high tumor, 24 h after adoptive transfer of OT-1 T cells untreated with AMD3100 (Fig. 6, *A* and *D*). In contrast, T cell recruitment was clearly visible in the B16/OVA.MSCV as well as in the B16/OVA.CXCL12-high tumor when OT-1 $CD8^+$ T cells were pretreated with AMD3100 (Fig. 6, *B* and *E*). Quantitation of the T2 fit value in the right and left tumors gave a ratio of 0.55 ± 0.13 (mean ± SD; $n = 3$) in the AMD3100-untreated setting compared with a ratio close to 1 (1 ± 0.12) when OT-1 T cells were pretreated with AMD3100 before adoptive transfer ($p = 0.01$). To rule out variability between mice, a second adoptive transfer with OT-1 T cells pretreated with AMD3100 was performed in the same group of mice who received AMD3100-untreated OT-1 T cells the day before (Fig. 6). Under these conditions T cells again infiltrated both tumors, confirming that CXCR4 antagonism restores T cell recruitment into tumors expressing high levels of CXCL12 (Fig. 6, *C* and *F*).

Quantitation of T cell infiltration by immunohistochemistry showed that incubation of OT-1 $CD8^+$ T cells with 0.08 μg/ml or 0.25 μg/ml AMD3100 before adoptive transfer resulted in a highly significant increase in $CD3^+$ T cell infiltration into B16/OVA.CXCL12-high tumors (101 ± 10.6 and 88.1 ± 16.9 cells/mm^2; $p = 0.009$ and $p = 0.011$), compared with 40 ± 4.1 cells/mm^2 in AMD3100-untreated B16/OVA.CXCL12-high tumors (Fig. 6, *M*, *N*, and *O*, and *I*, *J*, and *O*, respectively). In the same groups of mice, the number of T cells infiltrating B16/OVA.MSCV tumors (Fig. 6, *K*, *L*, and *O*) was not significantly changed (109 ± 3.2 and 93 ± 14.8) compared with the AMD3100-untreated B16/OVA.MSCV controls (130 ± 13.9 cells/mm^2; $p = 0.098$) (Fig. 6, *G*, *H*, and *O*).

To further support a bidirectional role of CXCR12 on T cell trafficking in and out of tumors, we examined the level of T cell infiltration in B16/OVA.CXCL12-low tumors in the context of the specific CXCR4 antagonist, AMD3100. As expected, a higher level of T cell infiltration was observed in B16/OVA.CXCL12-low compared with control B16/OVA.MSCV tumors in the absence of AMD3100 (162 ± 22 and 126 ± 16, respectively; $p = 0.0058$). This higher level of T cell infiltration into B16/OVA.CXCL12-low tumors was not observed when T cells were preincubated with AMD3100 at 0.25 μg/ml (Fig. 6*P*). In fact, no significant difference in the number of recruited T cells was observed in B16/OVA.SDF-1-low and B16/OVA.MSCV tumors treated with AMD3100 (B16/OVA.CXCL12-low 122 ± 21; B16/OVA.MSCV 126 ± 19; $p = 0.072$). These data confirm the finding that the primary effect of tumor secretion of CXCL12 is on T cell infiltration into the tumor, and that a chemoattractant or chemorepellent effect of the chemokine was abrogated by pretreatment of the tumor Ag-specific T cells with a CXCR4 antagonist.

Secretion of high levels of CXCL12 by tumor cells impairs infiltration and immune control by Ag-specific memory T cells

Having shown that early recruitment of tumor-specific effector T cells is impaired when CXCL12 is present at high levels in the tumor microenvironment, we explored whether expression of the chemokine had an effect on the long-term control of CXCL12-expressing tumors. Therapeutic efficacy of adoptively transferred tumor Ag-specific T cells is dependent, in part, on the ability of donor cells to persist as long-term memory T cells (40). It has also been shown that the response of T cells to CXCL12 can vary between naive, effector and memory $CD8^+$ cells, and that this may be due to the fact that effector $CD8^+$ T cells express higher levels of CXCR4 than memory T cells (41). Flow analysis of OT-1 $CD8^+$ cells after 5 days of in vitro stimulation and expansion showed that 67.2 ± 9.8% (mean ± SEM) of effector cells express CXCR4 compared with 15.5 ± 1.5% of naive OT-1 cells (data not shown). We therefore examined the role of high levels of CXCL12 in a model where OT-1 $CD8^+$ memory cells with potent antitumor activity are established after adoptive transfer (37). Twenty-one days following adoptive transfer of 1×10^7 OT-1 $CD8^+$ T cells, two groups of mice were challenged s.c. with 2×10^5 B16/OVA.MSCV or B16/OVA.CXCL12-high cells. As in the vaccination protocol, adoptively transferred mice initially controlled B16/OVA.MSCV as well as B16/OVA.CXCL12-high tumors, compared with control mice that did not receive adoptive transfer (Fig. 7, *A–D*). However, 20% of adoptively transferred mice ultimately developed B16/OVA.MSCV tumors by day 60 from challenge, compared with 60% of mice challenged with B16/

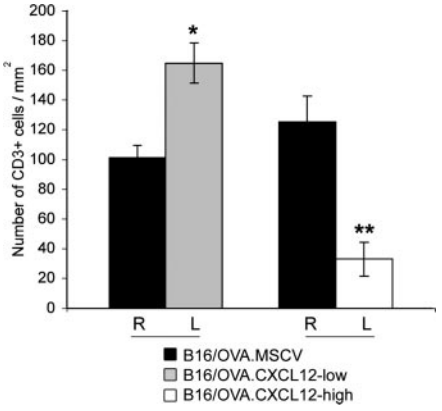

FIGURE 5. The recruitment of adoptively transferred OT-1 $CD8^+$ T cells is dependent on the level of CXCL12 in the tumor microenvironment. Two groups of mice ($n = 3$) were challenged with B16/OVA.MSCV cells in the right (R) flank and with B16/OVA.CXCL12-low or CXCL12-high cells in the left (L) flank. After 48 h from adoptive transfer of OT-1 $CD8^+$ T cells, tumors were collected and sections stained with polyclonal anti-CD3 Ab. A significantly higher number of tumor-infiltrating $CD3^+$ cells was found in B16/OVA.CXCL12-low compared with B16/OVA.MSCV tumors. As expected, mice bearing B16/OVA.MSCV and B16/OVA.CXCL12-high tumors showed a T cell infiltrate in the B16/OVA.MSCV but not in the B16/OVA.CXCL12-high tumor. The mean number (±SD) of $CD3^+$ cells per mm^2 from three animals is shown. *, $p = 0.032$; **, $p = 0.005$.

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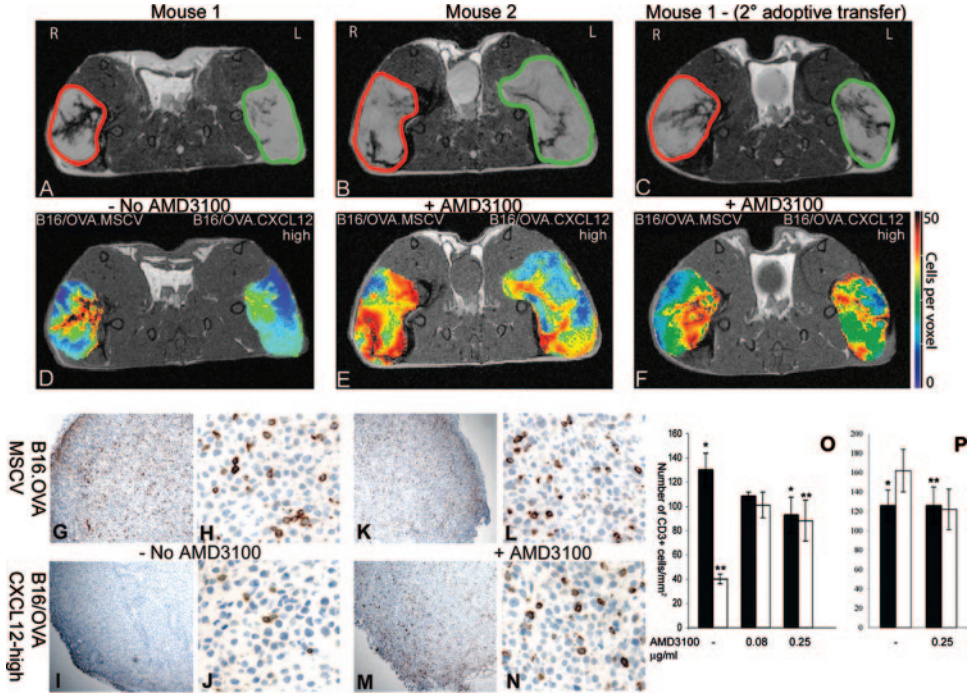

FIGURE 6. Blockade of CXCR4 by AMD3100 restores T cell infiltration B16/OVA.CXCL12-high tumors. CLIO-tat-labeled-activated OT-1 $CD8^+$ T cells were incubated with PBS (*A* and *D*) or AMD3100 (0.08 and 0.25 μg/ml) (*B* and *E* and *C* and *F*) before adoptive transfer into mice bearing bilateral B16/OVA.MSCV and B16/OVA.CXCL12-high tumors. Axial MRI shown in this figure was performed 24 h after adoptive transfer. *C* and *F* show MR images of a representative mouse (no. 1), which initially received T cells untreated with AMD3100 and then subsequently received a second adoptive transfer with AMD3100-treated OT-1 $CD8^+$ T cells 24 h from the first one. MRI images were obtained again at 24 h after this second adoptive transfer. Significant T2 reduction was observed in B16/OVA.MSCV as compared with B16/OVA.CXCL12-high tumors (*D*; low T2 fit value ratio). Equivalent T2 reduction was seen when OT-1 T cells were preincubated with AMD3100 with equivalent infiltration of T cells into B16/OVA.MSCV and B16/OVA.CXCL12-high tumors (*E*; T2 fit value close to 1). As expected, a second adoptive transfer of AMD3100-pretreated OT-1 $CD8^+$ T cells performed to mouse 1 showed a bilateral T2 signal reduction (*F*; T2 fit value close to 1). Tissues were collected 48 h after adoptive transfer and stained with anti-CD3 Ab (*G–J*, AMD3100 untreated; and *K–N*, AMD3100 treated). The mean number (±SD) of $CD3^+$ cells per mm^2 from three animals per group after adoptive transfer of OT-1 T cells pretreated with AMD3100 at 0.08 and 0.25 ng/ml concentration is shown (*O*). Magnification, ×40 and ×100. *, $p = 0.098$; **, $p = 0.011$. The recruitment of OT-1 T cells following CXCR4 blockade by AMD3100 was also evaluated in the context of tumors expressing low CXCL12 levels. B16/OVA.MSCV (▨) or B16/OVA.CXCL12-low (□) tumors were quantitated for $CD3^+$ T cell infiltration (*P*). The mean number (±SD) of $CD3^+$ cells per mm^2 from two animals per group is shown. *, $p = 0.0058$: B16/OVA.MSCV vs B16/OVA.CXCL12-low without AMD3100; **, $p = 0.072$: B16/OVA.MSCV vs B16/OVA.CXCL12-low with AMD3100.

OVA.CXCL12-high tumors ($p = 0.036$; Fig. 7, *B*, *D*, and *E*). Intratumoral OT-1 $CD8^+$ T cells from mice that developed tumors were recovered and analyzed by FACS. The total fraction of intratumoral OT-1 $CD8^+$ T cells was 7-fold lower in the B16/OVA.CXCL12-high tumor (Fig. 7*I*) compared with B16/OVA-MSCV tumor (Fig. 7*G*) (0.22% ± 0.4 vs 1.7 ± 0.7% (mean ± SD), respectively). When calculating the total number of $CD8^+$ T cells expressing the transgenic Vα2-Vβ5.1/5.2 TCR, a 4.5-fold reduction was found in B16/OVA.CXCL12-high compared with B16/OVA.MSCV tumors (12 ± 5.4 vs $55.5 \pm 17.6 \times 10^4$ (mean ± SD), respectively; $p = 0.005$) (Fig. 7, *G*, *I*, and *J*). No significant differences were found in the fraction of transgenic CD8 T cells isolated from the draining LN of both groups ($p = 0.7$; Fig. 7*J*). These data support the view that long-term protection from adoptively transferred OT-1 T cells is overcome when growing tumors express chemorepellent concentrations of CXCL12.

CXCL12 expressed at high levels by tumor cells induces CTL chemorepulsion and impairs cytotoxicity in vitro

We hypothesized that high expression of CXCL12 by tumor cells protects them from CTL-mediated lysis in vitro as a result of repulsion of effector cells, mediated via the chemokine receptor, CXCR4. We tested this hypothesis in a standard ^{51}Cr release assay, using OT-1 CTLs recognizing H-$2K^b$ MHC I and $OVA_{257–264}$ peptide and in a recently developed modified assay, which takes account of the influence of effector cell migration on cytotoxicity (9). OVA-specific CTLs killed B16/OVA.MSCV cells as effectively as they killed B16/OVA-CXCL12-high cells when target cells are pelleted together in the standard ^{51}Cr release assay performed in round-bottom wells (Fig. 8*A*; $p = 0.16$), supporting the view that the production of CXCL12 by tumor cells did not influence the susceptibility of B16/OVA cells to CTL killing. In contrast, when killing activity was quantitated in flat-bottom wells in which the linear density of cells could be decreased and therefore the distance between effector and target cell increased, Ag-specific CTLs were significantly less effective at killing B16/OVA cells expressing CXCL12-high compared with B16/OVA.MSCV cells (Fig. 8*B*; $p = 0.0004$). Preincubation of OT-1 $CD8^+$ T cells with the CXCR4 antagonist AMD3100 resulted in a significant increase in the killing of B16/OVA.CXCL12-high cells compared with the lytic activity observed when CXCR4 of effectors was not blocked ($p = 0.001$; Fig. 8*C*).

Because we found that low levels of CXCL12 enhance the in vivo recruitment of adoptively transferred OVA-specific CTL into tumors, we aimed to evaluate whether the bidirectional effect on T cell migration was maintained in the cytotoxicity

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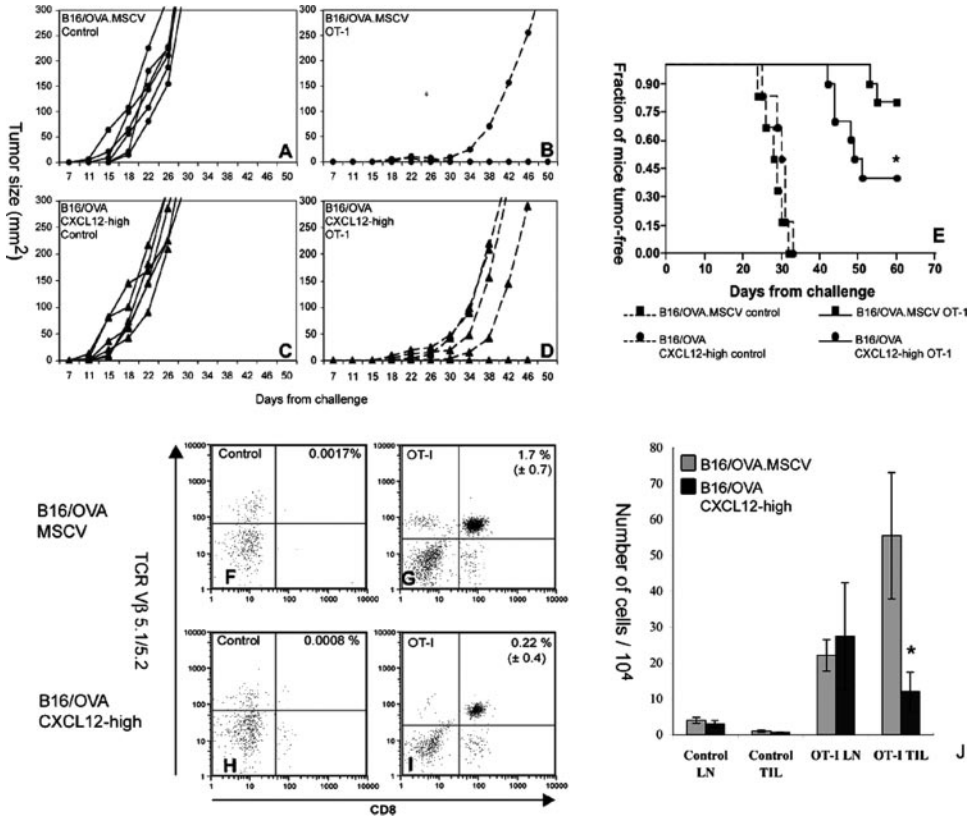

FIGURE 7. Potent antitumor activity of persistent adoptively transferred OT-1 CD8$^+$ cells is overcome by B16/OVA.CXCL12-high tumors. Groups of six mice were challenged with B16/OVA.MSCV (*A* and *B*) or B16/OVA.CXCL12-high (*C* and *D*) cells 21 days after i.v. adoptive transfer of 1×10^7 OT-1 CD8$^+$ T cells. Control mice were challenged 21 days after i.v. HBSS alone (*A* and *C*). Tumor growth in one representative experiment with six individual mice (*A–D*) and the cumulative survival analysis from two independent experiments (*E*) is shown. Sixty percent of B16/OVA.CXCL12-high tumor-bearing mice (*D* and *E*) developed tumors compared with 20% of mice that were challenged with B16/OVA.MSCV cells (*B* and *E*). *, $p = 0.036$. OT-1 CD8$^+$ T cells were recovered from tumors and LN and analyzed by FACS (*F–J*). Each dot plot shows the result of the CD8/Vβ-5-1 staining of cells gated on low SSC. Each dot plot reports the mean fraction (±SD) of CD8$^+$ cells expressing Vβ5.1/5.2 and Vα2 TCR in the total number of B16/OVA.MSCV or B16/OVA.SDF.1-high tumor cells analyzed from control (*F* and *H*) and adoptively transferred mice (*G* and *I*). The total number of Vβ5.1/5.2 and Vα2 CD8$^+$ cells in each tumor or in two draining LN was calculated from the percentage reported in each dot plot and the total number of cells ($\times 10^4$) recovered from 100 mg of tumor (*J*). All mice had OT-1 CD8$^+$ T cells in LN without any significant difference between groups bearing B16/OVA.MSCV or B16/OVA.CXCL12-high tumors ($p = 0.7$). There was a significant 4.5-fold reduction in the total number of OT-1 CTL in CXCL12-expressing tumors compared with B16/OVA.MSCV tumors (*, $p = 0.005$). Results are expressed as the mean ± SD from three or four mice per group from two independent experiments.

assay. Consistent with in vivo findings, B16/OVA.CXCL12-low cells were more efficiently killed by CTL compared with control B16/OVA.MSCV cells ($p = 0.019$; Fig. 8*D*), supporting a bidirectional role of the chemokine in CTL migration and killing activity. As for the repulsive effect, AMD3100 pretreatment of CTLs was effective in reversing the killing activity to the baseline (Fig. 8*D*).

High-level secretion of CXCL12 by tumors cells therefore impairs the efficacy of CTL killing in an assay in which CTL migration plays a critical role. These data are consistent with our finding in vivo that blockade of the CXCR4 receptor by AMD3100 restores the ability of CTL to infiltrate and engage a tumor cell expressing CXCL12 at high level.

CXCL12 does not increase apoptosis or impair activation, proliferation, and killing activity of stimulated OT-1 CD8$^+$ T cells or induce cross desensitization to a CCR5 binding chemokine

To rule out a direct effect of CXCL12 on T cell function, we studied the effect of CXCL12 on apoptosis, activation, and proliferation of effector OT-1 CD8$^+$ cells (Table II). The level of T cell apoptosis after 72-h incubation with different concentrations of recombinant CXCL12 did not differ from the level of apoptosis in the absence of CXCL12 (22.9 ± 0.8 and 21.3 ± 1.6%, in the presence of CXCL12 at 1000 or 0 ng/ml, respectively; $p = 0.22$). CXCL12 (1000 ng/ml) increased the surface expression of CD69 on OT-1 CD8$^+$ cells compared with untreated controls (89.3 ± 1.74 vs 79.4 ± 0.3.1% ($p = 0.031$), respectively; Table II). CXCL12 also increased the expression of CD25 in comparison to untreated controls (82.5 ± 1.08 vs 71.9 ± 1.7; $p = 0.011$; Table II). CXCL12 did not affect the fraction of dividing OT-1 CD8$^+$ T cells as analyzed by CFSE compared with untreated controls (95.2 ± 2.6 and 95.9 ± 1.9%, respectively, in presence and absence of CXCL12; $p = 0.13$).

We also tested whether the ability of OT-I CD8$^+$ T cells to kill B16/OVA cells was affected by the presence of CXCL12 (Table II). This question was addressed by exposing proliferating OT-I CD8$^+$ T cells in vitro to soluble CXCL12 or to CXCL12 produced by B16/OVA.CXCL12-high cells growing in the upper chamber of a Transwell system. OT-I CD8$^+$ T cells cocultured in the presence of CXCL12-high produced by melanoma cells showed the same level of killing activity against B16/OVA.MSCV or B16/OVA.CXCL12-high target cells compared with the killing activity of T cells that were exposed to B16/OVA.MSCV cells that were not producing CXCL12 ($p = 0.75$ and $p = 0.31$, respectively).

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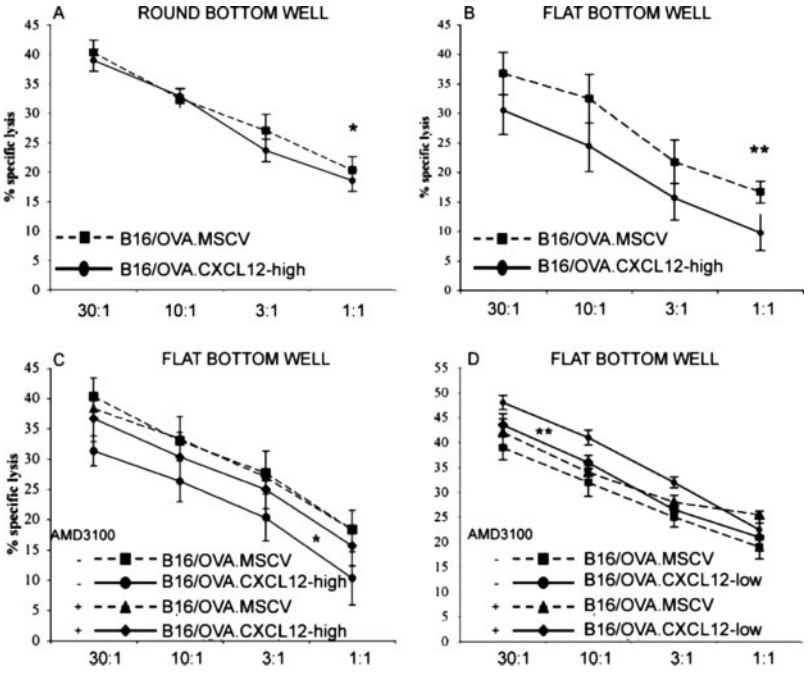

FIGURE 8. The killing activity of tumor-specific CTL against B16/OVA.CXCL12-high cells is reduced in a migration-dependent ^{51}Cr release assay. Quantitation of the cytotoxicity of OT-1 CD8$^+$ T cells against B16/OVA.MSCV (■) or B16/OVA.CXCL12-high (●) target cells was measured in a standard ^{51}Cr-release assay (*A*) or in a previously described modified assay performed in flat-bottom wells (*B*). *, $p = 0.16$; **, $p = 0.0004$. Results of six independent experiments are shown. Cytotoxicity of OT-1 CD8$^+$ T cells pretreated with the specific CXCR4 antagonist, AMD3100 (1 μg/ml) or untreated and incubated with B16/OVA.MSCV or B16/OVA.CXCL12-high (*C*) and B16/OVA.CXCL12-low (*D*) was also assessed in ^{51}Cr-release assays in flat-bottom wells. Mean values ± SEM from two or three independent experiments are shown. *, $p = 0.001$; **, $p = 0.019$.

Similar results were observed when incubating OT-I T cells with recombinant CXCL12 (data not shown). Therefore, the activation, proliferation, and functional antitumor activity of tumor-specific T cells were not impaired by high levels of CXCL12.

Finally, we explored the possibility that high levels of CXCL12 might interfere with the migratory responses of CD8$^+$ T cells to chemokines other than CXCL12 that are thought to be involved in T cell infiltration into tumors via the mechanism of heterologous receptor desensitization. Expression of the chemokine CCL3 within melanomas has been shown to increase the effector T cell infiltrate (42). First, we evaluated the migratory responses of T cells to the CCR5 binding chemokine, CCL3, in transmigration assays after pretreatment of cells with high concentrations of CXCL12. The percentage of input CD8$^+$ T cells migrating chemotactically to CCL3 at concentrations of 10 and 100 ng/ml were 22 ± 3.8 and 9 ± 1.1%, respectively. T cell chemotaxis to CCL3 at peak concentrations of 10 and 100 ng/ml was not significantly effected by preincubation with CXCL12 (1 μg/ml) (18 ± 1.6 ($p = 0.221$) and 11 ± 1.3 ($p = 0.118$), respectively). This finding was confirmed when we examined whether pre-exposure of CD8$^+$ T cells to chemorepellent concentrations of CXCL12 (1 μg/ml) reduced CCR5 expression on these cells. The fraction of T cells expressing CCR5 after exposure to CXCL12 at concentrations of 100 ng/ml and 1 μg/ml was 67 ± 10.8 and 72 ± 9.5% (MFI, 81 ± 8.6 and 60 ± 12.9), respectively. These values were not significantly different from the level of CCR5 expression in T cells not exposed to CXCL12 (68 ± 8.4%; MFI, 72 ± 13.8; $p = 0.581$ and $p = 0.492$, in comparison to exposure at CXCL12 100 ng/ml and 1 μg/ml, respectively). These data support the view that chemorepellent concentrations of CXCL12 do not induce heterologous desensitization of CCR5 or inhibit chemotactic responses of T cells to CCL3 in vitro and confirm the data of Honczarenko et al. (43).

Table II. *OT-1 and CD8$^+$ T cell activation, apoptosis, and killing activity in the context of CXCL12 or supernatants from B16/OVA.MSCV or B16/OVA.CXCL12-high cells*

	rCXCL 12 (ng/ml)		
	0	500	1000
CD25	71.9 ± 1.77	77.4 ± 0.65	82.5 ± 1.08
CD69	79.4 ± 3.1	85.2 ± 1.7	89.3 ± 1.74
CFSE (% Dividing cells)	95.9 ± 1.9	95.7 ± 1.27	95.2 ± 2.6

	rCXCL12 (ng/ml)			
	0	10	100	1000
% Apoptosis	21.3 ± 1.69	18.5 ± 0.7	21 ± 0.98	22.9 ± 0.84

	Activation in Presence of B16/OVA MSCV	Activation in Presence of B16/OVA CXCL12-high
Killing activity against B16/OVA.MSCV	32.8 ± 3.64	31.1 ± 3.5
Killing activity against B16/OVA.CXCL12-high	30.8 ± 0.9	30.5 ± 3.8

[a] OT-1 activation was assessed in proliferating cells by measurement of CD25 and CD69 expression and the percentage of cells undergoing division using CFSE. The fraction of apoptotic cells (propidium iodide negative and annexin V positive) was also determined in the presence or absence of CXCL12. Killing activity of OT-1 CD8$^+$ T cells to B16/OVA.MSCV cells or B16/OVA.CXCL12-high cells (expressed as lytic unit 30 (LU30) × 10^6 was determined after exposure of these CD8$^+$ T cells to supernatants generated from B16/OVA.MSCV cells or B16/OVA.CXCL12-high cells in a Transwell system.

Discussion

This study demonstrates that expression of high levels of CXCL12 by engineered B16 melanoma cells abrogates infiltration of the tumor by Ag-specific T cells and thereby allows the tumor to escape immune control. Furthermore, our data set supports the proposal that this occurs as a direct result of CXCL12-induced and CXCR4-mediated chemorepulsion of T cells, previously termed fugetaxis (3, 5). These data are consistent with previous demonstrations that recombinant CXCL12 and CXCR4 binding HIV-1 envelope protein, gp120, can repel Ag-specific T cells in vivo (3, 9). The direct effect of CXCL12 on T cell chemorepulsion was further substantiated in experiments in which tumor cell lysis in

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vitro and T cell infiltration into the tumor in vivo was restored when T cells were pretreated with the CXCR4 antagonist, AMD3100. We propose that CXCL12 exerts its chemorepellent action on T cells within the tumor microenvironment in view of the fact that melanomas are vascularized and because of the chemokine's high-affinity binding to matrix proteins and its inactivation in the blood stream (16, 44). In this study we also show that CTL killing of tumor cells secreting high levels of CXCL12 is impaired in an assay in which effector cell migration is critical for target cell killing (16, 44).

CXCL12 is known to regulate the local immune response and is a potent chemoattractant for T cells, pre-B lymphocytes, and dendritic cells (1, 45). Melanoma cells producing low levels of CXCL12 have been shown to attract CTLs in organotypic culture (46). This is consistent with the expected up-regulation of an immune response against tumor cells expressing CXCL12 in the low nanomolar range. Our data support the view that CXCL12 has a bimodal effect on T cell migration attracting T cells at low concentrations (up to 10 nM) and repelling at higher concentrations of the chemokine (above 10 and up to100 nM). The immune response to tumor cells, which have been engineered by other groups to express CXCL12, have also revealed a dose-dependent effect of the chemokine on tumor immunity. Dunussi-Joannopoulos et al. (19) demonstrated that low expression of CXCL12 (2 ng/ml) secreted at the tumor site by genetically modified B16.F1 melanoma cells resulted in delayed tumor growth and supported the development of long-lived tumor-specific CTL responses. However, therapeutic immunity against tumors was not observed when a high number ($>2 \times 10^5$) of irradiated tumor cells expressing CXCL12 was used in the vaccination protocol (19). Similarly, immunogenic MethA fibrosarcoma and HM-1 ovarian carcinoma secreting high levels of CXCL12 (90 and 55 ng/ml, respectively) were able to induce a significant immune response only when tumors were engineered to coexpress IL-2 or GM-CSF (17). Shrikant et al. (38) recently reported that Ag-specific T cells adoptively transferred into mice are able to migrate to the site of tumor challenge, where they proliferate and exert lytic activity only for a short time, but that they eventually fail to control tumor growth due to migration away from the tumor bulk.

Taken together, these results may also explain why vaccinated and adoptively transferred mice in our study were unable to reject B16 tumors in our model despite initial control of tumor growth. In addition, a bimodal effect of CXCL12 on tumor-specific T cell migration is also consistent with the recent immunotherapeutic paradigm that predicts that early tumors can be rejected by the transfer of tumor-specific T cells, whereas late tumors are resistant to immune control due to reduced infiltration of tumor-specific T cells into the tumor itself (33, 47). We propose a refinement of this paradigm to include the proposition that certain growing tumors initially generate CXCL12 at a level that induces T cell chemoattraction but ultimately establish an immune privileged site through the chemorepellent activity of high levels of CXCL12 on tumor-specific T cells. Our in vitro data, which are consistent with previously published studies (9), demonstrate that T cell chemorepulsion is active at the cell-to-cell level and impairs CTL-mediated lysis of tumor cells, which requires that the effector makes direct contact with the target cell.

Our data also support the idea that CXCL12 is only one of several chemokines cooperating in the migration and recruitment of immune cells. In this regard, we observed substantial T cell infiltration into control B16/OVA tumors not expressing CXCL12. The fact that we observed increased T cell infiltration in the presence of low levels of CXCL12 is consistent with the previous demonstration that expression of combinations of intratumoral chemokines may stimulate proliferation of $CD8^+$ T cells and to attract T cells more efficiently than any single chemokine alone (48).

It is possible that high concentrations of CXCL12 exert an effect on $CD8^+$ CTL function via mechanisms other than those involving its direct effect on cell migration, which would include the induction of apoptosis, TCR-mediated signaling, or adhesion (28–30, 49, 50). Although we cannot exclude that these mechanisms play a role in vivo, chemorepellent concentrations of CXCL12 did not affect the level of T cell apoptosis, activation, proliferation, and function of OT-1 T cells in vitro. These findings were consistent with previously published data (29, 30). The possibility that high levels of CXCL12 may abolish T cell infiltration as a result of the concomitant down-regulation of chemokine receptors other than CXCR4 was not supported by our data, which demonstrated that migratory responses to CCL3 and CCR5 expression were not inhibited or down-regulated, respectively, in the context of preincubation with high concentrations of CXCL12.

CXCL12 has been shown to promote the adhesion of lymphocytes to endothelial cells via the induction of the ICAM-1 (51). However, it has also been demonstrated that CXCR4 is not absolutely required for adhesion, rolling, and accumulation of T cells at a specific site (52, 53). The CXCL12/CXCR4 axis has also been shown to regulate cell growth via the activation of the ERK1/2 and Akt pathways and the stimulation of DNA synthesis through an autocrine/paracrine mechanism (13, 49). In this study, secretion of high levels of CXCL12 by essentially CXCR4-negative B16 melanomas or treatment of naive mice with AMD3100 did not affect B16/OVA tumor cell proliferation in vitro or tumor growth in vivo.

Our data support a dominant effect of CXCL12-induced chemorepulsion or fugetaxis on T cell recruitment, as demonstrated by the increase of T cell infiltration following blockade of CXCR4 by AMD3100. Furthermore, the finding that a small fraction of tumor-specific T cells are capable of infiltrating the tumor despite CXCL12 is consistent with the fact that ~70% of OT-1 T cells express CXCR4. Finally, the concomitant challenge of mice with both control and CXCL12-expressing tumors allows us to rule out any functional defect of tumor-specific T cells. Our data from combined MRI and immunohistochemical analysis supports the view that local dysregulation of the migration of tumor-specific T cells results in the failure of the immune system to control the growth of CXCL12-expressing tumor and not an effect of CXCL12 on T cell apoptosis, TCR-mediated signaling, or adhesion.

This study examined tumor cells that were engineered to express high levels of CXCL12. Primary human melanoma, ovarian and prostate carcinoma, and glioblastoma have been shown to constitutively express high levels of chemokines including CXCL12 and IL-8 (13, 15, 49, 54). Mellado et al. (49) reported that primary human melanomas produce up to 25.6 ng/ml (per 1×10^6 cells) CXCL12 in vitro, a level equivalent to that expressed by the B16/OVA.CXCL12-high cells in this study. Whether this level of CXCL12 may impair a tumor-specific immune response against a primary melanoma by inducing tumor-specific T cell chemorepulsion is currently under investigation.

In conclusion, these findings extend the range of the biological activities of CXCL12 in vivo and substantiate the action of this chemokine as a T cell chemorepellent or fugetaxin. The action of CXCL12 as a chemorepellent in vivo may have implications for immune evasion by certain tumors that elaborate high concentrations of CXCL12 including melanoma and ovarian cancer. Overcoming this potential mechanism, including the selective antagonism of chemokine/receptor interactions, using an anti-fugetaxin may ultimately prove to be a useful and novel adjunctive therapy to augment native tumor immunity and the efficacy of cancer vaccines and other anticancer immunotherapeutic approaches.

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Disclosures

The authors have no financial conflict of interest.

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