Radiation-Induced CXCL16 Release by Breast Cancer Cells Attracts Effector T Cells

Satoko Matsumura, Baomei Wang, Noriko Kawashima, Steve Braunstein, Michelle Badura, Thomas O. Cameron, James S. Babb, Robert J. Schneider, Silvia C. Formenti, Michael L. Dustin and Sandra Demaria

*J Immunol* 2008; 181:3099-3107; doi: 10.4049/jimmunol.181.5.3099
http://www.jimmunol.org/content/181/5/3099

**References**
This article cites 57 articles, 31 of which you can access for free at:
http://www.jimmunol.org/content/181/5/3099.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Radiation-Induced CXCL16 Release by Breast Cancer Cells Attracts Effector T Cells

Satoko Matsumura,* Baomei Wang,2* Noriko Kawashima,* Steve Braunstein,† Michelle Badura,‡ Thomas O. Cameron,*¶ James S. Babb,‡ Robert J. Schneider,¶ Silvia C. Formenti,§ Michael L. Dustin,†¶ and Sandra Demaria3*

Recruitment of effector T cells to inflamed peripheral tissues is regulated by chemokines and their receptors, but the factors regulating recruitment to tumors remain largely undefined. Ionizing radiation (IR) therapy is a common treatment modality for breast and other cancers. Used as a cytocidal agent for proliferating cancer cells, IR in combination with immunotherapy has been shown to promote immune-mediated tumor destruction in preclinical studies. In this study we demonstrate that IR markedly enhanced the secretion by mouse and human breast cancer cells of CXCL16, a chemokine that binds to CXCR6 on Th1 and activated CD8 effector T cells, and plays an important role in their recruitment to sites of inflammation. Using a poorly immunogenic mouse model of breast cancer, we found that irradiation increased the migration of CD8+CXCR6+ activated T cells to tumors in vitro and in vivo. CXCR6-deficient mice showed reduced infiltration of tumors by activated CD8 T cells and impaired tumor regression following treatment with local IR to the tumor and Abs blocking the negative regulator of T cell activation, CTLA-4. These results provide the first evidence that IR can induce the secretion by cancer cells of proinflammatory chemotactic factors that recruit antitumor effector T cells. The ability of IR to convert tumors into “inflamed” peripheral tissues could be exploited to overcome obstacles at the effector phase of the antitumor immune response and improve the therapeutic efficacy of immunotherapy. The Journal of Immunology, 2008, 181: 3099–3107.

Although in IT the therapeutic agent is a cell (i.e., an antitumor T cell), the requirements for IT to be successful are the same as those for other antitumor agents: the agent must be effective in the microenvironment of the tumor and it must reach the target cells in optimal quantities (4). The inability to meet one or both of these requirements is often responsible for the ineffective destruction of established vascularized solid tumors by tumor-specific CTL (5–8).

Chemokines and their receptors play a crucial role in T cell recruitment to different tissues and regulate both homeostatic and inflammation-dependent homing of T cells (9). The chemokine receptor(s) involved in recruiting effector T cells to a specific site are recognized as stimulus- and organ-dependent, but little is known about the receptors involved in the recruitment of effector T cells to tumors (3). The chemokine receptor CXCR6 is expressed at very low levels on naive T cells and is up-regulated upon activation under Th1-polarizing conditions (10). Importantly, CXCR6 is expressed at high levels by CD8 T cells with cytotoxic function (11, 12) and has been implicated in the recruitment of these cells to inflamed tissues (13–15).

Cancer cells produce several chemokines, largely to recruit leukocytes that promote tolerance and immune escape and to aid tumor growth by enhanced angiogenesis (16, 17). However, tumor cells engineered to express proinflammatory chemokines such as IFN-γ-inducible protein 10 (IP-10)/CXCL10 and RANTES/CCL5 have been shown to recruit lymphocytes that can reject the tumors (18, 19).

Evidence is accumulating that ionizing radiation (IR) therapy, a treatment modality routinely used to kill cancer cells, can modulate the expression of several receptors and cytokines by cancer cells and tumor stroma, resulting in modifications of the tumor microenvironment that can be exploited to enhance the effects of IT (reviewed in Refs. 20 and 21). Some of the IR-induced modifications appear to facilitate T cell recruitment to tumors, in part, by
promoting normalization of the vasculature and/or by up-regulating the expression of endothelial adhesion molecules (22–24). However, specific mechanisms by which IR regulates trafficking of T cells to solid tumors remain largely undefined.

We have used the 4T1 preclinical model of metastatic breast cancer to test the therapeutic potential of local IR combined with IT (25). When injected into syngeneic mice, 4T1 cells form highly angiogenic, metastatic, and poorly immunogenic tumors, hence recapitulating many of the characteristics of aggressive human breast cancer (26–28). Treatment of mice with established (day 13) 4T1 tumors by using local IR and CTLA-4 blockade in combination but not as single modalities induced CD8-mediated antitumor responses, inhibiting metastases and inducing regression of the primary irradiated tumors (25). In this study we show that most of the CD8 T cells infiltrating 4T1 tumors following the combination treatment were CXCR6⁺. Tumor irradiation increased the recruitment of tumor-specific activated CD8 cells and markedly enhanced the expression and release of the CXCR6 ligand, the proinflammatory chemokine CXCL16, by 4T1 cells. CXCR6-deficient mice showed reduced treatment-induced CD8 cell infiltration in tumors and reduced tumor inhibition. Taken together, these data indicate that IR has the ability to induce chemokines involved in the recruitment of effector T cells, effectively converting tumors into “inflamed” peripheral tissues that are rendered susceptible to the effector phase of the antitumor immune response.

Materials and Methods

Mice

Six- to 8-wk-old BALB/c mice were purchased from Taconic Animal Laboratory. CXCR6⁻/⁻ and CXCR6⁻/-/hph mice have been described (11), and were provided in the BALB/c background by D. Littman (New York University School of Medicine, New York, NY). BALB/c CL4-TCR mice transgenic for an influenza virus influenza hemagglutinin (HA) H2-Kd-restricted epitope (29) were purchased from The Jackson Laboratory. All experiments were approved by the Institutional Animal Care and Use Committee of New York University.

Cells and Abs

The 4T1, 4T07, 66c14, 168FARN, and 67NR BALB/c mouse-derived mammary carcinoma cell lines (26) and 4T1-HA derivatives (30), were grown in DMEM (Invitrogen) supplemented with 2 mM l-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 × 10⁻⁵ M 2-ME, and 10% FBS (Gemini Bio-Products) (complete medium). The human cell lines MCF10A, CRL2324, CRL1902, and HTB-20 were obtained from the American Type Culture Collection (ATCC) and were cultured as recommended by ATCC. Mouse Baf-3 cells were transduced with the control vector MSCV-IRE-EGFP or with MSCV-CXCR6-IRE-GFP expressing mouse CXCR6, selected by sorting for GFP⁺ cells, and cultured in the presence of 10 ng/ml IL-3.

Anti-CTLA-4 hamster mAb 9H10 was purified as previously described (31). The CXCL16-Fc fusion protein (10) was a gift of M. Matloubian (University of California, San Francisco, CA). Goat anti-mouse CXCL16 was stained at 4°C with various mAbs and analyzed using a FACScan flow cytometer and FlowJo version 6.4.4 software (Tree Star).

Adoptive transfer

WT mice bearing 4T1-HA tumors of ~5-mm average diameter were injected i.v. with 20 × 10⁵ CFSE⁺ labels, in vitro activated CL4 CD8 T cells or 4T1 CL4 CD8 T cells that expressed the congeneric marker Thy.1.1. After 24 h the number of adoptively transferred T cells infiltrating the spleen and tumors was determined as described (33). Briefly, to obtain sufficient cell numbers for analysis, tumors from four mice per group were pooled and digested with collagenase D as described above. Obtained cell suspensions were stained with PE-Thy.1.1 and PE-Cy5-CD8 and analyzed by flow cytometry. An equal number of 5-μm polystyrene beads (Polysciences) were added to each sample before analysis to estimate the total number of CD8⁺ Thy.1.1⁺ cells.

Immunohistochemistry

4T1 tumors were harvested 48 h after IR or mock treatment, fixed for 1 h at 4°C in 4% paraformaldehyde followed by overnight incubation in 30% sucrose, and frozen in OCT medium. Seven-micrometer sections were incubated with 0.3% H₂O₂ to quench endogenous peroxidase activity and seeded in the bottom of a 24-well plate. The adherent tumor cells were washed twice with PBS and changed to 600 μl of chemotactic buffer and mouse rCXCL16 (Peprotech) or 1 × 10⁴ 4T1 cells untreated or irradiated with 12 Gy and preseeded in the bottom of a 24-well plate. The adherent tumor cells were

Tumor challenge and treatment

Mice were injected s.c. in the right flank with 5 × 10⁴ 4T1 cells in 0.1 ml of DMEM medium without additives on day 0. Perpendicular tumor diameters were measured with a vernier caliper, and tumor volumes were calculated as length × width² × 0.52. Treatment was started on day 13 when tumors reached the average diameter of 5 mm (~65 mm³ in volume). Radiation was administered to a field including the tumor with <5-mm margins using a radion source by two fractions of 12 Gy each on days 13 and 14 as previously described (25). Control hamster IgG and 9H10 were given i.p. at 200 μg at 1, 4, and 7 days after IR.

Analysis of tumor-infiltrating lymphocytes (TIL)

Tumors were dissected carefully by removing surrounding normal tissue, minced into ~1-mm pieces, and digested with collagenase D (400U/ml) for 25 min at 37°C in a shaker. Aliquots of 10⁶ tumor-derived cells were stained at 4°C with various mAbs and analyzed using a FACScan flow cytometer and FlowJo software (Tree Star).

RT-PCR and real-time PCR

Total RNA isolated from carcinoma cells with TRizol (Invitrogen) was subjected to RT-PCR using specific primers for mouse CXCL16 (forward: 5'-GCTTTGGAACCCCTTGTCTTTG-3'); reverse: 5'-GTGTCGTAGCTCTACTGATCTGTCG-3'). mouse CL4 (forward: 5'-GGGCGGCTGCACGAGTCTCT-3'); reverse: 5'-ATATGCAGCTGTTGTGTTGTTGAGCA-3'). Real-time PCR was performed using the SYBR Green quantitative RT-PCR kit (Sigma-Aldrich) and LightCycler (Roche). The following primers were used: mouse CXCL16 (forward: 5'-CCTTGGTCCTCCTGGCTCCTTC-3'); reverse: 5'-TCCAAATGTACCCCTGCGTATC-3'). mouse ADAM10 (forward: 5'-AGCAGAATCCTGCCGAAAAC-3'); reverse: 5'-TGCGCCAGATTCAACAAAACA-3'). mouse ADAM17 (forward: 5'-GTACGGTCACTGGCAAAACA-3'); reverse: 5'-GACGCAACCATGACGCAGCAAC-3'). (where ADAM is “a disintegrin and metalloproteinase”). The estimated amount of the gene of interest was normalized to the amount of EF1GII (eukaryotic translation initiation factor 4G II).
CXCL16 was measured in supernatants after 4 h by ELISA (RayBiotech). The medium was changed to DMEM containing 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% FBS. Some wells contained 200 ng/ml PMA and/or 20 µM BB94 (batimastat). Released CXCL16 was measured in supernatants after 4 h by ELISA (RayBiotech).

Soluble CXCL16 measurement

Mouse and human carcinoma cells were plated at 1 × 10⁴ cells/well in duplicate wells of a 96-well plate 48 h after radiation or mock treatment. The medium was changed to DMEM containing 2 mM l-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% FBS. Some wells contained 200 ng/ml PMA and/or 20 µM BB94 (batimastat). Released CXCL16 was measured in supernatants after 4 h by ELISA (RayBiotech).

CXCL16 knockdown

Lentiviral-mediated knockdown of murine CXCL16 was achieved by insertion of a 58-bp DNA duplex oligonucleotide containing a specific 21-bp target region directed against the 3'-untranslated region of the CXCL16 gene, via EcoRI-AgeI cloning sites, immediately downstream of a U6 DNA-polymerase III promoter in the pLKO.1.puro vector, which ultimately generates a short hairpin (sh) RNA specifically disrupting generation of the target protein (sh-CXCL16). A control vector with a 58-bp nonsilencing (NS) sequence (sh-NS) was also created. Knockdowns were produced by transfection of the 293GP packaging line with the specific pLKO.1 vector plus pCI-VSV-G and pCMVΔ8.2R to generate active viruses. 4T1 cells transduced with control (sh-NS-4T1) and silencing (sh-CXCL16-4T1) were selected in puromycin at 1.5 µg/ml.

Statistical methods

Tumor volume data were acquired longitudinally from WT and CXCR6<sup>gfp/gfp</sup> mice. Random coefficient regression was used to model the square root of tumor volume as a function of genotype, treatment, and elapsed time from treatment onset. The square root of volume was modeled because the temporal change in this measure was well approximated as linear, thereby allowing a straightforward assessment of the interaction between treatment and genotype in terms of their effects on tumor growth. The covariance structure was modeled by assuming observations to be correlated only when obtained from the same animal and by allowing the error variance to differ across animal groups. All reported p values are two-sided and were declared significant at the 5% level. Statistical computations were conducted using SAS for Windows, version 9.0 (SAS Institute).

Results

Local IR increases recruitment of tumor-specific activated CD8 T cells to the tumor

We have previously shown in the mouse 4T1 carcinoma model that local IR used in combination with CTLA-4 blockade promotes immune-mediated tumor destruction (25). To test whether radiation-induced changes in the tumor microenvironment may facilitate the recruitment of tumor-specific effector T cells, mice were injected with 4T1-HA cells expressing the reporter Ag HA. HA-specific CD8 T cells were obtained by in vitro activation of CD8 T cells to the tumor.

Results

Local IR increases recruitment of tumor-specific activated CD8 T cells to the tumor

We have previously shown in the mouse 4T1 carcinoma model that local IR used in combination with CTLA-4 blockade promotes immune-mediated tumor destruction (25). To test whether radiation-induced changes in the tumor microenvironment may facilitate the recruitment of tumor-specific effector T cells, mice were injected with 4T1-HA cells expressing the reporter Ag HA. HA-specific CD8 T cells were obtained by in vitro activation of CD8 T cells purified from the spleen of CL4 TCR transgenic mice. Following activation with plate-bound anti-CD3 and anti-CD28 mAb and culture in IL-2, the majority of these cells expressed the activation marker CD69 and the chemokine receptor CXCR6 as detected by staining with CXCL16-Fc fusion protein (10), a characteristic of T cytotoxic 1 (Tc1) effector cells (12) (Fig. 1A). Consistent with this, activated CL4 cells exhibited Ag-specific effector functions as demonstrated by IFN-γ production in response to 4T1-HA, but not 4T1 cells (Fig. 1B). Next, activated CL4 T cells were CFSE labeled before i.v. injection into mice bearing 4T1-HA s.c. tumors 48 h after tumor irradiation or mock treatment. Mice were sacrificed 24 h after CL4 T cell transfer and spleen and tumors were harvested. The number of adoptively transferred T cells infiltrating the spleen and tumors was determined by staining with Thy1.1 and CD8 and using flow cytometry. As assessed by CFSE dilution, the transferred CD8 T cells did not proliferate within this time period (Fig. 1C). Whereas the numbers of adoptively transferred cells present in the spleen of irradiated and nonirradiated mice were similar (mean of 3.2 × 10⁶ and 3.6 × 10⁶ cells/mouse, respectively), irradiated tumors contained almost 2-fold more CD8 T cells (Fig. 1D). These
results are consistent with previous observations that radiation can enhance T cell infiltration of tumors (22, 23) and suggest that CD8 T cells of the Tc1 type are responsive to changes produced by tumor irradiation.

The majority of CD8 TIL in regressing 4T1 tumors from CXCR6<sup>−/−</sup>/gfp mice treated with local IR and CTLA-4 blockade expresses CXCR6

The antitumor response elicited by treatment with the combination of IR and CTLA-4 blockade in 4T1 tumor-bearing mice is mediated by CD8 T cells (25). To test whether CD8 cells that infiltrate regressing tumors following treatment express CXCR6, we injected 4T1 cells into mice with a heterozygous GFP knockin in the CXCR6 locus (CXCR6<sup>+/−</sup/>gfp). In these mice CD8 cells become strongly GFP<sup>+</sup> when they up-regulate CXCR6 expression (11). Mice were left untreated or treated with IR delivered exclusively to the tumor and with the CTLA-4 blocking mAb 9H10 (Fig. 2). H&E-stained tumor sections showed increased TIL in treated tumors and with the CTLA-4 blocking mAb 9H10 (Fig. 2). Mice were left untreated or treated with IR delivered exclusively to the tumor and with the CTLA-4 blocking mAb 9H10 (Fig. 2). Flow cytometric analysis of lymphocytes within dissociated tumors and TDLN of treated mice. D. Cells were stained with PE-Cy5-anti-CD8α and gated on CD8<sup>+</sup> cells. Numbers indicate the percentage of cells in each gate. E. Bars show the percentage of CD8<sup>+</sup>GFP<sup>+</sup> cells from tumors (red) and TDLN (black) expressing CD69 or CD62L. Results are from 4–8 mice per group and are representative of three experiments.

Radiation induces up-regulation of CXCL16 expression and release by 4T1 tumor cells

Migration of CXCR6<sup>+</sup> effector T cells to peripheral sites of inflammation has been shown to be dependent, in part, on up-regulation of the CXCR6 ligand, the chemokine CXCL16, in the target tissue (13, 14, 35). To determine whether CXCL16 is expressed in vivo in 4T1 tumors, immunohistochemistry for CXCL16 was performed 48 h after tumor irradiation or mock treatment. In mock-treated 4T1 tumors, weak CXCL16 immunoreactivity was detectable in some vessels, with a faint staining in tumor cells (Fig. 3A). In contrast, irradiated tumors showed more intense staining of vessels as well as strong staining in the majority of tumor cells. To confirm the expression of CXCL16 by the carcinoma cells, in vitro cultured 4T1 cells were tested by RT-PCR, which showed a clear positive band (not shown; see also Fig. 7A).

CXCL16 is expressed as a transmembrane molecule and is shed in soluble form from the cell surface by the disintegrin-like metalloproteinase (MPase) ADAM10 (36, 37). Therefore, we tested 4T1 cells for the expression of surface CXCL16 by immunofluorescence staining and flow cytometry. Surface expression of CXCL16 on 4T1 cells was detected only when its MPase-mediated cleavage was inhibited (Fig. 3B), indicating that CXCL16 is rapidly shed in soluble form from these cells.

Next, to investigate the effects of IR on CXCL16 expression, real-time RT-PCR was performed at different times after in vitro irradiation of 4T1 cells. Results indicated a >4-fold induction of CXCL16 mRNA peaking at 48 h post-IR (Fig. 3C). In contrast, mRNA levels of the MPases ADAM10 and ADAM-17, implicated in constitutive and PMA-induced cleavage of CXCL16, respectively (36–38), were unchanged. Measurement of released CXCL16 in 4T1 cell supernatants showed that, as expected, activation of the MPase by PMA enhanced CXCL16 release whereas incubation with the specific MPase inhibitor BB-94 reduced it (Fig. 3D). Remarkably, release of CXCL16 was markedly increased following irradiation of 4T1 cells. The radiation-induced
release was inhibited by BB-94. This indicates that the MPase mediated the CXCL16 release.

**IR-induced CXCL16 release attracts CXCR6⁺ cells to the 4T1 tumor cells**

Next, chemotaxis assays were performed to determine the ability of CXCL16 released by 4T1 cells to induce the migration of CXCR6⁺ cells. Baf-3 cells transduced to express the CXCR6 receptor (CXCR6⁺ Baf) migrated in a dose-dependent manner toward rCXCL16 (Fig. 4A). Remarkably, the migration of CXCR6⁺ Baf, but not Baf-3 cells, toward irradiated 4T1 cells was increased by almost 8-fold compared with nonirradiated 4T1 cells (4T1-IR), indicating that the enhanced production of CXCL16 by 4T1 cells following IR is functionally relevant. To determine the requirement of CXCL16 for chemotaxis induction, 4T1 cells with selective CXCL16 gene knockdown were generated by the transduction of cells with lentivirus shRNA expression vectors targeting CXCL16 (shCXCL16). Effective knockdown of >90% was detected by RT-PCR and Western blotting (data not shown), and shCXCL16–4T1 cells showed markedly reduced ability to release soluble CXCL16 and to induce chemotaxis of CXCR6⁺ Baf cells (Fig. 4, C and D).

To determine whether the migration of activated CD8 cells to 4T1 cells is enhanced by radiation, CD8 cells were purified from the spleen of naive WT and CXCR6⁺/⁺ (CXCR6⁺/⁻) mice. Cells were activated in vitro with plate-bound anti-CD3 and anti-CD28 mAb and cultured with IL-2; expression of CXCR6 was confirmed by staining with the CXCL16-Fc fusion protein (see Fig. 1A) and was found to be >90% on cells from WT mice, whereas a comparable percentage of CXCR6⁻/⁻ cells was GFP⁺. Migration of CD8 cells of both genotypes toward untreated 4T1 cells was minimal, although higher for WT cells (~3% vs 1%) (Fig. 4E). Migration of WT CD8 cells toward irradiated 4T1 cells...
Efficient tumor infiltration by effector CD8 cells requires expression of a functional CXCR6 receptor

To determine whether in vivo CXCR6/CXCL16 interactions can regulate the infiltration of 4T1 tumors by effector CD8 cells elicited by treatment with IR and CTLA-4 blockade, CXCR6+/gfp and CXCR6gfp/gfp mice were injected with 4T1 cells and treated as shown in Fig. 2A. The percentages of the CD4 and CD8 T cell subsets in the TDLN of CXCR6+/gfp and CXCR6gfp/gfp mice were similar (data not shown). In contrast, the absolute number of CD8, but not CD4, TIL was markedly lower in treated CXCR6gfp/gfp as compared with CXCR6+/gfp mice (Fig. 5A). The majority of CD8 TIL was positive for GFP and for the activation marker CD69 in both CXCR6+/gfp and CXCR6gfp/gfp mice (Fig. 5B), suggesting that CD8 T cells in CXCR6gfp/gfp mice are not defective in the ability to become activated. In mice of both genotypes, the majority of TIL expressing GFP were CD8+ cells (80 and 63% in CXCR6+/gfp and CXCR6gfp/gfp mice, respectively), with only a small percentage of CD4+ cells (13 and 23% in CXCR6+/gfp and CXCR6gfp/gfp mice, respectively).

To determine whether similar changes in TIL are induced by treatment in CXCR6+/gfp and CXCR6gfp/gfp mice, the percentages of TIL positive for each marker in treated vs untreated mice of each genotype were compared. As shown in Fig. 5C, in treated mice of both genotypes there was a reduction in CD4 TIL. In contrast, the treatment-induced increase in CD8 TIL observed in CXCR6+/gfp was not detected in CXCR6gfp/gfp mice, suggesting that a functional CXCR6 receptor is required for the accumulation of effector CTL in irradiated tumors.

Impaired tumor inhibition in CXCR6-deficient mice treated with IR and CTLA-4 blockade

To determine whether the reduced tumor infiltration by CD8 effectors in CXCR6gfp/gfp mice was reflected in reduced treatment-induced tumor inhibition, WT and CXCR6gfp/gfp mice were injected with 4T1 cells and left untreated or treated with IR and CTLA-4 blockade starting on day 12 postinjection. There was no significant difference in tumor growth (p = 0.9) between WT and CXCR6gfp/gfp mice receiving control Ig (Fig. 6). Treatment with IR and 9H10 significantly reduced the rate of tumor growth among animals of both genotypes (p < 0.0001). However, tumor growth in CXCR6gfp/gfp mice receiving IR plus 9H10 was significantly higher than in WT mice (p = 0.00086). The ability of treatment to cause tumor growth inhibition was significantly weaker (p = 0.017) in CXCR6gfp/gfp as compared with WT mice.
differing metastatic potential (26) were tested by RT-PCR. In ad-

breast, four additional mouse mammary carcinoma cell lines of

to radiation are common among epithelial malignancies of the

Expression of CXCL16 by mouse and human breast cancer cells

tumors and the overall tumor inhibition.

FIGURE 7. Expression of CXCL16 is common in mouse and human

breast cancer lines. A, CXCL16 was expressed in three of five mouse 
mammary carcinoma lines of differing metastatic potential (26) tested by 
RT-PCR. B, IR enhanced in a dose-dependent fashion the release of soluble 
CXCL16 by the nonmetastatic 67NR and the metastatic 4T07 cells. C, 
CXCL16 was expressed in four human cell lines derived from primary 
breast specimens of benign breast (MCF 10A) or invasive breast cancer 
(CRL-2324, CRL-1902, and HTB-20) tested by RT-PCR. D, Soluble 
CXCL16 was measured by ELISA in the supernatants of untreated or ir-

radiated (12 Gy, IR) MCF 10A and HTB-20 cells cultured for 6 h in the 
presence or absence of the MPase inhibitor BB-94, as indicated. Radiation 
increased the release of CXCL16 by the HTB-20 tumor cells but not by the 
epithelial MCF 10A cells derived from benign breast tissue. Data are the 
mean of duplicate wells and are representative of two experiments.

Overall, these data indicate that CXCR6/CXCL16 interactions 
regulate the recruitment of 9H10-activated CD8 cells to irradiated 
tumors and the overall tumor inhibition.

Expression of CXCL16 by mouse and human breast cancer cells is common

To determine whether the expression of CXCL16 and its response 
to radiation are common among epithelial malignancies of the 
breast, four additional mouse mammary carcinoma cell lines of 
differing metastatic potential (26) were tested by RT-PCR. In ad-
dition to the very aggressive and highly metastatic 4T1 cells, 
CXCL16 was found to be expressed by the nonmetastatic 67NR 
cells and by the weakly metastatic 4T07 cells (Fig. 7A). 67NR and 
4T07 cells responded to irradiation similarly to 4T1 cells, by up-
regulating the production of soluble CXCL16 (Fig. 7B).

Next, four human breast epithelial cell lines were tested and 
found to be positive for the expression of CXCL16 by RT-PCR 
(Fig. 7C). The MCF10A cells were derived by the cloning of spontane-
ously immortalized epithelial cells from a patient with fibro-
cystic disease and resemble a normal ductal epithelium (39), 
whereas the other three lines were derived from primary invasive 
breast cancers (40). Both MCF10A and HTB20 showed MPase-
mediated release of soluble CXCL16, but only HTB20 responded to 
IR with increased release (Fig. 7D). Overall, these data suggest 
that the expression of CXCL16 by breast epithelial cells is com-
mon and that the response to radiation may be influenced by neo-
plastic transformation.

Discussion

In this study, we show for the first time that mouse and human 
breast cancer cells express the chemokine CXCL16 and that IR 
strikingly up-regulates its expression and release. CXCL16, the 
only known ligand for CXCR6, is one of only two chemokines that 
are expressed as transmembrane molecules (10). The transmem-
brane form can mediate adhesion to CXCR6+ cells as well as 
function as a scavenger receptor for oxidized low-density lipopro-
teins, phosphatidylserine, bacteria, and dextran sulfate (37, 41). 
The chemokine domain of CXCL16 is cleaved from the cell sur-
face by the activity of the MPase ADAM10 (36). Although we 
cannot completely exclude the possibility that the transmembrane 
form plays some role as a receptor on 4T1 cells, analysis of cell 
surface expression showed that CXCL16 was undetectable unless 
the MPase activity was inhibited (Fig. 3B), suggesting that it does 
not accumulate on the surface of the cancer cells in significant 
quantities.

Soluble CXCL16 has been shown to induce strong chemotaxis 
of activated CD8 T cells, which express high levels of CXCR6, 
and, to a lesser degree, chemotaxis of activated CD4 cells, which 
have lower levels of the receptor (10). Consistent with this, our 
data showed that irradiated 4T1 tumor cells induced strong 
CXCL16-dependent chemotaxis of activated CD8 cells in vitro 
(Fig. 4E) and that local tumor irradiation in vivo enhanced the 
recruitment of tumor-specific CXCR6+ CD8 cells (Fig. 1). Import-
antly, comparison between CXCR6+/gfp and CXCR6gfp/gfp mice 
indicated that CXCR6 expression by CD8 cells is required for 
efficient infiltration of 4T1 tumors (Fig. 5). Because endogenous 
CD8 cell activation was triggered in these mice by treatment with IR 
and CTLA-4 blockade, it cannot be excluded that CXCR6-
deficient T cells were impaired in their ability to become activated. 
However, this is not very likely because TIL from CXCR6+/gfp 
and CXCR6gfp/gfp mice expressed similar levels of the activation 
marker CD69 (Fig. 5B), and CXCR6-deficiency has been shown to 
decrease homing to inflamed organs but not CD8 cell activation 
in other experimental systems (14).

CXCL16 is known to be expressed by dendritic cells, macro-
phages, and some endothelia and to be up-regulated during inflam-
mation in different tissues and organs (13, 14, 35, 42). In a recent 
report, Hojo et al. showed that human colon carcinoma cells also 
express CXCL16 (43). Importantly, patients with tumors express-

ing higher levels of CXCL16 had increased TIL and a significantly 
better prognosis than patients with low CXCL16 expression in the 
tumor (43), suggesting that high expression of CXCL16 enhances 
the effectiveness of immune-mediated tumor control by improving
the recruitment of antitumor T cells. Our data presented here provide support for this hypothesis by showing that the increased CXCL16 expression induced by IR in breast carcinoma cells enhanced the recruitment of antitumor CD8 cells and that tumor in vivo in mice deficient in CXCR6 expression was less efficient than that in WT mice.

It remains to be determined whether malignancies other than breast and colon cancer express CXCL16 or other chemokines capable of recruiting Th1 and Tc1 cells. The ability of radiation to enhance, albeit to a lesser extent, the migration of CXCR6-deficient CD8 cells toward 4T1 cancer cells (Fig. 4E) suggests that additional chemokines are induced by IR. The observation that preoperative IR correlates with enhanced infiltration of oral squamous cell carcinoma by activated CD8 cells (44) supports the hypothesis that the induction of T cell chemotactic factors may be a relatively common component of the stress response that follows radiation exposure of neoplastic cells. Interestingly, MCF10A cells, which are derived from normal breast epithelium, did not increase CXCL16 release in response to IR (Fig. 7D). However, it remains to be determined whether this differential response is a characteristic of nonneoplastic cells. Up-regulation of some proinflammatory chemokines has been reported in normal lung and skin after IR at doses that cause inflammation and fibrosis in susceptible mouse strains (45, 46). However, it is unclear whether this is a primary effect of IR or is secondary to other inflammatory stimuli, and the cell type that is responsible for this chemokine production is also unknown.

It is intriguing that CXCL16 has been reported to stimulate proliferation, chemotaxis, and tube formation in HUVECs, suggesting that it may act as an angiogenic factor (47). Because the increased secretion of proangiogenic factors is a major part of the response of neoplastic cells to radiation (48), it is possible that CXCL16 plays a dual role in tumor growth by contributing to angiogenesis while at the same time attracting antitumor T cells. Improved understanding of the molecular changes that are induced by IR in tumors opens the possibility of exploiting the tumor response to radiation with a timely immunotherapeutic intervention that, by enhancing antitumor T cell activation, will contribute to immunemediated tumor destruction.

The molecular mechanism(s) responsible for up-regulation of CXCL16 following IR of breast cancer cells are presently undefined. A possible candidate is the PI3K/Akt signaling pathway, which is activated by IR in tumor and endothelial cells (49). This pathway promotes survival of tumor cells and has been recently linked to the induction of CXCL16 in a mouse model of mammary tumorigenesis (50).

IR is a standard treatment modality in oncology and can now be very precisely targeted to tumors (51) to minimize immunosuppressive side effects, an advantage in comparison to systemic chemotherapy. Recently, the possibility of combining local IR with IT has been explored by us and others in preclinical and some clinical studies (reviewed in Refs. 20 and 21). IR has been shown to induce changes in both the surviving cancer cells and the surviving tumor stromal cells that promote their recognition by antitumor CD8 cells (52–56). In this study we provide evidence for another mechanism that enhances immune-mediated tumor destruction, the IR-induced up-regulation of chemokines that recruit activated T cells to the tumor. We propose that IR should be further considered as an easily translatable strategy to overcome immune barriers at the effector phase of the antitumor immune response (57).

Acknowledgments

We thank Dan Littman for CXCR6Cre/Cre mice, Mehrdad Matloubian for the CXCR6-Fc construct, James P. Allison for 9H10 Ab, Fred Miller for mouse breast cancer cell lines, Ivan Borrello for 4T1-HA cells, and the personnel of NYU Cancer Institute Flow Cytometry facility for expert assistance. We are grateful to William H. McBride for critical reading of the manuscript.

Disclosures

The authors have no financial conflict of interest.

References


