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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 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 cytotoxic 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 effector 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 pro-inflammatory 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\(^{+/+}\) mice have been described (11), and were provided in the BALB/c background by D. Littman (New York University, New York, NY). BALB/c, C57BL/6, and C57BL/6 Tg(CXCR6\(^{+/+}\)) mice transgenic for an influenza virus influenza hemagglutinin (HA) H2-K\(^{+}\)-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, 66Cl4, 168FARN, and 67NR BALB/c mouse-derived mammary carcinoma cell lines (26) and 4T1-HA derivatives (30), were purchased 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-IRE6-GFP or with MSCV-CXCR6-IRE6-GFP expressing mouse CXCR6, selected by sorting for GFP\(^{+}\) cells, and cultured in the presence of 10\(\mu\)g/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. Mattilobian (University of California, San Francisco, CA). Goat anti-mouse CXCL16 and isotype control were purchased from R&D Systems. FITC-donkey anti-goat IgG was from Jackson ImmunoResearch Laboratories. PE-Cy5, PE-Cy5-CD8, PE-Cy5-CD3, PE-CD4, PE-CD8, PE-CD69, PE-CD62L, and PE-Thy1.1 were from BD Pharmingen.

**In vitro CD8 T cell activation and expansion**

CD8 T cells were purified from spleen single cell suspensions with CD8\(\alpha\) (Ly-2) MACs beads (Miltenyi Biotec). Cells were cultured in 24-well tissue culture plates coated with 2 mg/ml anti-CD3 (clone 145-2C11; eBioscience) and 5 mg/ml anti-CD28 (clone 37.51; BD Pharmingen) for 48 h, followed by culture in fresh RPMI 1640 medium supplemented with 2 mM t-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 \(\times\) 10\(^{-5}\) M 2-ME, and 10% FBS (Gemini Bio-Products) (complete medium). The human cell lines U2OS, HT1080, MDA-MB-231, MDA-MB-435S, and A549 were maintained by ATCC. Mouse Baf-3 cells were transduced with the control vector MSCV-IRE6-GFP or with MSCV-CXCR6-IRE6-GFP expressing mouse CXCR6, selected by sorting for GFP\(^{+}\) cells, and cultured in the presence of 10\(\mu\)g/ml IL-3.

**Chemotaxis assays**

Chemotaxis assays were performed as described (32) using 5\(\mu\)m Transwell filters for Bal-3 cells and 3\(\mu\)m Transwell filters for T cells (Corning Costar). Briefly, 3 \(\times\) 10\(^{3}\) BaF3 or 5 \(\times\) 10\(^{3}\) T cells were added to the upper chamber in 100 \(\mu\)l of DMEM with 1% BSA (chemotaxis buffer). The lower chamber contained 600 \(\mu\)l of chemotaxis buffer and mouse rCXCL16 (Peprotech) or 1 \(\times\) 10\(^{4}\) CD8 cells untreated or irradiated with 12 Gy and pre-seeded in the bottom of a 24-well plate. The adherent tumor cells were washed twice with PBS and changed to 600 \(\mu\)l of chemotactic buffer 16 h before the assay. For neutralization of CXCL16 activity, 1 \(\mu\)g/ml anti-mouse CXCL16 or control Ab was added to the lower chamber 30 min beforehand. After 4–6 h of incubation at 37\(^\circ\)C, transmigrated GFP\(^{+}\) or CFSE\(^{+}\) cells were counted by flow cytometry. An equal number of 5\(\mu\)m polystyrene beads (Polysciences) were added to each sample before analysis to correct for variability in volume aspirated by the cytometer.

**Tumor challenge and treatment**

Mice were injected s.c. in the right flank with 5 \(\times\) 10\(^{3}\) 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 \(\times\) width\(^2\) \(\times\) 0.52. Treatment was started on day 13 when tumors reached the average diameter of 5 mm (~65 mm\(^3\) in volume). Radiation was administered to a field including the tumor with <5-mm margins using a \(^{60}\)Co radiation 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 \(\mu\)g 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 (400 U/ml) for 25 min at 37\(^\circ\)C in a shaker. Aliquots of 10\(^{4}\) tumor-derived cells were stained at 4\(^\circ\)C with various mAbs and analyzed using a FACSscan 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 \(\times\) 10\(^{5}\) CFSE-labeled, in vitro activated CL4 CD8 T cells 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-Thy1.1 and PE-C5y-CD8 and analyzed by flow cytometry. An equal number of 5\(\mu\)m polystyrene beads (Polysciences) were added to each sample before analysis to estimate the total number of CD8\(^{+}\)Thy1.1\(^{+}\) cells.

**Immunohistochemistry**

4T1 tumors were harvested 48 h after IR or mock treatment, fixed for 1 h at 4\(^\circ\)C in 4% paraformaldehyde followed by overnight incubation in 30% sucrose, and frozen in OCT medium. Seven-micrometer sections were incubated with 0.3% H\(_2\)O\(_2\) to quench endogenous peroxidase activity and stained overnight at 4\(^\circ\)C with 1 \(\mu\)g/ml polyclonal goat anti-mouse CXCL16 or control Ab followed by FITC-donkey anti-goat IgG. Subsequently, slides were incubated with peroxidase-conjugated anti-FITC Ab (Roche), visualized with 3,3-diaminobenzidine (DAB substrate kit; BD Pharmingen), and counterstained with hematoxylin.

**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'-GCTTTGGACCCTTGTCTCTTGC-3'; reverse: 5'-TCCAAAGTACCCTGCGGTATC-3'), mouse ADAM10 (forward: 5'-GACTATGTGC-3'; reverse: 5'-TGGCCAGATTGACCTC-3'), mouse ADAM12 (forward: 5'-AGCAAA-3'; reverse: 5'-TTGGCCAGATTGACCTC-3'), and the internal control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (forward: 5'-AGCAGGTGCGTGATGCAG-3'; reverse: 5'-GCTTTGGACCCTTGTCTCTTGC-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'-CCCTGTGCTCCTGGGCTTCTTCCTC-3'; reverse: 5'-TCCAAGATTTCCCTGGTATTGC-3'). mouse CXCL16 (forward: 5'-GGGAGCAGCTACCGAAGTC-3'; reverse: 5'-ATTAACCGCGGTGTTGTTGGAGCAAGG-3').
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 MB94 (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 × 10⁶ 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 MB94 (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 viri. 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 viri. 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 viri.

**Statistical methods**

Tumor volume data were acquired longitudinally from WT and CXCR6gfp/gfp 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 within days after treatment. 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 within days after treatment.

**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 × 10⁵ and 3.6 × 10⁵ cells/mouse, respectively), irradiated tumors contained almost 2-fold more CD8 T cells (Fig. 1D). These differences were significant at the 5% level. Statistical computations were conducted using SAS for Windows, version 9.0 (SAS Institute).
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 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. 2A), and tumor and tumor-draining lymph nodes (TDLN) were analyzed by day 26 when the initial IR-mediated tumor growth delay is followed by an immune-mediated regression phase (Fig. 2B). H&E-stained tumor sections showed increased TIL in treated tumors, many of which were GFP<sup>+</sup> as shown by fluorescence microscopy analysis (Fig. 2C). Flow cytometric analysis of lymphocytes within dissociated tumors showed that 70–80% and 45–50% of GFP<sup>+</sup> TIL were CD8 cells in treated and untreated mice, respectively (not shown). The majority of CD8 cells in tumors, but not in the TDLN of treated mice, expressed high levels of CXCR6 (Fig. 2D). The CD8<sup>+</sup> CXCR6<sup>+</sup> cells within the tumors were negative for the lymphoid homing receptor CD62L and positive for the activation marker CD69, whereas in TDLN this subset was less activated (Fig. 2E). Some of the CD8<sup>+</sup> CXCR6<sup>−</sup> cells in TDLN retained high expression of CD62L, although the percentage was lower than that in CD8<sup>+</sup> CXCR6<sup>−</sup> cells (27 vs 88%, respectively; data not shown), as previously reported (12). Overall, these results suggest that CD8 T cells that home to 4T1 tumors following treatment with IR and CTLA-4 blockade are Tc1 effectors expressing high levels of CXCR6. In TDLN the CD8<sup>+</sup> CXCR6<sup>+</sup> cells may represent an early effector population (34).

**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 metalloprotease (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 ADAM-10 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. 4). 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 transduced with control (sh-NS; open bars) and silencing (sh-CXCL16; filled bars) retroviral vectors were left untreated (None) or irradiated (IR). CXCL16 release was tested in supernatants after 4 h of culture in the presence or absence of BB-94. Migration of Baf (open bars) and CXCR6^+ Baf-3 (filled bars) to 4T1 cells transduced with sh-NS and sh-CXCL16 (Fig. 4D), 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^gfp/gfp (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...
imilar (data not shown). In contrast, the absolute number of CD8,
ated by treatment with IR and CTLA-4 blockade, CXCR6
expression of a functional CXCR6 receptor
Efficient tumor infiltration by effector CD8 cells requires
large part, the migration of CD8 cells toward irradiated 4T1 car-
these data indicate that CXCR6/CXCL16 interactions drive, in
was increased by >10-fold. This increased migration was mark-
edly reduced by incubation with CXCL16 blocking Ab and by
CXCL16 knockdown (Fig. 4E). Interestingly, although the migration
of CXCR6−/− CD8 cells toward irradiated 4T1 cells was also
increased, it was markedly lower as compared with that of WT
cells. Blocking CXCL16 reduced the migration of WT CD8 cells
to the level observed for CXCR6−/− CD8 cells. Taken together,
these data indicate that CXCR6/CXCL16 interactions drive,
In contrast, the treatment-induced increase in CD8 TIL observed in
treated CXCR6gfp/gfp mice was markedly lower in treated CXCR6gfp/gfp as
impaired tumor inhibition in CXCR6-deficient mice treated with
CTLA-4 blockade
To determine whether the reduced tumor infiltration by CD8 ef-
fectors in CXCR6gfp/gfp mice was reflected in reduced treatment-
induced tumor inhibition, WT and CXCR6gfp/gfp mice were in-
jected with 4T1 cells and left untreated or treated with IR and
CTLA-4 blockade starting on day 12 post-injection. There was no
significant difference in tumor growth (p = 0.9) between WT and
CXCR6gfp/gfp mice receiving IR plus 9H10 (open triangles, n = 7) mices receiving the control IgG. In
contrast, CXCR6gfp/gfp mice receiving IR plus 9H10 (open triangles, n = 7) had a significantly (p = 0.00086) higher tumor volume than WT mice receiving IR plus 9H10 (full triangles, n = 7). Tumor volume differences between radiation therapy plus 9H10 and control (IgG) mice were statistically significant (p < 0.0001) within each genotype.

Impropead tumor inhibition in CXCR6-deficient mice treated with
IR and CTLA-4 blockade
To determine whether the reduced tumor infiltration by CD8 ef-
fectors in CXCR6gfp/gfp mice was reflected in reduced treatment-
induced tumor inhibition, WT and CXCR6gfp/gfp mice were in-
jected with 4T1 cells and left untreated or treated with IR and
CTLA-4 blockade starting on day 12 post-injection. 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.

FIGURE 6. Comparison between WT and CXCR6gfp/gfp mice with es-
hablished 4T1 carcinoma in the response to treatment with local IR and
CTLA-4 blockade. Treatment was started on day 12 after s.c. inoculation
of 4T1 cells in the flank. Radiation therapy was delivered in two fractions
of 12 Gy to the s.c. tumors on days 12 and 13. Ab were given i.p. 1, 4, and
7 days post-IR. Tumor volume is shown as the mean ± SE in each treat-
ment group up to day 28, when all animals were alive. Tumor growth

covered by flow cytometry analysis. The lymphocyte gate was set based on
the scattered plots in TDLN. A, The percentage of cells in the lymphocyte
gate positive for CD3, CD3 and CD8 (CD8), CD3 and CD4 (CD4), or
GFP was multiplied by the percentage of cells in the lymphocyte gate
and by the total number of viable cells isolated from the tumors and
divided by the tumor weight to obtain the number of cells per milligram
of tumor. B, TIL isolated from treated mice were gated on CD8+ cells
and analyzed for the expression of GFP and CD69 as indicated. Num-
bers are the percentage of cells in each quadrant. C, For each marker,
the percentage of positive cells in the lymphocyte gate in tumors iso-
lated from treated mice was divided by the percentage of cells isolated
from untreated mice. A ratio of one (line) indicates no change, >1
indicates an increase in treated tumors, and <1 indicates a decrease in
treated tumors. Data are the mean of eight mice of each genotype per
treatment group. Errors bars are absent because the pooling of tumors
within each group was necessary to count the T cells. Results are re-
representative of two experiments.
differing metastatic potential (26) were tested by RT-PCR. In breast cancer, four additional mouse mammary carcinoma cell lines of To determine whether the expression of CXCL16 and its response to radiation are common among epithelial malignancies of the Expression of CXCL16 is common in mouse and human breast cancer cells. FIGURE 7.

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 spontaneously immortalized epithelial cells from a patient with fibrocystic 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 common and that the response to radiation may be influenced by neoplastic 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 transmembrane form can mediate adhesion to CXCR6 receptors as well as integrins and phosphatidylserine, bacteria, and dextran sulfate (37, 41). The chemokine domain of CXCL16 is cleaved from the cell surface 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). Importantly, comparison between CXCR6+/−/− and CXCR6+/+/− 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+/−/− and CXCR6+/+/− 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, macrophages, and some endothelia and to be up-regulated during inflammation 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 expressing 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 inhibition 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).

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Disclosures

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References