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Radiation-induced CXCL16 release by breast cancer cells attracts effector T cells[1]

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Abstract

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 pre-clinical studies. Here 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. Employing 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 antibodies 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 pro-inflammatory chemotactic factors that recruit anti-tumor 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 anti-tumor immune response and improve the therapeutic efficacy of immunotherapy.

Keywords

Tumor immunity; Cell trafficking; Chemokines; T Cells; Cytotoxic

INTRODUCTION

The molecular identification of tumor-associated antigens has provided many potential targets for the development of therapeutic cancer vaccines (1). Much research in the field of immunotherapy (IT)[4] is focused on optimizing vaccination strategies to boost pre-existing and/or prime de novo anti-tumor responses. However, successful vaccination as measured by expansion of vaccine-specific T cells does not necessarily translate into clinical tumor responses (2), emphasizing the need to overcome obstacles also at the effector phase of the anti-tumor immune response (3).

Although in IT the therapeutic agent is a cell (i.e., an anti-tumor T cell), the requirements for IT to be successful are the same as for other anti-tumor agents: the agent must be effective in the microenvironment of the tumor, and it must reach the target cells in optimal quantities

[4]**Non Standard Abbreviations**: HA, hemagglutinin; IR, ionizing radiation therapy; IT, immunotherapy; MPase, metalloproteinase; Tc1, T-cytotoxic 1; TDLN, tumor-draining lymph nodes; TIL, tumor infiltrating lymphocytes; shRNA, short hairpin RNA.

(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 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 pro-inflammatory chemokines such as 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 employed 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 references 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 employed 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 with local IR and CTLA-4 blockade in combination but not as single modalities induced CD8-mediated anti-tumor responses inhibiting metastases and inducing regression of the primary irradiated tumors (25). Here we show that most of the CD8 T cells infiltrating 4T1 tumors following the combination treatment were $CXCR6^{+}$. Tumor irradiation increased recruitment of tumor-specific activated CD8 cells and markedly enhanced the expression and release of 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 recruitment of effector T cells, effectively converting tumors into “inflamed” peripheral tissues that are rendered susceptible to the effector phase of the anti-tumor immune response.

MATERIALS AND METHODS

Mice

Six to eight week old BALB/c mice were purchased from Taconic Animal Laboratory (Germantown, NY). $CXCR6^{+/gfp}$ and $CXCR6^{gfp/gfp}$ mice have been described (11), and were provided in the BALB/c background by Dan Littman (New York University School of Medicine, NY). BALB/c CL4-TCR mice transgenic for an influenza virus influenza hemagglutinin (HA) H2-K^{d}-restricted epitope (29) were purchased from Jackson Laboratory (Bar Harbor, ME). All experiments were approved by the Institutional Animal Care and Use Committee of New York University.

Cells and antibodies

4T1, 4T07, 66cl4, 168FARN, and 67NR BALB/c mouse-derived mammary carcinoma cell lines (26), and 4T1-HA derivatives (30), were grown in DMEM medium (Invitrogen Corporation, Carlsbad, CA) supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5×10^{-5} M 2-mercapthoethanol, and 10% FBS (Gemini Bio-Products Woodland, CA) (complete medium). Human cell lines MCF10A, CRL2324, CRL1902, and HTB-20 were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were cultured as recommended by ATCC. Mouse Baf-3 cells were transduced with control vector MSCV-IRES-GFP or MSCV-CXCR6-IRES-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). CXCL16-Fc fusion protein (10) was a gift of M. Matloubian. Goat anti-mouse CXCL16 and isotype control were purchased from R&D System, (Minneapolis, MN). FITC-Donkey anti-goat IgG was from Jackson ImmunoResearch (West Grove, PA). PE-Cy5-CD8, PE-Cy5-CD3, PE-CD4, PE-CD8, PE-CD69, PE-CD62L, and PE-Thy1.1 were from BD PharMingen, (San Diego, CA).

In vitro CD8 T cell activation and expansion

CD8 T cells were purified from spleen single cell suspensions with CD8α (Ly-2) MACS beads (Miltenyi Biotec, Auburn, CA). Cells were cultured in 24-well tissue culture plates coated with 5μg/ml anti-CD3 (145-2C11 clone, eBioScience, San Diego, CA) and 5μg/ml anti-CD28 (37.51, BD Pharmingen, San Diego, CA) for 48 h, followed by culture in fresh RPMI 1640 medium supplemented with 2mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50nM 2-mercapthoethanol, 10% FBS (T cell medium) and 50μU/ml human rIL-2 (provided by the National Cancer Institute BRB Preclinical Repository) (10). After 8 to 10 days of expansion, activated CD8 cells were used for phenotype analysis, chemotaxis assays, or adoptive transfer. For chemotaxis assays and adoptive transfer, CD8 cells from WT mice were labeled with 5μM or 10μM CFSE (Molecular Probe, Eugene, OR), respectively. IFNγ production by CL4 CD8 cells was measured in supernatants by ELISA (Diaclone Tepnel, Lifecodes Corp. Stamford, CT) after 20 h culture with irradiated (100 Gy) 4T1 or 4T1-HA cells at E/T ratio of 10 in duplicate wells.

Chemotaxis assays

Chemotaxis assays were performed as described (32) using 5-μm transwells for Baf-3 cells and 3-μm transwells for T cells (Corning Costar Corp., Corning, NY). Briefly, 3×10^5 BaF3 or 5×10^5 T cells were added to the upper chamber in 100μl of DMEM 1% BSA (chemotaxis buffer). The lower chamber contained 600μl of chemotaxis buffer and mouse rCXCL16 (Peprotech, Rocky Hill, NJ) or 1×10^5 4T1 cells untreated or irradiated with 12 Gy and pre-seeded in the bottom of 24-well plate. The adherent tumor cells were washed twice with PBS and changed to 600μl of chemotactic buffer 16 hours before the assay. For neutralization of CXCL16 activity, 1μg/ml anti-mouse CXCL16 or control Ab was added to the lower chamber 30 min beforehand. After 4 to 6 h incubation at 37°C transmigrated GFP+ or CFSE+ cells were counted by flow cytometry. An equal number of 5μm polysterene beads (Polysciences, PA) was added to each sample prior to 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 5×10^4 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 × width^2 × 0.52. Treatment was started on Day 13 when tumors reached the average diameter of 5 mm (approximately 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 μg at 1, 4 and 7 days after IR.

Analysis of TIL

Tumors were dissected carefully removing surrounding normal tissue, minced into ~1 mm pieces, and digested with collagenase D (400U/ml) for 25 minutes at 37 °C in a shaker. Aliquots of 10^6 tumor-derived cells were stained at 4°C with various mAbs and analyzed using a FACScan flow cytometer and FlowJo version 6.4.4 (Tree Star, Ashland, OR).

Adoptive transfer

WT mice bearing 4T1-HA tumors of ~5 mm average diameter were injected i.v. with 20×10^6 CFSE-labeled *in vitro* activated CL4 CD8 T cells which express the congenic marker Thy1.1. 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 4 mice per group were pooled and digested with collagenase D as described above. Obtained cell suspensions were stained with PE-Thy1.1 and PE-Cy5-CD8, and analyzed by flow cytometry. An equal number of 5μm polysterene beads (Polysciences, PA) was added to each sample prior to 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°C in 4% paraformaldehyde followed by overnight incubation in 30% sucrose, and frozen in OCT medium. 7μm sections were incubated with 0.3% H_2O_2 to quench endogenous peroxidase activity and stained overnight at 4°C with 1μg/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, Indianapolis, IN), visualized with 3,3-diaminobenzidine (DAB Substrate Kit, BD Pharmingen, San Diego, CA), and counterstained with hematoxylin.

RT-PCR and real-time PCR

Total RNA isolated from carcinoma cells with TRIZOL (Invitrogen, CA) was subjected to RT-PCR using specific primers for mouse CXCL16 (forward: 5′ – GCT TTG GAC CCT TGT CTC TTG C – 3′; reverse: 5′ – GTG CTG AGT GCT CTG ACT ATG TGC – 3′); or human CXCL16 (forward: 5′ – GGG GGC AGT CAC CGC AGT CCT– 3′; reverse: 5′ – ATT AGC CGG GTG TGG TGG TGA GCA– 3′).

Real time PCR was performed using SYBR Green Quantitative RT-PCR Kit (SIGMA, MO) and Light Cycler (Roche, IN). The following primers were used: mouse CXCL16 (forward: 5′ – CCT TGT CTC TGG CGT TCT TCC – 3′; reverse: 5 – TCC AAA GTA CCC TGC GGT ATC – 3′); mouse ADAM10 (forward: 5 – AGC AAC ATC TGG GGA CAA AC – 3′; reverse: 5′-TGG CCA GAT TCA ACA AAA CA – 3′); mouse ADAM17 (forward: 5′ – GTA CGT CGA TGC AGA GCA AA – 3′; reverse: 5′ – AAA CCA GAA CAG ACC CAA CG – 3′). The estimated amount of the gene of interest was normalized to the amount of eIF4GII.

Soluble CXCL16 measurement

Mouse and human carcinoma cells were plated at 1×10^4 cells/well in duplicate wells of a 96-well plate 48 h after radiation or mock treatment. The medium was changed to DMEM containing 2mM 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, Norcross, GA).

CXCL16 knockdown

Lentiviral mediated knockdown of murine CXCL16 was achieved by insertion of a 58-bp DNA duplex oligo containing a specific 21-bp target region directed against the 3′UTR of the CXCL16 gene, via EcoR1-AgeI cloning sites, immediately downstream of a U6, DNA-polIII promoter in the pLKO.1.puro vector, which ultimately generates a shRNA specifically disrupting generation of target protein (sh-CXCL16). A control vector with a 58-bp non-silencing 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 pCMVΔ 8.2R′ to generate active virions. 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 was acquired longitudinally from WT and CXCR6$^{gfp/gfp}$ mice. Random coefficients 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 since 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 carried out using SAS for Windows, version 9.0 (SAS Institute, Cary, NC).

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 recruitment of tumor-specific effector T cells, mice were injected with 4T1-HA cells expressing the reporter antigen 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) (Figure 1A). Consistent with this, activated CL4 cells exhibited antigen-specific effector functions as demonstrated by γIFN production in response to 4T1-HA, but not 4T1 cells (Figure 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 harvested. The number of adoptively transferred T cells infiltrating the spleen and tumors was determined by staining with Thy1.1 and CD8, and flow cytometry. As assessed by CFSE dilution, the transferred CD8 T cells did not proliferate within this time (Figure 1C). Whereas the number of adoptively transferred cells present in the spleen of irradiated and non-irradiated mice was similar (mean 3.2×10^6 and 3.6×10^6 cells/mouse respectively), irradiated tumors contained almost 2-fold more CD8 T cells (Figure 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 Tc-1 type are responsive to changes produced by tumor irradiation.

The majority of CD8 TIL in 4T1 tumor-bearing mice treated with IR and CTLA-4 blockade expresses CXCR6

The anti-tumor 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 heterozygous GFP-knockin in the CXCR6 locus ($CXCR6^{+/gfp}$) mice. In these mice CD8 cells become strongly GFP^+ when they up-regulate CXCR6 expression (11). Mice were left untreated or treated with IR delivered exclusively to the tumor and CTLA-4 blocking mAb 9H10 (Figure 2A), and tumor and tumor draining lymph nodes (TDLN) were analyzed at Day 26 when the initial IR-mediated tumor growth delay is followed by an immune-mediated regression phase (Figure 2B). H&E-stained tumor sections showed increased tumor infiltrating lymphocytes (TIL) in treated tumors, many of which were GFP^+ by fluorescence microscopy analysis (Figure 2C). Flow cytometric analysis of lymphocytes within dissociated tumors showed that 70–80% and 45–50% of GFP^+ TIL were CD8 cells in treated and untreated mice, respectively (not shown). The majority of CD8 cells in tumors, but not in TDLN of treated mice, expressed high levels of CXCR6 (Figure 2D). The $CD8^+CXCR6^+$ 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 (Figure 2E). Some of $CD8^+CXCR6^+$ cells in TDLN retained high expression of CD62L, although the percentage was lower than in $CD8^+CXCR6^-$ cells (27% *versus* 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 Tc-1 effectors expressing high levels of CXCR6. In TDLN the $CD8^+CXCR6^+$ cells may represent an early effector population (34).

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

Migration of $CXCR6^+$ effector T cells to peripheral sites of inflammation has been shown to be dependent, in part, on up-regulation of 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 (Figure 3A). In contrast, irradiated tumors showed more intense staining of vessels, as well as strong staining in the majority of tumor cells. To confirm 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 Figure 6A).

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 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 (Figure 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 an over fourfold induction of CXCL16 mRNA peaking at 48 h post-IR (Figure 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 (Figure 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 towards rCXCL16 (Figure 4A). Remarkably, the migration of CXCR6$^+$Baf, but not Baf-3 cells, towards irradiated 4T1 cells was increased by almost 8-fold compared to non-irradiated 4T1 cells (Figure 4B), indicating that the enhanced production of CXCL16 by 4T1 cells following IR is functionally relevant. To determine the requirement for CXCL16 for chemotaxis induction, 4T1 cells with selective CXCL16 gene knockdown were generated by transduction of cells with lentivirus short hairpin RNA (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 (Figure 4C and D).

To determine if migration of activated CD8 cells to 4T1 cells is enhanced by radiation, CD8 cells were purified from the spleen of naïve WT and CXCR6$^{gfp/gfp}$ (CXCR6$^{-/-}$) mice. Cells were activated in vitro with plate-bound anti-CD3 and anti-CD28 mAb and culture in IL-2, and expression of CXCR6 was confirmed by staining with CXCL16-Fc fusion protein (see Figure 1A), and 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 towards untreated 4T1 cells was minimal, although higher for WT cells (~3% versus 1%) (Figure 4E). Migration of WT CD8 cells towards irradiated 4T1 cells was increased by over 10-fold. This increased migration was markedly reduced by incubation with CXCL16 blocking antibody and by CXCL16 knockdown (Figure 4E). Interestingly, although the migration of CXCR6$^{-/-}$ CD8 cells towards irradiated 4T1 cells was also increased, it was markedly lower as compared to 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 large part, the migration of CD8 cells towards irradiated 4T1 carcinoma cells, while additional chemotactic factors may be induced by radiation and contribute to increased T cell migration.

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 CXCR6$^{gfp/gfp}$ mice were injected with 4T1 cells and treated as shown in Figure 2A. The percentage of the CD4 and CD8 T cell subsets in TDLN of CXCR6$^{+/gfp}$ and CXCR6$^{gfp/gfp}$ mice was similar (data not shown). In contrast, the absolute number of CD8, but not CD4, TIL was markedly lower in treated CXCR6$^{gfp/gfp}$ as compared to CXCR6$^{+/gfp}$ mice (Figure 5A). The majority of CD8 TIL was positive for GFP and for the activation marker CD69 in both CXCR6$^{+/gfp}$ and CXCR6$^{gfp/gfp}$ mice (Figure 5B), suggesting that CD8 T cells in CXCR6$^{gfp/gfp}$ 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 CXCR6$^{gfp/gfp}$ mice, respectively) with only a small percentage of CD4$^+$ cells (13 and 23% in CXCR6$^{+/gfp}$ and CXCR6$^{gfp/gfp}$ mice, respectively).

To determine whether similar changes in TIL are induced by treatment in CXCR6$^{+/gfp}$ and CXCR6$^{gfp/gfp}$ mice, the percentage of TIL positive for each marker in treated versus untreated mice of each genotype was compared. As shown in Figure 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 CXCR6$^{gfp/gfp}$ mice, suggesting that a functional CXCR6 receptor is required for 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 CXCR6$^{gfp/gfp}$ mice reflected in reduced treatment-induced tumor inhibition, WT and CXCR6$^{gfp/gfp}$ mice were injected 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 CXCR6$^{gfp/gfp}$ mice receiving control Ig (Figure 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 CXCR6$^{gfp/gfp}$ mice receiving IR+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 CXCR6$^{gfp/gfp}$ as compared to WT mice.

Overall, theses 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 addition to the very aggressive and highly metastatic 4T1 cells, CXCL16 was found to be expressed by the non-metastatic 67NR cells and by the weakly metastatic 4T07 cells (Figure 7A). 67NR and 4T07 cells responded to irradiation similarly to 4T1 cells, by up-regulating the production of soluble CXCL16 (Figure 7B).

Next, four human breast epithelial cell lines were tested and found to be positive for expression of CXCL16 by RT-PCR (Figure 7C). The MCF10A cells were derived by cloning of spontaneously immortalized epithelial cells from a patient with fibrocystic disease and resemble 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 (Figure 7D). Overall, these data suggest that expression of CXCL16 by breast epithelial cells is common, and 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^{+} cells, as well as function as a scavenger receptor for oxidized low density lipoproteins, 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 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 (Figure 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 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* (Figure 4E), and that local tumor irradiation *in vivo* enhanced the recruitment of tumor-specific CXCR6^{+} CD8 cells (Figure 1). Importantly, comparison between CXCR6$^{+/gfp}$ and CXCR6$^{gfp/gfp}$ mice

indicated that CXCR6 expression by CD8 cells is required for efficient infiltration of 4T1 tumors (Figure 5). Since 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 since TIL from $CXCR6^{+/gfp}$ and $CXCR6^{gfp/gfp}$ mice expressed similar levels of the activation marker CD69 (Figure 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*., have shown 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 recruitment of anti-tumor 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 recruitment of anti-tumor CD8 cells, and that in mice deficient in CXCR6 expression tumor inhibition was less efficient than in WT mice.

It remains to be determined if malignancies other than breast and colon cancer express CXCL16 or other chemokines capable of recruiting Th1 and Tc-1 cells. The ability of radiation to enhance, albeit to a lesser extent, the migration of CXCR6-deficient CD8 cells towards 4T1 cancer cells (Figure 4E) suggests that additional chemokines are induced by IR. The observation that pre-operative IR correlates with enhanced infiltration of oral squamous cell carcinoma by activated CD8 cells (44) supports the hypothesis that 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 (Figure 7D). However, it remains to be determined if this differential response is a characteristic of non-neoplastic cells. Up-regulation of some pro-inflammatory chemokines has been reported in normal lung and skin after IR at doses that cause inflammation and fibrosis in susceptible mice strains (45, 46). However, it is unclear if this is a primary effect of IR, or it is secondary to other inflammatory stimuli, and which cell type is responsible for chemokine production.

It is intriguing that CXCL16 has been reported to stimulate proliferation, chemotaxis, and tube formation in human umbilical vein endothelial cells, suggesting that it may act as an angiogenic factor (47). Since increased secretion of pro-angiogenic 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 anti-tumor 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 anti-tumor T cell activation, will contribute to immune-mediated 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 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) minimizing immunosuppressive side effects, an advantage to systemic chemotherapy. Recently, the possibility of combining local IR with IT has been explored by

us and others in pre-clinical and some clinical studies (reviewed in references 20 and 21). IR has been shown to induce changes in both, the cancer and tumor stromal cells that survive, that promote their recognition by anti-tumor CD8 cells (52–56). Here we provide evidence for another mechanism that enhances immune-mediated tumor destruction, the IR-induced up-regulation of a chemokine recruiting 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 anti-tumor immune response (57).

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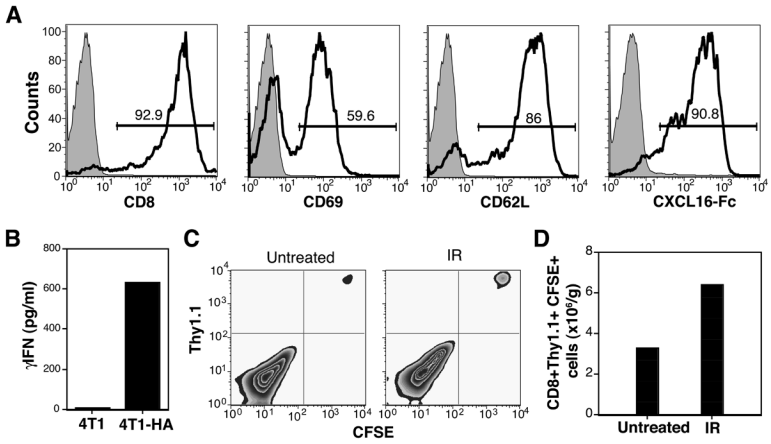

Figure 1. Irradiation of 4T1 tumors enhances recruitment of adoptively transferred tumor-specific CD8 T cells

WT mice were injected with 4T1-HA cells. Local IR or mock treatment (Untreated) was delivered to the tumors in two 12 Gy fractions at 24 h interval. 48 h after last IR mice received adoptive transfer of CFSE-labeled *in vitro* activated CL4 T cells. (A) Phenotype of activated CL4 T cells. Shaded histograms are cells stained with control antibody. Numbers indicate the percentage of cells in the gate. (B) Activated CL4 T cells produced IFNγ in response to 4T1-HA ut not parental 4T1 cells. (C, D) Tumors were harvested 24 h after adoptive transfer, and obtained single cell suspensions stained with PE-Cy5-anti-CD8 and PE-anti-Thy1.1 mAbs to identify the transferred T cells. (C) T cells recovered from the tumors did not proliferate within this time as indicated by lack of CFSE dilution. (D) Recruitment of T cells to tumors was enhanced by radiation. Data are from 4 mice of each group. Errors bars are absent because pooling of tumors within each group was necessary to count the CD8 T cells. Results are representative of two experiments.

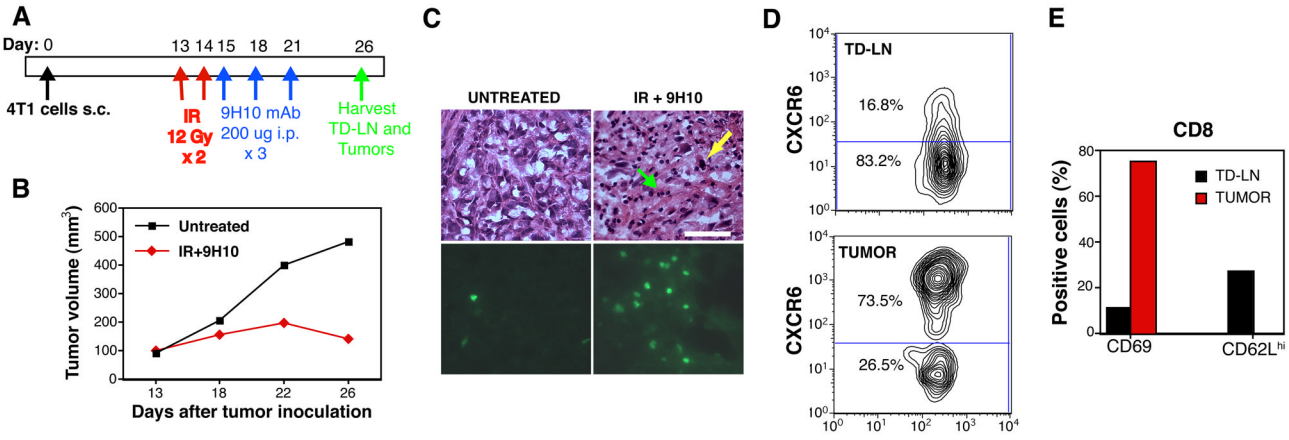

Figure 2. CD8 TIL in regressing 4T1 tumors from CXCR6$^{+/gfp}$ mice treated with local IR and CTLA-4 blockade express CXCR6

(A) Treatment schedule. (B) Growth of representative tumors from untreated (black squares) and IR+9H10-treated (red diamonds) mice. (C, D) CXCR6^{+} (as detected by GFP-positivity) TIL in treated tumors. Each tumor was bisected and half was processed for microscopic evaluation whereas the other half was used for flow cytometric analysis. For the latter, tumors from mice in the same group were pooled. (C) Sequential sections of representative tumors from untreated or treated (IR+9H10) mice were stained with H&E (upper panels) or analyzed by fluorescence microscopy for the presence of GFP^{+} cells (lower panels). In tumors from treated mice residual tumor cells (yellow arrow) were admixed with TIL (green arrow), and many of the TIL were GFP^{+}. Bar, 50 μm. (D, E) Flow cytometric analysis of lymphocytes within dissociated tumors and TD-LN of treated mice. (D) Cells were stained with PE-Cy5-anti-CD8α and gated on CD8^{+} cells. Numbers indicate the percentage of cells in each gate. (E) Bars show the percentage of CD8^{+}GFP^{+} cells from tumors (red) and TDLN (black) expressing CD69 or CD62L. Results are from 4 to 8 mice per group, and are representative of 3 experiments.

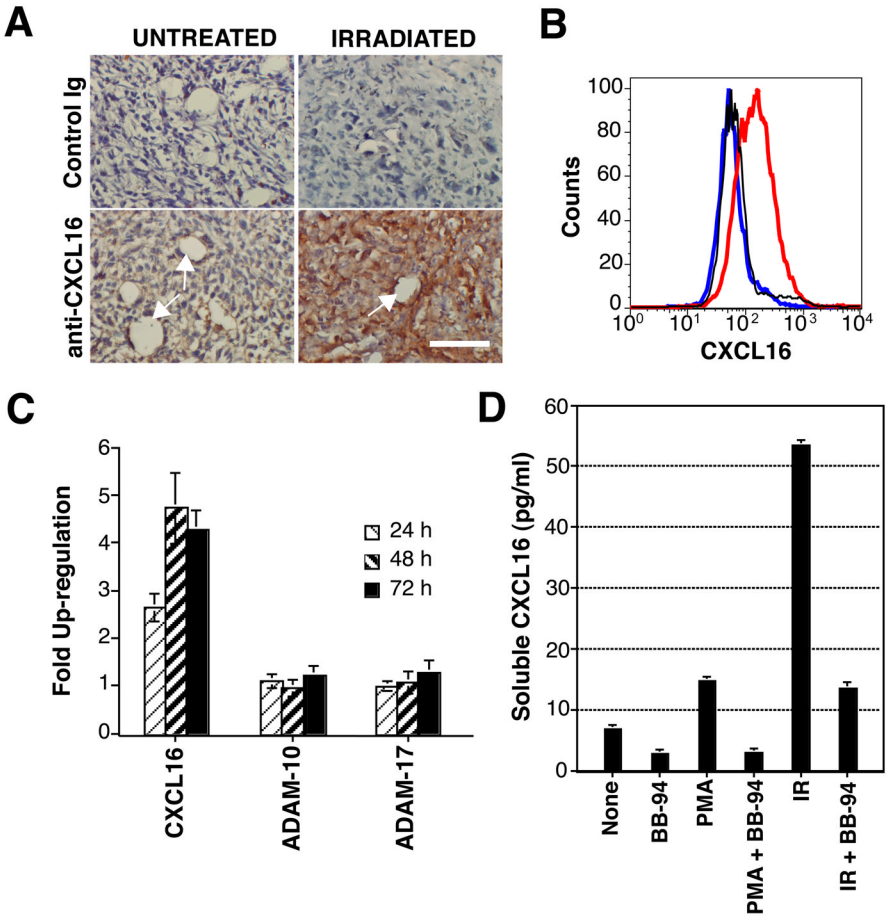

Figure 3. Release of CXCL16 by 4T1 cells is mediated by a MPase and enhanced by radiation

(A) Up-regulation of CXCL16 by local IR of 4T1 tumors *in vivo*. Tumors were harvested 48 h after IR (IRRADIATED) or mock treatment (UNTREATED) and stained with anti-CXCL16 or control Ig to detect expression of CXCL16. White arrows indicate blood vessels. Bar, 50 μm. (B) MPase inhibition leads to accumulation of CXCL16 molecules on the surface of 4T1 cells. Cells were incubated for 2 h at 37°C in the presence (red line) or absence (blue line) of BB-94 (20 μM), and stained with anti-CXCL16, or with control Ig (black line), followed by FITC-Donkey anti-Goat Ab. (C) Real time RT-PCR measurement of the expression of CXCL16, ADAM-10 and ADAM-17 in 4T1 cells at different times post-radiation. Samples were normalized to eIF4G II, and expression on untreated cells was assigned a relative value of 1.0. Data are expressed as the mean ±SD (n=3). (D) Soluble CXCL16 was measured by ELISA in supernatants of untreated or irradiated (12 Gy, IR) 4T1 cells cultured for 4 h in the presence or absence of MPase activators/inhibitors, as indicated. Data are expressed as the mean ±SD (n=3).

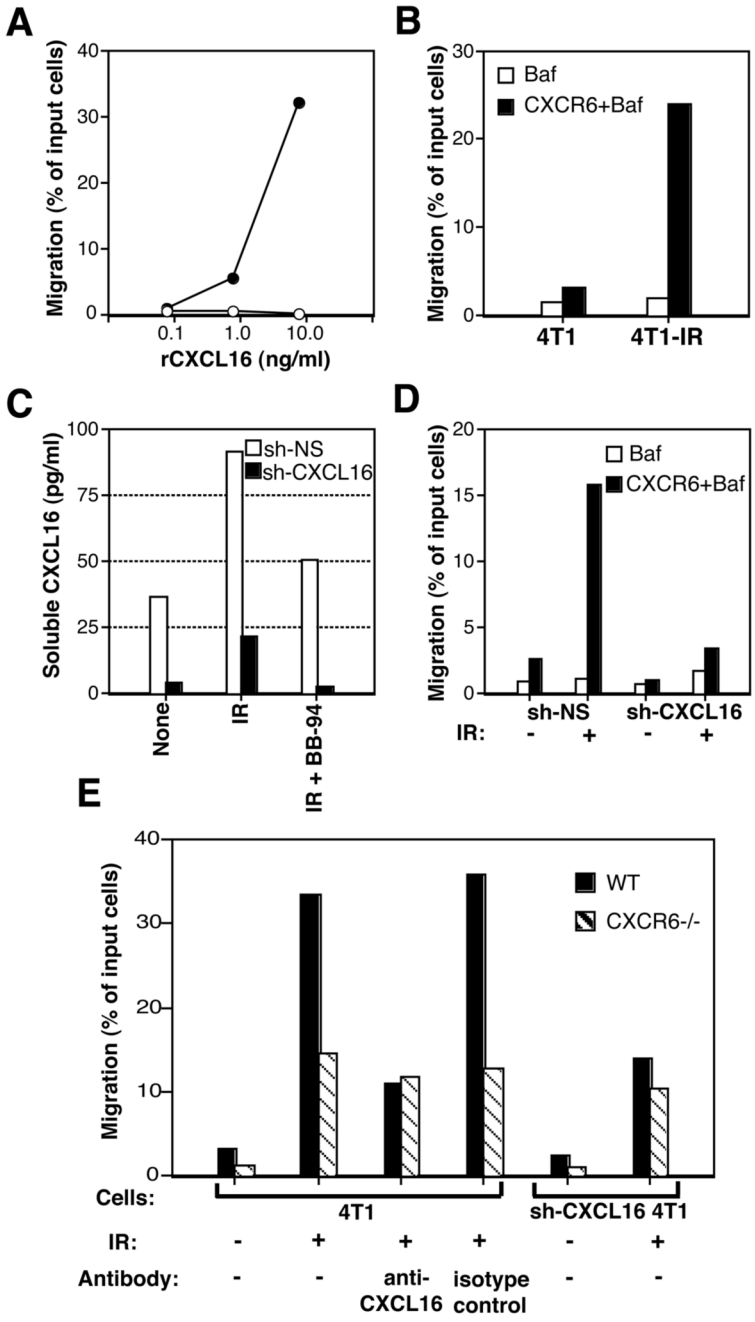

Figure 4. CXCL16/CXCR6 interactions regulate the migration of CD8 activated T cells to irradiated 4T1 cancer cells *in vitro*

Results of migration assays are presented as the percentage of input cells migrating to the lower chamber of a transwell filter. (A) Response of Baf-3 (empty circles) and CXCR6^{+}Baf-3 (full circles) cells to various concentrations of rCXCL16. (B) Migration of Baf (white bars) and CXCR6^{+}Baf-3 (black bars) cells towards untreated (4T1) or irradiated with 12 Gy 48 h earlier (4T1-IR) 4T1 cells. (C) 4T1 cells transduced with control (sh-NS, white bars) and silencing (sh-CXCL16, black bars) retroviral vectors were left untreated (None) or irradiated (IR). CXCL16 release was tested in supernatants after 4 h culture in the presence or absence of BB-94. (D) Migration of Baf (white bars) and CXCR6^{+}Baf-3 (black bars) to 4T1 cells

transduced with sh-NS and sh-CXCL16. (E) Migration of *in vitro* activated CD8 T cells from WT (black bars) and CXCR6$^{gfp/gfp}$ (CXCR6$^{-/-}$, shaded bars) mice to 4T1 and sh-CXCL16-4T1 cells, untreated (−) or irradiated with 12 Gy 48 hours earlier (+). In some wells 1 μg/ml of anti-CXCL16 or isotype control Ab was added, as indicated. Data are representative of two experiments.

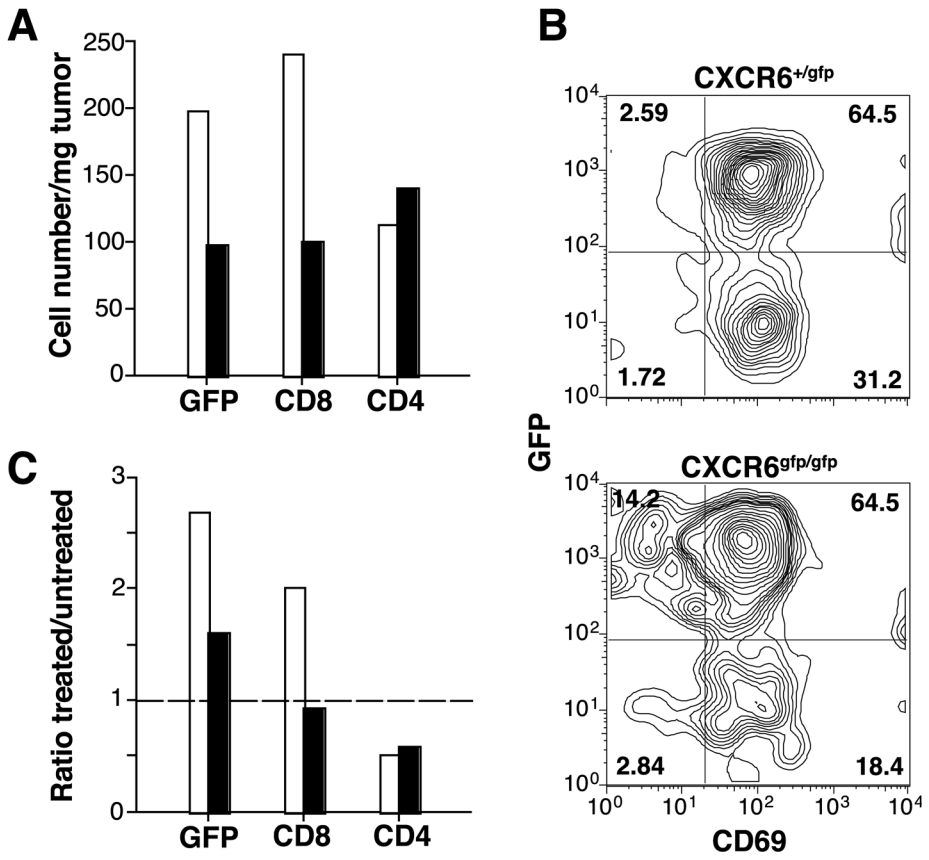

Figure 5. CXCR6-deficient mice cannot increase CD8 TIL following treatment with IR and CTLA-4 blockade

$CXCR6^{+/gfp}$ (white bars) and $CXCR6^{gfp/gfp}$ (black bars) mice were injected s.c. with 4T1 cells and left untreated or treated as described in figure 2A. On Day 26 tumors and TDLN were collected and obtained single cell suspensions stained with PE-Cy5-anti-CD3, and PE-anti-CD8 or anti-CD4 mAbs, followed 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), and GFP was multiplied for the percentage of cells in the lymphocyte gate and for the total number of viable cells isolated from the tumors, and divided for the tumor weight to obtain the number of cells per mg of tumor. (B) TIL isolated from treated mice were gated on $CD8^+$ cells and analyzed for the expression of GFP and CD69, as indicated. Numbers are the percentage of cells in each quadrant. (C) For each marker, the percentage of positive cells in the lymphocyte gate in tumors isolated 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 8 mice of each genotype per treatment group. Errors bars are absent because pooling of tumors within each group was necessary to count the T cells. Results are representative of two experiments.

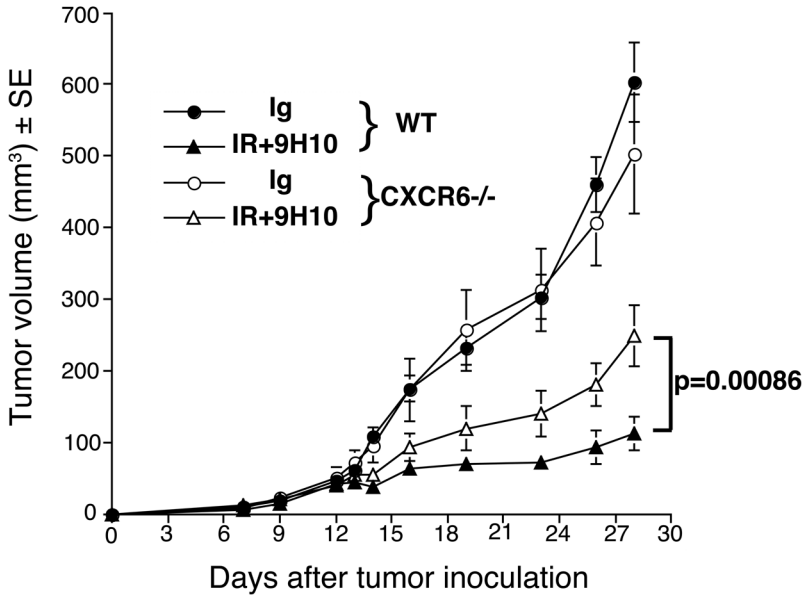

Figure 6. Comparison between WT and CXCR6$^{gfp/gfp}$ mice with established 4T1 carcinoma in the response to treatment with local IR and CTLA-4 blockade

Treatment was started on Day 12 post-s.c. inoculation of 4T1 cells in the flank. RT was delivered in two fractions of 12 Gy to the s.c. tumors on Day 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 treatment group up to Day 28 when all animals were alive. Tumor growth was not significantly different (p=0.9) in WT (full circles, n=8) and CXCR6$^{gfp/gfp}$ (empty circles, n=7) mice receiving the control IgG. In contrast, CXCR6$^{gfp/gfp}$ mice receiving IR+9H10 (empty triangles, n=7) had a significantly (p=0.00086) higher tumor volume than WT mice receiving IR+9H10 (full triangles, n=7). Tumor volume differences between RT+9H10 and control (IgG) mice were statistically significant (p<0.0001) within each genotype.

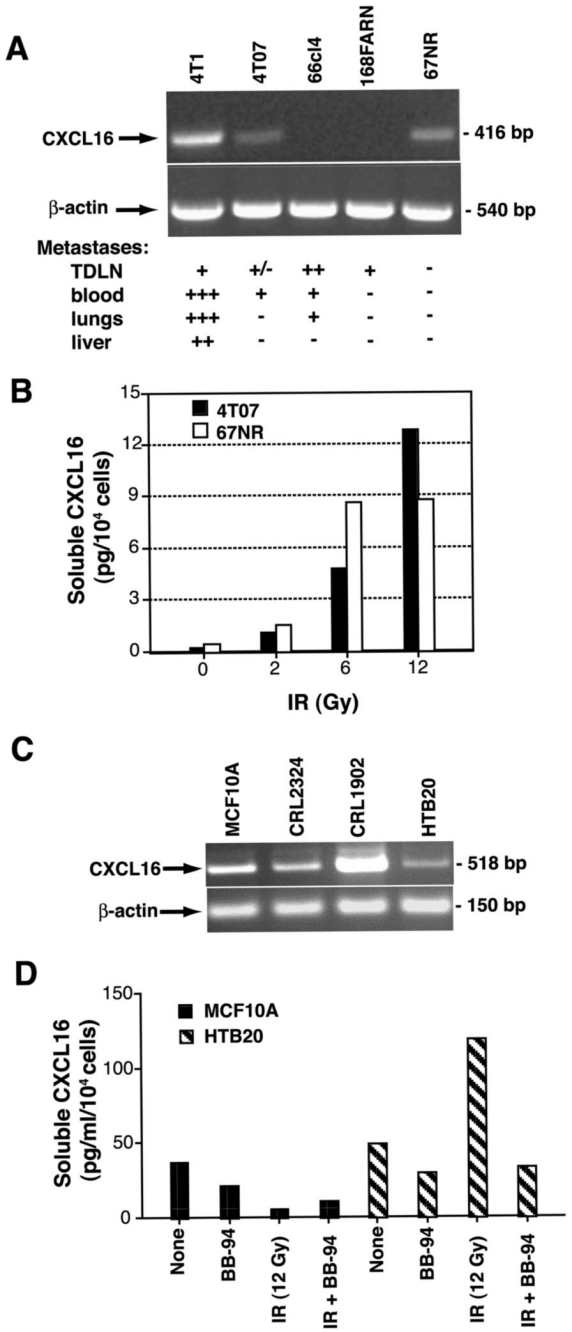

Figure 7. Expression of CXCL16 is common in mouse and human breast cancer lines

(A) CXCL16 was expressed in 3 out of 5 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 non-metastatic 67NR and the metastatic 4T07 cells. (C) CXCL16 was expressed in 4 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 supernatants of untreated or irradiated (12 Gy, IR) MCF 10A and HTB-20 cells cultured for 6 h in the presence or absence of 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.