Research Article

TH2-Polarized CD4\(^+\) T Cells and Macrophages Limit Efficacy of Radiotherapy

Stephen L. Shiao\(^1\), Brian Ruffell\(^2\), David G. DeNardo\(^3\), Bruce A. Faddegon\(^4\), Catherine C. Park\(^4\), and Lisa M. Coussens\(^2\)

Abstract

Radiotherapy and chemotherapy following surgery are mainstays of treatment for breast cancer. Although multiple studies have recently revealed the significance of immune cells as mediators of chemotherapy response in breast cancer, less is known regarding roles for leukocytes as mediating outcomes following radiotherapy. To address this question, we utilized a syngeneic orthotopic murine model of mammary carcinogenesis to investigate if response to radiotherapy could be improved when select immune cells or immune-based pathways in the mammary microenvironment were inhibited. Treatment of mammary tumor-bearing mice with either a neutralizing mAb to colony-stimulating factor-1 (CSF-1) or a small-molecule inhibitor of the CSF-1 receptor kinase (i.e., PLX3397), resulting in efficient macrophage depletion, significantly delayed tumor regrowth following radiotherapy. Delayed tumor growth in this setting was associated with increased presence of CD8\(^+\) T cells and reduced presence of CD4\(^+\) T cells, the main source of the TH2 cytokine IL4 in mammary tumors. Selective depletion of CD4\(^+\) T cells or neutralization of IL4 in combination with radiotherapy phenocopied results following macrophage depletion, whereas depletion of CD8\(^+\) T cells abrogated improved response to radiotherapy following these therapies. Analogously, therapeutic neutralization of IL4 or IL13, or IL4 receptor alpha deficiency, in combination with the chemotherapy paclitaxel, resulted in slowed primary mammary tumor growth by CD8\(^+\) T-cell–dependent mechanisms. These findings indicate that clinical responses to cytotoxic therapy in general can be improved by neutralizing dominant TH2-based programs driving protumorigenic and immune-suppressive pathways in mammary (breast) tumors to improve outcomes. Cancer Immunol Res; 3(5); 518–25. ©2015 AACR.

Introduction

Breast cancer is the most prevalent malignant disease of women in the developed world and remains one of the leading causes of death (1). Radiation and chemotherapy form the mainstay of treatment for patients with breast cancer, and more than half of all patients diagnosed with breast cancer will receive some form of radiotherapy (2). Although the antitumor properties of radiotherapy have long been considered a neoplastic cell-intrinsic process, evidence from multiple murine tumor models has demonstrated a critical role for immune cells in mediating radiotherapy response (3–6). Although much of the immune response against tumors has been attributed to T cells, myeloid cell bioactivity is also altered by radiotherapy and can regulate response to therapy (5, 6).

The presence of macrophages in multiple types of human cancer, including breast, ovarian, non–small cell lung cancer, mesothelioma, and Hodgkin lymphoma, correlates with poor clinical outcome, and it was associated with the ability of macrophages to enhance angiogenesis and promote the invasive and metastatic potential of malignant cells (7–11). The cytokine colony-stimulating factor (CSF)-1 plays a key role in recruitment and activation of macrophages following interaction with the CSF-1 receptor (CSF-1R), a high-affinity transmembrane receptor tyrosine kinase (12). Accordingly, in human breast cancer, colon cancer, and leiomyosarcoma, a CSF-1–response gene signature has been identified that predicts the risk of recurrence and metastasis (13–15). The ability to preferentially target macrophages via inhibiting the CSF-1/CSF-1R signaling pathway has led to several preclinical studies demonstrating therapeutic efficacy in glioblastoma, melanoma; and mammary, prostate, pancreas, and cervical carcinoma, either in combination with chemotherapy and radiotherapy or as monotherapy (16–20).

We and others have previously described a transgenic mouse model of aggressive mammary adenocarcinoma development in which late-stage carcinogenesis and pulmonary metastasis are regulated by CSF-1, tissue macrophages, and IL4-producing CD4\(^+\) T cells (21–23). Using this model, we recently reported that when macrophage infiltration into tumors was minimized via blockade of the CSF-1/CSF-1R pathway, platinum and paclitaxel–based chemotherapy responses were improved in a CD8\(^+\) T-cell–dependent manner (16) regulated by IL12-expressing dendritic cells (24). Based on these studies, we hypothesized that response to radiotherapy, like response to chemotherapy, could be improved by either reducing the presence of macrophages in tumors or by blocking induction of their TH2 polarization. Herein, we report...
that regrowth of mammary tumors following radiotherapy correlates with influx of T_{h}2-polarized macrophages and inhibiting macrophage recruitment after radiotherapy by neutralizing CSF-1 or blocking CSF-1R kinase activity significantly slows tumor regrowth. Moreover, our data indicate that radiotherapy promotes T_{h}2 polarization of infiltrating IL4-expressing CD4^{+} T cells, and that either depleting these or neutralizing IL4 slows tumor regrowth following radiotherapy, thus demonstrating that therapeutic targeting of T_{h}2 cytokines and the myeloid cells they regulate can enhance response to cytotoxic therapy.

**Materials and Methods**

Preclinical mouse models and animal husbandry

FVB/n strain background mice harboring the polyoma middle T (PyMT) transgene under the control of the mouse mammary tumor virus (MMTV) promoter have been described previously (23, 25). To generate a syngeneic orthotopic implantable tumor model, we prepared single-cell suspensions of tumor cell pools isolated from mammary tumors of 100-day-old MMTV-PyMT mice, and injected 1.0 million (total) tumor cells diluted in medium and basement membrane extract (Matrigel; BD Phar-mingen) orthotopically into unclered mammary fat pads (4th gland) of 10-week-old virgin FVB/n female mice (16). Following implantation, tumors were allowed to grow to an average diameter of 1.0 cm before randomization and enrollment into studies. Mice were randomized into treatment groups, e.g., PLX3397, a neutralizing anti-CSF1 mAb (5A1), or CD4 mAb (GK1.5), or CD8 (YTS169.4), or IL4 mAb (11B11), based on tumor size and subsequently irradiated with a single fraction of 5 Gy focal gamma irradiation (Mark III Cesium Irradiator) generated from a cesium source with mice mounted in a custom shielding apparatus (Fig. 1A). PLX3397 (16) was formulated in mouse chow so that the average dose per animal per day was 40 mg/kg, and chow containing vehicle alone was used as a control. Antibodies were injected i.p. at 1.0 mg to start, followed by 500 μg every 5 days. Depletion of CD4^{+} and CD8^{+} cells was confirmed by flow cytometry of peripheral blood before initiating therapy. Pharmaceutical grade paclitaxel (Hospira) was administered i.v. every 5 days at 10 mg/kg as described (16, 24). Neutralization of IL13 was achieved via administration of a rat anti-mouse IL13 mAb (54D1) injected i.p. at 1.0 mg to start, followed by 500 μg every 5 days. Tumor burden was evaluated by caliper measurement on anesthetized mice every 2 to 5 days. Before tissue collection, mice were cardiac perfused with PBS to clear peripheral blood. As described previously (23), homozygous null IL4Rα mice were obtained from The Jackson Laboratories. To generate PyMT mice on the IL4Rα-/- background, IL4Rα-/- mice were backcrossed into the FVB/n strain to N6 and then intercrossed with PyMT mice to generate breeding colonies. Mice were maintained either within the University of California, San Francisco Laboratory for Animal Care barrier facility or the Oregon Health and Science University Department of Comparative Medicine barrier facility. Experiments involving animals were approved by the Institutional Animal Care and Use Committees at the respective institutions.

**Flow cytometry analysis**

Single-cell suspensions were prepared from mammary tumors dissociated by manual mincing and enzymatic digestion for 40 minutes at 37°C using collagenase A (3.0 mg/mL; Roche) and DNase I (Roche) dissolved in DMEM (Invitrogen) under stirring conditions. Digestion mixtures were quenched by adding DMEM containing 10% FBS and then filtered through 0.7-μm nylon strainers (Falcon). Cells were then incubated for 10 minutes at 4°C with rat anti-mouse CD16/CD32 mAb (BD Biosciences) at a 1:100 dilution in PBS containing 1.0% of BSA (Sigma) to prevent nonspecific antibody binding. Subsequently, cells were washed...
Immune cell isolation

Immune cells were isolated from tumors using a dual-purification strategy, including magnetic purification followed by flow sorting. Single-cell suspensions from tumors were generated as described above. Cells were incubated for 10 minutes at 4°C with rat anti-mouse CD316/CD32 mAb (BD Biosciences) at a 1:100 dilution in PBS/BSA and then washed twice in PBS/BSA and incubated for 20 minutes with appropriate fluorescent primary antibodies that included CD45-APC (30-F11), CD4 (145-2C11), CD8α (53-6.7), CD11b (M1/70), CD11c (N418), CD14 (Sa2-8), CD19 (MB19-1), Ly6C (HK1.4), Ly6G (1A8), CD44 (IM7), CD45 (30-F11), CD80 (16-10A1), CD86 (GL1), CD115 (AF598), F4/80 (BM8), and/or MHCII (M5/114.15.2; all from eBioscience) followed by two washes with PBS/BSA. 7-AAD (BD Biosciences) was added (1:10) to discriminate between viable and dead cells, or alternatively live/dead aqua was used (Invitrogen). Data acquisition and analysis were performed on a LSRII (BD Biosciences) using the FlowJo version 8.8 software (Tree Star).

Quantitative RT-PCR

Total tissue RNA was extracted from cells using an RNeasy Mini kit (Qiagen). cDNAs were synthesized using Superscript III first-strand synthesis (Invitrogen). Primers specific for β-actin, GAPDH, 18S, IFNγ, IL2, IL4, IL12p35, IFNε, ARG1, and IL34 (Superarray) were used, and relative gene expression was determined using RT2 Real-Time SYBR Green/ROX PCR master mix (Superarray) on an ABI 7900HT quantitative PCR machine (ABI biosystems). Data were normalized to β-actin and GAPDH and/or 18S as reference genes. Alternatively, real-time PCR for gene expression was performed using individual TaqMan Assays following a preamplification step (Life Technologies), and data normalized to Tbp expression. The comparative threshold cycle method was used to calculate fold change in gene expression for all experiments.

Results

CSF-1/CSF-1R blockade delays primary tumor regression following radiotherapy

Using a syngeneic orthotopic murine explant model of mammary carcinoma growth, we first evaluated varying doses of radiation for effects on tumor growth and immune composition. Similar regression kinetics were observed following a single dose of 2, 5, or 8 Gy (Fig. 1A; Supplementary Fig. S1A); however, only 5 Gy provided a sufficient dose to induce a period of tumor stasis, followed by tumor regression approximately 8 to 12 days later (Fig. 1A). Analysis of immune infiltrates in mammary tumors at distinct time points following radiotherapy revealed reduced presence of CD45+ cells immediately following radiotherapy (day 2), with infiltration restored shortly thereafter (day 4), and markedly elevated 2 weeks later (day 14; Fig. 1B). Although CD11b+ F4/80+ macrophage infiltration was not altered during tumor regression (day 2) or stasis (day 4), macrophages were significantly increased during the tumor regression phase (day 14), even as compared with untreated tumors growing at a similar rate (Fig. 1B). Macrophages were also significantly increased on day 14 by 8 Gy, but not by 2 Gy radiation (Supplementary Fig. S1B). Gene expression analysis of infiltrating macrophages revealed increased acute expression of biomarkers, representing both classically and alternatively activated macrophages after radiotherapy, that returned to baseline as tumors initiated regrowth around day 10 (Fig. 1C), thus indicating that one consequence of acute radiotherapy is altered gene expression programs in resident leukocytes.

In a previous study, we reported that radiotherapy promotes CSF-1 expression by MMTV-PyMT-derived mammary epithelial
cells in vitro (16); herein, we sought to evaluate whether delivery of immunologic or pharmacologic agents neutralizing the CSF-1/CSF-1R pathway could provide a therapeutic benefit to radiotherapy. To address this question, mice bearing syngeneic orthotopic mammary explant tumors were treated with either a neutralizing mAb against CSF-1 (clone 5A1) or a competitive ATP inhibitor selective for CSF-1R and cKIT receptor tyrosine kinases (PLX3397) currently being evaluated clinically (16, 20, 26, 27) 2 days following radiotherapy. Importantly, as low-dose radiotherapy has been reported to promote T-cell responses via macrophage repolarization (28), we initiated treatment 2 days following radiotherapy, with full depletion of macrophages anticipated approximately 3 days later (17). Treatment of mice with either αCSF-1 mAb (Fig. 2A) or PLX3397 (Fig. 2B) resulted in significantly delayed/slowed tumor regrowth following radiotherapy, with no effect observed with either agent as monotherapy. Similar to our previous findings with these agents when delivered in combination with chemotherapy (16), the majority of tumor-infiltrating macrophages were efficiently eliminated by αCSF-1 mAb or PLX3397, and this was associated with an approximately 2-fold increase in CD8^+ T-cell presence (Fig. 2C; Supplementary Fig. S2).

**TH2 Polarization Limits Radiotherapy**

A and B, mRNA levels of Il4 (B) and Il13 (C) in FACS-sorted populations of epithelial and stromal populations from untreated MMTV-PyMT mammary tumors, as determined by real-time PCR. Data are normalized to Tbp expression and displayed as mean ± SEM with n = 8 per cell type. DC, dendritic cell; M/M0, macrophage; mono, monocyte. C, CD4^+ CD8^+ T cells were FACS sorted from orthotopic PyMT-derived explant tumors at days 1 and 10 following radiotherapy (5 Gy). mRNA expression from sorted cells was then analyzed using quantitative real-time PCR for the indicated genes. Treatment schematic is depicted at the top, and data are expressed as mean fold change ± SD compared with untreated tumors (4 mice/group). Statistical significance was determined by an unpaired t test relative to untreated controls, or between days 1 and 10 as indicated. D, orthotopic PyMT-derived explant tumors were grown to a median diameter of 1 cm, and then the tumor-bearing mice were enrolled in the study. On day 1, mice were subjected to localized gamma irradiation (5 Gy), and total tumor burden/animal assessed every 3 days until endpoint. αCD4-depleting antibody was administered 2 days before radiotherapy and then every 5 days for the duration of the study. Treatment schematic is depicted at top, and data are displayed as mean tumor burden ± SEM (>8 mice/group). Statistical significance was determined by two-way ANOVA. One of two experiments is shown.

For all figures, statistical significance is shown as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001.

**Figure 3.**

**TH2-polarized CD4^+ T cells promote tumor regrowth after radiotherapy (RT).** A and B, mRNA levels of Il4 (B) and Il13 (C) in FACS-sorted populations of epithelial and stromal populations from untreated MMTV-PyMT mammary tumors, as determined by real-time PCR. Data are normalized to Tbp expression and displayed as mean ± SEM with n = 8 per cell type. DC, dendritic cell; M/M0, macrophage; mono, monocyte. C, CD4^+ CD8^+ T cells were FACS sorted from orthotopic PyMT-derived explant tumors at days 1 and 10 following radiotherapy (5 Gy). mRNA expression from sorted cells was then analyzed using quantitative real-time PCR for the indicated genes. Treatment schematic is depicted at the top, and data are expressed as mean fold change ± SD compared with untreated tumors (4 mice/group). Statistical significance was determined by an unpaired t test relative to untreated controls, or between days 1 and 10 as indicated. D, orthotopic PyMT-derived explant tumors were grown to a median diameter of 1 cm, and then the tumor-bearing mice were enrolled in the study. On day 1, mice were subjected to localized gamma irradiation (5 Gy), and total tumor burden/animal assessed every 3 days until endpoint. αCD4-depleting antibody was administered 2 days before radiotherapy and then every 5 days for the duration of the study. Treatment schematic is depicted at top, and data are displayed as mean tumor burden ± SEM (>8 mice/group). Statistical significance was determined by two-way ANOVA. One of two experiments is shown. For all figures, statistical significance is shown as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure 4.
IL4 blockade slows tumor regrowth following radiotherapy (RT) in a CD8⁺ T-cell-dependent manner. A and B, surface expression of IL4Rα (A) and IL13Rα1 (B) as measured by mean fluorescence intensity (MFI) minus background in cells isolated from MMTV-PyMT mammary tumors as indicated, and as compared with equivalent populations in the spleens of non-tumor-bearing animals (lower graphs). (Continued on the following page.)
contrast with our previous findings in which CD4\(^+\) T-cell infiltration was unaffected by CSF-1/CSCf-1R blockade as monotherapy, or in combination with chemotherapy (16, 24). That said, others have reported that a consequence of radiotherapy is altered expression of macrophage-expressed chemokines regulating T-cell infiltration in radiation-induced pneumonitis (29). In agreement with our previous results (16), CD4\(^+\) T cells in mammary tumors of MMTV-PyMT mice were main sources of IL4 (Fig. 3A) and IL13 (Fig. 3B), which are both significant cytokines regulating macrophage polarization. We therefore examined gene expression profiles of CD4\(^+\) T cells from mammary tumors by FACS-sorting cells 1 and 10 days after radiotherapy. We found that mRNA for Th2 cytokines, including IL4, IL5, and IL13, and the Th2 transcription factor GATA-3, were elevated at least 3-fold on day 1 after radiotherapy, with levels extending until endpoint.

IL4 neutralization enhances antitumor immunity in response to radiotherapy

Based on these data, we next sought to evaluate the efficacy of neutralizing IL4 to determine if suppression of its Th2-polarizing effects on immune and/or nonimmune cells influenced tumor growth and/or response to radiotherapy. IL4 and IL13 bind multimeric receptor complexes involved in Th2 cell biology. Th2 cells express IL4/IL13R\(\alpha\) (type II receptors (IL4R\(\alpha/\gamma C\), IL13R\(\alpha 1\), or IL13R\(\alpha 2\) subunits, type I receptors (IL4R\(\alpha/\gamma C\)) are activated exclusively by IL4, whereas both IL4 and IL13 bind type II receptors (IL4R\(\alpha/IL13R\alpha 1\); ref. 30). In mammary tumors of MMTV-PyMT mice, both receptor types are broadly expressed by immune cells infiltrating tumors, as well as by fibroblasts (Fig. 4A and B). Increased IL4R\(\alpha\) was particularly pronounced in the MHCI\(^{\text{low}}\) subset of macrophages (Fig. 4A), which are known to exhibit enhanced tumor-promoting properties (24, 31).

Neutralization of IL4 following radiotherapy significantly slowed tumor growth following radiotherapy as compared with either radiotherapy or anti-IL4 mAb alone (Fig. 4C), similar to CSF-1/CSCf-1R blockade and CD4 depletion (Figs. 2 and 3).

Analysis of the immune composition of tumors following anti-IL4 mAb/radiotherapy similarly revealed a significantly decreased presence of CD4\(^+\) T cells and significantly increased CD8\(^+\) T cells in the tumor parenchyma (Fig. 4D), which was significant as CD8\(^+\) T-cell depletion before radiotherapy abrogated improved responses (Fig. 4E), similar to the responses following CSF-1/CSCf-1R blockade (16, 24). To determine if this improved response extended to chemotherapy, we evaluated mammary tumor growth in syngeneic IL4R\(\alpha\)-deficient mice and wild-type mice treated with anti-IL4 mAb, with and without paclitaxel, and found significantly reduced primary tumor growth as compared with mice treated with paclitaxel or anti-IL4 mAb alone (Fig. 4F and G).

Improved responses to paclitaxel were similarly dependent on induced CD8\(^+\) T-cell infiltration (Fig. 4G). Because IL4R\(\alpha\) acts as a receptor for both IL4 and IL13, we also evaluated the effects of IL13 neutralization and found that anti-IL4 and anti-IL13 mAb produced equivalent effects on de novo mammary adenocarcinoma growth in combination with paclitaxel in transgenic MMTV-PyMT mice, with no additive effects observed when anti-IL4/anti-IL13 were used in combination (Fig. 4H).

Discussion

Results reported herein from our study using a murine orthotopic explant model of primary mammary carcinoma reveal that a consequence of radiotherapy is induced recruitment of immunosuppressive F4/80\(^+\) macrophages that limit radiotherapy (and chemotherapy) efficacy. Recruitment of these protumorigenic immune cells is in part due to increased expression of CSF-1 and IL34 by mammary epithelial cells following cellular ‘damage’ induced by radiotherapy (and chemotherapy) observed in both murine mammary models and clinical breast tumors following therapy (16, 32). Selective macrophage depletion resulting from CSF-1/CSCf-1R blockade improved responses to radiotherapy and was associated with decreased presence of type 2 cytokine-expressing CD4\(^+\) T cells. Whether macrophages are directly responsible for CD4\(^+\) T-cell recruitment is unclear; that said, in other studies, we reported that therapeutically blocking humoral-based pathways regulating protumoral macrophage programming, in combination with chemotherapy, induced T\(_{H}1\)-type programing of
macrophages that recruit CD8+ T cells via increased expression of macrophage-derived chemokines (33), similar to results reported for radiation-induced pneumonitis implicating macrophage-derived CCL22 and CCL17 (ligands forCCR4; ref. 29). Depletion of CD4+ T cells or neutralization of the type 2 cytokines, i.e., IL4 or IL13 that they express, phenocopied CSF-1/CSF-1R blockade without depletion of macrophages. Improved responses to radiotherapy resulting from these treatments were dependent on the increased presence of CD8+ T cells in irradiated tumors as their selective depletion before radiotherapy abrogated the improved outcomes.

Based on subcutaneous xenograft models of head and neck cancer (34) and B16 melanoma (35), others have reported that high-dose radiotherapy leads to increased presence of CD11b+ myeloid cells that significantly affects outcome, as therapeutic use of CD11b-blocking mAbs or administration of clodronate liposomes restricted tumor regrowth associated with reduced myeloid-dependent angiogenesis (34, 36). Researchers from other studies in melanoma have reported that radiotherapy as monotherapy promotes CD8+ T-cell infiltration (35), and that CSF-1R blockade improves efficacy of adoptive T-cell transfer (18, 36). These results are in agreement with findings herein and elsewhere (16, 24, 33) that macrophages in tumor parenchyma limit CD8+ T-cell recruitment and effector function, and thereby restrict efficacy of cytotoxic therapies.

In mouse models of pancreatic neuroendocrine tumor growth, low-dose (2 Gy) radiotherapy improved efficacy of adoptive T-cell transfer by mechanisms involving polarization of macrophages from expressing high levels of Ym-1 and Arginase-1 to instead expressing elevated inducible nitric oxide synthase, changes that correlated with enhanced recruitment of cytotoxic CD8+ T cells (28). Using 60-Gy doses, others have reported that focal radiotherapy induces expression of genes associated with Tnfr2-type macrophage activation (37, 38). In the mammary carcinoma model used herein, low-dose (2 Gy) radiotherapy alone was without consequence (data not shown), whereas 5-Gy focal radiotherapy led to enhanced expression of genes associated with both Tnfr1-type (Il1a, Il1b, Tnfα) and Tnfr2-type (C16d, Mrct1, Arg1) macrophage activation. The polarization state of macrophages and other infiltrating cells is significant as blockade of IL4Rα signaling similarly improved radiotherapy (and chemotherapy) response by CD8+ T-cell–dependent mechanisms. In human breast cancer, CD4+ T cells are biased toward production of IL4 and IL13 (39), a phenotype that is recapitulated in MMTV-PyMT transgenic mice (23). Although our results revealed that type 1 and 2 IL4/13 receptors were both broadly expressed by tumor-infiltrating stromal cells, it remains unclear whether IL4 directly or indirectly suppresses CD8+ T-cell recruitment/function. We favor the hypothesis that IL4 suppresses CD8+ T-cell activity indirectly through regulation of macrophage polarization, as we and others have reported (23, 40), and consistent with a role for macrophages in mediating resistance to radiotherapy and chemotherapy. However, it is possible that IL4 directly suppresses CD8+ T-cell cytotoxic effector function (41), and in other tumor types, malignant cells also express IL4Rα (42, 43).

Based on compelling preclinical data revealing improved responses to chemotherapy and delayed/slowed solid tumor growth following CSF-1R blockade (16, 17, 44, 45), clinical trials evaluating efficacy of CSF-1R antagonists in several types of solid tumors are ongoing (NCT01596751, NCT01349036, NCT01004861, and NCT01346358). Salient for translation to the clinic regarding macrophage repolarization versus depletion is the fact that whereas macrophage depletion before radiotherapy was without consequence, IL4 neutralization before radiotherapy yielded an improved outcome. Based on data herein that IL4 neutralization also improved response to chemotherapy, we assert that IL4 and/or IL13 neutralization represents a compelling immune-based strategy for improving responses in patients receiving either chemotherapy or radiotherapy.

**Disclosure of Potential Conflicts of Interest**

C. C. Park has ownership interest (including patents) in OncoSynergy. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**

Conception and design: S. L. Shiao, B. Ruffell, D. G. DeNardo, L. M. Coussens


Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. L. Shiao, B. Ruffell, D. G. DeNardo, B. A. Faddegon, L. M. Coussens

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. L. Shiao, B. Ruffell, D. G. DeNardo, B. A. Faddegon, C. C. Park, L. M. Coussens

Writing, review, and/or revision of the manuscript: S. L. Shiao, B. Ruffell, C. C. Park, L. M. Coussens

Study supervision: L. M. Coussens

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**T\_h2-polarized CD4\(^+\) T cells and macrophages limit efficacy of radiation therapy**

**Supplemental Data**

Stephen L. Shiao\(^1\), Brian Ruffell\(^2\), David G. DeNardo\(^3\), Bruce A. Faddegon\(^4\), Catherine C. Park\(^4\), Lisa M. Coussens\(^2\).*

\(^1\)Department of Radiation Oncology, Cedars-Sinai Medical Center, Los Angeles, CA 90048
\(^2\)Department of Cell, Developmental & Cancer Biology and Knight Cancer Institute, Oregon Health & Science University, Portland, OR
\(^3\)Department of Medicine, Department of Pathology and Immunology, and Siteman Cancer Center, Washington University School of Medicine, St Louis, MO 63110.
\(^4\)Department of Radiation Oncology, Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, San Francisco, CA 94143

*Address for correspondence:
Lisa M. Coussens, Ph.D.
Cell, Developmental & Cancer Biology
Knight Cancer Institute
Oregon Health & Science University
3181 SW Sam Jackson Park Road
Portland, OR 97239-3098
Voice: 503-494-7811
Fax: 503-494-4253
email: coussenl@ohsu.edu
Supplemental Figure 1: Radiation dose response and immune profile. **A** Orthotopic MMTV-PyMT-derived explant tumors were grown to a median diameter of 1.0 cm for study enrollment (day 0), and one day later received localized gamma irradiation (2 or 8 Gy). Total tumor burden/animal was then assessed every 3 days until endpoint. Treatment schematic is depicted at top and data are displayed as mean tumor burden ± SEM (>5 mice/group). Significance was determined by two-way ANOVA. One of two experiments is shown. **B** Number of CD3⁺CD4⁺, CD3⁺CD8⁺, and CD11b⁺F4/80⁺ cells within tumors 14 days following RT in groups that were untreated, treated with RT (2 Gy), RT (5 Gy) or RT (8 Gy). Data are depicted as mean number of cells ± SEM as a % of CD45⁺ cells as analyzed by flow cytometry (>5 mice/group). Significance was determined by an unpaired t-test relative to untreated controls. For all panels, significance is shown as *p < 0.05, **p < 0.01, ***p < 0.001.

Supplemental Figure 2: Immune profile with RT in combination with αCSF-1 mAb or PLX3397. **A** Number of CD3⁺CD4⁺, CD3⁺CD8⁺, and CD11b⁺F4/80⁺ cells within tumors 4 days following RT (2 days after administration of αCSF-1 mAb) in groups that were untreated, treated with αCSF-1 mAb alone, RT (5 Gy) alone or treated with a combination of RT and αCSF-1 mAb. **B** Number of CD3⁺CD4⁺, CD3⁺CD8⁺, and CD11b⁺F4/80⁺ cells within tumors 4 days following RT (2 days after administration of PLX3397) in groups that were untreated, treated with PLX3397 alone, RT (5 Gy) alone or treated with a combination of RT and PLX3397. Data are depicted as mean number of cells ± SEM as a % of CD45⁺ cells as analyzed by flow cytometry (>5 mice/group). For all figures significance is shown as *p < 0.05, **p < 0.01, ***p < 0.001.
Shiao et al., Supplemental Figure 1

**A**

- Tumor volume (mm$^3 \times 1000$) vs. Days of treatment
- Graph showing tumor volume changes with and without RT (2 Gy and 8 Gy).

**B**

- % CD45$^+$ cells vs. Days of treatment
- Graph showing changes in % CD45$^+$ cells with different RT treatments (2 Gy, 5 Gy, 8 Gy).

Legend:
- Control
- 2 Gy
- 5 Gy
- 8 Gy

* and ** indicate statistical significance.