CSF1R Signaling Blockade Stanches Tumor-Infiltrating Myeloid Cells and Improves the Efficacy of Radiotherapy in Prostate Cancer

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Abstract

Radiotherapy is used to treat many types of cancer, but many treated patients relapse with local tumor recurrence. Tumor-infiltrating myeloid cells (TIM), including CD11b (ITGAM)⁺/F4/80 (EMR1)⁺ tumor-associated macrophages (TAM), and CD11b⁺Gr-1 (Ly6G)⁺ myeloid-derived suppressor cells (MDSC), respond to cancer-related stresses and play critical roles in promoting tumor angiogenesis, tissue remodeling, and immunosuppression. In this report, we used a prostate cancer model to investigate the effects of irradiation on TAMs and MDSCs in tumor-bearing animals. Unexpectedly, when primary tumor sites were irradiated, we observed a systemic increase of MDSCs in spleen, lung, lymph nodes, and peripheral blood. Cytokine analysis showed that the macrophage colony-stimulating factor CSF1 increased by two-fold in irradiated tumors. Enhanced macrophage migration induced by conditioned media from irradiated tumor cells was completely blocked by a selective inhibitor of CSF1R. These findings were confirmed in patients with prostate cancer, where serum levels of CSF1 increased after radiotherapy. Mechanistic investigations revealed the recruitment of the DNA damage-induced kinase ABL1 into cell nuclei where it bound the CSF1 gene promoter and enhanced CSF1 gene transcription. When added to radiotherapy, a selective inhibitor of CSF1R suppressed tumor growth more effectively than irradiation alone. Our results highlight the importance of CSF1/CSF1R signaling in the recruitment of TIMs that can limit the efficacy of radiotherapy. Furthermore, they suggest that CSF1 inhibitors should be evaluated in clinical trials in combination with radiotherapy as a strategy to improve outcomes. Cancer Res; 73(9): 2782–94. ©2013 AACR.

Introduction

Radiotherapy is one of the primary treatments for prostate cancer. Approximately 30% of patients are treated with radiotherapy either alone or in combination with other therapies (1). Data from Cancer of the Prostate Strategic Urologic Research Endeavor identified that 63% of patients experienced biochemical prostate-specific antigen (PSA) recurrence after radiotherapy (2). In fact, D’Amico and colleagues determined that a high rate of PSA velocity pretreatment is significantly associated with a shorter time to not only PSA recurrence but also prostate cancer-specific mortality after radiotherapy (3), and most of the recurrence is local (4). Several studies have addressed the importance of hypoxia and the SDF-1/CXCR4 axis in promoting tumor regrowth after radiotherapy in brain tumor and breast cancer (5, 6). However, a better understanding of the mechanisms of tumor regrowth is needed to achieve increased local control by radiotherapy in prostate cancer and improve the cure rate of this disease.

Solid tumors contain a significant population of tumor-infiltrating myeloid cells (TIM; ref. 7). TIMs are now recognized as important mediators of not only tumor progression and metastasis (8), but also therapeutic resistance (9, 10), through promoting angiogenesis and suppressing antitumor immune responses (11, 12). The protumorigenic role of “alternatively” activated macrophages has been well established (12). Recently, another specific subtype of TIMs, namely myeloid-derived suppressor cells (MDSC), is receiving great attention in cancer research. MDSCs comprise a heterogeneous population of immature myeloid cells that originate in the bone marrow and are recruited to the tumor by a diverse array of cytokine and chemokine signals. Similar to tumor-associated macrophages (TAM; ref. 8), MDSCs have been shown to generate an environment favorable for tumors by heightening immunosuppression, angiogenesis, and invasion (13–15). Various cell surface markers are used to identify TIM subsets. TAMs can be

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identified by CD11b and F4/80, and MDSCs by CD11b and Gr-1 coexpression in murine models (11, 16). Macrophage colony-stimulating factor (M-CSF or CSF1) is a potent growth factor that promotes the differentiation, proliferation, and migration of monocytes/macrophages via signaling through its receptor tyrosine kinase CSF1R (cFMS; refs. 17, 18). We recently showed that TAMs and MDSCs form a spectrum of bone marrow-derived myeloid cells dependent on CSF1/CSF1R signaling for recruitment into the tumor and that they play critical roles in tumor growth (15). In addition, DeNardo and colleagues highlighted the importance of CSF1/CSF1R signaling in the recruitment of TAMs in breast cancer and further showed that CSF1R blockade can inhibit TAMs in chemotherapy and improve treatment outcome (19).

ABL1 (c-Abl) is an ubiquitously expressed nonreceptor tyrosine kinase that has been implicated in many cellular processes including cell migration, differentiation, apoptosis, and gene regulation (20–22). ABL1 has also been implicated in the proliferation and metastasis of melanoma and breast cancer cells (23–25). In the present study, we show that the infiltration of TIMs is significantly enhanced by local irradiation of prostate cancer. In addition, CSF1 mRNA and CSF1 secretion is increased following radiotherapy through an ABL1-dependent mechanism. We further showed that blockade of CSF1/CSF1R signaling effectively reduces TIMs infiltration to tumors, thereby achieving more effective tumor growth suppression after irradiation. The rational combination therapy reported here may provide a more effective and durable treatment strategy for patients with prostate cancer.

Materials and Methods

Cell culture

Murine macrophage RAW264.7 cells (American Type Culture Collection), ras+myc-transformed RM-1, and RM-9 prostate tumor cells (kind gifts from Dr. Timothy C. Thompson, Baylor College of Medicine, Houston, TX), human glioblastoma cell lines U87 and U251 (a kind gift from Dr. Paul Mishel, University of California Los Angeles (UCLA), Los Angeles, CA), human breast cancer cell line MDA-MB-231, and mouse malignant peripheral nerve sheath tumor cells (MMPNST, a kind gift from Dr. Hong Wu, UCLA) were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) containing 10% FBS, 100 U/mL penicillin, and 15 mmol/L HEPES at 37°C with 5% CO2. Human prostate cancer cells line CWR and LNCaP, and the human carcinoma cell line A549 were cultured in RPMI-1640 medium containing 10% FBS, 100 U/mL penicillin, and 15 mmol/L HEPES at 37°C with 5% CO2. Cell lines were periodically authenticated by morphologic inspection and tested negative for mycoplasma contamination by PCR tests.

Chromatin precipitation

Treated Myc-CaP cells were cross-linked with 1% formaldehyde at room temperature for 15 minutes. The cells were then washed with PBS and processed according to the manufacturer’s instruction using Pierce Agarose ChIP Kit (Thermo Scientific). c-Abl antibody (Santa Cruz Biotechnology) and RNA polymerase II (Santa Cruz Biotechnology) were used for immunoprecipitation. The following primers were used for detecting CSF1 promoter sequences: Forward: 5’ATGTGT1CA GTGCCCTGTAGTTGTG3’, Reverse: 5’GCCAGGGTATTTCC-CATAAACCA 3’; CSF1 control sequences: Forward: 5’TGCAGA-GAGCACCCTGAAATGGC3’, Reverse: 5’ATGCCAAAGCCT-GCAGTTAACCC3’.

Human serum assessment

Sera, collected before and after radiotherapy, from human prostate cancer patients were obtained from by the Department of Radiation Oncology, UCLA Medical Center Hospital, with informed consent according to US federal law and are exempt from consideration by the UCLA Administrative Panel on Human Subjects in Medical Research. Analysis of CSF1 was done by EvE Technologies using the human CSF1 multiplex kit (Bio-Rad).

In vivo tumor models

C57BL6 male mice (4 to 8 weeks old) were purchased from Jackson Laboratory (Bar Harbor). RM-1 (2.5 × 10^5 cells), RM-9 (2.5 × 10^5 cells), or Myc-CaP (2 × 10^5 cells) were implanted subcutaneously in the thigh, and treatment was initiated when tumors reached 4 mm in diameter. All animal experiments were approved by the UCLA Institutional Animal Care and Use Committee and conformed to all local and national animal care guidelines and regulations. Tumor size was measured by digital calipers daily or every 2 days, depending on the model. Mice were sacrificed and tissues were analyzed at the ethical tumor size limit of 1.5 cm in diameter. For GW2580 treatment, mice were treated with control diluent (0.5% hydroxypropyl methylcellulose; Sigma-Aldrich; 0.1% Tween20 in distilled H2O) or GW2580 (160 mg/kg) by oral gavage beginning on the same day irradiation treatment started. PLX3397 was provided in food chow together with daily food consumption.

In vitro migration assay

RAW264.7 (1.5 × 10^5 cells) were seeded in cell culture inserts (8 µm pore size; BD Falcon) in DMEM containing 0.1% FBS with or without 1,000 nmol/L GW2580. Inserts were placed in 24-well plates with tumor-conditioned media collected 48 hours after irradiation treatment (3 Gy). After 6 hours, migrated cells were immediately fixed in 3% formaldehyde and stained with 4,6-diamidino-2-phenylindole (DAPI). Nine fields per well at ×4 magnification were quantified using ImageJ Version 1.34s (NIH).

Immunohistochemistry

Tissues were harvested and fixed in 3% paraformaldehyde overnight. Sections (5 µm) were stained with the following antibodies: anti-F4/80 (1:500; Serotec), anti-Gr-1 (1:100; eBioscience), or anti-CD31 (1:300; BD Biosciences) antibodies. Histology was conducted, processed, and quantified as previously described (15). The samples were analyzed using an Olympus BX41 fluorescent microscope fitted with a Q-Imaging QICAM FAST 1394 camera. Images were captured at ×4, ×10, or ×20 magnification using QCapture Pro Version 5.1 (Media Cybernetics), and quantified using ImageJ Version 1.34s (NIH).
Immunofluorescence microscopy

Cells seeded on cover slips were fixed with 3% formaldehyde and incubated with c-Abl antibody (K-12, 1:100; Santa Cruz Biotechnology) followed by AlexaFluor 488 rabbit anti-mouse (Invitrogen), and then AlexaFluor 568 phalloidin and DAPI (Invitrogen). Mounting medium (Pro-Long Gold Antifade Reagent; Invitrogen) was applied and cover slips were sealed with clear nail polish. Fluorescent images were acquired at room temperature on a confocal microscope, LSM710 (Carl Zeiss).

Flow cytometric analysis

To prepare single-cell suspensions for flow cytometry, harvested tissues (tumors or lungs) were dissected into approximately 1 to 3 mm³ fragments and digested with 80 U/mL collagenase (Invitrogen) in DMEM containing 10% FBS for 1.5 hours at 37°C while shaking. Spleens and lymph nodes were gently dissociated between 2 glass slides for single-cell isolation. Peripheral blood was isolated directly into BD Vacutainer K2 EDTA tubes (BD Biosciences). After red blood cell lysis (Sigma-Aldrich), single-cell suspensions were filtered and incubated for 30 minutes on ice with the following APC, PerCP-Cy5.5, PE, and APC-e780-conjugated antibodies (CD11b, Gr-1, CSF1R, and F4/80) were purchased from eBioscience (1:200). Ly6C (1:200) was purchased from BD Biosciences. DAPI was purchased from Invitrogen. Cells were washed twice before analysis on the BD LSR-II flow cytometer (Beckman Coulter). Data were analyzed with FlowJo software (TreeStar).

Local irradiation

Irradiation was carried out using a Gulmay X-ray machine (300 kV, 10 mA) with a dose rate of 1.84 Gy/min. When tumors reached 4 to 5 mm in diameter, mice were anesthetized and irradiated with a daily dose of 3 Gy for 5 days to the tumor area with the rest of body shielded.

Real-time reverse transcriptase-PCR analysis

Total cellular RNA was extracted from cells using TRI Reagent (Sigma Aldrich). RNA was isolated according to the TRizol procedure. RNA was quantified and assessed for purity by UV spectrophotometry and gel electrophoresis. RNA (1 μg) was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturer’s instructions. For each sample, 1 μL cDNA (~20 ng) was amplified using Syber Green 2× Master Mix (Bioline) and 10 μmol/L primers (Primer sequences are listed in Supplementary Table S1). The reaction was run on the My IQ single color iCycler real time PCR machine (Bio-Rad). Samples were amplified using the following cycling conditions: 40 cycles of 95°C/15 s, 60°C/30 s, and 72°C/30 s. Gene expression was determined by the ΔCt method and normalized to β-actin expression.

SDS-PAGE

For experiments using concentrated media, cells were plated in 150 mm tissue culture dish in 0% FBS DMEM overnight. Twenty milliliters of media were collected 48 hours after irradiation and subjected to media concentration using a 150 mm tissue culture dish in 0% FBS DMEM overnight. Twenty milliliters of media were collected 48 hours after irradiation and subjected to media concentration using a protein concentrator (Pierce Biotechnology) at a speed of 4,500 × g for 30 minutes. Total volume was normalized between samples and 40 μL was loaded onto 4% to 12% continuous gradient Tris–glycine gel (Invitrogen). For ABL1 cleavage experiments, cells were plated in 6-well plates overnight and collected after irradiation at the time indicated. Cells were lysed in radioimmunoprecipitation assay buffer (Upstate) containing protease inhibitor cocktail (Sigma), sonicated briefly, and centrifuged for 10 minutes at 12,000 × g. Twenty microliters of cell lysate was resolved on a 4% to 12% continuous gradient Tris–glycine gel (Invitrogen). The gels were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) and incubated with primary antibodies: anti-ABL1 (K-12, rabbit polyclonal; Santa Cruz Biotechnology), dilution 1:1,000; anti-CSF1 (H-300, rabbit polyclonal, dilution 1:20,000; Santa Cruz Biotechnology), dilution 1:1,000; anti-GAPDH (A-3, mouse monoclonal; Santa Cruz Biotechnology).

Statistical analysis

Data are presented as mean ± SEM. Statistical comparisons between groups were carried out using the Student t test.

Results

Local irradiation enhances myeloid cell infiltration to tumors

Abundant evidence points to the infiltrating myeloid cells exerting significant influences on tumor cell aggression and the immunologic environment. Therefore, we studied the recruitment of irradiation-induced TIMs in 2 immunocompetent murine prostate cancer models, namely RM-1 and Myc-Cell line syngeneic in the C57/BL6 and FVB strain, respectively. These models provide distinct host genetic background, tumor growth rate, degree of myeloid cells infiltration, and response to irradiation to broaden the perspectives on this issue. We first examined the recruitment of TIMs to tumors after irradiation in the RM-1, a Ras-, and Myc-transformed murine prostatic cancer model (26), with moderate level of TIMs infiltration. As shown in Fig. 1, irradiation effectively delayed the tumor growth by approximately 7 days (Fig. 1A). Control tumors and irradiated tumors were collected when they reached similar sizes (day 13 for control tumors and day 19 for irradiated tumors) and processed to assess their content of TIMs. Irradiation significantly induced the infiltration of F4/80+CD11b+ TAMs (Fig. 1B) and Gr-1+CD11b+ MDSCs (Fig. 1C) to the tumors. Immunohistochemical staining further confirmed this increase of TIMs in the tumors (Fig. 1D). Recent reports further distinguished the myeloid subsets within MDSCs as consisting of MO-MDSCs (CD11b+Ly6C+CD11c−) and PMN-MDSCs (CD11b+Ly6C−CD11c+), each with different functional characteristics (27). We found that both MO-MDSCs and PMN-MDSCs were significantly induced by irradiation, with MO-MDSCs showing a larger increase (Fig. 1E). Likewise, the irradiation-induced TIMs recruitment was also observed in the RM-9 tumor, a C57BL6 compatible model derived in the same manner as RM-1, and Myc-Cell line tumor, a myc oncogene-driven model implantable in FVB host (Supplementary Figs. 1 and 2). Taken together, these data show that local irradiation enhances...
recruitment of both TAMs and MDSCs to tumors in several murine prostate cancer models.

**Local irradiation enhances systemic myeloid cell expansion**

To better characterize the potential systemic impact of local tumor irradiation, we examined the infiltration of MDSCs and TAMs in peripheral tissues at different time points after irradiation, providing insights on the dynamics and kinetics of the myeloid cell recruitment process. The level of CD11b⁺F4/80⁺ macrophages were low in lungs, spleens, lymph nodes, and blood; therefore, we focused on CD11b⁺Gr-1⁺ MDSCs in the systemic sites and analyzed the content of CD11b⁺F4/80⁺ macrophages only in the tumor (Fig. 2B). As shown in Fig. 2, before irradiation, the baseline levels of MDSCs in tumors, blood samples, and spleens were 1.5 ± 0.9%, 30.1 ± 1.3%, and 3.4 ± 1.1%, respectively, and the levels in the lungs and lymph nodes were negligible. In untreated mice, MDSC levels stay the same or only mildly increase over time (Fig. 2). In irradiated mice, within 2 days after irradiation, MDSCs in the peripheral blood doubled to 69.0 ± 6.8% (Fig. 2C), whereas MDSCs stayed relatively stable in spleens, lymph nodes, and lungs (Fig. 2D–2F). In the irradiated tumor, there was a sustained low level of tumor MDSCs throughout and beyond the duration of irradiation although they increased nearly 4-fold (1.5 ± 0.9% to 6.5 ± 1.9%) in nonirradiated tumors. On day 12, 2 days after
cessation of tumor irradiation, MDSC levels reached their nadir in the tumors, but had begun to rise in the other organs. By day 15, a dramatic increase in the MDSC population was observed in the tumor, spleen, and lymph node, with levels reaching a peak of approximately 15% in all 3 sites (Fig. 2A, D, and F) before falling on day 17. In the blood and lungs, the trend of MDSC elevation continued from day 15 onward, reaching a remarkable level of 80.0/6.3% and 30.7/6.14.6%, respectively, on day 17. Collectively, these data suggest that local tumor irradiation induces the expansion of MDSCs and their subsequent influx into different organs in a time-dependent manner.

Irradiation induces macrophage migration and expression of protumorigenic genes

We next used an in vitro culture system to aid in dissecting the complex cross-signaling between different cellular components in the tumor microenvironment after irradiation. We first examined the effects of conditioned media from irradiated tumor cells on the migration ability of macrophages. RAW264.7 murine macrophages were analyzed in a Transwell migration assay with conditioned media from irradiated or nonirradiated murine prostate cancer cells (RM-1 and Myc-CaP) as the migration stimulus. Conditioned media from irradiated tumor cells induced a nearly 2-fold greater number of RAW264.7 cells to migrate across the Transwell filter compared with nonirradiated controls (Fig. 3A; data not shown for Myc-CaP).

A large volume of work points to the plasticity of tissue macrophages that are educated by tumor environmental cues to promote tumor growth (8). Therefore, we interrogated whether irradiation could skew bone marrow-derived macrophages (BMDM) toward the gene expression profiles of protumorigenic macrophages. Direct irradiation with 3 Gy (Fig. 3B) and indirect effects transmitted through coculturing BMDMs with irradiated tumor-conditioned media (Fig. 3C) both polarized BMDMs toward a protumorigenic phenotype, as we observed increased expression of Arg1, Fizz, IL-1β, IL-10, MMP-9, VEGF-A, CD206, and CSF1, and decreased expression of inflammatory genes such as iNOS and IL-12. As a reflection of their immunosuppressive roles in tumors, protumorigenic macrophages also typically exhibit lower MHCII expression (28). By fluorescence-activated cell sorting (FACS) analysis, all (100%) of the CD11b+/F4/80+ macrophages from irradiated tumors displayed low MHCII expression, whereas only 50% of the macrophages in nonirradiated tumors were MHCIIlow (Fig. 3D). These data suggest that both direct and indirect effects of irradiation can have profound effects on macrophages by skewing them toward a protumorigenic subtype with enhanced migration ability and gene expression profile that favor tumor growth.

CSF1 expression is increased by irradiation

Next, we examined whether tumor cell irradiation can alter the expression of cytokines known to participate in myeloid cell recruitment (6, 29). The expression of CSF1, CCL2, CCL5, and SDF-1 was examined in Myc-CaP cells 24 hours after 3 Gy of irradiation. Among these cytokines examined, CSF1 showed the highest expression and the most significant increase in irradiated over untreated tumor cells (Fig. 4A). We further evaluated secreted levels of CSF1 protein, which was elevated in conditioned media from irradiated tumor cells (Fig. 4B). IL-34 is a recently discovered second CSF1R ligand that functions similarly to CSF1 (30). However the expression of IL-34

Figure 2. A–F, local irradiation enhances systemic myeloid cell expansion. Control mice were sacrificed on days 6, 8, and 12. Irradiated mice were sacrificed on days 6, 8, 12, 15, and 17. Tumors, blood, spleens, lungs, and lymph nodes were collected for FACS analysis for MDSC and TAM population; n = 4 for each time point.
was 100-fold lower than CSF1 in our 2 prostate cancer systems (RM-1 and Myc-CaP model, data not shown) and, thus, we did not pursue IL-34 further. The ability of irradiation to augment CSF1 expression appeared to be a general phenomenon. In total, we tested 9 murine and human cancer cell lines and 8 of 9 showed an increase in CSF1 after irradiation (Fig. 4A and E; Supplementary Fig. S3). Consistent with results from cell culture experiments, irradiated tumors (the same cohort as Fig 1A) showed a significant increase in CSF1 gene expression compared with untreated tumors (Fig. 4C). Importantly, an increase in CSF1 was also observed in the serum of patients with prostate cancer after radiotherapy (Fig. 4D), supporting the clinical relevance of CSF1 increase seen in our murine models.

To explore the issue of cross-talk between tumor cells and macrophages on the CSF1 axis, we cocultured RAW264.7 macrophages or BMDMs with RM-1 tumor cells and observed an increase in the magnitude of CSF1 expression more than that in either cell grown alone, especially in the irradiation setting (Fig. 4E and F). Furthermore, the addition of a highly selective CSF1R kinase inhibitor GW2850 (31) resulted in a complete negation of increased macrophage migration toward irradiated tumor-conditioned media (Fig. 4G). Collectively, these data show that irradiation increases CSF1 expression in tumors, which is amplified by tumor–macrophage interactions. The heightened CSF1 production induced by irradiation in turn drives macrophage migration and recruitment into irradiated tumors.

Irradiation enhances CSF1 production through an ABL1-dependent mechanism

Given our finding shown above that irradiation boosts tumoral CSF1 expression, we sought to interrogate a signal transduction pathway implicated in irradiation-induced transcriptional regulation of CSF1. ABL1, a nonreceptor tyrosine kinase, is known for mediating apoptosis and cycle arrest after irradiation (32). It has been reported that ABL1 can also be recruited to the promoter region of CSF1 and regulates CSF1 gene expression in concert with AP-1 (33). Thus, we examined in detail the kinetics of irradiation-induced CSF1 expression and ABL1 activation in our system. First, detailed analysis of irradiation-induced gene expression of CSF1 in Myc-CaP cells showed the increase in CSF1 RNA initiated at 4 hours after irradiation (Fig. 5A). Next, we profiled the activation of ABL1 protein in response to irradiation. We observed that two ABL1 cleavage products, 75 kDa and 60 kDa product, emerged at 2 hours after irradiation (Fig. 5B). Further examination of the subcellular localization of ABL1 by confocal microscopy...
revealed that before the radiation insult, ABL1 was predominantly located in the cytoplasm with very little ABL1 immunochemical signal registered in the nucleus (Fig. 5C, left). As early as 1 hour after irradiation, a noticeable portion, but not all, of the ABL1 protein has translocated to the nucleus (Fig. 5C, middle), and this is maintained at 4 hours (Fig. 5C, right). To further substantiate the functional impact of ABL1 on the CSF1 gene expression, we analyzed the binding of ABL1 to the CSF1 promoter by a chromatin immunoprecipitation assay (ChIP). An increase in ABL1 binding to the CSF1 promoter was observed as early as 1 hour after irradiation and peaked at 2 hours, before slowly declining (Fig. 5D). When looking into the kinetics of RNA polymerase II binding to the CSF1 promoter, it first showed a significant decrease at 1 hour and an increase starting 2 hours post-irradiation (Fig. 5E). This bell-shaped kinetics of RNA polymerase activity could be attributed to the radiation-induced DNA damage causing an initial inhibition on transcription (34). The timing of ABL1 nuclear translocation, its binding to the CSF1 promoter, and the binding of RNA polymerase II to the CSF1 promoter preceded the changes in CSF1 mRNA level. These findings support the involvement of ABL1 in the regulation of CSF1 expression in response to irradiation.

Next, we used an ABL1-targeted siRNA and a small-molecule inhibitor to further confirm its regulatory role on CSF1 in our system. The irradiation-induced CSF1 expression in MycCaP cells was significantly inhibited by the addition of the ABL1 kinase inhibitor, STI-571 (5 μmol/L; Fig 5F). A similar result was also observed in the human prostate cancer cell line CWR22Rv1 (Supplementary Fig. S4). Using a migration assay, we found that STI-571–treated conditioned media displayed decreased ability to promote macrophage migration (Fig. 5G). Furthermore, the addition of GW2580 to macrophages (top chamber) did not further retard macrophage migration throughout STI-571 treatment of tumor cells (Fig. 5G), suggesting STI-571 and GW2580 may be targeting the same pathway to regulate migration. Like most of the pharmacologic protein kinase inhibitors, STI-571 is not completely specific. STI-571...
also inhibits c-Kit, platelet-derived growth factor receptor, and CSF1R (35). Thus, we used siRNA-mediated knockdown of ABL1 to examine its effect on CSF1. Because of the low efficiency of transfecting murine prostate cancer cells, we used the human CWR22Rv1 cell line for this experiment. The ABL1 siRNA was able to decrease ABL1 mRNA expression to 30% of the normal level in the CWR22Rv1 cells (Fig. 5H, right). As expected, ABL1 siRNA effectively blocked the CSF1 mRNA induction by irradiation (Fig. 5H, left). These data suggest that upon irradiation, ABL1 is activated, translocates to the nucleus, binds to the promoter region of CSF1, and promotes its expression.

**Blockade of CSF1/CSF1R signaling retards tumor regrowth after local irradiation**

CSF1 is a potent cytokine well known to promote myeloid cell proliferation, differentiation, and migration. In a recent study, we showed that blockade of CSF1/CSF1R signaling can effectively inhibit TIM function and recruitment to tumors (15). Thus, in this study, we investigated whether blocking CSF1R can also reduce recruitment of TIMs and diminish their protumorigenic influences in the radiotherapy setting. The combined irradiation and CSF1R blockade treatment was first tested with the selective CSF1R inhibitor, GW2580. Significant reductions in TIM populations in RM-1 tumors were observed with GW2580 treatment (160 mg/kg/d), which augmented the efficacy of irradiation by achieving more effective suppression of tumor growth than irradiation alone (Supplementary Fig. S5A–E; data not shown). To further substantiate this rational combination strategy, we used a recently described small-molecule CSF1R kinase inhibitor PLX3397. This inhibitor was shown to be a highly potent inhibitor of CSF1R (cFMS) with IC50 of 20 nmol/L and it is under active clinical investigation for several types of cancers (19). Here, RM-1 prostate
tumor-bearing mice were treated with control (chow), local irradiation (3 Gy x 5 days), PLX3397 (drug chow), or the combination. As shown in Fig. 6A, PLX3397 alone has little effect on tumor growth compared with the control group. Irradiation reduced tumor size by 43% at day 10, 1 day after cessation of irradiation (P < 0.001). The irradiated tumor sizes were stabilized for a short duration, and subsequently resumed an aggressive tumor growth rate, whereas the combined irradiation-and-PLX3397-treated group maintained a much slower growth rate (Fig. 6A). Both flow cytometry and histologic analyses of tumors revealed a significant reduction of CD11b+Gr-1+ MDSCs and CD11b+ F4/80+ macrophages in tumors as well as in spleens of both PLX3397-treated groups, with more pronounced effects observed in the combination treatment (Fig. 6B–E, and G). Interestingly, both subsets of MDSCs, monocytic and polymorphonuclear, were reduced by PLX3397 (Fig. 6F), a result that differed from our previous findings with GW2580 treatment in 3LL tumors where only the monocytic subtype of MDSCs was inhibited (15). At the molecular level, CSF1R blockade significantly reduced radiotherapy-induced CSF1, MMP9, and Arg1 (Fig. 6H–J). The latter 2 genes are known to be involved in cancer progression and metastasis by promoting tissue remodeling, angiogenesis, and immunosuppression (15). Similar reductions in the expression of CSF1 and Arg1 were also observed in irradiated tumors treated with GW2580, along with a significant reduction in the macrophage chemotactic factor, CCL2 (Supplementary Fig. S3D and S3E). In summary, we observed that prostate tumor-directed irradiation can potently induce the influx of TIMs, which in turn can thwart treatment efficacy. The addition of potent CSF1R inhibitors such as PLX3397 and GW2580 can prevent the influx of TIMs and halt their protumorigenic functions, thus leading to more effective and durable tumor growth control.

Discussion

In the present study, we show that the recruitment of TIMs to prostate tumors is highly induced by local irradiation in several immunocompetent mouse models. We find elevated expression of CSF1, an important cytokine for macrophage survival, migration, and differentiation, in tumor cells after irradiation, which was also observed in the serum of prostate cancer patients after radiotherapy. Increased CSF1 expression was mediated, at least partially, by the ABL1 tyrosine kinase. On irradiation, ABL1 was activated and translocated to the nucleus, where it bound to the promoter region of CSF1 to upregulate its expression. We further showed that blockade of CSF1R with selective small-molecule kinase inhibitors, such as GW2580 and PLX3397, greatly inhibit TIMs infiltration and significantly delay tumor regrowth after irradiation. These results suggest that disrupting the protumorigenic contributions of host innate immune cells, namely MDSCs and macrophages, through blockade of the CSF1/CSF1R axis can be a promising approach for developing rational and more effective combination cancer therapies. A schema of irradiation-induced expression of CSF1 and recruitment of TIMs and the impact of CSF1R inhibition on TIMs’ modulation of tumor regrowth is shown in Fig. 7.

The use of immunocompetent murine prostate tumor models in this study allowed us to directly assess the contributions of host immune cells, in particular, the distinct myeloid subpopulations, to tumor progression after therapy. Our results showed that the major impact of CSF1R blockade is directed at the tumor microenvironment, namely TIMs. Interestingly, CSF1R has been shown to be expressed and can contribute to the oncogenesis of several types of cancer, including prostate cancer (36, 37). Therefore, the blockade of the CSF1/CSF1R axis could potentially have a direct, suppressive impact on tumor cells, albeit unlikely as the RM-1 and Myc-CaP tumors used here express negligible levels of CSF1R on the basis of sensitive RT-PCR analyses (data not shown). We also believe IL-34, a newly identified ligand for CSF1R having similar functions in stimulating macrophage proliferation and migration (30, 38), is unlikely to play a significant role as its expression level is 100-fold lower than CSF1 in our models (data not shown). Recent findings from Dr. Hong Wu and colleagues using the PTEN-knockout transgenic prostatic carcinoma model revealed that intratumoral MDSCs expansion contributes to tumor progression and that CSF1R blockade was an effective means to suppress the infiltration and function of MDSCs in this spontaneous murine prostate cancer model (data not shown).

It is promising that our initial exploratory study on 10 consecutive patients with prostate cancer, who recently underwent radiotherapy, also yielded data supportive of the CSF1 axis being involved. Although our data suggests that serum CSF1 could potentially be a biomarker of TIMs recruitment, there are several considerations that caution against this premature conclusion. First, a wide range of serum CSF1 level was detected in patients (Fig. 4D). This issue could likely be attributed to the different infection or inflammation status of the patients. Second, the timing of patient specimen procurement after radiotherapy was not uniform between patients in the small cohort tested. We did observe an increase in a MDSC population (CD11b+CD11c−) in the peripheral blood of a few patients whose serum CSF1 increased from pre- to postradiotherapy (data not shown). Because of the heterogeneity issues, data from a much larger cohort of patients will be needed to fully validate the concept put forth here. We are actively pursuing these studies with more standardized time points of peripheral blood collection in patients with prostate cancer undergoing radiotherapy.

Our findings are consistent with CSF1 being an important stimulus for the influx of TIMs to tumors especially in response to irradiation. However, our study does not exclude other pathways that may also be involved in this complex inflammatory cascade. For instance, several recent articles highlighted the role of the SDF-1/CXCR4 axis in local irradiation-induced influx of TIMs to tumors (5, 6). The authors showed that irradiation induced hypoxia through destruction of endothelial cells and the microvasculature, and the resultant increased expression of HIF-1α, in turn, induced the expression of CXCR4 and SDF-1, which then mediated the recruitment of TIMs to tumors. Likewise, CCL2 has also been...
implicated in the recruitment of bone marrow-derived myeloid cells into tumors and this axis can also modulate prostate cancer growth and metastasis to bone (39, 40). However, we observed negligible SDF-1 and CCL2 expression with or without irradiation in our tumor cells (Fig. 4A). The migration/recruitment of TIMs is a complex process that is likely regulated by several pathways, especially in the context of different tumor types, host genetic background, and stimulus induced...
by different therapeutic settings or progression status. Of interest, we consistently observed a decrease in CCL2 level with GW2580 or PLX3397 treatment (data not shown; Supplementary Fig. 5F). This finding is also in accordance with a recent study that showed the removal of CSF1 significantly decreases CCL2 (41). An intriguing possibility could be that CSF1 is an upstream regulator of other cytokines like CCL2. Clearly, the influence of the CSF1/CSF1R axis and its cross-talk with other cytokine/chemokine pathways in the recruitment of TIMs deserves further investigation.

A unique aspect of this study is the finding that the ABL1 pathway mediates the heightened CSF1 transcription induced by irradiation. Several factors have been implicated in the regulation of CSF1 gene expression, including PDGF (42), ABL1 (33), IFN-γ (43), and AP-1, CTF/NF-1, SPI, SP3 (44), and nuclear actin (45) in different cell types and contexts. Among these, ABL1 is known to respond to irradiation or DNA damage via the DNA-PK, ATM, and p53 pathway (46–48). ABL1 contains a catalytic domain as well as a NES and 3 NLS motifs. Several groups reported that ABL1 localizes to both nucleus and cytoplasm and can shuttle between these 2 compartments (49). In our study, we observed that a portion of cytoplasmic ABL1 is activated and translocated to the nucleus as early as 1 hour after irradiation (Fig. 5B). The ABL1 DNA-binding domain is critical for its biologic function (50), yet no classical DNA-binding motifs have been identified so far. The few ABL1 transcriptional targets identified include p21 and CSF1 (33, 51). Here, we showed that ABL1 binds to the promoter region of CSF1 and activates CSF1 gene transcription. A previous study suggested that ABL1 forms a complex with AP-1, a transcription factor composed of c-jun and c-fos, in the regulation of CSF1 (33). On the basis of this finding, a potential feedback loop in CSF1R-dependent cells is that the blockade of CSF1R signaling inhibits c-fos, which further downregulates CSF1 expression. This mechanism might explain why we observed a decrease in CSF1 expression in tumors after PLX3397 treatment (Fig. 6H).

In summary, the data presented in this study show that irradiation induces CSF1 through an ABL1-dependent mechanism in prostate cancer. The heightened CSF1 serves a critical role in the systemic recruitment of protumorigenic myeloid cells to irradiated tumors. Therefore, the blockade of TIMs in combination with local irradiation of prostate tumors displays an augmented and more durable response than irradiation alone in preclinical models. We believe that cotargeting the CSF1/CSF1R pathway with local irradiation of prostate tumors will be a promising strategy for clinical translation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

Conception and design: J. Xu, J. Escamilla, J. David, S.J. Priceman, L. Wu

Development of methodology: J. Xu, J. David

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Xu, J. David

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Xu, J. Escamilla, B.L. West
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