Microenvironment and Immunology

TLR9 Signaling in the Tumor Microenvironment Initiates Cancer Recurrence after Radiotherapy

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Abstract

Cancer radiotherapy may be immunogenic, but it is unclear why its immunogenic effects are rarely sufficient to prevent tumor recurrence. Here, we report a novel Toll-like receptor 9 (TLR9)–dependent mechanism that initiates tumor regrowth after local radiotherapy. Systemic inhibition of TLR9, but not TLR4, delayed tumor recurrence in mouse models of B16 melanoma, MB49 bladder cancer, and CT26 colon cancer after localized high-dose tumor irradiation. Soluble factors in the microenvironment of regressing tumors triggered TLR9 signaling in freshly recruited myeloid cells appearing within four days of radiotherapy. The tumorigenic effects of TLR9 depended on MyD88/NF-kB–mediated upregulation of interleukin (IL)-6 expression, which in turn resulted in downstream activation of Jak/STAT3 signaling in myeloid cells. In comparing global gene expression in wild-type, TLR9–, or STAT3-deficient myeloid cells derived from irradiated tumors, we identified a unique set of TLR9/STAT3–regulated genes involved in tumor-promoting inflammation and revascularization. Blocking STAT3 function by two myeloid-specific genetic strategies corrected TLR9-mediated cancer recurrence after radiotherapy. Our results suggest that combining localized tumor irradiation with myeloid cell–specific inhibition of TLR9/STAT3 signaling may help eliminate radio-resistant cancers.

Introduction

Radiotherapy relies on the increased radiation sensitivity of proliferating cells in comparison with surrounding patient tissues. As a noninvasive strategy, radiotherapy became one of the primary treatment modalities for about half of all patients with cancer (1, 2). However, many cancer types, such as melanoma, renal carcinoma, or advanced prostate cancers, are considered either completely or partly radioresistant (1, 2). More recently, emerging data have shown that long-term effects of radiotherapy are limited by intrinsic radioresistance of cancer cells and by extrinsic influence of the tumor stroma (3–5). Several tumor-associated but nonmalignant cell populations, including endothelial cells (6) and myeloid immune cells (7, 8), were implicated in promoting tumor recurrence after radiotherapy. Recent studies demonstrated that, within a few days after irradiation, regressing tumors recruit inflammatory monocytes/macrophages from the bone marrow (7–10). Tumor-associated macrophages (TAM) and other CD11b+ myeloid cell populations are known for supporting tumor growth, invasion, metastasis, and immune evasion (11–14). The CD11b+ monocytes recruited into the injured tissue can also recognize components of dying cells, which include TLR agonists (11, 15, 16). However, despite the abundance of immunostimulatory molecules, tumors frequently reoccur even after high-dose irradiation (17). Recent studies suggested that these undesirable effects might depend on myeloid cells, which stimulate restoration of tumor blood vessel after radiation-induced damage (9). However, the molecular mechanism(s) underlying tumor recurrence after radiotherapy were not defined.

Toll-like receptors (TLR) play crucial roles in driving inflammatory responses of myeloid cells in reaction to tissue stress and injury (11, 18, 19). TLR signaling is tightly controlled by multiple and interconnected layers of negative regulatory pathways (20). STAT3 seems to play a central role in the regulation of TLR-induced inflammatory processes in normal wound healing and in tumorigenesis (20, 21). Previous studies revealed that STAT3 is a negative feedback inhibitor for proinflammatory signaling by TLR4 and TLR9 in myeloid cells (22, 23). In addition, STAT3 activation was shown to enhance angiogenic activity of myeloid cells, whereas limiting antigen presentation (24, 25). Persistent STAT3 activity within the tumor microenvironment results from stimulation by cytokines, growth...
factors, and sphingolipids secreted by cancer and tumor stroma cells (20, 21). High-dose tumor radiotherapy eliminates the majority of cancer cells and tumor-associated stroma, disrupting the immunosuppressive signaling network and generating potentially immunogenic signals. However, it ultimately fails to prevent tumor regrowth. In the current study, we investigated whether local triggering of innate immunity receptors, such as TLR9, can provide an emergency tumorigenic signal to jump-start tumor regrowth after radiotherapy.

Materials and Methods

In vivo experiments

Mouse B16 and CT26 cells were from American Type Culture Collection; MB-49 cells were generous gift from T. Ratliff (University of Iowa, Iowa City, IA) and were kept in culture for less than 6 months. Cells were not further authenticated. Tbr9fl/fl mice were obtained from The Jackson Laboratory, whereas Tbr9fl/fl mice were originally from S. Akira (Osaka University, Osaka, Japan). Generation of mice with Stat3fl/fl hematopoietic cells using inducible Mx1-Cre system was reported previously (33). Mice were backcrossed for 7 to 10 generations to make them C57BL/6 congenic. To generate poly(IC)-inducible MyD88fl/fl mouse, C57BL/6 MyD88fl/fl and Mx1-Cre mice (The Jackson Laboratory) were crossed to generate Mx1-Cre/MyD88fl/fl mice. Animal care was performed under pathogen-free conditions following approved protocols from the Institutional Animal Care and Use Committees (City of Hope, COH, Duarte, CA). Established subcutaneous tumors were irradiated using single collimated dose of radiation from Cs-137 source using MARK-4 irradiator (J.L. Shepherd). The radiation was 13.3 Gy (±0.5 Gy) at the tumor site and negligible (0.01–0.16 Gy) in the 1 cm distance as measured using dosimeters (n = 4). Mice were injected peritumorally with CpG-siRNA (1 mg/kg) or retroorbitally with TTAGGG ODN (5 mg/kg) every other day. Oligonucleotides were synthesized in the DNA/RNA Synthesis Core at COH; the design of CpG-siRNA was described (33, 50). Spleen or tumor tissues were dissociated to single-cell suspensions as reported before (33). For Matrigel experiments, CD11b+/CD11c− cells were enriched from spleens of wild-type (WT) or Tbr9fl/fl tumor-bearing mice with more than 90% purity using magnetic separation (STEMCELL Technologies). A total of 1 × 10^6 WT or Tbr9fl/fl myeloid cells were admixed with 1 × 10^6 B16 cells in growth factor-reduced Matrigel (BD Biosciences) and injected into WT mice. After 6 days, Matrigel plugs were removed for hemoglobin analysis using Drabkin reagent (Sigma-Aldrich).

Immunofluorescent staining

Flash-frozen tumor specimens were fixed and stained with antibodies specific to myeloid (CD11b), endothelial (MECA32), and endothelial progenitor cells (CD31, VEGFR2; BD Biosciences), then detected with fluorochrome-coupled secondary antibodies (Invitrogen) as described previously (33).

Western blotting

Cells were treated with supernatants from in vivo irradiated tumors following the pretreatment with 10 nmol/L NF-κB inhibitor (EMD461407; Millipore) or with neutralizing antibody to interleukin (IL)-6 (10 μg/mL; BD Biosciences). Total cellular lysates were prepared as previously reported (33) and analyzed using antibodies specific to tyrosine phosphorylated STAT3 (pSTAT3: Cell Signaling Technology), total STAT3 (Santa Cruz Biotechnology), and β-actin (Sigma-Aldrich).

Quantitative real-time PCR

Total RNA was extracted from primary cells using the mirVana miRNA Isolation Kit (Ambion) and then transcribed them into cDNAs with the RT² First Strand Kit (Qiagen). The qPCR was carried out using primers for IL-6 (Qiagen) using CFX96 Real-Time PCR Detection System (Bio-Rad).

Global gene expression analysis

Total RNA samples extracted from magnetically enriched tumor–infiltrating CD11b+ cells using the mirVana miRNA Isolation Kit (Ambion) were sequenced on Illumina HiSeq2000. Sequences that passed the default chastity filter were aligned with the mouse reference genome (Genome Browser; University of California, Santa Cruz, CA) using the TopHat software v.1.3.1 to identify differential gene expression. The results were converted to reads per kilo base of total exon length per million mapped (RPKM) reads using Genomics Suite v.6.12.0713 (Parak) and normalized to gene models in the National Center for Biotechnology Information RefSeq database with a stringent cutoff of 0.1 RPKM and the false discovery rate (FDR) less than 0.05. Differentially expressed mRNA had fold change cutoff of 1.5 and Pvalue with FDR cutoff of 0.05. The expression profiling data were submitted to the Gene Expression Omnibus (GSE45180).

Flow cytometry

For extracellular immunostaining, single-cell suspensions were incubated with fluorochrome-coupled antibodies to CD11b and Gr1 (BD Biosciences). Before intracellular staining for tyrosine pSTAT3 (BD Biosciences), cells were fixed and permeated as described previously (33). Fluorescence data were analyzed using Accuri C6 cytometer (BD Biosciences) and FlowJo software v.7.6.5 (Tree Star).

Statistical analysis

Unpaired t test was used to calculate two-tailed P value to estimate statistical significance of differences between two treatment groups. One- or two-way ANOVA plus Bonferroni after test was applied to assess differences between multiple groups or in tumor growth kinetics experiments, respectively. Statistically significant P values were indicated in Figures 1, 2, 3, 4, and 6 as follows: **, P < 0.01 and *, P < 0.05. Data were analyzed using Prism software v.4.0 (GraphPad).

Results

TLR9 triggering initiates tumor regrowth after local radiotherapy

TLR4 and TLR9 are reportedly involved in responses to tissue injury (11, 15), therefore, we assessed whether genetic ablation of either one of these receptors will influence the effect of local radiotherapy. WT, Tbr4fl/fl or Tbr9fl/fl mice were injected subcutaneously with B16 melanoma cells, which are highly radioresistant tumors (26). After tumors were
established (average diameter 8–10 mm), mice were treated with a single 13 Gy dose of radiation focally directed to the tumor site. Unexpectedly, Tlr9 deletion did not accelerate but instead significantly delayed the regrowth of B16 tumors after radiotherapy compared with WT mice. In contrast, B16 tumors in Tlr4−/− mice grew with similar kinetics as in controls (Fig. 1A). We found an even more pronounced effect of Tlr9 gene deletion on the recurrence of irradiated MB49 bladder carcinomas. The single dose of irradiation induced complete regression of MB49 tumors in the majority of Tlr9−/− mice, whereas only transiently inhibiting MB49 growth in WT mice (Fig. 1B). The Tlr−/− and control WT C57BL/6 mice

Figure 1. TLR9 activity in the tumor microenvironment accelerates tumor recurrence after local irradiation. A, B16 tumor growth in WT, Tlr4−/−, and Tlr9−/− mice after single dose of tumor irradiation (RT) at 10 days after tumor injection. B, MB49 tumor growth in WT and Tlr9−/− mice after irradiation. C–E, blocking TLR9 by TTAGGG inhibits tumor relapse after tumor irradiation. WT mice were implanted subcutaneously with 1 × 10^5 B16 (C), 1 × 10^6 MB49 (D), or 5 × 10^6 CT26 (E) cells. Tumor-bearing mice were locally irradiated and intravenously injected with TTAGGG or PBS every other day, starting at 10 days. Data shown are from one representative of two independent experiments using 8 to 10 mice per group; means ± SEM. Statistically significant differences between three (A) or two (B–E) treatment groups are indicated by asterisks (*). RT, tumor irradiation.
were congenic to limit the influence of genetic differences. We also verified these results in WT C57BL/6 and Balb/C mice using the TLR9 receptor antagonist, TTAGGG oligodeoxynucleotide (ODN; 27). Repeated intravenous injections of TLR9 antagonist significantly delayed recurrence of B16 (Fig. 1C), MB49 (Fig. 1D), or CT26 colorectal carcinoma (Fig. 1E) tumors in C57BL/6 or Balb/C mice, respectively. These results suggest that independently from the mouse genetic background, TLR9 triggering in the tumor microenvironment provides a common mechanism promoting tumor recurrence after radiotherapy.

**TLR9 supports reconstruction of tumor blood vessels after radiotherapy**

To understand the role of TLR9 in promoting tumor growth after radiotherapy, we first evaluated the rate of B16 tumor revascularization in WT and Tlr9−/− mice. We compared the average size of blood vessels formed in WT and Tlr9−/− mice within 10 days after tumor irradiation. At this time the average tumor volumes in both experimental groups were comparable, which allowed for the analysis of blood vessels before tumor regrowth usually occurring in the WT group by 12 days after radiotherapy. For unbiased assessment, we quantified fluorescently stained MECA32+ endothelial cells on whole tumor cross-sections using automated slide scanning (Fig. 2A and Supplementary Fig. S1). The analysis of WT and Tlr9−/− B16 tumor cross-sections revealed that tumor blood vessels in Tlr9−/− mice were small and mostly immature, in contrast with well-developed tumor vasculature in WT mice (Fig. 2A).

The average blood vessel size in Tlr9−/− mice compared with WT controls was reduced 3-fold (Fig. 2B). Recent studies linked formation of tumor vasculature to the recruitment of EPCs from the bone marrow, which usually occurs 4 days after tumor irradiation (9). To verify whether EPCs can contribute to poor tumor revascularization in Tlr9-ablated mice, we assessed VEGFR2+ EPCs in tumors early (day 4) and late (day 10) after radiotherapy. The immunofluorescent microscopy revealed that transient tumor infiltration by EPCs occurs at 4 days after irradiation only in WT but not in Tlr9−/− mice (Fig. 2C and D). At 10 days after radiotherapy, EPCs were rare in tumors from both groups. Our results suggest that transient EPC mobilization preceded restoration of tumor vasculature after radiation-induced tumor cell death.

**TLR9 signaling in myeloid cells promotes revascularization of irradiated tumors**

Bone marrow–derived myeloid cells support early stages of tumor growth (11, 28) and ionizing radiation is known to intensify the process of their recruitment (9). The microscopic examination of immunofluorescently stained B16 tumor sections showed increased numbers of CD11b+ myeloid cells in tumors at day 4 after irradiation compared with nonirradiated controls in both WT and Tlr9−/− mice (Supplementary Fig.
immature myeloid cells (CD11b−) also showed a significant reduction of blood vessels. CD11b− cells from WT or Tlr9−/− mice were admixed with B16 cells in Matrigel at 10:1 ratio and subcutaneously injected into WT mice. Right, hemoglobin content in Matrigel plugs at 7 days; means ± SEM (n = 5). *, P < 0.05; **, P < 0.01.

To assess percentages of tumor-infiltrating myeloid cells, we analyzed single-cell suspensions from whole tumors with or without prior irradiation using flow cytometry. As expected, numbers of tumor-infiltrating CD11b+ cells were significantly increased 4 days after irradiation (Fig. 3A). Most of the freshly recruited cells were CD11b−Gr1+ macrophages with less percentage of CD11b+Gr1+ immature myeloid cells.

Previous studies demonstrated that proangiogenic activity of TAMs and other myeloid cells largely depends on signaling by STAT3 (11, 24, 25). We therefore assessed whether irradiation can activate STAT3 in myeloid cells mobilized into irradiated B16 tumors. Fluorescence-activated cell sorting analysis of activated, tyrosine pSTAT3, showed elevated levels of pSTAT3 in TAMs from WT but not from Tlr9−/− mice at day 4 after radiotherapy (Fig. 3B). Similar effects were observed also in immature myeloid cells (CD11b−Gr1+), although the overall levels of STAT3 phosphorylation were lower than in macrophages (Supplementary Fig. S2B). We further verified these data using sorted immune cell populations from in vivo irradiiated tumors as well as on in vitro–treated splenocytes (Supplementary Fig. S3C–S3E). Altogether, our results indicate that in the microenvironment of irradiated tumor, TLR9 activates STAT3 signaling specifically in CD11b−F4/80+ TAMs. To verify whether TLR9+/pSTAT3+ tumor–associated myeloid cells play proangiogenic role in the irradiated tumor microenvironment, we performed in vivo Matrigel plug assays. Splenic CD11b+ cells isolated from B16 tumor-bearing WT or Tlr9−/− mice were suspended in supernatant derived from in vivo irradiated tumors, admixed with B16 cells in Matrigel and injected subcutaneously into mice. Six days later, Matrigel plugs were harvested to assess formation of early blood vessels and measure the hemoglobin content, which corresponds with the extent of neovascularization. Matrigel plugs containing WT myeloid cells showed significantly increased hemoglobin levels compared with Matrigel plugs with Tlr9−/− myeloid cells (Fig. 3C). Our control experiments confirmed that under the same experimental conditions vascularization of Matrigel plugs with B16 tumors is increased nearly twice by WT myeloid cells (Supplementary Fig. S2C). These results indicate that TLR9 expression is required for myeloid cell–specific STAT3 activation as well as for the tumor revascularization after radiotherapy.

IL-6 couples TLR9 to STAT3 signaling in myeloid cells accumulated in irradiated tumors

Next, we assessed whether soluble factors released from in vivo irradiated tumors can stimulate TLR9-mediated STAT3
activity in normal myeloid cells. The B16 tumors established in C57BL/6 mice were locally irradiated as described in Fig. 1. After 4 days tumors were harvested and gently dispersed into single-cell suspensions. The soluble fractions collected from irradiated or nonirradiated B16 tumor suspensions were then used to treat freshly isolated WT or Tlr9−/− cells. As shown in Fig. 4A, supernatants from in vivo irradiated B16 tumors were more potent in inducing STAT3 phosphorylation in WT splenocytes than supernatants from nonirradiated tumors. In contrast, Tlr9 gene deletion abrogated STAT3 activation by the irradiated tumor supernatant in primary myeloid cells (Fig. 4B). To verify whether STAT3 activation is induced by soluble TLR9 ligands present in tumor supernatants, we used a competitive TLR9 inhibitor (TTAGGG) as in Fig. 1. The specific TLR9 blocking prevented upregulation of pSTAT3 in splenocytes treated using irradiated B16 tumor supernatant (Fig. 4B and Supplementary Fig. S3B). In contrast, the TLR9 inhibitor did not affect STAT3 activation induced by supernatant derived from nonirradiated B16 tumors. This is consistent with the well-known role of multiple cytokines and growth factors in stimulating STAT3 activity in the intact tumor microenvironment (29).

To understand the molecular mechanism(s) leading to STAT3 activation in the irradiated tumor microenvironment, we first assessed whether these effects depend on MyD88, a critical adaptor molecule downstream from TLR9 (30). We have generated mice with inducible deletion of MyD88 (Mx1-Cre/MyD88loxP/loxP). As shown in Fig. 4C, STAT3 activation by supernatants derived from irradiated tumors was reduced in splenic CD11b+ cells from MyD88-deficient mice compared with WT mice. We further verified that STAT3 induction through TLR9/MyD88 signaling involves NF-kB. In fact, small molecule NF-kB inhibitor abrogated STAT3 activation by the irradiated tumor supernatant in primary myeloid cells (Fig. 4D). Our initial studies indicated that TLR9 stimulation leads to release of soluble factor(s) that activates STAT3 in target cells (Supplementary Fig. S4A). TLR9/MyD88/NF-kB signaling is known to induce expression of various cytokines and growth factors, which could contribute to STAT3 activation in myeloid cells. The B16 tumors established in C57BL/6 mice were locally irradiated as described in Fig. 1. After 4 days tumors were harvested and gently dispersed into single-cell suspensions. The soluble fractions collected from irradiated or nonirradiated B16 tumor suspensions were then used to treat freshly isolated WT or Tlr9−/− cells. As shown in Fig. 4A, supernatants from in vivo irradiated B16 tumors were more potent in inducing STAT3 phosphorylation in WT splenocytes than supernatants from nonirradiated tumors. In contrast, Tlr9 gene deletion abrogated STAT3 activation by irradiated TMSN. Splenic CD11b+ cells were preincubated for 0.5 hours with transcriptional NF-kB inhibitor, then treated as indicated and analyzed for STAT3 activity. E, NF-kB inhibition prevents IL-6 upregulation by irradiated TMSN in CD11b+ myeloid cells. Splenic CD11b+ cells were treated using TMSN with or without the NF-kB inhibitor as in D for 1.5 hours. Shown are the qPCR results normalized to Actb expression; IL-6 mRNA level in control group was set as 1. F, IL-6 neutralization abrogates STAT3 activation by irradiated but not control TMSN. Total splenocytes treated with supernatants (TMSN) from irradiated or nonirradiated B16 tumors. Total splenocytes from WT or Tlr9−/− mice were treated using TMSN or left untreated for 3 hours, then pSTAT3/total STAT3 levels were examined. D, blocking NF-kB in myeloid cells abrogates STAT3 activation by irradiated TMSN. Splenic CD11b+ cells were preincubated for 0.5 hours with transcriptional NF-kB inhibitor, then treated as indicated and analyzed for STAT3 activity. E, NF-kB inhibition prevents IL-6 upregulation by irradiated TMSN in CD11b+ myeloid cells. Splenic CD11b+ cells were treated using TMSN with or without the NF-kB inhibitor as in D for 1.5 hours. Shown are the qPCR results normalized to Actb expression; IL-6 mRNA level in control group was set as 1. F, IL-6 neutralization abrogates STAT3 activation by irradiated but not control TMSN. Total splenocytes were incubated with TMSNs in the presence of IL-6 neutralizing or control IgG antibodies. Left, the representative results of the Western blot for pSTAT3; right, densitometric quantification of three independent experiments using ImageJ software (v. 1.47 hours). Shown are representative results from one of three (A, B, and F) or two (C, D, and E) independent experiments; β-actin was used as a loading control.
of numerous soluble proinflammatory mediators, which are expressed rapidly after stimulation and can activate STAT3 (31, 32). In addition, our in vitro experiments on splenocytes indicated that STAT3 activation is sensitive to broad specificity Janus–activated kinases (Jak) inhibitors (Supplementary Fig. S4B). Further gene expression analysis using qPCR identified that TLR9/NF-κB signaling in immune cells upregulates IL-6 expression, which is as a potent activator of STAT3 signaling (Fig. 4E and Supplementary Fig. S4C; ref. 32). We confirmed these results using tumor supernatants from both irradiated and nonirradiated tumors. As shown in Fig. 4F, the antibody-mediated neutralization of IL-6 in irradiated and nonirradiated tumors. As shown in Fig. 4F, the antibody-mediated neutralization of IL-6 in irradiated tumor supernatants reduced STAT3 activation by nonirradiated supernatants. The neutralization of type I IFNs, which are also upregulated by TLR9 signaling, did not prevent STAT3 activation (Supplementary Fig. S4D; ref. 30). These results suggest that IL-6 bridges signaling from TLR9 to Jak/STAT3 in myeloid cells accumulated in tumors after radiotherapy.

TLR9/STAT3 signaling orchestrates tumorigenesis-promoting gene expression in myeloid cells

Our results uncovered a potential molecular mechanism jump-starting tumor recurrence after radiotherapy. To assess whether STAT3 plays a role in these TLR9-induced effects, we compared global gene expression in CD11b⁺ myeloid cells isolated from tumors irradiated in genetically matched WT, Tlr9⁻/⁻ and Stat3-deficient C57BL/6 mice (33). The expression levels of more than 22,000 transcripts were analyzed in RNA samples from 3 to 4 individual mice sequenced on Illumina HiSeq2000. Genes that were differentially expressed in comparison with WT with a stringency cutoff of FDR less than 0.05 were identified as regulated either by TLR9 or STAT3. The hierarchical clustering of differentially expressed genes in individual mice revealed molecular signatures for WT, Tlr9⁻/⁻ and Stat3⁻/⁻ groups of mice (Fig. 5A), whereas confirming the specificity of TLR9 and STAT3 gene ablation (Supplementary Fig. S5A and data not shown). Differentially regulated (>1.5-fold) transcripts found in Stat3⁻/⁻ myeloid cells (2,145 up- and 786 downregulated) were twice the number

Figure 5. TLR9/STAT3 signaling modulates global gene expression in tumor-infiltrating myeloid cells after irradiation. A, overview of the gene expression pattern in the hierarchical clustering analysis. The red or green color indicates up- or downregulated expression, respectively. B, the number of commonly regulated genes is shown using Venn diagram comparing Tlr9⁻/⁻ with WT and Stat3⁻/⁻ to WT groups. C, top functional downregulated gene targets in Tlr9⁻/⁻ and Stat3⁻/⁻ compared with WT as assessed by IPA analysis. D and E, heatmaps showing differentially expressed genes related to angiogenesis (D) or myeloid cell differentiation (E).
of transcripts identified in Tlr9−/− cells (1,165 up- and 343 downregulated), thus underscoring the role of Stat3 as a point of convergence for tumorigenic signaling networks in immune cells (Fig. 5B; refs. 20, 29). Importantly, in comparison with WT controls, 993 transcripts were commonly regulated in Tlr9−/− and Stat3−/− myeloid cells (796 up- and 197 downregulated; Fig. 5B). These results suggest that Stat3 is directly or indirectly involved in the transcription of 68% (796/1,165) of all negatively, or 57% (197/343) of all positively TLR9-regulated genes during tumor regrowth after radiotherapy.

Top functional gene categories common for Tlr9−/− and Stat3−/− tumor–associated myeloid cells are likely related to cellular growth, survival, angiogenesis, and inflammatory immune responses as suggested by the ingenuity pathway analysis software (IPA; Fig. 5C). Among the transcripts downregulated in Tlr9−/− and Stat3−/− myeloid cells compared with WT cells, were many genes known for promoting tumorigenic inflammation and angiogenesis, including IL-6, IL-6st, IL-23, Lif, Hbegf, and Ccl1 and its receptor (Ccr8), Hdc, Slpr3, Plaur, Trem1, and Tgfb1 (Fig. 5D). Several of these genes encoded soluble ligands or receptors, which are known as activators of Stat3 in myeloid cells (Fig. 5E). In contrast, the expression of several mediators of innate and adaptive antitumor immunity such as Ifnb, Il-15, and Il-18 was elevated in Tlr9−/− and Stat3−/− myeloid cells compared with WT cells (Fig. 5E).

The other prominent functional gene category targeted by TLR9/STAT3 signaling comprised several transcription factor genes critical for monocyte/macrophage differentiation. Tumorigenic genes, which are activated in macrophages under hypoxia, such as Ets2 oncogene, as well as Egr1, Egr3 (34), were reduced in Tlr9− and Stat3−deficient myeloid cells compared with WT controls. In contrast, several genes promoting terminal differentiation and growth arrest (Gadd45a, Gadd45b, Gadd45g, and Rock1) or apoptosis (Bclaf1, Pdcd5, and Tmffl) were elevated in Tlr9− and Stat3−deficient macrophages. Several genes selected on the basis of RNA sequencing data were validated using qPCR (Supplementary Fig. S2B). These results suggest that STAT3 signaling downstream from TLR9 allows for concerted regulation of myeloid cell differentiation, survival, and proangiogenic activity in tumors recurring after radiotherapy.

Targeting STAT3 in TLR9−/− myeloid cells augments the efficacy of local tumor irradiation

We next assessed whether blocking STAT3 in the myeloid compartment would compromise tumor regrowth following local radiotherapy. B16 cells were injected into littermate mice with or without deletion of Stat3 in myeloid cells. At 8 days, tumors established in both Stat3+/− and Stat3−/− mice (average volume of 34 mm3) were treated locally irradiated or left untreated. As shown in Fig. 6A, the combination of Stat3 ablation in myeloid cells with radiotherapy effectively prevented tumor regrowth. Stat3 deletion alone and tumor irradiation alone delayed though did not abrogate B16 tumor growth. Mice with prolonged (>4 weeks) Stat3 ablation in hematopoietic cells develop autoinflammatory disorders, which limit experimental timeframe (33). Thus, we decided to use CpG-Stat3 siRNA as an alternative strategy to target Stat3 specifically in TAMs. We previously demonstrated that siRNA conjugated to CpG ODNs are actively internalized by TLR9-positive cells and induce gene silencing in both mouse and human systems (35). Mice with established B16 tumors (average volume of 198 mm3) were injected peritumorally with CpG-siRNAs every other day starting at 10 days after tumor inoculation or left untreated. Mice were irradiated at 12 days as indicated. Results represent one of two independent experiments; means ± SEM; *P < 0.05, **P < 0.01, ***P < 0.001.

Discussion

Recent clinical reports indicated that radiotherapy has the potential to generate abscopal effects and systemic antitumor immunity (36). Paradoxically, tumor irradiation was also shown to enhance cancer cell repopulation and recurrence (8, 10, 37). Our study reconciles these observations identifying a novel molecular mechanism, which controls the outcome of...
Recent reports suggested that the endogenous trigger for TLR9 signaling might be mitochondrial DNA released from dying cells during injury (38, 39). Whether mitochondrial DNA acts in the naked form or complexed with DNA binding molecules, such as HMGB1, is still unclear (42, 43). Other nucleic acid receptors, such as RNA-sensing TLR7/8 but not TLR3, could also contribute to the tumorigenic effect of radiotherapy through STAT3 activation (C. Gao and M. Kortylewski; unpublished data). We found TLR9 ligands in the tumor microenvironment after radiotherapy but not before irradiation. Tumor damage after radiotherapy can disintegrate the cytokine/growth factor network, which sustains STAT3 activity in growing and established tumors (20, 21). TLR9 triggering is likely to jump-start tumorigenic signaling in newly recruited myeloid cells. Our antibody-mediated neutralization experiments suggest that IL-6 is a critical STAT3 activator present in the irradiated tumor microenvironment. IL-6 is known for inducing rapid STAT3 phosphorylation through the IL-6R–associated Janus family kinases, primarily Jak1 rather than Jak2 or Tyk2 (32). Correspondingly, our in vitro experiments using various kinase inhibitors indicated that STAT3 activity was sensitive to broadly specific Jak inhibitors, but it was unaffected by targeting Jak2 only, EGFR, PI3K/Akt, or MEK/ERK. The crucial role of IL-6 in the inflammation-driven carcinogenesis is well established (44). Secretion of IL-6 by monocytes/macrophages translates initial inflammatory responses into tumorigenic STAT3 signaling in both malignant and immune cells. Previous studies performed in tumor-free mice indicated the dominant role of IFN-γ and TNF-α-dependent gene expression downstream of TLR9 (45). Our results demonstrate that in TAMs TLR9 signaling is skewed toward IL-6 and STAT3-dependent gene regulation. In addition to IL-6, we uncovered multiple potential STAT3 activators, such as Lif, IL-23, Hbegf, or Sfrp3. It is likely that IL-6 initiates a feed-forward mechanism that restores and maintains STAT3 activity by multidirectional stimulation (21). Furthermore, TLR9/STAT3 signaling coordinates expression of genes supporting angiogenesis, such as Ets2 (46), and repression of genes involved in macrophage differentiation and apoptosis (47). TLR9/STAT3 signaling was critical for tumor-promoting functions of CD11b+ cells, potentially through the recruitment of VEGFR2+ EPCs. Previous reports demonstrated the role of EPCs in tumor vasculogenesis after irradiation or under normal conditions (8, 28, 48). Nevertheless, we cannot rule out that residual endothelial cells also contribute to the reconstructive function of tumor vasculature as suggested by others (9).

The proof-of-principle experiments in mice using both genetic Stat3 deletion and CpG-Stat3 siRNA approach confirmed the negative role of TLR9/STAT3 signaling in determining the outcome of radiotherapy. Targeting STAT3 in myeloid cells augmented the proinflammatory effect of TLR9 triggering after radiotherapy, thereby preventing tumor recurrence. Recent phase I/II study in B-cell lymphoma demonstrated that intratumoral injections of CpG ODN could synergize with the effect of radiotherapy emphasizing the need for combination strategies (2, 49). Collectively, these findings suggest that TLR9 signaling might be involved in microenvironmental regulation of STAT3 activity in myeloid cells, potentially promoting tumor recurrence after radiotherapy. We demonstrate that TLR9, the innate immune receptor, triggers MyD88/NF-κB–mediated expression of IL-6, which in tumor induces tumorigenic signaling in myeloid cells freshly recruited in response to tumor irradiation (Fig. 7). We show that tumorigenic activity of TLR9+ myeloid cells depends on the IL-6–induced activation of STAT3, a master regulator of tumor angiogenesis and immune evasion (21). We demonstrate for the first time that the TLR9/IL-6/STAT3 signaling axis promotes expression of a unique set of genes in myeloid cells that contribute to neovascularization of irradiated tumors.

Our studies provide evidence that TLR9 activation in myeloid cells depends on soluble factors released into the tumor microenvironment after irradiation. Blocking TLR9 stimulation with an antagonistic oligonucleotide mimicked the effect of TLR9 ablation both in vitro and in vivo. These results agree with recent studies demonstrating that sterile tissue injury can cause TLR9-mediated inflammatory responses in vivo (38, 39). TLR9 gene expression is also the most sensitive to cellular stress caused by DNA damage through ionizing radiation or chemotherapy of all TLR gene family members (40). Although TLR9 expression in humans is more restricted than in mice, recent studies reported TLR9 expression in activated immune cells, such as monocyte-derived dendritic cells, macrophages or neutrophils (31, 41). In agreement with this study, we also observed upregulation of TLR9 levels in lymph node macrophages from patients with cancer that underwent radiotherapy compared with tumor-free individuals (Supplementary Fig. S6). Whether there is a causal relationship between TLR9 expression and STAT3 activation in human system remains to be established in more extensive studies.
imply that myeloid cell–specific inhibition of TLR9/IL-6/Jak/STAT3 signaling can generate novel, safer, and more effective strategies to combat cancer radiotherapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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