The dark side of Toll-like receptor signaling

TLR9 activation limits the efficacy cancer radiotherapy

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Accumulating evidence suggests that immunostimulation often contributes to the overall effects of conventional cancer therapies based on use of cytostatic, cytotoxic, or anti-angiogenic agents.1 Ionizing radiation and certain types of chemotherapy can indeed induce the release of immunogenic molecules from dying cancer cells, eventually resulting in the elicitation of antitumor immune responses. Such immunostimulatory effects often rely on the activation of Toll-like receptors (TLRs), critical mediators of inflammatory responses to tissue stress and injury.2 Radiation therapy (RT) has previously been shown to induce the TLR4-mediated maturation of dendritic cells (DCs), hence promoting the elicitation of T cell-mediated antitumor immune responses that are indispensable for preventing disease relapse.1,3 Nevertheless, such long-term effects of RT are rare.3 Rather, tumors initially regressing in response to RT recruit inflammatory CD11b+ myeloid cells from the bone marrow that gradually restore the growth, immune evasion, and metastatic dissemination of malignant cells.4 However, the molecular mechanisms that underlie the ability of myeloid cells to promote tumor recurrence upon RT are not well characterized. Signal transducer and activator of transcription 3 (STAT3) is known as a central regulator of inflammatory processes, supporting physiological wound healing as well as oncogenesis.5 The activation of STAT3 in myeloid cells enhance their pro-angiogenic potential while limiting antigen presentation.5 Such a conversion in the functions of myeloid cells results from multiple STAT3 inducers that are released in the tumor microenvironment (Fig. 1A). In addition, specific Toll-like receptors (TLR) such as TLR4 and TLR9 rapidly activate STAT3 in myeloid cells in the context of a negative feedback signaling loop.6 Hence, in tumors regressing upon RT (in which the majority of cells are dying), danger signals may provide an emergency cue to restore STAT3 activity in the tumor microenvironment. To explore this hypothesis, we recently set out to examine TLR signaling in the microenvironment of solid tumors treated with high-dose ionizing radiation.6

Our initial studies based on TLR-deficient mouse strains revealed that the lack of TLR9 but not TLR4 delays the recurrence of aggressive and radioresistant tumors, such as melanoma, colon, and bladder carcinoma, upon RT. Although TLR9 signaling has not been previously associated with the immunogenic effects of RT, TLR9 was known to promote chronic inflammation in response to sterile tissue injury.7 Since TLR9 is commonly expressed by murine myeloid cells, which play an important role in tumor vascularization and disease progression, we compared angiogenesis in tumor-bearing wild-type (WT) and Tlr9−/− mice treated with RT. As expected the deletion of Tlr9 reduced tumor revascularization in this setting, most likely owing to the impaired recruitment of vascular endothelial growth factor receptor 2 (VEGFR2)+ endothelial progenitors from the bone marrow, which usually precedes angiogenesis.8 The levels of tumor-associated macrophages (TAMs) were similarly elevated in both mouse strains, but Tlr9−/− TAMs failed to activate STAT3 after colonizing irradiated tumors. We further demonstrated that TAMs recognize soluble TLR9 ligands released by malignant cells succumbing to RT, resulting in the activation of a myeloid differentiation primary response 88 (MYD88)- and NF-κB-dependent signaling pathway that promotes the secretion of interleukin (IL)-6 and hence the activation of JAK/STAT3 signaling (Fig. 1B). To obtain further insights into the molecular mechanism(s) underlying the ability of STAT3 to promoting tumor recurrence upon RT, we undertook a whole-genome expression profiling approach on myeloid cells isolated from irradiated tumors that
had developed in WT, Tlr9−/− or STAT3-deficient (Mx1-Cre-Stat3floxflo) mice. Our analysis revealed the molecular signature resulting from TLR9/STAT3-dependent signaling in myeloid cells that infiltrate irradiated tumors. We highlighted a key role for STAT3, which was involved in the transcriptional control of most TLR9-regulated genes. Several genes upregulated by TLR9/STAT3 signaling coded for oncogenic and pro-angiogenic factors, including chemokine (C-C motif) ligand 1 (CCL1), histidine decarboxylase (HDC), transforming growth factor β1 (TGFβ1), and many known STAT3 activators, including IL-6, IL-6 receptor (IL-6R), IL-23, leukemia inhibitory factor (LIF), sphingosine-1-phosphate receptor 3 (S1PR3), and heparin-binding EGF-like growth factor (HBEGF), pointing to the activation of feed-forward mechanism that perpetuate STAT3 signaling. At the same time, TLR9/STAT3 signaling reduced the expression of multiple genes promoting the terminal differentiation or apoptotic demise of macrophages, including growth arrest and DNA-damage-inducible (Gadd45), Rho-associated, coiled-coil containing protein kinase 1 (Rock1) and BCL2-associated transcription factor 1 (Bclaf1).

Therefore, STAT3 seems to convert TLR9 signaling into a transcriptional program that prevents myeloid cell differentiation while promoting their survival and angiogenic activity. We further verified these findings in vivo, demonstrating that Stat3 ablation or silencing using oligonucleotides that specifically target TLR9+ myeloid cells, i.e., CpG-Stat3 small-interfering RNAs (siRNAs), is sufficient to correct aberrant TLR9 signaling and prevent cancer recurrence upon RT.

The identity of TLR9 agonists released by irradiated tumor cells is yet unclear, but previous reports suggested that the mitochondrial DNA, alone or complexed with small peptides, can trigger TLR9 activation in vivo. In fact, preliminary experiments performed in our lab indicate that the plasma levels of mitochondrial DNA are elevated shortly after RT not only in mice bearing mouse and human tumors but also in prostate cancer patients subjected to standard RT regimen (unpublished data). Further studies are still necessary to verify whether TLR9/STAT3 signaling contributes to the recurrence of human tumors after RT. If this turned out to be the case, targeting the TLR9/STAT3 signaling axis could provide several therapeutic opportunities to improve the clinical outcomes of RT. Approaches in this sense include the combination of RT with therapeutics that currently in clinical

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Figure 1. TLR9-dependent “emergency” signaling restores STAT3 activity in the tumor microenvironment, thereby accelerating cancer recurrence after radiation therapy. (A) Multiple cytokines, growth factors, and sphingolipids promote the activity of signal transducer and activator of transcription 3 (STAT3) in tumor-associated macrophages (TAMs) upon the engagement of JAK and SRC kinases. STAT3 is a critical mediator of the pro-angiogenic and immunosuppressive activities of myeloid cells in the tumor microenvironment. (B) High-dose radiation therapy results in the death of most malignant cells, hence mediating robust therapeutic effects. However, danger signals delivered by dying cells can generate an “emergency” cue to restore STAT3 activity. In particular, the activation of Toll-like receptor 9 (TLR9) results in the activation of a myeloid differentiation primary response 88 (MYD88)- and NF-κB-dependent signaling pathway that promotes the secretion of interleukin-6 (IL-6) and hence the activation of JAK/STAT3 signaling in freshly recruited TAMs. Active STAT3 drives a transcriptional program which promotes the pro-angiogenic functions of TAMs, hence supporting tumor recurrence.
trials such as TLR9 antagonists, humanized IL-6R-blocking antibodies, and small molecule inhibitors of NF-κB or JAK/STAT3 signaling. The overall efficacy of strategies relying on the systemic blockade of TLR9/IL-6/STAT3 signaling may be limited by unexpected toxicities resulting from both on-target and off-target effects. The local and myeloid cell-targeted delivery of STAT3 inhibitors stands out as a safe but effective approach to support RT, as demonstrated by our proof-of-principle experiments based on CpG-Stat3 siRNAs. The availability of CpG-STAT3 siRNAs optimized for targeting human TLR9+ immune cells further increase the clinical potential of this strategy. The combination of local TLR9 agonist (CpG oligonucleotides alone) with RT has already shown promise in Phase I/II clinical trials enrolling B-cell lymphoma patients. Therefore, we believe that the targeted inhibition of TLR9/STAT3 signaling in the tumor microenvironment may constitute a novel approach to support the efficacy of RT in cancer patients.

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

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