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Radiotherapy Combined with Intralesional Immunostimulatory Agents for Soft Tissue Sarcomas

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Abstract

Immunotherapy has shifted the treatment paradigm for many types of cancer. Unfortunately, the most commonly used immunotherapies, such as immune checkpoint inhibitors (ICI), have yielded limited benefit for most types of soft tissue sarcoma (STS). Radiotherapy (RT) is a mainstay of sarcoma therapy and can induce immune modulatory effects. Combining immunotherapy and RT in STS may be a promising strategy to improve sarcoma response to RT and increase the efficacy of immunotherapy. Most combination strategies have employed immunotherapies, such as ICI, that derepress immune suppressive networks. These have yielded only modest results, possibly due to the limited immune stimulatory effects of RT. Combining RT with immune stimulatory agents has yielded promising preclinical and clinical results but can be limited by the toxic nature of systemic administration of immune stimulants. Using intralesional immune stimulants may generate stronger RT immune modulation and less systemic toxicity, which may be a feasible strategy in accessible tumors such as STS. In this review, we summarize the immune modulatory effects of RT, the mechanism of action of various immune stimulants, including toll-like receptor agonists, and data for combinatorial strategies utilizing these agents.

Introduction

Over the past decade, immunotherapy has become a cornerstone in treating various malignancies. Chimeric antigen receptor (CAR) T-cells and immune checkpoint inhibitors (ICI) have shifted the treatment paradigm for leukemia, lymphoma, lung cancer, melanoma,[1–4] and many other cancers. Despite these revolutionary advances, most cancer patients will not benefit from immunotherapy and additional progress is needed. The effectiveness of CAR T-cells remains limited in treating solid tumors, and ICI are less successful at generating clinically meaningful anticancer responses as monotherapy in solid tumors with low mutational burdens, such as soft tissue sarcomas (STS).[5,6] Therefore, it is

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important to explore other therapies that can work synergistically with immunotherapy to induce a robust anti-cancer response in other histologies.

Radiotherapy has long been proposed to have the potential to work synergistically with immunotherapy to enhance antitumor response in primary tumors and promote a systemic antitumor immune response. There are numerous immune modulating mechanisms of radiotherapy, some enhancing antitumor effects and some promoting immunosuppression.[7] In-situ vaccination has been a mechanism of particular interest, in that it could generate synergy with ICI. The induction of immunogenic cell death increasing the availability and presentation of tumor antigens coupled with increased trafficking of lymphocytes into the tumor microenvironment (TME) could induce new and expand existing antitumor immune responses. Clinical evidence supports this concept in that on rare occasions, an abscopal effect of radiotherapy has been reported where a systemic antitumor effect is observed outside the radiation field.[8–11] In these rare instances, it is likely that radiotherapy depends, in part, on this in-situ vaccination effect for its efficacy. However, the rarity of abscopal events suggests that radiotherapy generally does not generate a sufficient in-situ vaccination effect strong enough to eradicate systemic disease. As with other vaccine approaches, it is likely that vaccine adjuvants will be required to realize the full capacity of radiotherapy induced in-situ vaccination. This may be of particular relevance in diseases such as STS where the antitumor T cell response is rarely strong enough to generate clinical response from ICI monotherapy.

Toll-like receptor (TLR) agonists and other immunotherapies such as Fms-like tyrosine kinase 3 ligand (FLT3L), stimulator of interferon genes (STING) agonists, CD40 agonists, oncolytic viruses, and cytokines have shown promising antitumor effects in preclinical and clinical studies. Local injection of these immunostimulants in STS with radiotherapy could produce a robust in-situ vaccination effect and allow potent activation of both innate and adaptive immune systems while minimizing side effects that are commonly associated with systemic therapies.

The objective of this review is to explore the antitumoral effectiveness of combining intratumoral injection of TLR agonists or other immunostimulants into STS with adjuvant radiotherapy to generate local and systemic antitumor immune responses. We will examine both preclinical and clinical trial data to provide a comprehensive overview of the potential and limitations of such therapeutic strategies. The ultimate goal is to shed light on the scientific rationale and the clinical feasibility of integrating locally administered immunostimulants with radiotherapy for the treatment of STS.

Immunomodulatory Effects of Radiotherapy

Historically, the principle of radiotherapy involves the precise delivery of ionizing radiation to a focal tumor while minimizing damage to the surrounding healthy tissues for a localized effect in cancer directed care. Determining the optimal timing, dose, delivery method, and combination of RT and immunotherapy remains a challenge when balancing desired antitumor immune response and therapeutic toxicity.[12] Recent advances in our understanding of the mechanism of action of ionizing radiation on the immune system has

resulted in increasing attention to radiotherapy's ability to stimulate the immune system, locally, at the level of the TME, and systemically to cause antitumor effects manifesting as regression in distant, unirradiated sites.[13] A detailed review of the immune modulatory effects of radiotherapy has been performed elsewhere[7] and is beyond the scope of this discussion, but we will provide a brief synopsis of points central to our topic. An idealized and simplistic account of radiotherapy induced in-situ vaccination is presented below (Fig. 1).

First, radiotherapy is primarily a local modality and the effects of radiation on the immune system begin directly at the site of the tumor irradiation (and draining lymph nodes). Radiotherapy can affect the TME which consists of various immune system cells, an intricate network of fibroblasts, blood vessels, lymphatics, and the cancer cells themselves. These components of the TME are enclosed within a dense structure formed by collagen and elastin fibers, collectively known as the extracellular matrix. The TME has been shown to be a vital factor in affecting the signaling pathways that influence tumor characteristics that promote or suppress antitumor immunity and ultimately disease progression.[14] Radiotherapy can alter the TME structure to increase diapedesis of immune cells. Infiltrating antigen-presenting cells, such as dendritic cells, can take up antigens from dying tumor cells and present them to T cells to activate the adaptive immune response against the tumor.[15,16] Thus, the first local action of radiotherapy is to alter the stroma and the TME to allow influx of immune cells. Second, due to DNA and RNA alteration and protein misfolding, cancer cells generate new nonself antigens, referred to as neoantigens, which the immune system can identify as foreign.[17] Irradiated cancer cells undergo immunogenic cell death, resulting in the uptake and presentation of tumor-specific antigens by the increased infiltration of immune cells. The dying cancer cells also release damage-associated molecular patterns (DAMPs), resulting in the upregulation of interferons and activation of the inflammatory cascade.[15] Radiotherapy augments type-1 IFN signaling and TLR expression.[18,19] These effects can increase the functionality of antigen presenting cells to present tumor neoantigens more efficiently and they can also upregulate antigen presentation machinery on cancer cells. Finally, the increased antigen presentation can generate new and bolster pre-existing antitumor T cell responses. The chemokine and cytokine changes in the TME can help support T cell functionality and increased expression of MHC and stress ligands on tumor cells can sensitize them to T cell killing.[20] Only when T cells are generated with sufficient function and numbers to enter the systemic circulation and eradicate distant tumors can this be considered a true in-situ vaccination resulting in an abscopal response.

While data clearly supports the radiation-induced immune events outlined above, the full story is more complex as the TME consists of a milieu of immune cells that promote and suppress an antitumor immune response. Radiation can increase the release of immunosuppressive cytokines, such as VEGF, TGF-β, and IL-10. These cytokines can promote suppressive immune cell function in the TME and reduce the overall lymphocyte count in systemic circulation.[21,22] Radiotherapy can also upregulate other immune suppressive factors such as PD-L1 and the enzyme indolamine-2,3-dioxygenase (IDO).[23] While the exact kinetics are model dependent and not completely deciphered,

it generally appears that radiotherapy introduces a brief window of pro-inflammatory opportunity before these immune suppressive networks set in.

While local stimulation of the immune system from immunotherapy may enhance the response of a tumor to RT, the potential systemic effects of radiotherapy and immunotherapy create an opportunity for even greater clinical impact. The interplay between radiotherapy and immunotherapy based on evidence suggesting that radiotherapy can trigger an anti-tumor immune response has been summarized in prior reviews dating back over 50 years ago.[24–26] Yet, as mentioned above, the antitumor immune response induced by radiation alone may not be sufficient, likely due to a limited in-situ vaccination effect which is unable to induce meaningful systemic T cell responses, the upregulation of immune suppressive networks, and a hostile TME at distant unirradiated sites.

Combining Radiation and Immunotherapy in Sarcomas

Treatment of STS typically involves a multimodal approach. Radiotherapy for STS is often administered to lower the risk of local recurrence. Neoadjuvant RT can be delivered prior to surgery to try to reduce tumor size and improve operability. An unmet need in STS is improved therapies to prevent and treat systemic disease. About half of patients with high-grade STS will develop distant metastases and current chemotherapy approaches have response rates in the 15% to 20% range and are highly toxic. An emerging approach being evaluated in clinical trials is a combination of radiotherapy and immunotherapy to bolster the immune response against STS. Several studies have reported an improved immune response when systemic immunotherapy and radiotherapy are combined.[27–29] Developing strategies combining local radiotherapy and immunotherapy to promote an in-situ vaccination robust enough to prevent or treat systemic disease would have a significant impact on patient care.

There are limited preclinical studies examining the synergistic effects of radiotherapy with immunotherapies to enhance the immune response against STS, but it is an area of increasing research. Wisdom et al.[30] investigated the response of STS to RT and anti-PD-1 immunotherapy in a primary mouse model of STS and transplanted STS derived from the primary tumor. Transplanted STS had a marked synergistic response to RT + anti-PD-1, but the primary STS was resistant to the same combination therapy.[30] These results highlight the difference between the tumor immune microenvironment in a cancer that develops after cell line injection into syngeneic mice (ie transplanted tumors) and autochthonous tumors that coevolve with the immune system. They also emphasize the importance of testing RT + immunotherapies in primary sarcoma preclinical models and studying the TME in human STS.

Studies of the TME in human STS have been performed at several institutions. A study from Memorial Sloan Kettering has shown that PD-L1 expression in STS was relatively low compared to other tumors, and was not able to demonstrate correlation between PD-L1 expression and tumor type, size, or grade.[31] This study was limited by sample size. Kim et al.[32] investigated PD-L1 expression in STS and found that STS patients with elevated PD-L1 expression experienced lower event-free survival suggesting a potential role for the immune

system in patient outcomes. Kirtesh et al.[33] explored the role of neoadjuvant radiotherapy in modulating PD-L1 expression in STS and suggested that radiotherapy may improve efficacy of agents that target the PD-1/PD-L1 immune cascade, though this study was limited by a small sample size.

A critical question for STS is which type(s) of immunotherapy and what administration route of immunotherapeutic agent will maximize the efficacy of combined radiotherapy and immunotherapy. This review will examine available preclinical and clinical studies related to intratumoral injection of TLR agonists and other immunostimulants for STS to activate the immune system. The systemic administration of immunotherapies can result in potentially severe side effects. This is particularly true of immunostimulatory agents such as systemic IL-2, which can cause a capillary leak syndrome and adversely impact the lungs, heart, kidneys, and CNS. Advancements in interventional techniques have increased the feasibility of intratumoral delivery of immunotherapeutic agents, such as TLR agonists, allowing for high local drug exposure in the TME to induce strong local and potentially systemic antitumor response while minimizing the risk of systemic adverse effects.[34] The TME plays a critical role in immune response against STS triggered by the combination of immunotherapy and radiotherapy. Thus, direct targeting of the TME with intralesional immunotherapy in combination with radiotherapy is an area of particular interest.

TLR Agonists

TLRs play a fundamental role in the activation of innate immunity and can act as a bridge to adaptive immune responses. TLRs are membrane-bound receptors that are usually constitutively expressed on innate immune cells and identify pathogen-associated molecular patterns (PAMPs).[35] PAMPs are conserved molecular motifs that are commonly associated with various pathogens, but not present in humans.[36,37] Commonly seen motifs include lipoteichoic acid and peptidoglycan from bacteria activate TLR2; double-stranded RNA (dsRNA) fragments from viruses are usually detected by TLR3; lipopolysaccharides (LPS) from gram-negative bacteria bind to TLR4; Flagellin from bacteria activates TLR5; TLR7; or TLR8 recognizes single-stranded RNA (ssRNA) fragments from viruses; unmethylated CpG motifs from viruses, bacteria, and protozoa activate TLR9. Many endogenous ligands for TLRs have also been identified. These are generally molecules expressed or released during times of stress and have been referred to as damage-associated molecular patterns (DAMPs).

TLR agonists bridge the innate and adaptive immune system by activating and upregulating antigen presentation by innate immune cells to T cells and B cells. A few TLR agonists were listed as immunotherapeutic agents with the highest potential to treat cancer by the National Cancer Institute, such as polyinosinic-polycytidylic acid (polyI:C) (TLR3 agonist), monophosphoryl lipid A (MPLA) (TLR4 agonist), imidazoquinoline compounds (TLR7 agonist), and CpG oligodeoxynucleotides (CpG-ODN) (TLR9 agonist) (Fig. 2).[36,38] These TLR agonists have been studied both pre-clinically and in clinical trials for different types of cancer. They can be used as stand-alone agents or in combination with other therapies such as chemotherapy, radiotherapy, and other forms of immunotherapy to augment therapeutic responses. Preclinical models have shown promising results in activating antitumor immune

responses, while early-phase clinical trials have demonstrated tolerable safety profiles and initial signs of efficacy, especially when combined with other treatment modalities.[39–41] Further research is needed to explore the full therapeutic potential of TLR agonists in STS when combined with radiotherapy.

TLR3

TLR3 is expressed in multiple cell types, including myeloid DCs, macrophages, NK cells, neurons, astrocytes, fibroblasts, and epithelial cells.[42–45] TLR3 is expressed both on the cell surface and intracellularly in most cell types, but it is exclusively localized to endosomes in myeloid DCs.[43] TLR3 primarily recognizes double-stranded RNA (dsRNA), which is commonly associated with viruses. Upon binding to its ligand, TLR3 activates a downstream signaling cascade via the toll/interleukin-1 receptor domain-containing adapter-inducing interferon-β (TRIF) pathway. This leads to the activation of NF-κB, interferon regulatory factor 3 (IRF3), and mitogen-activated protein kinase (MAPK) which induce the expression of type I interferons and other proinflammatory cytokines.[43,46,47] It should be noted that cytokine production via TLR3 is myeloid differentiation factor 88 (MyD88)-dependent, but DC maturation and activation of NF-κB and MAPKs are MyD88-independent, unlike other receptors in the TLR family where MyD88 is an essential signaling adapter.[45,46]

Poly I:C is the synthetic analog to dsRNA and a potent agonist for TLR3.[46] TLR3 agonist is not usually employed as monotherapy in cancer treatment. One preclinical paper showed that poly(I:C) monotherapy is sufficient to reduce tumor burden by 51% in mice with heterotopic lung cancer.[48] However, most studies utilize TLR3 agonist as an adjuvant therapy to stimulate DC activation and antigen-presentation. It is also often administered as an intralesional therapy.

Hammerich et al. combined poly-lysine and carboxy-methylcellulose (pICLC), FLT3L, and radiotherapy as an in situ vaccine (ISV), to recruit and activate DCs in the intratumoral areas in mice with transplanted A20 lymphoma. pICLC (or Hiltonol) is a clinical-grade formulation of poly I:C.[49,50] They observed tumor-free survival in 40% of mice with an abscopal effect. After anti-PD1 was added to the treatment regimen, about 80% of mice survived for at least 3 months with systemic tumor burden reduction.[50] The promising treatment effect in preclinical settings prompted a clinical trial for eleven patients with advanced-stage indolent non-Hodgkin’s lymphomas.[50] About 72% of the patients had partial or complete regression of the treated tumor, while 27% of the patients showed significant tumor reduction at treated and distal sites, achieving total remission.

Other studies have demonstrated the combination of TLR3 agonists with other treatment modalities improves outcomes in various pre-clinical models. Lee et al. showed that a combination treatment of TLR2 agonist, TLR3 agonist, anti-PD1, and CTLA-4 induced complete tumor regression in 90% of mice with orthotopic B16F10 melanoma. They also showed that tumor-specific immune memory was preserved after treatment as mice rechallenged with the same B16F10 tumor cells rejected tumor implantation, but MC38 tumors were able to grow in the same mice.[51] Chemotherapy combined with TLR3 agonists

demonstrated enhanced tumor cytotoxicity in preclinical models of pleural mesothelioma and colon cancer.[52,53]

Several clinical trials have been conducted with TLR3 agonists. NCT01188096 is a phase I study evaluating pICLC in the management of recurrent pediatric low-grade gliomas, and preliminary results suggest an overall response rate of 74% at 6 months following treatment (source: www.clinicaltrials.gov). Most of the clinical trials involve some combination of anti-PD1/anti-PD-L1 and TLR3 agonists.[54] The efficacy of such treatment is generally reported with clinical benefits in various cancers, except for a study in high-grade glioma patients.[55,56]

Only 3 clinical trials to date involve the utilization of TLR3 agonists and RT to treat cancers. NCT01976585 was conducted in patients with advanced-stage indolent non-Hodgkin's lymphomas, and 8 out of 11 patients experienced partial or complete lymphoma regression as mentioned above.[50] NCT03789097 is currently recruiting patients with non-Hodgkin's lymphoma, metastatic breast cancer, and, head and neck squamous cell carcinoma to undergo treatment with pICL, radiation, FLT3L, and pembrolizumab. NCT03835533 recently completed recruitment of patients with metastatic castration-resistant prostate cancer for different interventions with stereotactic body radiation + CDX-301 + Poly-ICLC + nivolumab as one of the experimental arms. The combination of TLR-3 agonists, RT, and adjuvant immunotherapy warrants further investigation in solid tumors with convenient intralesional injections such as STS, and the outcomes of some of these trials will help further guide development of this strategy. Local administration of TLR3 agonists is usually well tolerated with low toxicity. Patients generally experience grade 1 adverse events or no side effects.[50] Several clinical trials have observed mild side effects with few treatment-limiting toxicities.[56]

TLR4

TLR4 is localized to the cell surface and is well-known for its ability to recognize lipopolysaccharide (LPS), a critical component of the cell wall of gram-negative bacteria. When activated, TLR4 recruits toll/interleukin-1 receptor domain containing adaptor protein (TIRAP) or TRIF-related adapter molecule (TRAM) to the plasma membrane and initiates a complex intracellular signaling cascade that involves the MyD88-dependent and the MyD88-independent pathways, respectively.[47,57] MyD88 leads to the activation of MAPKs, AP1s, and NF-κB. The MyD88-independent pathway activates the transcription factor IRF-3. Both pathways lead to the production of proinflammatory cytokines and type I interferons. High mobility group box 1 (HMGB1), a DNA binding protein which is released by dying irradiated cells and helps induce immunogenic cell death, may be an endogenous TLR4 agonist.

LPS induces the activation of various innate immune cells, which leads to antigen presentation and activation of cellular and humoral immune responses.[58,59] However, it should be noted that DCs actually express low levels of TLR4 mRNA and surface receptors in mice and humans; macrophages, on the other hand, express high levels of TLR4.[60–63] LPS can activate DCs through a bystander mechanism whereby LPS-responsive cells induce

upregulation of CD86 in DCs through IFN-γ and enhanced responsiveness through the TLR9 pathway.[64] It is also interesting to note that human CD4 and CD8 T cells express TLR4 receptors and can respond to LPS directly. Murine CD4 T cells also express and respond to TLR4, but murine T cells appear not to respond to most TLR agonists directly.[65–67]

LPS is the natural ligand of TLR4, which is an essential component of gram-negative bacteria. It was first utilized in cancer immunotherapy in the early 1940s, when it was identified as an active ingredient in Coley's toxin.[68] LPS has since been studied in a few preclinical studies to treat various cancers. Mariani and his colleagues treated rats with RG-2 gliomas using LPS and single-fraction external beam radiotherapy (EBRT) or LPS and Zymosan A. Both combination treatments resulted in reductions in tumor burdens and the development of immunologic memories that protected rats from tumor rechallenge.[69] Chicoine et al. also studied the effectiveness of LPS in treating gliomas but using murine models. They injected LPS intratumorally into subcutaneously implanted mouse gliomas and observed complete tumor regression in most mice. However, higher doses of LPS injection resulted in treatment-related deaths in about 40% of animals.[70] LPS is also often associated with more severe "flu-like symptoms" in humans.[71,72] Therefore, researchers tried replacing LPS with the lipid A subunit or its synthetic analogs. Farias and his colleagues used Brucella lumazine synthase (BLS), a homodimeric protein, as the TLR4 agonist. They treated cutaneous melanoma with BLS and anti-PD1, which delayed tumor growth significantly.[73]

Many lipid A analogs that have been tested in clinical trials produce limited antitumor responses.[74–76] However, glucopyranosyl lipid A (GLA) or G100 has shown promise as a potent immunostimulant with superior tumor suppression effects. Intradermal injection of G100 in human skin explant model enhanced human skin DCs' capacity to activate T cells.[77] Pollack et al. conducted a phase I clinical trial of radiation therapy and intratumoral stable emulsion formulation of GLA (GLA-SE) in 12 patients with metastatic STS (NCT02180698).[78] Patients were treated with 8 weekly intratumoral injections of GLA-SE starting one to two weeks before radiotherapy (23 Gy to 40 Gy delivered over 2 weeks). All of the tumors that were treated with TLR4 agonist and radiotherapy achieved local control with a pooled mean tumor size reduction of 69%. In contrast, in patients with concomitant lesions that were treated with radiotherapy alone or with unirradiated tumors, the pooled mean tumor size decreased (−39%) and increased (+69%) respectively.[78] Taken together, these results suggest that intratumoral injection of GLA-SE enhances the efficacy of radiotherapy, but is not able to activate a systemic antitumor immune response to cause tumor regression. Additional clinical trials are needed to study GLA-SE as an adjuvant immunotherapy with radiotherapy.

TLR7

TLR7 is an endosomal sensor that is primarily expressed in plasmacytoid dendritic cells (pDCs) and involved in the recognition of ssRNAs. A notable characteristic of pDC is its ability to secrete large amounts of type I interferon during viral infections.[45,47,79] TLR7 has 2 binding sites. One site is for small ligands that are conserved in both TLR7 and TLR8.

The other binding site is strictly for binding with ssRNA and enhances activation of the first binding site.[80] Activation of TLR7 leads to the recruitment of interleukin-1 receptor-associated kinase 4 (IRAK4). MyD88 is then also recruited to form a complex containing TRAF3, TRAF6, IRAK4, IRAK1, IKKa, OPNi, and Dock2. The complex activates IRF7 which results in the transcription and translation of type I interferons.

A few TLR7 agonists have been developed over the years. Imiquimod is one of the earliest and most utilized TLR7 agonists since its approval by the Food and Drug Administration (FDA) as a treatment for basal cell carcinoma and genital warts.[81,82] TLR7 agonist was first utilized as a cancer therapy because TLR7 is upregulated in oral squamous cell carcinoma (OSCC).[83] Imiquimod effectively induced necrosis and apoptosis in OSCC cells. Imiquimod has since been found to be effective in suppressing tumor growth in murine models of bladder cancer, colon cancer, lymphoma, and melanoma.[84–86] Other TLR7 agonists have also shown promising results in preclinical models. DSR-6434, DSR-29133, and R848 have synergistic antitumor effects when combined with RT to treat murine sarcoma and colon cancers.[87–89] Adlard and her colleagues showed that RT + DSR-6434 delayed sarcoma growth by approximately 7.5 days when compared to mice receiving RT alone.[87] Dovedi et al.[88] also reported that a combination of DSR-29133 and RT cured 80% of mice with colon cancer. Another study by Dovedi et al. also showed superior treatment effects in mouse models of lymphoma with combined TLR7 agonist (R848) and radiotherapy. All 3 studies demonstrated the capacity of RT to work synergistically with TLR7 agonists and activate tumor antigen-specific CD8 T cell response in mice.[87,88]

Due to the encouraging data demonstrated in preclinical studies, a series of clinical trials testing TLR7 agonists have been conducted. In 1 study in early-stage cutaneous T-cell lymphoma, 92% of patients experienced improvements in more than 50% of body surface area and 2 patients had complete remission of the disease after treatment with TLR7 agonist.[90] In a clinical study in patients with breast cancer and cutaneous metastases, topical imiquimod, and Nab-paclitaxel resulted in a complete response in 36% of patients and partial response in 36% of patients.[91] However, the response duration was limited with analysis showing increased levels of PD-1+ T cells after treatment.[91] Therefore, it is possible that the addition of anti-PD1 to the treatment regimen might improve response duration. A search of clinicaltrials.gov does not reveal a clinical trial that utilizes radiotherapy and TLR7 agonists to treat solid tumors, despite positive preclinical data suggesting synergistic effects between the 2 treatments. This combination treatment represents a potential strategy for future investigation.

TLR9

TLR9 is another endosomal receptor that is predominantly expressed in pDCs. TLR9 is mainly involved in the recognition of unmethylated CpG motifs commonly found in bacterial and viral DNA. Upon activation, TLR9 also signals through the MyD88-dependent pathway in the pDCs, similar to that of TLR7. IRAK1, within the complex recruited by MyD88, phosphorylates IRF7, which is constitutively expressed at high levels in pDCs.[79,92,93] IRF7 then translocates into the nucleus and upregulates the expression of type I interferons, thereby initiating a robust immune response.[94] TLR9 agonists, such as

CpG-ODN, have been evaluated in murine, canine, and clinical trials for their safety profiles and antitumor effects.[23,41,95,96]

There are 3 major classes of CpG-ODN, Class A, B, and C, corresponding to preferred stimulation of pDCs, B cells, or dual activation.[97] The A-class CpG is characterized by phosphorothioate-modified 3′ ends with poly G and a phosphodiester palindromic center.[98,99] The A-class CpG induces strong IFN-α expression in pDCs, but weaker stimulation of B cells. The B-class CpG predominantly activates B cells and NK cells with the specific hexamer motif 5′-GTCGTT-3′ for humans and 5′-GACGTT-3′ for mice.[100–102] The C-class CpG is designed to stimulate both B cells and pDCs at the same time, thus it contains both a complete phosphorothioate backbone and a CpG palindromic center.[97]

In 1995, Krieg et al.[103] described how CpG motifs in bacterial DNA induced more than 95% splenic B cells proliferation and activation in mouse models. This elicited increased research interest in utilizing CpG in cancer treatment. Akira's group later identified that TLR9 is responsible for mediating the immuno-stimulatory effect of CpG.[104] CpG has since been utilized in several preclinical studies as a single treatment modality, administered intratumorally or systematically, and demonstrating promising therapeutic effects.[105–107] However, clinical trials employing TLR9 agonist as monotherapy reported suboptimal treatment effects with low overall response rates.[108–115] Therefore, researchers explored combined therapies with TLR9 agonists in the hope of achieving greater treatment efficacy.

Levy's group explored the combined therapeutic effects of local TLR9 agonist and systemic agonistic OX40 antibody administration on murine models with spontaneous mammary gland tumors. OX40 is a receptor from the tumor necrosis receptor superfamily that is induced in activated T cells and acts as a costimulatory immune checkpoint molecule. They observed significant tumor burden reduction not only at the TLR9 agonist injection site, but also at distant tumor sites.[116] Milas et al. were amongst the first to examine the combination therapy effect of CpG-ODN and RT in STS. In a preprelinical transplanted FSa sarcoma model in syngeneic mice, they used peritumoral injection of a B-class CpG ODN 1826 once before, and twice after, single fraction radiotherapy to increase tumor growth delay by at least 2.5 fold and decreased the radiation dose required to achieve local control (TCD_{50} assay) by approximately 2-fold.[95] They observed that only 20.5 Gy of radiation therapy was needed to eliminate tumors in 50% of mice when combined with CpG treatment vs 39.6 Gy of radiation without CpG. When sarcomas were treated with 3 doses of CpG ODN 1826 and 20 Gy radiation, 43% of tumors were cured while the remainder of the tumors had prolonged tumor growth delay.[95] Even more potent radiosensitization was observed by Mason and colleagues with fractionated radiation therapy.[117] They delivered CpG ODN by peri-tumoral or intratumoral injection of transplanted fibrosarcomas in mice and found that the fractionated dose of radiotherapy required to achieve local control (TCD_{50} assay) decreased by 3.6-fold. As discussed above, transplanted tumor models may stimulate an antitumor immune response that primes preclinical models to respond to radiotherapy and immunotherapy in a manner that does not reflect the response in a tumor model system that coevolves with the immune system.[30] Therefore, Su and colleagues tested the combination of intratumoral injection of CpG ODN and a single dose of radiotherapy in an autochthonous mouse model of STS.[118] In this model where the sarcoma coevolves with the

immune system and does not show increased response by adding anti-PD-1 immunotherapy to RT, they observed increased tumor growth delay with combination CpG ODN and RT mediated by CD8+ T cells.[30,118]

Monjazeb et al. combined intratumoral CpG-ODN, local radiotherapy, and systemic indolamine-2,3-dioxygenase (IDO blockade) in spontaneous canine malignancies and observed robust antitumor responses. Five canines with spontaneous sarcoma or melanoma and distant metastases to the lung were enrolled in the study. All 5 canines received four 8 Gy fractions of radiotherapy with adjuvant intratumoral CpG-ODN and daily oral IDO blockade over a 4-week treatment period. They were followed up to 20 weeks after treatment. The local tumor response rate to treatment was 100%, and the systemic response rate was 60%. One canine had stabilized metastases, and only 1 had progressive metastatic disease despite a reduced local tumor burden.[23] The significant antitumor effect achieved through 4 weeks of treatment warrants future studies with longer courses of therapy in spontaneous canine tumors and human patients.

Due to the positive treatment outcomes with radiation therapy and TLR9 agonists observed in preclinical models and in canine patients, several clinical trials have been conducted. Levy's group explored the use of radiation in combination with TLR9 agonists in treating mycosis fungoides and indolent B-cell lymphoma in patients. All 3 clinical trials have demonstrated meaningful reductions in not only tumor burdens at the treatment site, but also systemically.[96,119,120] The findings are highly promising, especially since instances of abscopal effects have been previously reported but are seldom observed. Achieving systemic tumor reduction through localized treatment could have significant clinical implications if these results are replicated and expanded to sarcomas and other solid cancers.

FLT3

FLT3 (CD135) is a transmembrane tyrosine kinase receptor that is expressed on monocyte and dendritic cell progenitors, as well as terminally differentiated conventional DCs (cDCs) and plasmacytoid CDs (pDCs).[121,122] Upon binding with FLT3 ligand (FLT3L), the cytoplasmic domain of FLT3 associates with phosphoinositol-3-kinase (PI3K), and activates downstream pathways such as PI3K/protein kinase B (Akt) and MAPK pathways, which ultimately drive cellular proliferation, survival, and differentiation.[123–125] In humans and murine models, FLT3L drives proliferation of various bone marrow progenitor cells that are crucial for differentiation of B cells, NK cells, monocytes, DCs, and innate lymphoid cells.[126–133] Furthermore, FLT3 signaling is especially important to the expansion and differentiation of conventional dendritic cells and plasmacytoid dendritic cells.[127–130] These mature populations of dendritic cells preserve FLT3 expression unlike most other immune cells.[134] Due to its potent immunostimulatory effects, particularly on dendritic cells, FLT3L has garnered interest in the field of cancer immunotherapy. It has been studied as an adjuvant to enhance the efficacy of cancer vaccines and other immunotherapeutic strategies, including the use of TLR agonists such as TLR3, TLR7, and TLR9, by bolstering antigen presentation, plasmacytoid dendritic cell activation, and T-cell priming.[50,135,136] Hammerich et al.[50] developed a treatment regimen combining FLT3L, radiotherapy, a TLR3 agonist, and anti-PDl that demonstrated abscopal cancer remissions in indolent non-Hodgkin's lymphoma

patients. This group is currently conducting another clinical trial NCT02839265, utilizing the same treatment regimen in patients with nonsmall cell lung cancer. There are currently 7 ongoing clinical trials utilizing recombinant FLT3L according to clinicaltrials.gov. Most of these trials utilize combinations of FLT3L with TLR3 agonists or radiotherapy. Future preclinical and clinical studies combining FLT3L and TLR7 or TLR9 agonists in cancer therapy are worthy of investigation because TLR7 and TLR9 are constitutively expressed on plasmacytoid dendritic cells, suggesting potential activity for this combination.[137]

CD40

CD40 is a member of the TNF (tumor necrosis factor) receptor superfamily. It is a costimulatory receptor that is crucial in mediating activation and maturation of DCs.[138] CD40 is usually expressed on antigen presenting cells such as DCs, B cells, and macrophages.[139,140] The ligand of CD40, CD40L, is usually expressed by activated T cells and activated platelets.[141] However, CD40 can also sometimes be expressed by macrophages, B cells, and other immune cells under inflammatory conditions.[141] The interaction between CD40 and CD40L is crucial to cell-mediated immunity.[142] When CD40 is expressed on DCs and binds to CD40L, it allows DCs to express costimulatory molecules CD80/86 and produce IL-12 and IFN-γ.[143] It also upregulates expression levels of major histocompatibility complex (MHC) molecules on DCs, which allows for more efficient antigen presentation and activation of CD8+ T cells.[144] Given its potential to prime the immune system, CD40 agonists have been investigated as immunotherapeutic agents in cancer treatment, often in combination with other modalities such as radiotherapy, chemotherapy, and TLR agonists.

Nowak and colleagues found that the combination of cis-platin and CD40 agonist cured malignant mesothelioma in murine models.[145] Byrne and Vonderheide observed similar promising treatment efficacy of combined CD40 agonist, gemcitabine (Gem), and nab-paclitaxel (nP) in an autochthonous pancreatic ductal adenocarcinoma (PDA) model that closely mimics the immune microenvironment in patients.[146] They observed clonal CD8 T cell expansion against PDA after the addition of anti-CD40. They also reported that treatment efficacy does not require innate immune sensor pathways (TLR).[146] This promising result indicates that the addition of a CD40 agonist antibody can convert a known immunologically cold tumor into a “hot” one, and TLR agonists might be used as an adjuvant therapy to further stimulate the innate immune sensor pathways, because CD40 agonists activate the immune system through a separate TLR-independent pathway. Broomfield et al.[147] indeed demonstrated systemic malignant mesothelioma regression in mice when intratumoral TLR7 agonist was combined with CD40 agonist antibody. Vonderheide’s group also showed that the addition of radiotherapy and ICI to CD40 agonist antibody significantly reduced tumor burden and improved overall survival in preclinical models.[148] These results have led to several clinical trials (NCTs) NCT03165994, NCT04491084, and NCT03123783, that are currently underway.

Most clinical trials utilizing CD40 agonist antibodies as a single agent have not yielded a strong objective response rate in most patients.[149,150] However, clinical trials utilizing combination therapies with agonist CD40 antibodies and chemotherapy, or other

immunotherapies, have produced promising clinical outcomes. Nowak et al.[151] showed a 40% ORR in patients with malignant pleural mesothelioma when treated with cisplatin/pemetrexed and anti-CD40 agonist antibodies. O'Hara MH et al.[152] demonstrated a 54% ORR in patients with metastatic pancreatic cancer when treated with gemcitabine, nab-paclitaxel, and anti-CD40 agonist antibodies. Based on preclinical and clinical data, CD40 agonists have demonstrated notable activity in cancer treatment, at least when incorporated in combination therapies. Combining CD40 agonists with other cancer in situ vaccination treatments, such as radiation therapy, might further improve clinical responses.

STING

Developing chemotherapies and immunomodulators that in combination with radiotherapy induce T cell immunity is challenging. Immune responses triggered by STING pathway activation in irradiated tumors have the potential for systemic effects that could lead to the abscopal effect. Cytosolic accumulation of dsDNA following radiotherapy allows for cyclic GMP-AMP (cGAMP) oligomerization, and subsequent activation of STING.[153] Once activated, STING initiates transcription of cytokines important for T-cell response, including type I IFN, IL-6, and TNF. Type I IFN is critical for anticancer immune response initiation. This can potentially improve the overall effectiveness of radiotherapy and contribute to controlling metastatic disease. Harnessing the synergistic effects of radiotherapy and immune activation through the STING pathway holds promise for improving the outcomes of cancer treatment.

Pharmacologic STING agonists are under development.[153] Flavone acetic acid (FAA) was serendipitously developed as the first STING agonist.[154] Initially thought to disrupt the tumor vascular bed, FAA has been modified into more potent agonists such as 5,6-dimethylxanthenone-4-acetic acid (DMXAA, also known as ASA404 or vadimezan), which synergizes with other anticancer regimens such as radiation, chemotherapy (especially taxanes), immunomodulators, and immunotherapy. DMXAA promotes endothelial cell apoptosis, and phase I trials show an excellent safety profile without myelosuppression,[155] providing the opportunity for combination with chemotherapy regimens. Phase II and III clinical trials with DMXAA are evaluating intravenous DMXAA with chemotherapies, rather than radiotherapy. Thus far, this combination has had poor preliminary efficacy, which may be a consequence of DMXAA binding murine, but not human, STING.[156]

Other agents are under development to activate STING in human tumors. Macrocycle-bridged STING agonist (MBSA) agent E7766 is the only drug undergoing evaluation as a single intravenous agent for solid tumors. GSK3745417 is undergoing evaluation as a single agent or in combination with pembrolizumab, with a favorable safety profile. MK-2118 is the only STING agonist in clinical trials administered via intratumoral injection and is currently being studied with pembrolizumab. Le Naour et al.[153] have summarized clinical trials for other STING agonists (ADU-S100, SB11285, and BMS-986301), which are being studied in combination with immunologic agents.

A non-DMXAA derivative with STING agonist activity is phosphatidylserine coated (PS-coated) liposome loaded with cyclic guanosine monophosphate-adenosine monophosphate

(NP-cGAMP).[157] This agent is inhalable and allows direct uptake into lung antigen presenting cells (APCs), resulting in a proinflammatory tumor microenvironment, which can synergize with lung radiotherapy resulting in systemic anticancer immunity. Lastly, indirect STING activation and cytokine release is also initiated by other anticancer treatments such as radiotherapy, poly(ADP)-ribose polymerase (PARP) inhibitors, taxanes, and experimental ATR serine/threonine kinase (ATR) inhibitors. Collectively, developing multimodal anticancer regimens that directly or indirectly activate STING are promising.

Cytokines

Specific cytokines are known to modulate the tumor microenvironment and immune response, and cytokine-based therapies have demonstrated promise in STS. Determining the optimal timing, dosage, combination, and delivery method remains a challenge when balancing desired immune response and systemic toxicity. In this section, we will review data for intratumoral injection of IL-2, IL-12, and adenoviral vectors that express proteins to enhance radiotherapy response in mice and humans.

Systemic interleukin therapy is hampered by an unfavorable therapeutic index.[158] Side effects associated with systemic intravenous administration are related to high peak serum concentrations.[159] Intratumoral injection allows for drug/gene delivery in the tissue of interest (usually at a lower dose) and decreases the likelihood of systemic toxicity and allergic reactions. Intralesional IL-2 has been used clinically for in-transit and metastatic melanoma, sometimes in combination with radiotherapy. As opposed to systemic IL-2 that requires in-patient administration due to toxicity, intralesional IL-2 can be administered safely on an outpatient basis with limited toxicity and excellent efficacy in stimulating an immune response.[160,161]

Gene transfer methods further allow for confinement of cytokine therapy to the tumor microenvironment, limiting side effects. Less drug/gene is also required with local administration to achieve the desired treatment response. Limitations of cytokine intratumoral injection are primarily related to drug delivery to the lesion by percutaneous or endoscopic approach, as well as periprocedural complications (bleeding, infection, sedation, adverse events, etc.). With advanced imaging and improved minimally invasive interventional techniques, these risks continue to decline.

Initial preclinical studies of intratumoral injection of an adenoviral vector encoding IL-12 began in transplanted mouse models of sarcoma.[162,163] Murine fibrosarcoma insensitive to systemic IL-12 was treated with single dose intratumoral injection of IL-12-encoding fiber-mutant adenoviral vector (AdRGD-IL-12). Successful infection in Cox-sackie adenovirus receptor (CAR) deficient mice was also possible with AdRGD-IL-12, possibly via integrins. Intratumoral injection caused pronounced antitumor activity, increased mouse survival, induced T cell accumulation in the tumor, and exhibited an antimetastatic effect in addition to long-term antitumor immunity.[163] The antitumor mechanism of AdRGD-IL-11 depended on TME alterations and systemic immunity. Intratumoral injection was dependent on T-cells (primarily CD8+) and promoted cellular immunity (IFN-c positive cells in local lymph

nodes), thereby adapting the TME. Lymphocyte activation markers increased, as did the expression of cell adhesion molecules.

Adenoviral vectors encoding interleukins were further advanced by studying their effect in combination with radiotherapy in mice.[164,165] Distant microscopic metastatic disease was suppressed following intratumoral adenoviral vector expressing IL-12; locally, the antiangiogenic effects of IL-12 improved tumor response to radiation therapy, and T-cell mediated immunity helped suppress disease distant from the irradiated transplanted tumor. In combination, adenoviral vector intratumoral injection and radiotherapy were superior to individual treatments.

Similarly, neoadjuvant radiation and recombinant IL-2 intratumoral injection into a subcutaneous murine rectal cancer model showed augmented local and abscopal effects of concurrent hepatic metastases.[164] The administration of IL-2 significantly improved radiation response, and completely eradicated the transplanted subcutaneous lesion. Radiotherapy reduced hepatic metastasis formation, whereas IL-2 and radiotherapy in combination inhibited hepatic metastasis formation. A positive association existed between CD8+ T cell infiltration into the transplanted rectal cancer and lower metastatic frequency, presumably through systemic T cell induction as evident in higher populations of CD4+ cells in splenocytes. Collectively, the augmented immune response by intratumoral recombinant IL-2 injection improved radiotherapy of advanced rectal cancer.

A phase I study combining SBRT and systemic IL-2 in patients with metastatic melanoma (n = 7) or RCC (n = 5) resulted in 1 CR and 7 PR; of the 7 PR on CT RECIST criteria, 6 had CR when evaluated on PET-CT.[11] SBRT sites were metastatic lesions in either the chest or liver. SBRT and IL-2 were administered without clinically significant toxicity. The greater than expected response of melanoma patients suggested host immunity as a likely contributing factor, as evidenced by higher populations of circulating CD4+ effector memory T cells.

Based on phase I outcomes, a phase II study comparing systemic IL-2 alone with IL-2 and SBRT in metastatic melanoma (n = 44) was performed.[166] The ORR in combination and monotherapy were 54% and 35% (Table 1). Seven patients crossed over to the combination group. While PFS and OS were not different, the disease control rate was higher in the combination group; this may be related to pre-clinical findings that intratumoral injection has improved efficacy via changes in the TME when compared to systemic interleukin therapy. It is important to note that the combination of radiotherapy with intratumoral injection of recombinant or adenoviral vector interleukin for STS has not been studied to date in humans - this is an intriguing prospect.

Conclusions

The treatment landscape for STS is complex and often involves a multimodal approach that can include radiotherapy, surgery, and in some cases chemotherapy. During the past decade, a growing body of preclinical evidence supports clinical trials of immunotherapies for STS to try to enhance treatment outcomes. While systemic administration of immunotherapies

such as PD-1/PD-L1 inhibitors has shown promise in various cancers, the effectiveness of ICI is limited to a minority of patients with STS.[6,167] TLR agonists and other immunomodulators are promising immunotherapeutic approaches for STS. Intratumoral delivery of these immunostimulants to irradiated lesions could enhance local radiation response and have the potential to induce systemic responses while mitigating the risks associated with systemic administration. The TME serves as a critical target for immune engagement and is particularly amenable to localized therapeutic strategies, including radiotherapy and intratumoral immunotherapies. In combination with radiotherapy, these intralesional immunostimulants may help improve an in-situ vaccine effect and have the potential to induce distant immune-mediated responses.

TLRs occupy a unique space in this paradigm, serving as key initiators of both innate and adaptive immunity against STS.[35–37] TLR3, TLR4, TLR7, and TLR9 have received increasing attention in oncological research for their immunotherapeutic potential.[39–41] These agents have shown excellent safety profiles in pre-clinical models and clinical trials, and the studies generally demonstrated encouraging treatment efficacies, particularly when administered alongside other treatment modalities such as radiotherapy.[23,50,95,96] Other immunomodulators such as FLT3L, CD40, STING agonist, and cytokine therapy also offer exciting potential in the field of cancer immunotherapy. Combination strategies, particularly those involving radiation therapy, appear to be especially promising avenues for enhancing the local response to radiation therapy and have the potential to promote control of systemic cancer.

The location of many extremity and trunk wall STS provide a unique advantage for the utilization of combined intratumoral immunotherapy and RT. STS most commonly occurs in the extremities, accounting for about 50%-60% of all STS.[168,169] The remainder of the STS usually arises in trunks and retroperitoneum.[169] The primary tumor sites of STS are usually easily accessible for intratumoral injections and radiation, which is associated with a much lower chance of developing severe toxicity.

In summary, available data support the need for further rigorous, adequately powered studies in STS to dissect the intricate interplay between radiotherapy and localized immunotherapies. Exploring these avenues may not only deepen our understanding of STS pathophysiology but also open up new therapeutic regimens to improve the prognosis and quality of life for patients suffering from this challenging disease. As ongoing and future studies continue to unravel mechanistic interactions, optimal dosing, and potential synergies, a new horizon of targeted and less toxic cancer treatments may become available. Combination strategies, particularly those involving localized immunotherapies and radiation therapy, appear to be especially promising avenues for improving local control and have the potential to promote a systemic antitumor effect.

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Declaration of Competing Interest

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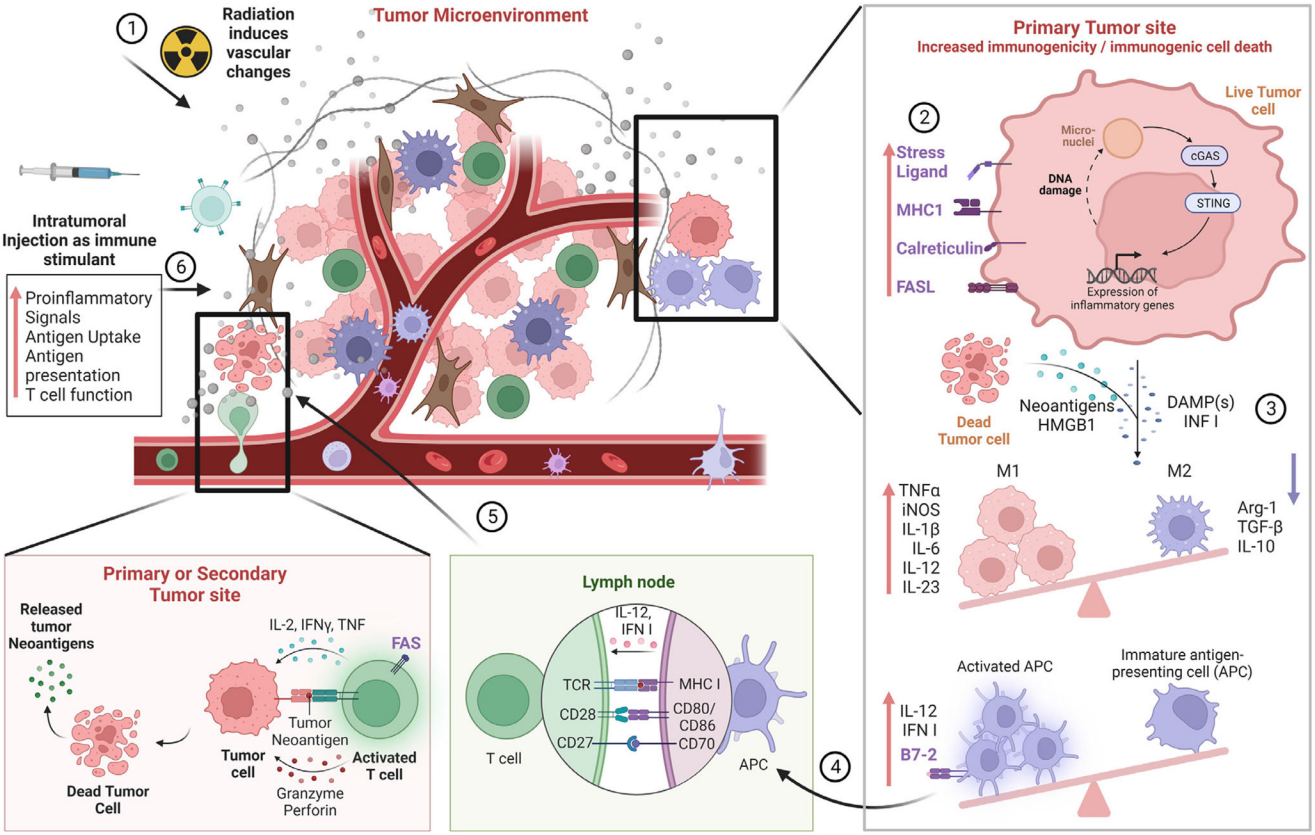

Figure 1.

Combined Effects of Radiotherapy and Intralesional Immunotherapy. (1) Radiotherapy can alter the TME by inducing vascular changes and increasing immune cell infiltration. (2) Radiotherapy increases immunogenic cell death (expression of calreticulin on cell surface and release of HMGB1) and the immunogenicity of surviving cancer cells through cell surface ligands (MHC1, FASL, stress ligands) which sensitize these tumor cells to immune mediated killing and secreted proinflammatory factors. (3) Immunogenic cell death and pro-inflammatory signals alter macrophage polarization and antigen presentation cell (APC) activation and antigen presenting function. (4) Activated APCs presenting neoantigens from dying irradiated cancer cells migrate to draining lymph nodes to activate T cells. (5) Activated T cells migrate to irradiated and non-irradiated tumors and enhance anti-tumor immunity. (6) Intratumoral injection of immune stimulants acts as an adjuvant and intensifies the radiotherapy in-situ vaccine effect by potentiating many of the mechanisms in 1-5. Created with BioRender.com.

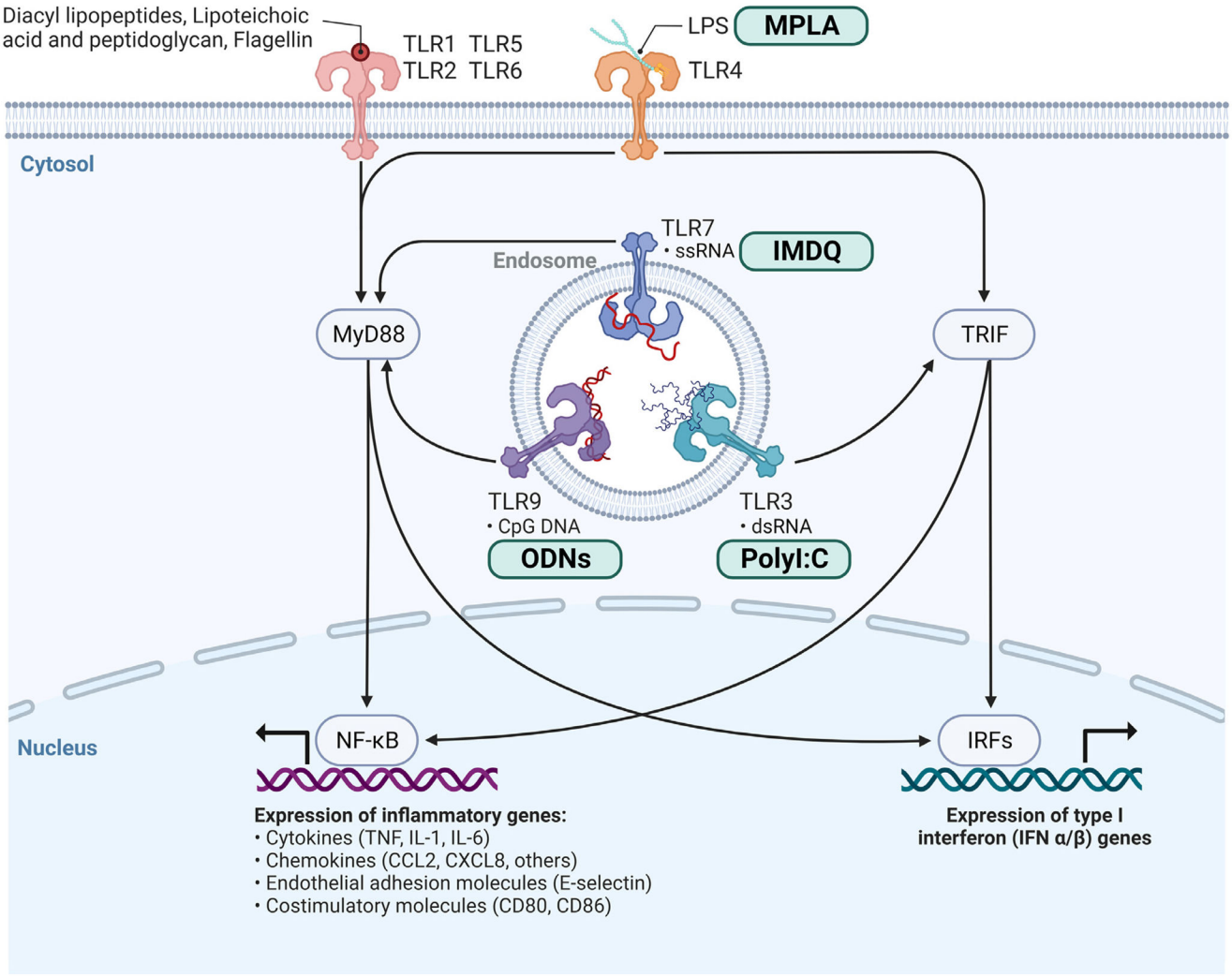

Figure 2.

Toll-like receptor (TLR) signaling. Cell surface and endosomal TLRs listed with their putative ligands. Downstream signaling pathways for the TLRs are outlined. Selected pharmaceutical agonists of TLRs are listed in cyan. Created with BioRender.com.

Table 1

IL-2 Monotherapy Versus Combination Therapy ORR in NCT01416831

	CR (%)	PR (%)	SD (%)	PD (%)
IL-2 monotherapy	15	20	25	40
IL-2 + SBRT	21	33	21	25