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Stimulating innate immunity to enhance radiation therapy-induced tumor control

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Abstract

Novel ligands that target Toll-like receptors and other innate recognition pathways represent a potent strategy for modulating innate immunity to generate anti-tumor immunity. While many of the current clinically successful immunotherapies target adaptive T-cell responses, both pre-clinical and clinical studies suggest that adjuvants have the potential to enhance the scope and efficacy of cancer immunotherapy. Radiation may be a particularly good partner to combine with innate immune therapies, since it is a highly efficient means to kill cancer cells, but may fail to send the appropriate inflammatory signals needed to act as an efficient endogenous vaccine. This may explain why although radiation therapy is a highly used cancer treatment, true abscopal effects – regression of disease outside the field without additional systemic therapy – are extremely rare. This review focuses on efforts to combine innate immune stimuli as adjuvants with radiation, creating a distinct and complementary approach from T cell targeted therapies to enhance anti-tumor immunity.

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Introduction

Ionizing radiation has the ability to induce various types of cell death, including apoptosis, necrosis, necroptosis, and autophagy, which have been shown to have both immunosuppressive or immunogenic effects[1]. Radiation directed to one tumor site can induce the regression of tumor(s) at other distant site(s), a phenomenon known as the abscopal effect. However, despite approximately 500,000 radiation treatments per year in the USA, Abuodeh *et al*. recently described that there have been only 21 cases of abscopal tumor regression of solid tumors reported in the literature over the past 45 years, excluding recent reports of radiation therapy combined with systemic immunotherapy[2] This suggests that radiation therapy alone does not generate clinically significant systemic immunity. Since radiation therapy remains an effective means to induce cell death and provide antigen to the immune system, we have to consider why radiation does not generate systemic immunity as a single agent.

Most of the recent studies that have validated radiation therapy as an effective partner for immunotherapy in preclinical and clinical settings have utilized immunotherapies that block T cell checkpoint regulatory molecules, such as *antagonistic* anti-CTLA4 and anti-PD1 antibodies that block *inhibitory* signals on T cells to unleash full T cell effector function[3–8], or *agonistic* antibodies that target 4-1BB and OX40, *costimulatory* molecules that are present for a short period after antigen stimulation. Ligation of these co-stimulatory molecules results in expansion of antigen-stimulated T cells[9–14] including tumor-specific T cells and drive their differentiation into effector and memory T cells with anti-tumor potential[15–17]. One of the classic tenets in immunology states that T cells require three signals in order to generate effective immunity. *Signal 1* comes from antigen bound to MHC class I or MHC class II signaling through the T cell receptor. *Signal 2* is a co-stimulatory signal (B7.1 and B7.2 on antigen presenting cells (APC) binding to CD28 on T cells), and *Signal 3* is a cytokine that helps shape the subsequent immune response. Thus, antigen without adjuvant fails to generate effective adaptive immunity because it provides signal 1 without signals 2 or 3 resulting in tolerance or anergy. While radiation provides dying cells as a source of antigen (Signal 1), we propose that radiation does not provide sufficient co-stimulation (signal 2) or cytokine release (signal 3) to efficiently activate the adaptive immune system. While cytokines are induced in tumors following radiation therapy, the poor efficacy of radiation alone as an endogenous vaccine is most clear when compared to strong exogenous vaccines: in preclinical experiments, our work has shown that the T cell response to antigens from dying cancer cells can be an order of magnitude lower than the response to antigens expressed in bacteria or viruses.

Dying cells can provide adjuvant in the form of DAMPs (danger associated molecular patterns) by expressing heat shock proteins[18–20], releasing HMGB1[21,22] or translocating calreticulin[23], and lysis of tumor cells has been associated with the adjuvant activity of

IL-33[24] and uric acid[25,26]. However, M2 polarized macrophages, the dominant myeloid cell in most tumor environments, respond to adjuvant by secreting cytokines such as VEGF, IL-10 and TGFβ[27–29], which are viewed as tumor supporting or immunosuppressive molecules. Moreover, irradiated cancer cells have been shown to drive undifferentiated macrophages towards M2 polarization[27,30–32] despite the adjuvant content of irradiated cells. These macrophages limit the efficacy of radiation therapy in a range of mouse models[33–37], and preventing M2 polarization enhances radiation tumor control by radiation therapy[27,38]. Taken together, these data suggest that the endogenous adjuvant activity of irradiated cancer cells is often insufficient to overcome the preexisting suppressive environment of the tumor and may even enhance the suppressive M2 macrophage environment. Since any immune response generated following tumor radiation very rarely influences tumors outside the treatment field[2], it is logical to increase the immunogenicity of radiation therapy via the exogenous delivery of adjuvant.

Toll-like receptors (TLR) are pattern recognition receptors capable of recognizing microbial products[39]. Signaling through distinct TLR can share downstream pathways such as MyD88 and TRIF, but TLR expression varies across cell types (Figure 1). Therefore, the consequence of TLR ligation can vary according to the cell type and their differentiation[40,41]. More recently characterized STING and RIG-I-like receptors have also been shown to trigger the release of key innate cytokines such as type I IFN and TNFα. This review will focus on the synergy between activation of innate immune receptors and radiation therapy.

TLR3

TLR3 is particularly expressed in DC and macrophage subsets (Figure 1) and mediates the host response to double stranded RNA in infectious agents[42]. Interestingly, the infected cell does not need to respond to the presence of double stranded RNA, but it is critical that dendritic cells express TLR3 in order to efficiently cross-present antigens from infected cells[43]. This is applicable to our goal to generate adaptive immune priming following radiation therapy, as the $CD8^+$ DC population is critical for cross-presentation of cell-associated antigens to generate T cell responses[44], and dying cells are efficiently tolerogenic without adjuvant[45,46]. TLR3 is unusual amongst TLRs because it uses TRIF rather than MyD88 to signal[41,47].

The synthetic TLR3 ligand poly(I:C) has been used as a cancer immunotherapy for over 45 years. Intraperitoneal administration of poly(I:C) first showed clinical promise in treating a mouse model of melanoma[48]. Poly(I:C) has been widely used in vaccines, providing adjuvant signals to a range of antigen sources, including free peptides and tumor-derived apoptotic cells (reviewed in[49]). Early studies with poly(I:C) showed limited benefit due to its short half-life[50], but a modified, degradation-resistant poly(ICLC)[51] has shown increased cytokine induction, but also has produced increased toxicity in patients (reviewed in[49,50]). Poly(I:C) can also activate RIG-I-like receptors when poly(I:C) gains access to the cytosol. For this reason, as will be discussed below, some of the activities formerly ascribed to TLR3 are also dependent on RIG-I like receptors such as MDA5[52–54].

A few studies have examined poly(ICLC) in combination with radiation therapy. A phase II study of intramuscular poly(ICLC) and fractionated radiation following surgery in patients with glioblastoma showed improved survival compared to historical controls[55]. Similarly, a phase II study combining fractionated chemoradiation with intramuscular poly(ICLC) reported that the combination was well tolerated and that median survival was longer than prior studies with chemoradiation alone[56]. However, thus far there are no reports demonstrating efficacy of the combination of radiation therapy with poly(ICLC) in randomized clinical trials.

TLR4

TLR4 is expressed in neutrophils and macrophages (Figure 1), and ligation of TLR4 by lipopolysaccharide (LPS) or endogenous damage associated molecular patterns (DAMPs) such as hyaluronan, heat shock proteins (HSP), and HMGB1[41] results in signaling through MyD88 dependent and independent pathways[57,58]. Many of these factors can be released after radiotherapy and pre-clinical studies have demonstrated the importance of this endogenous TLR signaling to the success of radiotherapy. Apetoh *et al.* demonstrated that the release of HMBG1, resulting from radiotherapy- or chemotherapy -induced cell death, triggered TLR4, which in turn increased the processing and presentation of tumor antigens[21]. This process of “immunogenic cell death” was found to be critical to the anti-tumor effects of cytotoxic therapy because TLR4 knockout or blocking HMGB1 drastically abrogated the efficacy of therapy. Additionally, endogenous activation of TLR by microbiota may also play a role in the anti-tumor effects of radiotherapy since translocation of gut microbiota and subsequent TLR4 activation is critical to the efficacy of total body irradiation and adoptive transfer in mouse models[59].

Early experiments demonstrating the efficacy of LPS as a therapy were performed in the late 1960s, and these early experiments recognized a tight balance between toxicity and efficacy[60]. A range of purified LPS preparations have been tested in clinical studies for cancer (reviewed in[61]) with moderate efficacy, however, the systemic toxicities of LPS are often due to the systemic consequences of IL-1 and TNFα production. Intratumoral administration of LPS has been shown to permit complete regression of tumors[62]. However, few studies have tested the addition of exogenous TLR4 ligands as partners for radiation therapy. BCG can be used to trigger TLR4 and increase the radiosensitivity of HCT-116 colon carcinoma cells by increasing autophagy[63]; however, many cancer cells cannot directly respond to TLR4 and as with other TLR ligands, it is more important that the stromal and immune cells can respond to the TLR ligand than the cancer cell[64].

While LPS can induce potent pro-inflammatory responses from macrophages in particular contexts, as discussed above LPS treatment of tumor macrophages following radiation therapy results in secretion of cytokines that are anti-inflammatory and support tumor growth and tissue repair[27–29,65]. These data suggest that while LPS is a potent immune adjuvant, and while endogenous TLR4 adjuvants contribute to tumor control following radiation therapy, TLR4 ligation can have anti-inflammatory as well as proinflammatory effects, depending on the differentiation of the responding cells.

TLR7

TLR7 is highly expressed in monocyte and macrophage lineages (Figure 1), and the synthetic TLR7 ligand Imiquimod has been used successfully as an immunotherapy for dermatological malignancies and pre-malignancies[66]. Imiquimod has been shown to have direct cytotoxic effects on squamous cell carcinoma cell lines *in vitro*[67], but when administered *in vivo*, the control of squamous cells is not direct and is dependent on the cytokines and inflammation generated by immune cells[68–71]. Topical administration of Imiquimod can alter the immune environment of skin metastases of breast cancer with some evidence of local tumor response[72]. In a preclinical mammary carcinoma model, topical administration of Imiquimod changed the immune environment in underlying subcutaneous tumors, and led to growth delay of both the primary treated tumor and a distant tumor[70]. In addition, delivery of radiation (8Gy × 3) significantly increased the number of complete local responses. Regression of distant unirradiated tumors was also observed if they were also treated with topical Imiquimod[70].

However, the solubility profile of Imiquimod has limited its clinical application. There are TLR7 ligands that can be applied systemically[73]. Dovedi *et al.* demonstrated that systemic application of a novel TLR7 ligands synergized with RT for control of preclinical B and T cell lymphoma models[74]. This effect was entirely dependent on CD8 T cells and resulted in antigen-specific T cell immunity. Following from this work, Adlard *et al.* demonstrated that systemically delivered TLR7 agonist significantly improved survival in combination with RT in preclinical models of solid tumors[75] while systemic delivery without RT did not impact tumor growth or progression. Improved survival was observed both with a fractionated dose of 2Gy × 5 and a single fraction of 15Gy.[75]. By contrast, in the T cell lymphoma model, systemic administration of the TLR7 agonist was more effective with fractionated radiation (2Gy × 5) than with a single fraction of 10Gy[74]. These data show that novel TLR7 ligands have significant therapeutic potential to treat cancer, particularly in conjunction with radiation therapy.

TLR9

TLR9 has a broad expression profile, in B cells, dendritic cells, macrophages and monocytes, and signals primarily through MyD88- dependent pathways. TLR9 recognizes unmethylated CpG oligonucleotide sequences that are present in microbial DNA but not mammalian DNA, as well as endogenous DAMPs including chromatinDNA complexes and ribonucleoproteins[4176,77]. Synthetic CpGs that activate TLR9 have been demonstrated to be superior to bacterially derived products in tumor therapy[78]. A side-by-side comparison demonstrated that CpG oligonucleotides were the most effective single-agent cancer therapeutic compared to other TLR ligands[64]. In general, CpGs induce the activation and maturation of DCs resulting in secretion of type I interferon and up-regulation of co-stimulatory molecules such as CD80 and CD86[79], leading to the activation of natural killer (NK) cells[79] and the expansion of cytotoxic T lymphocytes. CpGs also enhance the differentiation of B cells into antibody-secreting plasma cells which can eradicate tumor cells through antibody dependent cellular cytotoxicity[80–82].

A number of studies have shown that TLR9 agonists have anti-tumor effects in murine models when initiated while tumors are small[76,83–90]. Direct intratumoral or local injection of CpG appears to be more effective and less toxic than systemic administration[91]. Certain cancers can be refractory to single agent CpG due to low expression of MHC-I and the abundant expression of immunosuppressive TGFβ[92]. Overall, the most potent effects of CpG therapy are seen when used in combinatorial strategies. CpGs have been tested in combination with ionizing radiotherapy using *in vitro* models, animal models and in clinical trials[91–101] and found to be superior in anti-tumor activity compared to either modality alone. Initial studies performed by Milas and colleagues demonstrated significant local synergy for single fraction and fractionated radiotherapy regimens in combination with CpG[95,96]. In a mouse fibrosarcoma model they demonstrate that the TCD50 for fractionated radiotherapy is reduced almost 4-fold (from 83.1 Gy to 23 Gy) when combined with CpG. The local synergy of radiotherapy and CpG was independently verified by a second group in a glioma model[97]. Although the exact mechanism of CpG-mediated radiosensitization is not known, studies have suggested that CpG increases both mTOR activation and autophagy[102], decreases expression of Oct-4- mediated renewal[103], and increases NF-kB signaling and nitric oxide production[94].

In vivo, the efficacy of radiation in combination with CpG caused both improved local control and the generation of systemic immunity. Guha and colleagues demonstrated synergistic effects of CpG and radiation against both local irradiated tumor and systemic lung metastases in a 3LL tumor model[98]. This correlated with an increase in a humoral anti-tumor immune response and increased activation of dendritic cells. Clinical studies combining intralesional CpG with local radiotherapy have confirmed the safety and efficacy of this combinatorial approach in humans. A series of clinical trials by Levy and colleagues in low-grade lymphomas and demonstrated that this combinatorial approach is capable of inducing objective responses outside of the irradiated and injected lesion in about 20% of patients and disease stability in about another 20% of patients with heavily pretreated systemic[91] or cutaneous lymphoma[99]. Many of the responses were durable and lasted for months or years.

Despite the potent immune stimulation of TLRs, studies using CpG as a therapeutic have highlighted that the increased immune effect may be a doubled edged sword. TLR activation combined with radiotherapy can generate immune responses, but as acute inflammation becomes chronic, immune suppression will ensue. Thus, chronic endogenous TLR9 signaling instigated by radiotherapy induces chronic inflammation and increases tumor recurrence[104]. In the clinical studies of radiotherapy combined with CpG described above, patients whose tumors induced immunosuppressive regulatory Tregs responded poorly to therapy and had a poor prognosis[107]. Radiotherapy combined with CpG was found to upregulate indolamine 2,3-dioxygenase (IDO) expression, which was termed “rebound immune suppression”[105]. This upregulation of IDO occured in response to the inflammation induced by radiotherapy and TLR activation, generating an immune suppression that included Tregs. The addition of IDO blockade reversed immune suppression and significantly improved the local and systemic efficacy of radiotherapy combined with CpG in murine models as well as companion canines with late stage metastatic spontaneous melanomas or sarcomas[105]. As discussed below, this rebound immune suppression may be a

common feature of potent IFN-inducers[106–108]; thus a triple therapy approach which includes blockade of immune suppression to achieve maximal efficacy may be required to increase the clinical impact of these combinatorial approaches.

RIG-I like receptors

RIG-I, MDA5 and LGP2 are cytoplasmic sensors of viral RNA and signal though MAVS to activate type I IFN responses in infected cells. RIG-I recognizes dsRNA containing 5′ triphosphates and biphosphates[109], which are present in the nucleus of cells early following transcription, but are removed before entry into the cytoplasm. During infection, the entry of unmodified RNA into the cytoplasm, triggers these sensors and activates the production of type I IFN[110]. RIG-I[111] and LGP2[112], but not MDA5,[113] also can detect endogenous nuclear material that has translocated to the cytoplasm following radiation therapy. Irradiated cells activate type I IFN production in a RIG-I dependent manner and mice lacking RIG-I were protected against gastrointestinal epithelial cell death following total body radiation[111]. By contrast, LGP2 has an opposite effect and suppresses type I IFN induced by radiation therapy[112].

While we now know that specific sequences activate RIG-I like receptors, these sequences are present in poly(I:C), which was initially thought to be exclusively a TLR3 ligand. Knockout studies using a range of synthetic ligands on dendritic cells demonstrated that MDA5 activates type I IFN responses to poly(I:C) but RIG-1 does not[53]. The receptor that recognizes poly(I:C) can be influenced by the route of administration – for example, RIG-I like receptors may require that the ligand access the cytoplasm. However, the cell types and pattern of receptor expression also will influence the use of these various ligands. Thus, conventional dendritic cells rely on RIG-I like receptors while plasmacytoid DCs rely on TLR3 binding to the same ligand[54]. Therefore, many of the studies discussed above that used poly(I:C) as a therapeutic agent may have activated a TLR3 pathway, a RIG-I like receptor pathway, or both.

Although differential activation of the two receptors (RIG-I and TLR3) may lead to different outcomes, it seems that the presence of both pathways leads to maximal response to poly(I:C). In the TRAMP murine model of prostate adenocarcinoma, poly(I:C) administered systemically resulted in complete control of tumor growth, and although much of this was lost in TLR3 knockout mice, some activity remained[114]. The authors attribute this to direct activity of the systemic poly(I:C) on the cancer cells, but this could also represent the activity of poly(I:C) on RIG-I like receptors. Similarly, in a model of poly(I:C)-induced lung pathology, TLR3 knockout mice exhibited similar responses with a reduced magnitude, and this may represent the activity of RIG-I like receptors[115]. Interestingly, a non-hematopoeitic stromal MDA5 response was shown to be required for the full efficacy of poly(I:C) as a vaccine adjuvant[52], so it is possible that local inflammation driven by stromal cells is critical to support antigen-specific responses. Further studies will be necessary to determine the relative value of selective targeting of TLR3 and RIG-I like receptors, but at the moment, the data suggests that the dual response to poly(I:C) is advantageous in cancer therapy.

STING

Recently, there has been a surge of interest in STING (STimulator of INterferon Genes) for its role as a cytosolic sensor of DNA. Initially, the functions carried out by STING were attributed to TLR9, which can recognize CpG DNA and drive type I IFN responses to microbial DNA. However, double stranded DNA also activated type I IFN responses in cells lacking TLR signaling pathways and RIG-I, and this suggested that there was an unrecognized DNA-sensing mechanism that remained to be discovered[116]. The STING-dependent cytosolic DNA sensing pathway was discovered by two independent groups using a cDNA screen to identify proteins that induced type I IFN or IRF[117,118]. STING is widely expressed both among hematopoetic cells (Figure 1) and non-hematopoetic cells including cancer cells[119].

The STING pathway likely evolved as an intracellular sensor of pathogen DNA such as bacterial cyclic-di-nucleotides (CDN)[120,121]. Mice deficient in STING also show impaired clearance of DNA viruses due to impaired generation of a type I IFN-driven immune response[122]. However, as has been described for RIG-I like receptors and unmodified RNA, STING has also been shown to sense the presence of endogenous DNA introduced into the cytoplasm[123]. These data suggest that STING may be able to sense endogenous DNA released within irradiated cancer cells; however Deng *et al.* demonstrated that the major mechanism of STING activation following radiation of cancer cells resulted following with cross-presentation of cell-associated antigens to dendritic cells[124]. In these experiments, expression of STING by the cancer cells was not required for radiation therapy-induced tumor cure, which instead was entirely dependent on STING expression in host dendritic cells[124]. In highly immunogenic tumors, host expression of STING is necessary for spontaneous tumor regression[125]. In these models, cancer cells killed by a range of methods including radiation and freeze thaw were unable to active type I IFN activation in dendritic cells; however, tumor-derived DNA transfected into the cytoplasm of DC was a potent STING-mediated activator of type I IFN production. Although it is unclear how tumor DNA is transferred to the DC cytoplasm in vivo, mice deficient in STING were unable to generate type I IFN following tumor challenge, failed to generate effective anti-tumor immunity, and were less responsive to immunotherapy[125]. These data suggest that the inflammatory component of cross presentation at tumor challenge was critical to generate T cell responses to tumor antigens. In a different model system using less immunogenic tumors, STING activation in the host following tumor challenge was shown to *inhibit* endogenous anti-tumor immunity[106]. The mechanisms was again via induction of type I IFN but this time also resulted in subsequent induction of the immune suppressive enzyme IDO, resulting in increased tumor growth rates. However, other studies have not found a change in tumor growth rate in wild type versus STING knockout animals that are otherwise untreated[119,124,126]. It is possible that some feature of the cultured cancer cells, their preparation, or their route of delivery at transfer differentially affects the likelihood and consequence of STING activation in the host.

Since CDN activation of STING results in potent induction of type I IFN, CDN have been shown to be effective vaccine adjuvants[127,128]. In addition, direct injection of CDN into tumors has been shown to cause dramatic regression in a range of tumor models[126].

Interestingly, STING was recently found to be the gene activated by DMXAA[129], which is a highly active vascular disrupting agent resulting in hemorrhagic necrosis of tumors, though with variable results depending on the tumor model[130]. DMXAA activates mouse, but not human STING[131], potentially explaining the failure in clinical translation of DMXAA. Novel CDN have been engineered for increased potency against mouse STING and human STING isoforms[126,128], and novel small molecule agonists of the STING pathway have been identified[132]. While cancer cells can express STING[119], tumor therapy with CDN is ineffective in mice lacking STING[126], indicating that host STING is critical for anti-tumor activity. This also explains why DMXAA was able to cause vascular disruption in xenografts of human tumors in immunodeficient mice[133], since cancer cell expression of STING was not relevant to treatment outcome. These data from immunodeficient mice also suggest that the anti-tumor efficacy of STING ligands is not necessarily dependent on functional adaptive immunity.

Both DMXAA and CDN have shown efficacy in combination with radiation therapy. DMXAA administered systemically resulted in increased tumor control by RT in a radiation and DMXAA dose-dependent manner[134,135], and with fractionated radiation[135]. In the MC38 colorectal model that was not affected by intratumoral injection of CDN, the combination of CDN and RT resulted in significantly increased tumor control over RT alone[124]. Similarly, in a range of tumor models and mouse strains the combination of CDN and RT was shown to result in therapeutic synergy[119]. These effects were dependent on STING expression in the host, and resulted in TNFα-induced hemorrhagic necrosis in the tumor[119]. In these models, radiation therapy was necessary to produce CD8 T cell immunity and control distant disease, but optimal tumor control was dependent on both an early T cell independent, TNFα-dependent rapid tumor regression and a later, CD8 T cell dependent mechanism that contributed to the durable response of treated tumors[119]. These data demonstrate that STING ligands create synergy with radiation therapy for immune-mediated control of cancer.

Type I IFN

Type I IFN is induced downstream of many of the innate immune activators mentioned above. Type I IFN includes IFNα and IFNβ, which signal through the receptors IFNAR1 and IFNAR2[136,137] (Figure 1). This contrasts with type II IFN, or IFNγ, which signals through IFNGR1 and IFNGR2, and type III IFN, that includes IFN-lambda and IL-10[136,137] and signals through two α subunits and two β subunits. The ability to produce and respond to type I IFN is shared by almost all cell types, though plasmacytoid dendritic cells are able to secrete higher levels of type I IFN than any other cell type[130,139]. Activation of IFN receptors leads to a multifaceted response. They generate an innate anti-viral and anti-microbial state among infected and bystander cells, limiting the spread of pathogens, and also promote NK cell function and antigen presentation by dendritic cells to activate T cells (reviewed in[136,137]). However, type I IFN can also induce immune suppressive mechanisms, such as induction of the immune suppressive enzyme IDO[106]. IDO tolerizes adaptive immune responses[140] and can be harnessed to treat T cell-mediated autoimmunity[141]. Thus, as with many immune stimuli, there is a contextual and dose-related consequence of type I IFN activation *in vivo*. As has been shown for IFNγ, type I IFNs also result in upregulation

of PDL1 by stromal cells and cancer cells[142], and therefore a role in feedback regulation of adaptive immunity.

The expression of type I IFN and IFN-responsive genes have been correlated with favorable outcome in cancer patients. A type I IFN gene signature is induced by chemotherapy and radiation therapy in some patients. This type I IFN gene signature is associated with improved outcome following neoadjuvant chemotherapy in breast cancer patients[111]. However, this interferon signature is expressed in a broad array of cancer cell lines, and predicts a poor response to chemotherapy and radiation therapy in patients with breast cancer[143] and glioblastoma[144]. In preclinical models, the efficacy of radiation therapy was shown to be dependent on induction of type I IFN in murine tumor models[145]. Using bone marrow chimeras it was demonstrated that a functional response to radiation was dependent on IFNAR1 expression in hematopoietic cells, but not T cells, and IFNAR1 knockout mice lacked functional cross-presentation of tumor antigens by dendritic cells[145]. Type I IFN has been shown to be required for priming of T cell responses and recruitment of tumor-specific T cells to treated sites[146].

Type I IFN has been well studied as an adjuvant therapy for melanoma, resulting in improved recurrence-free survival[147,140], but no improvement on overall survival 149. Type I IFN has long been known to radiosensitize cancer cells *in vitro*[150], and some of the earliest studies of immunotherapy combined with radiation therapy involved local or systemic application of type I IFN. This has shown mixed signs of efficacy in a range of tumor types, but toxicities have limited application of this therapy. Preclinical studies in pancreatic cancer have shown improved outcome with type I IFN and chemotherapy in pancreatic cancer[151,152], and early phase studies suggested improved outcome in patients receiving type I IFN along with adjuvant chemoradiation[153]. Multicenter studies suggested an improved outcome; however, they also resulted in grade III or IV toxicity in 90% of patients[154]. A multicenter, randomized phase III trial of adjuvant chemoradiation plus type I IFN did not demonstrate a benefit of combinatorial therapy versus 5-FU alone and resulted in ≥ grade III toxicity in 85% of patients[155]. These data indicate that systemic delivery of type I IFN is significantly limited by toxicities and does not provide a favorable therapeutic ratio.

To minimize systemic consequences, type I IFN can be injected in the local tumor environment to generate tumor control[156], and intratumoral injection of an adenoviral vector expressing murine IFNβ can produce T cell-dependent control of a murine melanoma model[145]. Alternatively, IFN can be engineered to accumulate in the vicinity of cancer cells using immune-conjugates. Anti-CD20 coupled to type I IFN was shown to be an effective therapy against lymphoma[157] but hematological malignancies therapies require direct action of type I IFN on the cancer cells[157]. An anti-EGFR-IFNβ conjugate was effective against murine solid tumors expressing human EGFR, and in these models tumor clearance was dependent on functional adaptive immunity[158]. Further refinement of type I IFN delivery systems could potentially expand the use of this cytokine for cancer therapy.

TNFα

TNFα was identified as the factor induced by Coley's toxins responsible for necrosis of tumors[159,160]. Sufficient TNFα can produce rapid hemorrhagic necrosis as a result of vascular endothelia activation and death. In addition, TNFα can induce cancer cell apoptosis. As described above, TNFα-mediated hemorrhagic necrosis of tumors has been described following administration of STING ligands, LPS, poly(I:C) and CpG[119,130,161,162]. However, the lower levels of endogenous TNFα produced by macrophages in the tumor environment has been shown to promote tumor regrowth following radiation therapy[163]. In addition, TNFα produced downstream of pattern recognition receptor ligation is the main factor contributing to systemic shock[164,165] and can be causative of other toxicities. For example, pulmonary administration of CpG resulted in TLR9-dependent alveolitis and pneumonitis in mice, and this toxicity was lost in TNFα knockout mice[166].

To minimize systemic toxicity, TNFα has been engineered into an adenoviral vector with expression controlled by a radiation-responsive promoter (reviewed in[167]). This promoter has been optimized to generate up to a 3-fold induction of gene expression following radiation therapy[168] and this construct has been successfully used to induce vascular thrombosis in human tumor xenografts in immunodeficient mice[169]. Similarly, cancer cells modified to express TNFα constitutively or in response to radiation therapy have been shown to improve the clinical response to implanted radioactive seeds[170]. In immunocompetent mice, gene delivery of TNFα using an adenoviral vector resulted in improved tumor control following a single fraction of 20Gy, with a large portion of the effect due to CD8 T cells[171]. These data suggest that responses incorporate both a direct effect of TNFα on vascular cells, and a CD8-mediated adaptive immune response.

In the clinic, radiation-inducible TNFα gene therapy showed evidence of efficacy in preclinical and Phase II studies (reviewed in[167]); however, randomized phase III studies in locally advanced pancreatic cancer failed to show efficacy[172]. These data suggest that while TNFα is an extremely important effector cytokine that can have dramatic effects on tumors, it is very difficult to target TNFα clinically because of its pleiotrophic effects within the immune system, and subsequent toxicities.

Conclusions

Though cancer cells contain endogenous adjuvants with the potential to stimulate innate sensors, radiation therapy is not an effective stimulator of systemic immunity without the addition of immunotherapy[2]. It is possible that irradiated cells are only able to generate type I IFN responses in some patients, explaining variation in outcome according to the IFN gene signature[143,144]. This may be due to genetic variation in the endogenous DNA sensors, as has been proposed for TLR4[21], or due to the particular immune tumor environment. Nevertheless, the variable response presents an important opportunity to deliver the appropriate exogenous adjuvants to deliver missing signals and tap into the potential of radiation therapy as a patient-specific endogenous cancer vaccine (Figure 2).

When using adjuvants in addition to RT, we must dedicate efforts to learning how to best balance efficacy and toxicity. In addition, we must address the problem of local versus systemic delivery. Local delivery can maximize local tumor control while minimizing systemic toxicity, but this may also limit its clinical application. In addition, we must always consider how these therapies have the potential to interfere/suppress the adaptive immune response. As we have discussed, over-activation of inflammatory mechanisms by adjuvants can lead to adaptive immune suppression[140,141], requiring additional intervention such as inhibition of IDO[105]. Adaptive immunity requires a critical sequence of signals and any deviation in the appropriate timing and degree of inflammation has the potential to limit antigen-specific immunity[173]. The appropriate timing of adjuvant delivery in relation to radiation needs to be determined in order to ensure the success of this approach[174]. While adjuvant might be the oldest cancer immunotherapy, the therapeutic potential of adjuvant remains to be fully exploited.

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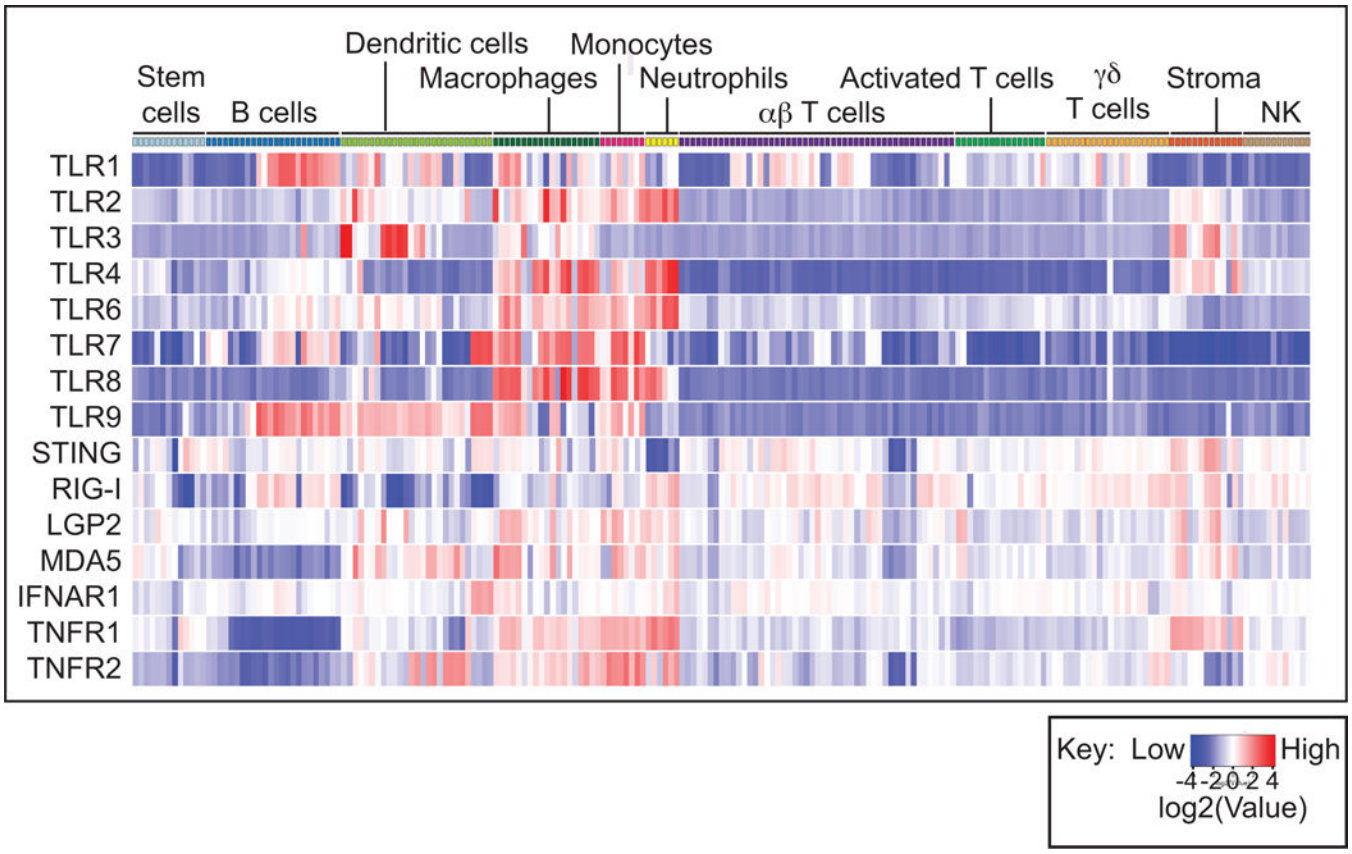

Figure 1. Distribution of innate receptors across cell types in the ImmGen dataset

The graph shows gene expression of innate receptors in sorted cell types clustered into broad immune populations. Expression of each gene is normalized across cell populations and color-coded according to the key. There is significant variation in expression of innate sensors across immune cells. While receptors such as TLR4 are most highly expressed by macrophages and neutrophils, TLR9 shows extended expression into dendritic cells and B cells. Broadly, T cells exhibit low expression of TLR. By contrast, the innate sensor STING and the type I IFN receptor are very evenly expressed across many cell populations. This analysis is a result of data assembled by the ImmGen Consortium. Full analysis of gene expression patterns can be visualized at www.immgen.org.

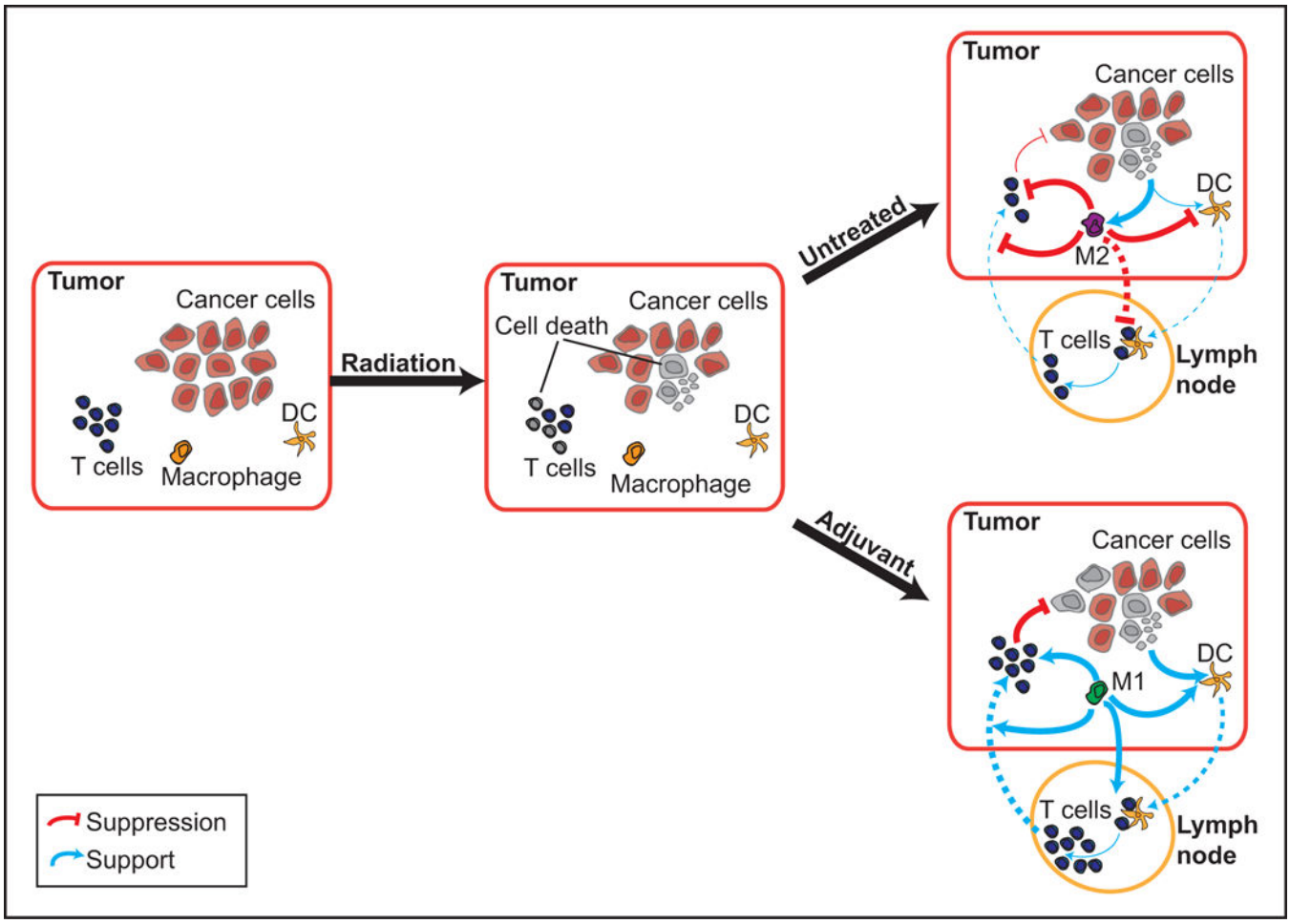

Figure 2. Effect of adjuvant on immune cell relationships in the tumor and draining lymph nodes

In the absence of adjuvant, cell death mediated by radiation therapy drives M2 responses that suppress DC maturation and effector T cell function. Innate immune adjuvants can drive proinflammatory M1 responses, enhance DC cross presentation of tumor antigens to T cells in the lymph node in a supportive cytokine environment, and support effector T cell control of residual disease.