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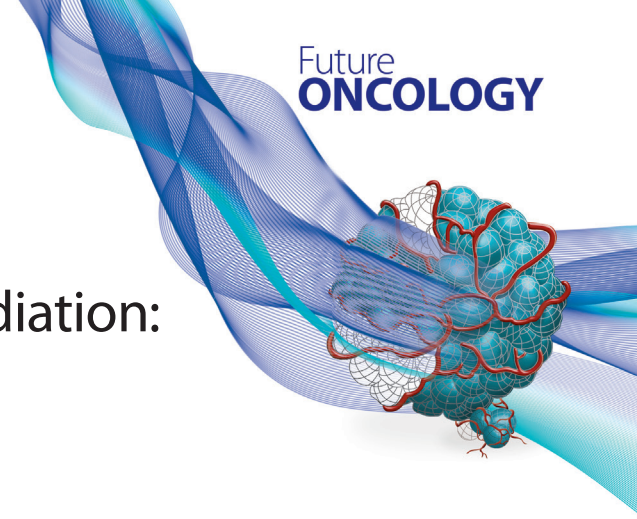

Immunomodulatory effects of radiation: what is next for cancer therapy?

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Despite its former reputation as being immunosuppressive, it has become evident that radiation therapy can enhance antitumor immune responses. This quality can be harnessed by utilizing radiation as an adjuvant to cancer immunotherapies. Most studies combine the standard radiation dose and regimens indicated for the given disease state, with novel cancer immunotherapies. It has become apparent that low-dose radiation, as well as doses within the hypofractionated range, can modulate tumor cells making them better targets for immune cell reactivity. Herein, we describe the range of phenotypic changes induced in tumor cells by radiation, and explore the diverse mechanisms of immunogenic modulation reported at these doses. We also review the impact of these doses on the immune cell function of cytotoxic cells *in vivo* and *in vitro*.

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• cancer • cytotoxic cells • immunogenic modulation • immunotherapy • low-dose radiotherapy • radiation • single-dose radiotherapy • T cells

The ability of ionizing radiation (IR) to influence the immune response against tumors has become more and more attractive as the development of novel cancer immunotherapies (CIT) has rapidly expanded and these agents have come into wider clinical use. Beginning in 2010, Provenge (Sipuleucel-T), Yervoy (ipilimumab), Keytruda (pembrolizumab) and Opdivo (nivolumab) have all received US FDA approval for the treatment of cancer. While only four CITs have received FDA approval at this time, there are numerous others under preclinical study and in clinical development. As a result, the immune enhancing powers of radiation are becoming a valued aspect and important use of radiotherapy (RT) [1].

Radiation can be used as an adjuvant to immunotherapies in several ways. First, it can induce a type of cell death in a subset of susceptible tumor cells, which can then activate antigen uptake, cell maturation and presentation by antigen-presenting cells (APCs). This immunogenic cell death (ICD) is identified by three main hallmarks on tumor cells; calreticulin exposure, ATP release and HMGB1 release [2]. APCs responding to ICD can subsequently induce other immune cells that are capable of attacking the surviving tumor cells. This body of work has been highlighted in a number of excellent reviews [3–7]. Second, radiation can cause molecular alterations in tumor cells in a manner that directly sensitizes tumor cells to immune cell-mediated killing. This property of radiation is referred to as immunogenic modulation (IM) of tumor cells [8,9]. Pre-existing (endogenous) immune cells, or those induced or activated by vaccine, may not be able to act once they reach tumors if the tumor microenvironment (TME) is immunosuppressive or the tumor cells themselves are suppressive or suboptimal targets. Modulated tumor cells surviving exposure to radiation, either because they are radio-resistant or because they receive sublethal doses, can become better targets

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for antitumor immune cells. Third, radiation can alter the activity and function of immune cells directly. Which of these three situations occurs is likely influenced by the dose and delivery scheme used (single dose vs separated into smaller fractions), though they are likely not mutually exclusive.

The immune enhancing effects of RT, and the different ways that RT has been combined with CITs clinically and preclinically, have been recently reviewed elsewhere [10,11]. Exciting outcomes have been observed in patients receiving RT for palliation with no intent to cure [12,13]. Similar success has been reported when higher doses of RT were used [14–16]. These clinical outcomes are even more exciting because they report immune mediated regression of not only the irradiated tumor, but also of distant tumors outside of the radiation field (i.e., abscopal response). Though these abscopal responses and clinical outcomes are thrilling, there remains significant room for improvement [10]. It is not fully understood what induces such responses on the cellular and molecular level, and it is not yet possible to routinely recapitulate these outcomes.

The focus of this review is on IM of tumor cells and the influence of radiation dose on the phenotype of tumor cells. The use of radiation for IM would not rely on RT to induce a *de novo* immune response, but would instead be used to specifically complement the elaborate CITs already in development [17,18]. At this time, RT is not routinely incorporated into most CIT approaches specifically for its IM properties. Our review will consider the effect of both low doses of radiation (≤2 Gy; see the 'Immunomodulation by low-dose radiotherapy' section), and hypofractionated doses (2–25 Gy; see the 'Immunomodulation by hypofractionated doses of radiation' section), on gene expression in cells surviving radiation, focusing on changes that can directly enhance cellular attack of tumor cells. We also consider the impact of these radiation doses directly on immune cells themselves both phenotypically (see the 'Immunomodulation by low-dose radiotherapy' and 'Immunomodulation by hypofractionated doses of radiation' sections) and functionally (see the 'Immunomodulation at work' section). Studies comparing responses to single dose (SD) versus multifraction (MF) delivery have been recently reviewed by others [19,20]. Here we leave our review to observations made in tumor cells surviving SD radiation, or systems where radiation alone has no observable tumor control.

• Immunomodulation by low-dose radiotherapy – ≤2 Gy

IM of tumor cells following low-dose radiotherapy doses

There are many reports on the ability of IR to modulate the expression of genes that are important for immune attack of tumor cells. However, much of the information available is from cells receiving high doses of radiation intended to kill tumor cells, and much less information is available from tumors treated with low or sublethal doses of radiation; yet, changes in several important immune genes have been reported in tumor cells treated with low-dose radiotherapy (LD-RT). MHC-I is responsible for presentation of endogenous antigens to cytotoxic T lymphocytes (CTLs). Reits *et al.* exposed a human melanoma cell line (MelJuSo) to radiation and quantified cell surface MHC-I complexes 18 h later. Radiation induced a dose-dependent increase in MHC-I expression *in vitro* and increases in MHC-I were observed beginning at 1 Gy. Furthermore, they observed more polyubiquitinated proteins available for proteasomal degradation after radiation doses as low as 1 Gy. This and other studies revealed that radiation can lead to more peptide diversity for MHC-I antigen presentation, in addition to enhanced MHC-I expression [21–23]. The stress ligands MICA/B can bind to cognate receptors (NKG2D) on cytokine-inducible lymphocytes, NK cells and T cells causing activation of these cells [24]. Liu *et al.* irradiated human myeloma cells (KAS-6/1) at various doses and analyzed the cells for expression of MICA/B 24 h postirradiation [25]. A dose-dependent increase in both MICA/B expression at doses as low as 2 Gy was observed. After appropriate activation, CTLs and NK cells can kill tumor targets expressing death receptors such as Fas (CD95) on their surface, and there is some evidence of increased Fas expression following treatment of tumors with LD-RT. In one study, Sheard *et al.* treated human colorectal (HCT116) and human breast cancer (MCF7) cells with 2 Gy IR and observed moderate upregulation of Fas [26]. The ligand for Fas, FasL, sends apoptotic signals into Fas expressing cells. Abdulkarim *et al.* irradiated a human nasopharyngeal cancer (C15) cell line

at various doses and measured FasL induction [27]. Fas is constitutively expressed in this NPC cell line prior to irradiation, but following 2 Gy IR there was an increase in FasL on these cells. FasL expressing cells could possibly kill themselves and/or nearby Fas-expressing cells, including infiltrating T cells. This is a rare example of LD-RT upregulating expression of a gene that could negatively impact T cells.

Mechanism of IM in tumor cells following LD-RT

Radiation has been paradoxically reported to be both proinflammatory and anti-inflammatory. As the key transcription factor for numerous immune factors, NF-κB has been investigated for its involvement in gene expression following LD-RT. The use of LD-RT to treat benign inflammatory or hypoproliferative conditions is well known [28,29], and inhibition of NF-κB is thought to be responsible for these anti-inflammatory activities. In this regard, Lodermann *et al.* observed a decrease in p38 (an upstream molecule of NF-κB) and AKT (a downstream molecule of NF-κB) following 0.5 Gy and 0.7 Gy of IR. Pajonk and colleagues reported that the 26S proteasome was a direct target of IR, and hypothesized that radiation-induced inhibition of the proteasome was a mechanism of NF-κB inhibition. This type of inhibition could modulate expression of numerous proinflammatory molecules, such as TNF-α, IL-1β and IL-6, at the transcriptional level. Indeed, bladder cancer cells (ECV304) treated with 0.17–2 Gy induced a rapid, dose-dependent decrease in 26S proteasome activity. In turn, this inhibition prevented the phosphorylation, ubiquitination and subsequent degradation of the IκB regulatory complex leading to reduced translocation of NF-κB complex to the nucleus. While altered proteasome function induced by LD-RT is an attractive explanation for many of the observed transcriptionally regulated effects, via inhibition of NF-κB, more investigation is needed in this area as chronic low dose exposure has resulted in both stimulation and inhibition of NF-κB regulated genes [30]. Further, most studies have utilized doses higher than 2 Gy to investigate radiation-induced changes in gene expression in tumor cells and NF-κB activation is observed at higher doses. Thus IR appears to be able to both activate and inhibit NF-κB and the outcome may be dependent on dose, and perhaps tissue, under study.

Modulation of immune cells following LD-RT

The efficacy of RT is known to be influenced by the TME, including local expression of agents such as HIF-1 and VEGF [31]. However, the reciprocal is also true and LD-RT can directly modulate the efficacy of immune cells including macrophages, DCs, NK and lymphocytic cells. LD-RT *in vitro* (0.075 Gy) increases NK cell secretion of IFN-γ and TNF-α [32]. Nontumor bearing mice treated with 0.2 Gy × 4 total body irradiation (total 0.8 Gy) had an increase in NK and NK T cell numbers at 21 days post IR [33]. Increased amounts of IFN-γ, IL-12 and TNF-α from macrophages were detected 10 days post IR in these animals. Klug *et al.* observed that 2 Gy irradiation induced iNOS expression (and reduced HIF-1, Ym-1, Fizz-1 and arginase expression) indicating an induction of the M1 (proinflammatory) macrophage phenotype [34]. They further showed that iNOS inhibition blocked VCAM-1 expression on CD31+ endothelial cells, indicating endothelial cell activation, and that the capacity to support leukocyte transmigration was enhanced post IR via iNOS. Lodermann *et al.* observed a decrease in IL-1β secretion from LD-RT-induced macrophages, and found that this downregulation was correlated with reduced nuclear translocation of the p65 (RelA) subunit of the NF-κB complex [35]. In another study, direct treatment of macrophages with IR decreased IL-1β (0.5–2 Gy) and increased TGF-β (0.1–0.5 Gy) expression with a decreased in nuclear localization of NF-κB also observed [36]. This demonstrates that LD-RT can change macrophage phenotype. Moreover, Liu *et al.* observed increased expression of CD80 and CD86 on macrophages following LD-RT suggesting an increased capacity for costimulation of T cells [37]. LD-RT can also impact other APCs such as DCs. Shigematsu *et al.* treated murine DCs with various doses of IR (0.02, 0.05, 0.1, 0.5, 1 Gy) and observed increased secretion of IL-12 and increased expression of *IL-2* and *IFN-γ* mRNA at doses as low as 0.05 Gy from DCs [38]. They further observed that LD-RT had no effect on proliferation or maturation of the DCs. Whole body irradiation of non-tumor bearing mice with 0.075 Gy caused an increase in CD28 expression concomitant with a reduction in CTLA-4 expression on splenic lymphocytes [37]. Conversely, treatment with 2 Gy produced reciprocal changes in these same cells. Treatment of T_{REGS} from rats with 0.15 Gy

of radiation *in vitro* reduced the expression of CTLA-4 [39], which is associated with the suppressive function of these cells. These findings demonstrate that LD-RT can directly alter the state of some immune cells with important roles in antitumor immunity.

The ability of lower dose ionizing radiation (<0.2 Gy) to activate immune cells has been recently reviewed by Farooque *et al.* [40], and we now present a table summarizing important immune relevant changes in both tumor cells and immune cells following low to intermediate doses of IR in **Table 1**.

• Immunomodulation by hypofractionated doses of radiation

IM of tumor cells within the hypofractionated dose range

Doses between 2 and 25 Gy have been evaluated extensively for enhancement of antitumor immune attack. In the context of radiation biology, a radiation dose of 5 Gy is in the moderate hypo-fractionated range, and 10 Gy dose is considered in the extreme hypo-fractionated range [41]. Stereotactic body radiation therapy has been administered in fractions up to 15 Gy [42]. As a result, the majority of IM studies have been conducted within these dose ranges, and there is much information available about the diverse immune relevant genes that are changed post IR **(Table 2)**. Though typically delivered in multiple fractions over time to reach a higher cumulative delivery dose to patients, here we review the impact of mostly single dose (SD) exposure on the modulation of surviving cells.

Signal 1

CTLs must see target tumor-associated antigens (TAA) displayed in MHC-I, and several studies have shown that IR can modulate this signal within tumor cells. Lugade *et al.* demonstrated that irradiated tumors (15 Gy) had an increased

Table 1. Low-dose radiotherapy range (≤2 Gy).

IM gene	Tissue (up/down)	Dose (Gy)	mRNA or protein	Role	Ref.
Tumor cells					
MHC-I	Melanoma (Hu); Up	1	Protein	Antigen presentation	[21]
FAS	CRC, BrCa (Hu); Up	2	Protein	Transmits apoptotic signals into cells	[26]
FasL	NPC (Hu); Up	2	Protein	Inducer of apoptosis	[27]
NKG2DL (MICA/B)	Myeloma (Hu); Up	2	mRNA/protein	Increased recruitment and function of NK cells	[25]
Immune cells					
IFN-γ	NK cells; Up	0.75	Protein	Activation of CD8 T cells; Th1 promoting	[32]
	Macrophages; Up	0.8	mRNA		[33]
	DCs; Up	0.05	mRNA		[38]
TNF-α	NK; Up	0.75	Protein	Inducer of tumor apoptosis	[32]
	Macrophages; Up	0.8	mRNA		[33]
IL-12	Macrophage; Up	0.8–0.075	mRNA/protein	Th1 promoting cytokine	[33,37]
	DCs; Up	0.05	mRNA/protein		[38]
IL-10	Macrophages; Down	0.075	Protein	Immunosuppressive cytokine	[37]
IL-1β	Macrophages; Down	0.5–0.7	Protein	Proinflammatory action	[35,36]
		0.5–2	Protein		
TGF-β	Macrophages; Up	0.1–0.5	Protein	Inhibits CTL function	[36]
iNOS	Macrophage; Up	2	Protein	M1 associated, inducer of cytotoxic mediator NO	[34]
Arginase	Macrophages; Down	2	Protein	M2 associated enzyme	[34]
HIF1	Macrophages; Down	2	Protein	Proangiogenic; tumor promoting	[34]
CD80/86	Macrophages; Up	0.075–2	Protein	Increased T-cell costimulation	[37]
IL-2	DCs; Up	0.05	mRNA	T-cell proliferation cytokine	[38]
CD28	Lymphocytes; Up	0.075	Protein	Transmits positive signal into T cells	[37]
	Lymphcytes; Down	2	Protein		
CTLA-4	Lymphocytes; Down	0.075	Protein	Inhibitory signal for cytotoxic cells	[37]
	Lymphocytes; Up	2	Protein		
	T_{REGS}; Down	0.15 Gy	Protein		[39]

BrCa: Breast carcinoma; CRC: Colorectal carcinoma; CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; Hu: Human; Ms: Mouse; NPC: Nasopharyngeal carcinoma.

Table 2. Hypofractionated dose range (>2 to <25 Gy).

IM gene	Tissue (up/down)	Dose (Gy)	mRNA or protein	Role	Ref.
Signal 1					
MHC I	Mel (Hu, Ms); Up	1–25	Protein	Antigen presentation	[21]
	Mel (Ms); Up	15	Protein		[43]
	CRC, Lu, PCa (Hu); Up	10–20	Protein		[44]
	Glioma (Ms); Up	4	Protein		[45]
	PCa (Hu); Up	25	Protein		[46]
TAA peptides	(Hu, Ms); Up	10–25	Peptide saturation	T-cell target	[21]
TAA	Tumor lines (Hu); Up	10–20	Protein		[44,46–47]
Effector signals					
OX40L, 4-1 BBL	CRC lines (Hu); Up	10	mRNA/ protein	Positive co-stimulation	[48]
	PCa Lines (Hu); Up	5–15	Protein		[49]
CD70, ICOSL	PCa lines (Hu); Up	5–15	Protein	Positive co-stimulation	[49]
PD-L1	PCa; Down	5–15	Protein	Inhibition of T cells	[49]
	BrCa tumor (Ms-TUBO); Up	12	Protein		[50]
	PCa tumor; Up/down	10	mRNA		[51]
CTLA-4	PCa tumor (Hu); Down	5–15	Protein	Inhibition of T cells	[49]
ICAM-1	CRC, Lu, PCa, HN (Hu); Up	10–20	mRNA protein	Mediates leukocyte adhesion to tumor cells	[44,52]
	BrCa (Ms); Up	2 × 12 Gy	Protein		[53]
	BrCa (Hu); Up	10	Protein		[47]
	Tumor lines (Hu); Up	10–20	mRNA/protein		[54]
MICB, ULBP1/2	Tumor lines (Hu); Up	20	RNA Protein	NKG2DLs, trigger cytotoxic cells	[55]
RAE-1	BrCa tumor (Ms); Up	2 × 12 Gy	Protein	NKG2DL, trigger cytotoxic cells	[53]
Death receptors					
FAS	Tumor lines (Hu); Up	10–20	Protein	Transmits death signal into cells	[44]
	Tumor line (Ms); Up	8–10	Protein		[56]
	BrCa, CRC (Hu); Up	10–16	Protein		[26,57]
	Esophageal lines; Up	3–6	Protein		[58]
DR4	CRC (Hu); Up	2.5–10	Protein		[59]
	CRC, Lu (Hu); Up	10	Protein		[60]
DR5	CRC (Hu); Up	2.5–10	Protein		[59]
Cytokines					
TGF-β1	PCa tumor (Ms); Up	6–10	mRNA/protein	Immunosuppressive	[61]
GMC-SF, IL-6	PCa tumor (Hu); Up	10	Protein	DC maturation	[51,62]
	Lu (Hu); Up	25	Protein		
TNF-α	Sarcoma (Hu); Up	5	Protein	Inducer of apoptosis	[63]
Chemokines					
CXCL16	BrCa (Ms); Up	2 × 12 Gy	Protein	Recruits immune cells	[64]
CCR2, CCL2, CXCR6, CXCL16	HPV tumor; Up	14	mRNA		[65]
Immune cells					
CD70	DCs (Hu); Up	10	Protein	Costimulates T cells	[66]
	DCs (Ms); Up	10	Protein		[67]
CD86	DCs (Hu); No change	10	Protein	Costimulates T cells	[66]
	DCs (Ms); Up	10	Protein		[67]
	Macrophages (Hu); Up	2–20	Protein		[68]
CD40	Macrophages (Hu); Up	2–20	Protein	Costimulates T cells	[68]

[†]Dose outside the 2–25 dose range of the other studies.

BrCa: Breast carcinoma; CRC: Colorectal carcinoma; DC: Dendritic cell; HN: Head and neck; HPV: Human papilloma virus; Hu: Human; iNOS: Inducible NO synthase; Lu: Lung; Mel: Melanoma; Ms: Mouse; NO: Nitric oxide; PCa: Prostate cancer.

Table 2. Hypofractionated dose range (>2 to <25 Gy).

IM gene	Tissue (up/down)	Dose (Gy)	mRNA or protein	Role	Ref.
MHC-II	Macrophages (Hu); Up	2–20	Protein	Antigen presentation	[68]
IL-12	DCs (Hu); Up	10	Protein	Promotes Th1 responses	[66]
IL-23	DCs (Hu); Up	20	Protein	Promotes Th1 responses	[66]
FoxP3	T_{REGS} (Hu); Down	7.5–30	Protein	T_{REG} transcription factor	[69]
CD45RO	T_{REGS} (Hu); Down	7.5–30	Protein	T_{REG} activation marker	[69]
CD62L	T_{REGS} (Hu); Down	7.5–30	Protein	Promotes trafficking to lymph nodes	[69]
TGF-β1	T_{REGS} (Hu); Down	30†	Protein	Immunosuppressive	[69]
PD-L1	DCs, macrophages; Up	12	Protein	Inhibitor of T cells	[50]
Arginase and COX-2	Macrophages; Up	25	RNA/protein	M2 associated, cancer promoting	[70]
iNOS	Macrophages; Up	25	RNA/protein	M1 associated, inducer of cytotoxic mediator NO	[70]

†Dose outside the 2–25 dose range of the other studies.
BrCa: Breast carcinoma; CRC: Colorectal carcinoma; DC: Dendritic cell; HN: Head and neck; HPV: Human papilloma virus; Hu: Human; iNOS: Inducible NO synthase; Lu: Lung; Mel: Melanoma; Ms: Mouse; NO: Nitric oxide; PCa: Prostate cancer.

capacity for presenting antigen to specific T cells in an MHC-dependent manner [43]. RT upregulated MHC class I expression to high levels on glioma cells in mice [45]. Reits *et al.* observed a dose-dependent increase in MHC class I surface expression 18 h post IR *in vitro* in a human melanoma cell line (MelJuSo) at doses as high at 25 Gy [21]. Further, local irradiation (25 Gy) of mice *in vivo* resulted in similar increases in MHC within the irradiated tissue 24 h post IR. Additionally, cells exposed to 4 Gy IR exhibit increased TAP mobility when analyzed 1 h later and increased polyubiquitination following exposure to 10 Gy, indicating increased protein degradation. Further analysis revealed that prolonged peptide saturation levels are directly related to increased radiation dose [21]. *In vitro*, human colorectal, lung and prostate cancer cell lines showed increase expression of various surface molecules, including MHC-I [44], 72 h post irradiation (10 and 20 Gy). Eight of the 23 lines increased surface expression of MHC-I and 17 of 23 increased surface expression of one or more of the TAAs evaluated (CEA or MUC). Gameiro *et al.* examined radiation's ability to induce IM of human breast cancer (MDA-231), non-small-cell lung cancer (H522) and prostate cancer cells (LNCaP) 72 h after 10 Gy IR. Radiation significantly induced the surface expression MHC class I and TAAs in these diverse cell lines [47].

Signal 2

Following recognition of antigen in MHC-I, naive T cells must also receive co-stimulatory signals for full activation and expansion. Once activated, the activity and function of effector T cells are also enhanced by signaling through co-stimulatory molecules such as CD27, ICOS, OX-40 and 4–1BB [71]. Conversely, the actions of effector T cells can be inhibited by co-inhibitory signals through CTLA-4 or PD-1. We have recently demonstrated that colorectal tumor cells surviving 10 Gy radiation have increased mRNA and surface protein expression of both OX-40L and 4–1BBL [48]. This observation was further extended to prostate cancer cells, and an increase in the surface expression of 4–1BBL, ICOS-L, OX-40L and CD70 was observed in three human prostate cancer cell lines post IR [49]. Changes in OX-40L and 4–1BBL were dose-dependent (5–15 Gy) and increased expression was observable even after exposure to 15 Gy. By contrast, a decrease in the expression of the inhibitory molecule PD-L1 after exposure to 10 Gy IR was observed in some of these same cells, again in a dose dependent manner. Interestingly, reduced expression of PD-L1 remained stable even when evaluated 6 days post irradiation. Variable modulation of CTLA-4 expression in tumor cells (normally expressed on T cells themselves) was reported. One of the tumor cell lines decreased CTLA-4 expression (DU145), while the other cell lines increased expression (PC3 and LNCaP). In contrast to tumor cells, normal prostate cells (PrEC) exhibited much less modulation of the genes evaluated. Aryankalayil *et al.*, assessed the immunomodulatory changes in three human prostate cancer cell lines following exposure to 10 Gy fractionated (1 Gy × 10) or single dose (10 Gy × 1) radiation [19]. They found the mRNA for a variety of immune-related genes

to be modulated 24h post IR. Among the modulated genes only PD-L1 had an obvious and direct link to cell-mediated attack against tumor cells. Exposure to 10Gy SD radiation induced the downregulation of PD-L1 gene expression in PC3 cells. It is important to note that multifraction (MF) delivery (1 Gy × 10) had the opposite effect in DU145 and caused upregulation of PD-L1 mRNA. No change in expression of this gene was reported in LNCaP. These data are in contrast to Bernstein *et al.*, using SD, showing down-modulation of the protein from the cell surface at a later time post IR. Collectively, these studies could be highlighting major differences in IM response between SD and MF delivery. In the clinic, radiation is delivered in multiple smaller fractions to spare normal tissue, and the range of IM likely depends on fractions and fraction doses given [51]. *In vivo*, mice bearing breast cancer tumors (TUBO) were locally irradiated with 12 Gy of RT and PD-L1 expression was increased in tumor cells [50]. Overall, expression of these co-stimulatory and co-inhibitory molecules is particularly interesting because it suggests that modulated tumors are not simply rendered more sensitive to attack by T cells but may be signaling back into responding immune cells and modifying their biological response.

Cell adhesion molecules such as ICAM-1 can enhance CTL and NK cell interaction with target cells, and also appear to deliver co-stimulatory signals into the responding cells. More than half of 23 diverse human tumor cell lines evaluated (colorectal, lung, and prostate) increased expression of ICAM-1 following 10–20 Gy of external beam radiation (EBRT) [44]. Similar observations have been seen in other cancer cell types including breast [47], head and neck [52], and skin [54]. Apart from EBRT, Chakraborty *et al.* have reported that exposure to a radionucleotide can also alter the phenotype of tumor cells. Samarium-153-EDTMP, a bone-seeking radio nucleotide, is used in palliation of bone metastasis. This study demonstrated that exposure to 153Sm-EDTMP induced several immunostimulatory molecules, including ICAM-1, on prostate and lung cancer cells. Importantly, IM of ICAM-1 has also been reported 48 h after *in vivo* local RT (12 Gy × 2) in mice bearing breast tumors (4T1) [53].

Radiation has also been shown to modulate the expression of stress ligands that stimulate NK cell activation. These ligands (MICA/B and ULBP in humans and RAE-1 in mice) bind to NKG2D receptors on NK cells (and $CD8^+$ T cells) and stimulate their lytic activity [72]. Kim *et al.* observed an increase in MICB and ULBP1/2 following 20 Gy radiation in various cancer cell lines (melanoma, colon, cervical and lung) [55]. Increased expression of RAE-1 has similarly been reported following *in vivo* RT of tumor bearing mice [53].

In addition to antigen presentation and activating signals to cytotoxic immune cells, the expression of mediators of effector activities such as death receptors, can also be modulated in tumor cells by IR. Chakraborty *et al.* observed a marked increase in surface Fas expression in MC38-CEA+ murine tumors treated with 8 Gy [56]. There are many reports of increased expression of Fas in human tumor cells as well [26,44,57–59]. Increased sensitivity to the receptors for TRAIL-mediated apoptosis has been reported post IR [73,74], and it has further been demonstrated that expression of both DR4 and DR5 can be modulated by radiation (2–10 Gy) in human tumor cells [59,60].

Cytokines can enhance immune activities of immune cells responding to tumor cells, and chemokines can facilitate the recruitment of immune cells into tumor tissue. IR can also modulate the expression of these molecules in tumor cells. Cytokines such as GM-CSF and IL-6 have been reported to be increased in the supernatants of two of three prostate cancer cell lines 48–72 h post IR (10 Gy) [51]. Another study reported an increase in the levels of GM-CSF, IL-1α and IL-6 in a human lung cancer cells line (AOI) 24 h post IR [62]. TNF-α was increased after treatment with 5 Gy in 5 of 13 human sarcoma cells [63]. Most recently, Wu and colleagues witnessed a significant increase in TGF-β1 in murine prostate cancer cells (TRAMP) both *in vitro* (6 Gy) and *in vivo* (10 Gy), suggesting that this immunosuppressive cytokine may also be induced from some tumors [61]. In addition to cytokines, IR can also alter the expression of chemokines from some tumor cells. The upregulation of CXCL16 (CXCR6 ligand) within tumors and blood vessels of 4T1 tumor-bearing mice following local irradiation (12 Gy × 2) *in vivo* has been demonstrated. CXCL16 is quickly shed by 4T1 tumor cells and markedly increased the recruitment of $CXCR6^+CD8^+$ T cells into the tumor [64]. A significant increase in *CCR2* and *CCL2* mRNA was observed in HPV-associated tumors of mice locally irradiated with 14Gy [65]. CCR2 mediates monocyte

chemotaxis and CCL2 recruits monocytes, MDSCs and DCs to sites of tissue injury and inflammation. The authors also reported significant upregulation of CXCR6 (regulates metastasis and progression of cancer via interaction with soluble CXCL16) and CXCL16 (induces migration of several subsets of T cells and NK cells) after radiation. Collectively, these results reveal that hypo-fractionated doses of IR can modulate soluble signals that influence the behavior of diverse immune cells as well as chemokines and ligands involved in homing of immune cells. Overall, the reports of phenotypic changes induced in tumor cells receiving the doses of radiation discussed in this review are summarized in **Figure 1**, and are overwhelmingly positive in favor of promoting effective antitumor immune responses.

Mechanism of modulation in tumor cells within the hypofractionated dose range

NF-κB

Several mechanisms have been described for how IR can modulate expression of immune relevant genes in tumor cells **(Figure 2)**. While LD-RT has been shown to reduce NF-κB via proteasome inhibition, hypofractionated doses of IR have conversely demonstrated increased activity of NF-κB. NF-κB can be activated in different cell types within an irradiated tumor mass including the tumor cells, stromal cells, cells of the vasculature, and immune cells. In tumor cells, genotoxic stress induced NF-kB activation is initiated by DNA double strand breaks (DSB) in the nucleus and can stimulate gene expression. NF-κB activation has been reported to occur in this manner in diverse tumor cell lines in an ATM-dependent pathway, mostly at doses above 3 Gy. ATM signaling alone is not sufficient; post-translational modification of NEMO is needed, and is also induced by IR. Fully composed ATM-NEMO complexes (reviewed in [30]) can phosphorylate IkB resulting in its degradation by the 26S proteasome. This frees NF-κB to translocate into the nucleus and activate NF-κB target genes. Subsequent expression of NF-κB-induced cytokines can sustain this signaling pathway in a positive feed forward loop. Not surprisingly, some of the genes modulated by IR in tumor cells, as mentioned above, are known NF-κB target genes (*MHC-I, ICAM1*, cytokines) [30]. ATM signaling is also a well-known activator of p53. p53 target genes, however, are related mostly to cell cycle, apoptosis and DNA repair pathways (reviewed in [75]). While most p53 inducible genes are not typical immune relevant genes, apoptosis inducing death receptors could be important for immune cell mediated signals transmitted from death ligands on cytotoxic immune cell. Unfortunately, less than 50% of tumor cells retain functional p53.

mTOR

While NF-κB may be responsible for MHC-I upregulation early post IR, the kinase mTOR has been shown to be responsible for the radiation-induced increase in MHC-I expression at later times post IR (24 h) [21]. mTOR is a critical regulator of protein translation by causing enhanced ribosomal translation. As overall translation is increased, so is the intracellular pool of peptides available for binding to MHC-I. This increase in peptides appears to be responsible for the increased MHC. Interestingly, the benefit of combination immunotherapy with RT was lost when an mTOR inhibitor was utilized demonstrating the therapeutic relevance of this pathway post IR [74].

Epigenetic

It has recently been reported that IR-induced DNA hypomethylation induced gene expression in CRC cells treated with 2–5 Gy [76]. Our studies have found that increased *4–1BBL* expression following 10 Gy radiation is mediated by increased histone acetylation at the promoter for this gene in human CRC cells [48]. More recently, we have reported that radiation (5 Gy) similarly increases histone acetylation at both the *Fas* and *DR5* promoters in addition to the *4–1BBL* promoter. Further, less HDAC2, HDAC3 and DNMT1 were bound to the promoter regions of both *4–1BBL* and *Fas*, but not other genes following IR of human tumor cells [77]. These observations suggest that radiation can also regulate the expression of specific genes by epigenetic mechanisms. This could be important for combination radiation-immunotherapy approaches, as epigenetic changes can be maintained for quite some time in the cell population. At this time, it is unclear how IR is altering the binding of these enzymes at specific gene promoters.

miRNAs

miRNAs are key regulators of gene expression. Several miRNAs have been reported to be modulated following exposure to IR [78,79]. There is

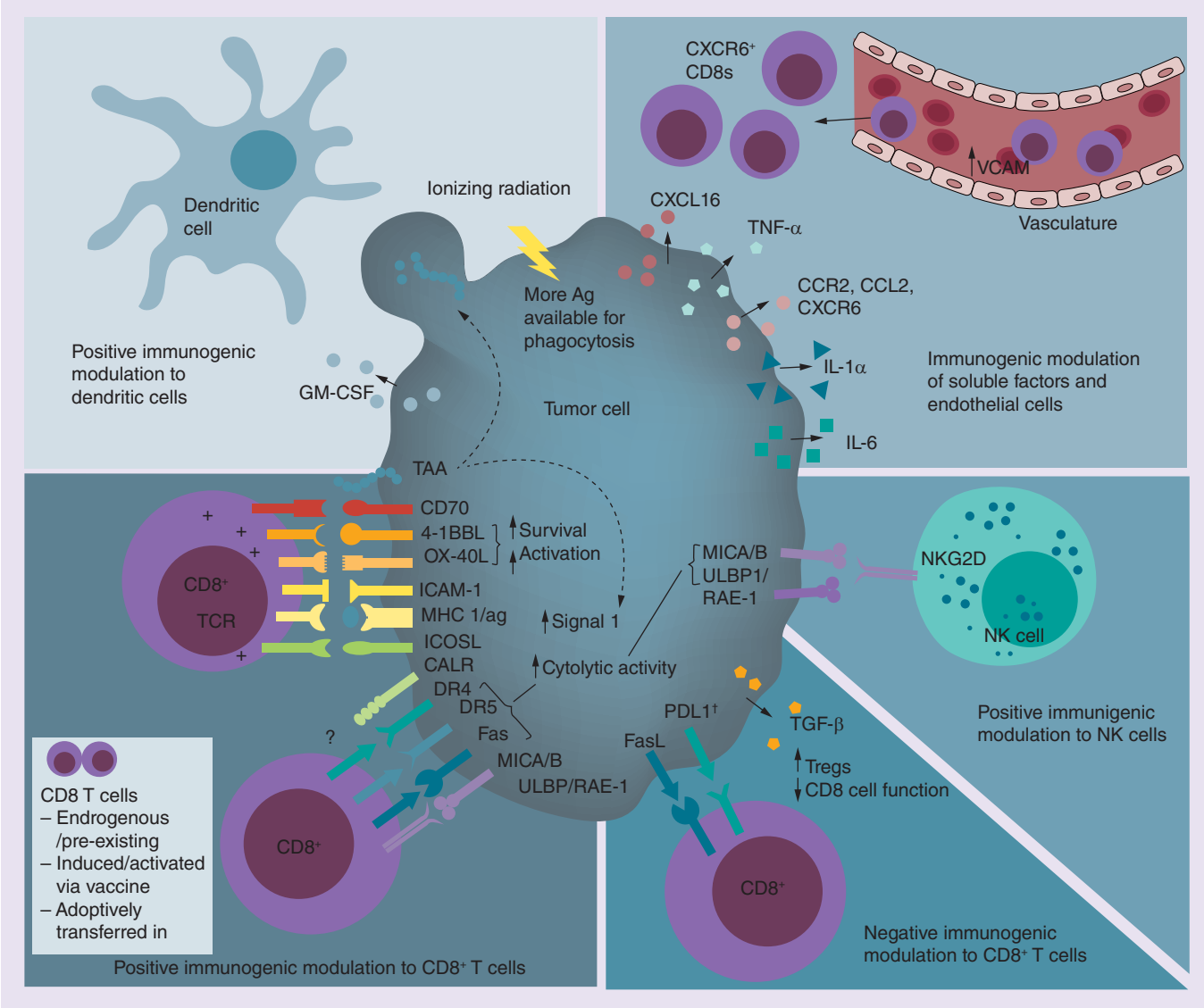

Figure 1. Immunogenic modulation of tumor cells by ionizing radiation. Tumors have been reported to be modulated in several ways, which could directly enhance the function, activity or recruitment of CD8+ T cells, as well as the function of NK and dendritic cells. Increased TAA could result in increased presentation on MHC-I to effector cytotoxic T lymphocytes but could also make more antigen available for uptake by antigen-presenting cells. There have also been limited reports of modulation of tumors in a manner that could negatively impact CD8+ T-cell activities. Note: Ionizing radiation-induced immunogenic cell death mechanisms that enhance dendritic cell activities are not depicted in this figure.

†PD-L1 has been reported to be both increased, and decreased, by ionizing radiation.

Ag: Antigen.

also evidence for regulation of gene expression via miRNAs in normal endothelial cells exposed to IR [80]. Interestingly, *miR16* was shown to be downregulated following exposure to IR (10 Gy delivered in fractions), and *4-1BBL* (TNFSF9) was identified as one of the target genes whose expression was increased in response to this miRNAs reduction. If this same mechanism of regulation operates in irradiated tumor cells needs to be determined. These data highlight the fact that molecules co-stimulatory to effector T cells seem to be induced by radiation in a variety of cell types, including both normal and tumor.

Modulation of immune cells within the hypofractionated dose range

Cells of the immune system can be rapidly dividing, and therefore vulnerable to radiation.

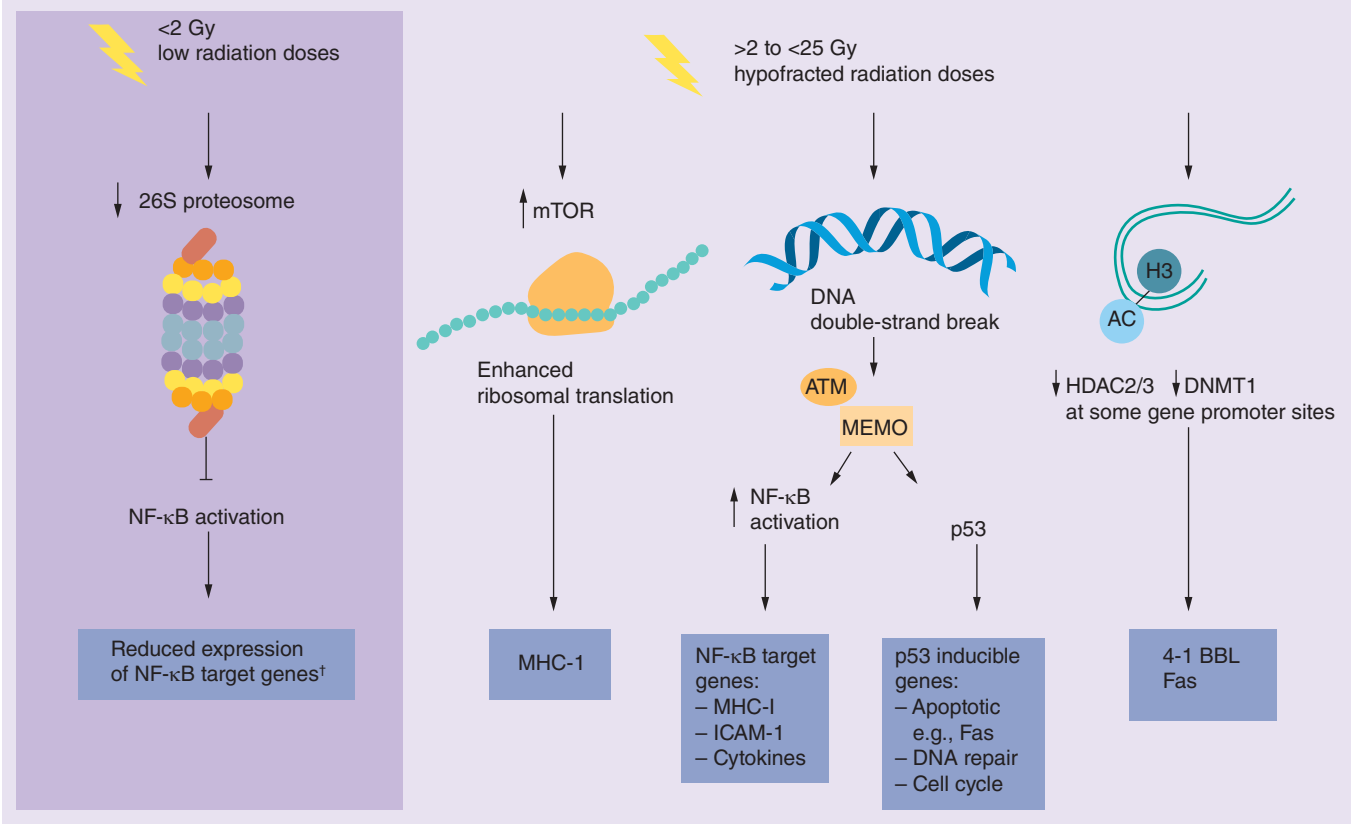

Figure 2. Diverse mechanisms of immunogenic modulation reported in tumor cells surviving radiation.
†NF-κB has been reported to be both inhibited and activated at lower radiation doses depending upon tissue and length of exposure.

Individuals receiving heavy doses of radiation (atomic bomb survivors) exhibit severe damage to mature lymphocytes and bone marrow stem cells, resulting in depletion of several immune cell subsets [81]. This has led to the long-standing perception that radiation beyond the low dose range is generally immune suppressive. In recent years, however, there have been several reports of higher dose radiation treatment directly modulating the phenotype of immune cells both positively and negatively.

Evidence demonstrating positive modulation of immune cells, in a manner that would make them more effective for antitumor activity, has been accumulating and is summarized in **Table 2**. For example, Huang *et al.* observed the upregulation of CD70 on mature dendritic cells and $CD20^+$ B cells isolated for human PBMCs following exposure to 10 Gy *in vitro* [66]. Further analysis showed no change in CD80 and CD86 expression or the maturation marker CD83 on DCs. Irradiation of DCs also increased IL-12 and IL-23 cytokine secretion with doses as high as 10 Gy and 20 Gy, respectively. Following local irradiation (10 Gy) of mice bearing B16 tumors, Gupta *et al.* detected significant upregulation of CD70 and CD86 on live DCs ($CD45^+CD11c^{high}MHC\text{-}II^{high}$) by flow cytometry 48 h post RT [67]. Radiation has also been reported to increase markers of activation in monocytes [68]. *In vitro* treatment with 20 Gy (and 2 Gy to a lesser degree) induced significantly higher expression of CD40, MHC-II (HLA-DR) and CD86 48 h post IR on the monocyte cell line U937 in an NF-κB dependent manner. APCs modulated in such ways would be better able to stimulate subsequent antitumor immune responses.

Conflicting data exist regarding the radiosensitivity of different subsets of lymphocytes. Thus, it is very important to elucidate how different components of the immune system are altered by IR. In particular, there are many conflicting reports on the impact of radiation on $CD4^+$ T_{REGS}, which play a major role in immune tolerance [82]. In cancer patients, T_{REGS} inhibit the generation of successful antitumor immune responses and increased infiltration of T_{REGS} is often detected within the TME [83,84]. One study suggests that irradiation may modulate the phenotype

(and function) of human T_{REGS} *in vitro* [69]. $CD4^+CD25^+$ T_{REGS} from PBMCs were irradiated and evaluated for expression of T_{REG} specific markers. There was reduced expression of CD62L, FOXP3 and CD45RO and increased expression of GITR 12 h post irradiation (1.875, 7.5 and 30 Gy). By contrast, there was no change in the expression of CD4, CD28 and CD45RA on T_{REGS}. The expression of membrane TGF-β, a suppressive effector molecule of T_{REGS}, was also decreased after irradiation of T_{REGS} with 30 Gy as compared with nonirradiated T_{REGS}. Here, reduced expression of T_{REG} effector molecules could correlate with reduced suppressive activity thus allowing effective antitumor immune responses.

There have also been reports of radiation modulating immune cells in a manner that could be inhibitory to effective antitumor immune responses. After exposing TUBO breast cancer cells to 12 Gy RT, PD-L1 expression was increased in DCs after 72 h [85]. There was also a slight increase in PD-L1 seen on macrophages but no observable change in MDSC expression of PD-L1. Curiously, PD-1 expression on $CD8^+$ T cells was slightly reduced 3 days post-irradiation in this same study. It is unclear, however, if this was direct modulation by RT or modulation in response to factors produced by other cells in the local environment. Irradiation of mice bearing murine prostate tumors (TRAMP-C1) induced phenotypic changes in macrophages associated with cancer promotion [70]. Higher dose RT with 25 Gy induced increased arginase and COX-2 and promoted tumor growth [70]. iNOS was produced at later times in these cells and at low levels. This is the opposite of findings discussed above, by Klug *et al.* using LD-RT, and highlights the importance of understanding the impact of IR dose on immune cell activities. The impact of radiation specifically on MDSC phenotype has not been extensively studied, but there are reports of both expansion [86] and reduction [87] in MDSC numbers post IR.

• Immunomodulation at work (functional outcomes)

In vitro evidence of IM & functional enhancement of cytotoxic immune cells

It is clear that there is much more to RT than its traditional use for direct tumor cell destruction or palliation of pain. IR has the ability to modulate gene expression in tumor cells in ways that make them better stimulators of immune cell function [9]. This ability of IR has been demonstrated in several *in vitro* model systems where the function of immune cells is enhanced as a direct consequence of interactions with irradiated tumor cells. Sublethal irradiation of colorectal carcinoma cells (CRC) lines resulted in enhanced susceptibility to lysis by tumor specific CTLs following 10 Gy of irradiation [44]. CTL killing was MHC restricted as irradiated MHC mismatched CRC were not killed by the TAA specific T cells. Moreover, increased killing by CTLs was also observable against other cancer types including prostate [47] and head neck squamous carcinoma [52], revealing that enhanced killing of irradiated tumor cells is not restricted to a single cancer type. More recently we have reported that interaction of CTLs with irradiated human CRC cells enhances the survival and activation of T cells [48]. Enhanced CTL function against irradiated tumor cells is not limited to EBRT, as Chakraborty *et al.* have reported that exposure to 153Sm-EDTMP enhanced CTL killing of irradiated prostate tumor cells (LNCaP) [46]. Despite different molecular profiles of IM, studies by Gameiro and colleagues reveal that radiation-induced tumor sensitivity to CTL lysis was equally augmented with single or fractionated doses of radiation, suggesting that either regimen could elicit effective attack by T cells [47]. In addition to modulated tumor cells, immune cells modulated by RT also have the ability to enhance T cell function. For example, irradiated DCs have been shown to increase the proliferation and IFN-γ production from T cells as a direct consequence of increased expression of CD70 [66]. There is also some evidence that the ability of T_{REGS} to suppress cells is reduced following both high-dose [69] and low-dose irradiation *in vitro* [39].

IM of tumor cells by RT has also been shown to enhance functional activity of NK cells. NK cell-mediated recognition and cytolysis of human tumor cells is governed by ligation of the NKG2D receptor on NK cells with MICA and MICB (MICA/B) molecules on tumor cells. Human melanoma cells and non-small-cell lung cancer cell lines that increase expression of MICA/MICB after 20 Gy IR also exhibited enhanced sensitivity to NK mediated killing [55]. Liu *et al.* reported that upregulation of MICA/B on the tumor cell surface led to increased recruitment, activation and survival of NK cells [25]. Direct exposure of human NK cells to radiation can result in increased NK cytotoxicity that seems to peak at an optimal

dose (6 Gy) and decline after the peak (range: 1–16 Gy). Radiation-treated NK cells from cancer patients have been reported to have higher lytic activity than NK cells from normal controls [88]. Radiation also enhanced the cytotoxic effects of NK cells when they were inoculated, as a mixture with tumor cells, into mice after irradiation [89]. This synergistic effect was not observed when the lymphoid cells were inoculated after irradiation, indicating that lymphoid cells needed to be in contact with tumor cells before irradiation. Anti-asialo GM1 reversed this effect implicating NK cells as the effectors here. Purified NK cells exposed to 0.2 Gy LD-RT *in vitro* displayed no significant difference in cell viability or proliferation. However, significant augmentation of cytotoxic function was detected when NK cells were stimulated with low-dose IL-2 prior to irradiation [90]. Others have similarly reported that radiation-treated NK cells exhibit increased function [91–93]. Collectively, these studies demonstrate the ability of IR to enhance NK cell activity by both direct treatment with IR and as a consequence of radiation-induced IM of the tumor.

In vivo evidence of IM & functional enhancement of cytotoxic immune cells

All cells in the TME, including the target tumor cells, endothelial cells, stromal cells and infiltrating immune cells, may respond uniquely and further influence the response of neighboring cells. While immune cells are thought to be more radiosensitive than other cells in the TME, there are numerous accounts of local tumor irradiation *in vivo* culminating in effective immune attack of tumors by both adaptive and innate cells. RT has been shown to enhance tumor-specific CTL numbers and function in the intact TME. Chakraborty *et al.* demonstrated enhanced killing of CEA$^+$MC38 tumors by CEA specific CD8$^+$ T cells in mice irradiated with 8 Gy. The ability of RT to enhance attack of tumor cells was demonstrated using both a therapeutic vaccine to induce T cell expansion [94] as well as when tumor specific T cells were adoptively transferred into the animals [56]. In the latter study, modulation of the Fas receptor on tumor cells was shown to be required for the ability of RT to enhance immune attack. Using another tumor model system, adoptive cell transfer of *ex vivo* activated OVA-specific OT-1 CD8$^+$ T cells led to increased infiltration of transferred T cells to tumor sites (and not merely expansion of localized T cells) following RT (15 Gy) to the tumor [95]. This study suggested that the mechanism of radiation-induced lymphocyte infiltration into the TME involved upregulation of chemo-attractants MIG and IP-10. Though it was not determined which cells were producing these factors, these chemo-attractants appeared to promote IFN-γ responses by conditioning the tumor microenvironment for enhanced CTL trafficking. Moreover, the tumor-infiltrating lymphocytes (TILs) isolated from *in vivo* irradiated tumors killed better than TILs from nonirradiated hosts.

There are similar reports of radiation modulating tumor cells *in vivo* and enhancing tumor-specific NK cell numbers and function. Ruocco *et al.* demonstrated that RT (12 Gy × 2) synergized with CTLA-4 blockade by upregulating RAE-1 expression on the surface of irradiated tumor cells and that the therapeutic effect of the combinatorial regimen were blocked by antibodies targeting the RAE-1 receptor (NKG2D) [53]. IL-12/1L-15/1L-18 preactivated NK cells showed increased frequencies and persistent effector functions inside established murine tumors following RT (5 Gy TBI), highlighting the enhanced therapeutic efficacy of combination NK cell therapy and RT [96]. In patients, NK cells from uterine cervix carcinoma patients undergoing radiotherapy demonstrated increased cytotoxicity, suggesting that it is possible for RT to enhance immune cell function in the human TME [97].

RT can also influence other noncytotoxic immune cells in a way that could positively influence antitumor attack. There have been limited reports of RT, in the dose ranges discussed here, modulating APCs in the TME to enhance cross-presentation of TAA [98] or recruiting additional immune cells including CD8$^+$ T cells into the TME [99]. T_{REG} mediated suppressive activity from irradiated (8.5 Gy) melanoma (D5) tumor bearing mice has been reported to be significantly reduced when compared with T_{REGS} from untreated mice indicating that RT impairs function of T_{REGS} [100]. However, there have also been contrasting reports revealing that RT (10 Gy) can induce suppressive immune cells following local RT to some tumors (TRAMP) [61]. Thus, it remains unresolved if IM of tumors by radiation can directly influence T_{REG} number and function.

Conclusion

The most exciting reports of RT-CIT in combination have been of the abscopal response following RT both preclinically [101,102] and clinically [12–16,103]. The abscopal response likely

involves cytolytic cells that retain the functional ability to mount a sustained response at a distant, nonmodulated, tumor site. The mechanism(s) of the abscopal effect has not been definitively determined. Molecules that can alter the lytic capacity or enhance the sustainability of effector CTLs or NK cells are likely candidates for promoting this type of effect. For a long time increased expression of death receptors such as Fas was thought to be the sole molecular mechanism responsible for enhanced immune attack of irradiated tumors [56]. However, very simple *in vitro* systems demonstrated that human tumor cells with deficient Fas signaling were still killed better by CTLs after radiation suggested that alternate mechanisms exist and contribute to this effect [44]. Use of these *in vitro* systems has allowed us to directly test the impact of irradiated tumors on CTL biology, and has revealed that CTLs also survive better and are activated better following interaction with irradiated tumor cells [48]. Examination of irradiated tumor cells for changes in expression of genes that could provide survival, activation and enhanced lytic activity in effector CTLs revealed that the co-stimulatory molecules OX-40L and 4–1BBL were upregulated on irradiated tumor cells [48,49]. Increased MHC-I and antigen expression following IR have been demonstrated by several groups. Increased signal 1, however, must work in concert with other signals to T cells. Increased antigen in a toleragenic or immunosuppressive environment where robust costimulation is not present leads to suboptimal immune responses such as T-cell anergy. Thus, many proposed mechanisms of RT-enhanced immune responses (more antigen presentation, immunogenic death, increased death receptor, increased stress ligands or increased cell adhesion expression) would still require appropriate co-stimulation to induce optimal and sustainable CTL responses. Furthermore, these changes all occur local to the irradiated tumor and it is difficult to envision how many of the reported changes at the local irradiated tumors could promote a response that is sustainable at a distant tumor unless the change was retained within the responding effector cells. Receiving signals from OX-40L or 4–1BBL, or perhaps effector activity programming cytokines, from irradiated tumor cells could leave a lasting impression on T cells leaving the tumor and impact future activity beyond the irradiated tumor [104,105]. This argument seems plausible given the fact that the mere presence of antigen specific T cells (or induction of greater numbers by vaccine) is not enough to induce curative T cell activity against many tumors. Indeed, there are situations where T cells of known TAA-specificity already exist but are not effectively attacking the tumor, even when large numbers are transferred into tumor bearing hosts [56]. In these situations radiation is not needed to 'prime' an initial T cell response to the TAA via ICD because large numbers of effector cells are being transferred in. Local RT does, however, result in greatly enhanced activity of T cells suggesting that the existing T cells may now be receiving different signals from the irradiated tumor [43,106]. Moreover, irradiated tumor cells alone, and in the absence of APCs, are killed better by effector CTLs [44,46–47,52], and are able to enhance T cell survival and activation [48]. This mechanism would be relevant when effector CTLs are already present but have less than optimal functional or killing capacity.

At this time, RT is routinely applied for palliation or tumor debulking in patients where it is not curative. Given the substantial evidence of the IM activities of IR, perhaps now is the time to begin utilizing RT specifically for IM in combo with CIT strategies. Though this review focused on radiation doses of 25 Gy or below, the influence of various doses within this range should be considered a critical factor moving forward [107,108] in defining the most effective dose for promoting IM activities. For example, NK cells are sensitive to high doses of radiation while lower dose radiation enhances cytotoxicity of NK cells [93]. Lower doses may help achieve a more favorable balance between tumor-infiltrating cytotoxic cells, and the unfavorable suppressive cells, allowing for tumor elimination [109]. Many RT-CIT studies use much higher doses (>25 Gy total) [87,110–111], and it is unclear if the immune outcomes reported occur similarly at lower doses. To this point, many differences are seen in the modulation of MHC-I following low dose [45] versus higher dose [112]. Lower doses may also be fundamentally better for use as an adjuvant to CIT because of lower toxicity as well as reduced clinical visits for the patient. In addition to dose range, single dose delivery versus multiple fraction delivery seem to differentially influence IM outcomes [19,20].

Future perspective

What is next regarding immunomodulation and radiation therapy for cancer? IM-induced phenotypic changes in tumor cells have been

studied extensively. However, given the differential radio-sensitivities of immune cell subsets, it seems imperative to more fully elucidate the direct effects of LD-RT and radiation doses within the hypofractionated dose range on immune cell function. It will also be important to determine which phenotypic changes occur most broadly and can thus be capitalized on across diverse human tumor types. Regarding the TME, how stromal cells and local endothelial cells are modulated and contribute to antitumor activity or immune suppression at these doses needs to be elucidated both *in vitro* and *in vivo*. Given the recent evidence that IM of some genes is occurring via epigenetic mechanisms, determining how long these changes are retained in tumor cells as well as what other immune relevant genes are regulated this way will be helpful for defining the therapeutic usefulness of RT-induced IM. It is perhaps most important to determine which changes occur in instances where abscopal responses are seen. The mechanisms reported for induction of the abscopal effect are diverse, coming from both tumor immunologists and radiation biologists, and it will be important to define which mechanism(s) are at play at these doses so that we can induce this type of response more consistently. Last, which of the many and diverse immunotherapies (checkpoint blockade, therapeutic vaccines, positive co-stimulatory agonist abs, adoptive cell transfer, TLR agonists, among others) will benefit the most from the IM action of RT needs to be defined. Overall, such data will allow for determination of which CITs should routinely incorporate RT for its IM activities, and at what dose, allowing for the rationale incorporation of RT into CIT approaches.

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EXECUTIVE SUMMARY

Immunomodulation by low-dose radiotherapy

- Low-dose radiotherapy (LD-RT) can modulate tumor phenotype.
- Immune cells can be directly modulated by LD-RT in ways that have been reported to be beneficial for antitumor efficacy.
- Single dose LD-RT inhibits NF-κB-mediated gene expression via proteasome inhibition.
- LD-RT and hypofractionated doses of RT appear to modulate NF-κB activity via different mechanisms in tumor cells.

Immunomodulation by hypofractionated doses of radiation

- Single dose RT (8–10 Gy) has been shown to modulate phenotype *in vitro* resulting in enhanced function of effector cytotoxic T lymphocytes (CTLs).
- Tumors irradiated with 10 Gy single dose (SD) enhance effector CTL viability and activity.
- Single dose RT in the hypofractionated dose range has been demonstrated to modulate tumor phenotype and gene expression by several diverse mechanisms.
- Antigen-presenting cells and T_{REGS} exposed to SD radiation appear to be modulated in ways that could enhance the activities of cytotoxic immune cells.

Immunomodulation at work (functional outcomes)

- SD RT (8–10 Gy) has been shown to modulate phenotype *in vivo* and result in enhanced function of transferred CTLs and NK cells.
- 10 Gy SD can synergize with therapeutic cancer vaccines to induce an abscopal response.
- *In vivo*, RT has been shown to influence other noncytotoxic immune cells in a way that could positively influence antitumor attack by effector cells.

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Review

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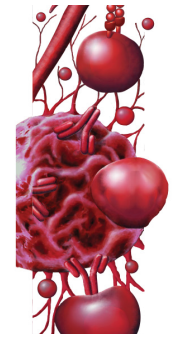
Immunotherapy



Immunotherapy in prostate cancer: challenges and opportunities

Although treatment options for castration-resistant prostate cancer (CRPC) have increased over the last decade, there remains a need for strategies that can provide durable disease control and long-term benefit. Recently, immunotherapy has emerged as a viable and attractive strategy for the treatment of CRPC. To date, there are multiple strategies to target the immune system, and several approaches including therapeutic cancer vaccines and immune checkpoint inhibitors have been most successful in clinical trials. With regard to this, we report the results of the most recent clinical trials investigating immunotherapy in CRPC and discuss the future development of immunotherapy for CRPC, as well as the potential importance of biomarkers in the future progress of this field.

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Prostate cancer remains highly prevalent and has a poor clinical outcome once metastatic. Localized prostate cancer can be managed with active surveillance, brachytherapy, external beam radiotherapy and radical prostatectomy with or without androgen deprivation therapy (ADT), depending on the individual prognosis [1]. Patients treated with ADT alone for metastatic disease usually respond well, but ultimately biochemical relapse and disease progression may occur. Disease that progresses despite ADT is referred to as castration-resistant prostate cancer (CRPC) and most prostate cancer-related deaths occur in patients with metastatic CRPC (mCRPC). During the past 5 years, the US FDA has approved five new agents (sipuleucel-T, cabazitaxel, abiraterone acetate, enzalutamide and radium-223) based on demonstrated overall survival (OS) benefit in Phase III studies [2–6].

Despite the improved therapeutic options for CRPC, there remains a need for treatments that can provide durable disease control and long-term survival benefit. Among the many strategies that are being investigated to address this need, immunotherapy is now an established treatment approach for prostate cancer with multiple clinical trials, such as randomized vaccination trials with sipuleucel-T and another with a recombinant virus-based vaccine, PROSTAVAC, demonstrating improvements in OS [7]. Although traditional treatment mostly focuses on androgen deprivation and chemotherapy, immunotherapy rely on activating the host's immune cell to specifically attack prostate cancer cells and generate tumor-specific immunity. Based on this mechanism, immunotherapeutic approach for prostate cancer may have broad applicability to all clinical states of the disease in the setting of neoadjuvant, biochemical relapse post-primary therapy, castrate nonmetastatic and castrate metastatic. Improved understanding of the interactions between the immune system and prostate cancer has generated renewed

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interest in treating prostate cancer with immunotherapy. This review presents the rationale for using immunotherapy to treat prostate cancer, summarizes recent clinical trial progress in particular CRPC, and discusses the future development of immunotherapy for patients with prostate cancer.

Principles of immunotherapy in prostate cancer

The primary aim of immunotherapy is to harness the immune system's ability to recognize and destroy tumor cells. Cell types central to the recognition and destruction of tumor cells include macrophages, antigen-presenting cells (APCs), $CD8^+$ cytotoxic T lymphocyte (CTL) cells and NK cells. Pathophysiologically, tumors are adept at developing pathways to suppress immune responses and escape from immune destruction, culminating in evasion and clinical progression [8]. Potential mechanisms of evasion include the modulation of immune-inhibitory (checkpoint) pathways to suppress T-cell activity and the disruption of antigen processing and presentation [9]. Tumors can also recruit and promote the developing immunosuppressive cells, such as Treg and myeloid-derived suppressor cells (MDSC) [10]. Tumors may directly or indirectly mediate the release of immunosuppressive factors, such as TGF-β and IL-10, which contribute to the development of an immunosuppressive microenvironment in and around the tumor [11,12]. In addition, indole 2,3-dioxygenase, which can be expressed by dendritic cells, MDSCs and cancer cells, promotes the naïve T cells to Treg and increases IL-6 expression with augmenting MDSC function [13]. Potential immunotherapeutic targets include these inhibitory factors in the tumor microenvironment, such as Treg, MDSC, IL-6 and indole 2,3-dioxygenase. Figure 1 shows the descriptive mechanism of immune responses in the tumor microenvironment.

Research suggests that prostate cancer is an immunologically modulated malignancy, and thus may be sensitive to immunotherapy. For example, data from studies that have evaluated the cellular composition of prostate tumors suggest that immune cell populations infiltrate the prostate gland [14,15]. Infiltrating leukocytes detected in prostate tumors include NK cells, effector cells and Treg cells, suggesting that both the innate and the adaptive branches of the immune system may play a role in mounting an attack against prostate cancer cells [16]. In addition, prostate cancer is particularly well suited to immunotherapeutic approaches for three reasons [17]. First, the relatively slow growth pattern of prostate cancer allows time to develop an immune response. Second, the prostate is a highly differentiated, gender-specific organ and prostate cancer offers a variety of suitable antigen targets for cancer immunotherapy, such as prostate-specific antigen (PSA), prostatic acid phosphatase (PAP), prostate-specific membrane antigen (PSMA) and prostate stem-cell antigen. Third, the use of PSA for the early detection of recurrent disease allows for the initiation of vaccine immunotherapy while the tumor burden is still minimal.

Vaccine-based immunotherapy

The goal of vaccine-based immunotherapy is to stimulate a specific antitumor immune response against tumor antigens while minimizing collateral damage to normal tissues [11]. The five main types of vaccine-based immunotherapies studied in CRPC can be classified as autologous, cell-based, viral-based, peptide-based and DNA vaccines. The important clinical trials of vaccine-based immunotherapy for CRPC are summarized in Table 1.

Sipuleucel-T

Sipuleucel-T is an autologous dendritic-cell vaccine prepared using the patient's own peripheral blood mononuclear cells, obtained with leukapheresis and *ex vivo* incubated with a recombinant fusion protein consists of PAP and granulocyte-macrophage colony-stimulating factor (GM-CSF) [18,25]. Phase I/II clinical trials have shown that sipuleucel-T is well-tolerated and the patients develop appreciable antigen-specific T-cell responses and antibodies against the fusion protein after the treatment [19,20]. Three Phase III clinical trials have been completed and showed promising findings of this autologous vaccine. The first two studies compared patients with asymptomatic mCRPC assigned to placebo or sipuleucel-T. There was no difference in time to tumor progression, but there was a significant increase of median OS (25.9 vs 21.4 months and 19.0 vs 15.7 months) [26,27]. A third Phase III clinical trial known as the Immunotherapy for Prostate Adenocarcinoma Treatment (IMPACT) trial showed a 4.1-month improvement in median OS [2], and this result led to the approval of sipuleucel-T as the first therapeutic cancer vaccine by the FDA in 2010. These trials demonstrated that sipuleucel-T was well-tolerated and that most common adverse events were injection-site reactions, transient fever and flu-like symptoms. The biological activity was reported in a study of immune responses in prostate tumor tissue following neoadjuvant sipuleucel-T in patients with localized prostate cancer [28]. Prostatectomy specimens from vaccinated patients showed a greater than twofold increase in $CD3^+$ and $CD4^+$ T cells at the tumor interface. Similar results were also reported in a neoadjuvant peptide vaccine study, in which prostatectomy specimens showed rapid infiltration of $CD45RO^+$ activated/memory lym-

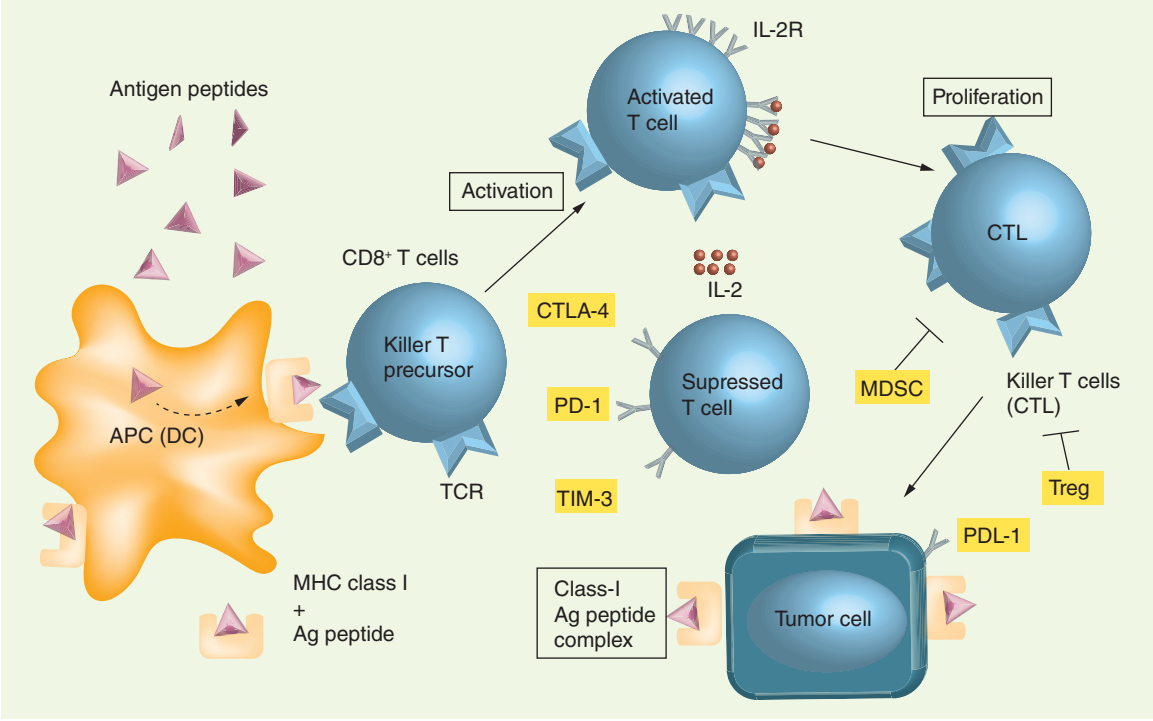

Figure 1. Mechanism of immune responses in the tumor microenvironment.

phocytes into tumor sites after the vaccination [21]. Retrospective analysis of the IMPACT trial suggested that sipuleucel-T has more clinical benefit in patients with a lower baseline PSA value than in patients with a higher baseline PSA value [22]. Patients with a lower baseline PSA had a median OS of 41.3 months with 13 months improvement than placebo, while patients with a higher baseline PSA had a median OS of 18.4 months with only 2.8 months improvement. These results demonstrate that a greater treatment benefit with sipuleucel-T occurs in patients with earlier-stage disease and more favorable baseline prognostic factors.

GVAX

GVAX is a cell-based vaccine derived from irradiated hormone-sensitive (LNCaP) and hormone-resistant (PC3) cell lines genetically modified to secrete GM-CSF. In a Phase I/II dose-escalating study of patients with metastatic prostate cancer, GVAX was associated with a tolerable safety profile, PSA decrease and stabilization, and a median OS time of 35.0 months with high-dose treatment [29]. These results prompted the initiation of two Phase III trials of CVAX. VITAL-1 randomized patients with asymptomatic mCRPC to GVAX or docetaxel-prednisone, while VITAL-2 randomized symptomatic patients to receive docetaxel or docetaxel plus GVAX. In the first Phase III study (VITAL-1), futility analysis indicated that the trial had a <30% chance of improved OS with GVAX, so that trial was terminated [30]. In the second Phase III study (VITAL-2), a planned interim analysis demonstrated a trend toward more deaths in the GVAX arm and shorter median OS, leading to the trial's closure as well [31]. Despite these results, GVAX continues to be explored in combination regimens and in other tumor types [32].

PROSTVAC (PSA-TRICOM)

Viral vectors are attractive for use in cancer immunotherapies as they can mimic natural infection and lead to the induction of immune response against the tumor antigen that they encode. PROSTVAC is a PSA-targeted poxvirus-based vaccine consisting of a heterologous prime-boost (vaccinia or fowlpox virus vector) and three costimulatory molecules (TRICOM; B7.1, ICAM-1 and LFA-3) that serve to increase the PSA-specific immune response [33]. Several early trials showed that the treatment of PROSTAVAC was well-tolerated with minimal toxicities (fever and injection-site reactions) [34–37]. In a multicenter Phase II trial of PROSTAVAC, 125 patients with minimally symptomatic mCRPC were randomized 2:1 to receive vaccine or placebo, respectively. As in the sipuleucel-T trials, there was no difference in terms of time to tumor progression. However, PROSTAVAC resulted in an 8.5-month survival advantage (median OS: 25.1 months for the PROSTAVAC group vs 16.6 months for controls; p = 0.0061) and

Table 1. Important clinical trials of vaccine-based immunotherapy for castration-resistant prostate cancer.

Agent (trial ID/ Ref.)	Phase	n	Setting	Target antigen	Platform	Comparison treatment	PFS	OS	Ref.
Sipuleucel-T	III	127	Asymptomatic mCRPC	PAP/GM-CSF fusion	Cultured autologous PBMCs	Placebo (autologous cells)	11.7 versus 10 weeks (HR: 1.45; 95% CI: 0.99–2.11; p = 0.052)	25.9 versus 21.4 months (HR: 1.70; 95% CI: 1.13–2.56; p = 0.01)	[18]
Sipuleucel-T	III	98	Asymptomatic mCRPC	PAP/GM-CSF fusion	Cultured autologous PBMCs	Placebo (autologous cells)	10.9 versus 9.9 weeks (HR: 1.09; 95% CI: 0.69–1.70; p = 0.719)	19.0 versus 15.7 months (HR: 1.27; 95% CI: 0.78–2.07; p = 0.331)	[19]
Sipuleucel-T	III	512	Asymptomatic mCRPC	PAP/GM-CSF fusion	Cultured autologous PBMCs	Placebo (autologous cells)	14.6 versus 14.4 weeks (HR: 0.95; 95% CI: 0.77–1.17; p = 0.63)	25.8 versus 21.7 months (HR: 0.775; 95% CI: 0.614–0.974; p = 0.032)	[20]
GVAX	III	626	Asymptomatic mCRPC	Allogeneic tumor lines	GM-CSF transduced cell lines	Docetaxel plus prednisone	NA	20.7 versus 21.7 months (HR: 1.03; 95% CI: 0.83–1.28; p = 0.78)	[21]
GVAX plus docetaxel	III	408	Symptomatic mCRPC	Allogeneic tumor lines	GM-CSF transduced cell lines	Docetaxel	NA	12.2 versus 14.1 months (HR: 1.70; 95% CI: 1.15–2.53; p = 0.008)	[22]
PROSTAVAC-VF	II	125	Minimally symptomatic mCRPC	PSA	Vaccine-fowl pox	Control vector	3.8 versus 3.7 months (HR: 0.884; 95% CI: 0.568–1.375; p = 0.6)	25.1 versus 16.6 months (HR: 0.56; 95% CI: 0.37–0.85; p = 0.006)	
PROSTAVAC plus GM-CSF (NCT01322490)	III	1200	Asymptomatic or minimally symptomatic mCRPC	PSA	Vaccine-fowl pox	Placebo GM-CSF	Ongoing	Ongoing	
PPV plus low-dose EMP	II	57	mCRPC	Various TAAs (including PSA, PAP, PSMA, MDRP and other epithelial antigens)	Multiple peptides	EMP	8.5 vs 2.8 months (HR: 0.28; 95% CI: 0.14–0.61; p = 0.012)	Not reached versus 16.1 months (HR: 0.28; 95% CI: 0.14–0.91; p = 0.033)	[23]

EMP: Estramustine phosphate; GM-CSF: granulocyte-macrophage colony-stimulating factor; mCRPC: Metastatic castration-resistant prostate cancer; MDRP: Multidrug resistance protein; OS: overall survival; PAP: Prostatic acid phosphatase; PBMC: Peripheral blood mononuclear cell; PFS: Progression-free survival; PPV: Personalized peptide vaccination; PSA: Prostate-specific antigen; PSMA: Prostate-specific membrane antigen; TAA: Tumor-associated antigen.

Table 1. Important clinical trials of vaccine-based immunotherapy for castration-resistant prostate cancer (cont.).

Agent (trial ID/ Ref.)	Phase	n	Setting	Target antigen	Platform	Comparison treatment	PFS	OS	Ref.
PPV	II	20	Docetaxel resistant mCRPC	Various TAAs (including PSA, PAP, PSMA, MDRP and other epithelial antigens)	Multiple peptides	Historical control	NA	17.8 versus 10.5 months (p = 0.166)	[24]
PPV (UMIN000011308)	III	333	Docetaxel resistant mCRPC	Various TAAs (including PSA, PAP, PSMA, MDRP and other epithelial antigens)	Multiple peptides	Placebo	Ongoing	Ongoing	

EMP: Estramustine phosphate; GM-CSF: granulocyte-macrophage colony-stimulating factor; mCRPC: Metastatic castration-resistant prostate cancer; MDRP: Multidrug resistance protein; OS: overall survival; PAP: Prostatic acid phosphatase; PBMC: Peripheral blood mononuclear cell; PFS: Progression-free survival; PPV: Personalized peptide vaccination; PSA: Prostate-specific antigen; PSMA: Prostate-specific membrane antigen; TAA: Tumor-associated antigen.

3-year survival of 30% for PROSTAVAC versus 17% for controls [38]. These trials suggested tumor-specific CTL responses and prolonged OS in patients treated with PROSTAVAC. A global Phase III randomized, placebo-controlled trial of PROSTAVAC (NCT01322490) is currently enrolling 1200 patients with asymptomatic or minimally symptomatic mCRPC, with OS as the primary endpoint. Patients are randomized to receive PROSTAVAC with GM-CSF, PROSTAVAC with placebo GM-CSF or double placebo. Data on the primary endpoint of OS are expected in December 2015.

Personalized peptide vaccination

Vaccine antigens that are selected and administered without considering the host immune cell repertoires could not efficiently induce beneficial antitumor immune responses. In view of the complexity and diversity of the immunological characteristics of tumors and the immune cell repertoires of hosts, a new concept of personalized peptide vaccination (PPV) has been developed [39]. In this 'personalized' cancer vaccine formulation, appropriate peptide antigens for vaccination are screened and up to four peptides are selected from a list of vaccine candidates in each patient, based on pre-existing host immunity. In several Phase I studies of PPV for CRPC, PPV was well-tolerated with toxicities consisting mainly of injection-site reactions and cellular and humoral immune responses, and decreases in the PSA levels in some patients have been reported [23–24,40]. In a randomized, crossover, Phase II trial of PPV plus low-dose estramustine phosphate (EMP) comparing standard-dose EMP in HLA–$A2^+$ or HLA–$A24^+$ patients with CRPC, median progression-free survival (PFS) was 8.5 months in the PPV group and 2.8 months in the EMP group ($p = 0.0012$), and the median OS for the PPV plus low-dose EMP group was not reached within 22.4 months, while the median OS for the standard-dose EMP group was 16.1 months ($p = 0.033$) [41]. These results suggest that this combination with a low-dose cytotoxic drug produces additional antitumor effects with minimum immunosuppression. In another Phase II study, the OS in docetaxel-resistant CRPC patients treated by PPV ($n = 20$) was compared with that of historical control ($n = 17$) [42]. Median OS from the first day of progressive disease was 17.8 and 10.5 months in docetaxel-based chemotherapy-resistant CRPC patients receiving PPV and not receiving PPV, respectively ($p = 0.166$). These encouraging preliminary studies suggested that PPV warrants further study as a novel therapy for CRPC patients with progressive disease after docetaxel chemotherapy. A Phase III, randomized, placebo-controlled trial of PPV (UMIN000011308) is currently enrolling 333 docetaxel-resistant CRPC patients, with OS as the primary endpoint. The results of this trial are eagerly anticipated.

DNA vaccine

Vaccines based on nucleic material from tumor-associated antigens have also been studied in prostate cancer. For example, DNA vaccine pTVG-HP has produced immunological responses in patients with recurrent, localized prostate cancer, and some evidence of clinical responses [43]. Of eight patients who had a ≥200% increase in PSA doubling time, six had detectable long-term PAP-specific IFN-γ-secreting T-cell responses [44]. An ongoing Phase II trial (NCT00849121) evaluating the immunogenicity of pTVG-HP with GM-CSF as an adjuvant is ongoing.

Immune-checkpoint inhibitor

Immune-checkpoint inhibitors have generated excitement recently because of their successful use in the treatment of multiple tumor types including metastatic melanoma. This newly developed class of agents interfere with the immune system's autoregulatory mechanisms, thereby enhancing T-cell activity and potentiating antitumor effects using antibodies targeting immunological checkpoint regulators, such as CTL-associated antigen 4 (CTLA-4), programmed death (PD)-1 and its ligand (PDL-1), which down-regulate the immune response pathway [45]. This treatment strategy is considered to be a more active immunotherapeutic approach than vaccine-based immunotherapies.

Ipilimumab is a fully human monoclonal antibody that targets CTLA-4 to turn the immune response back on and augment T-cell-mediated immune responses [45]. Ipilimimub was approved by the FDA in 2011 for the treatment of advanced melanoma and is currently being investigated for the treatment of non-small-cell lung cancer, metastatic renal cell cancer and ovarian cancer. Regarding mCRPC, a Phase I/II report of a dose-escalating study of ipilimumab with and without radiation therapy to a single site in bone showed stable disease and several dramatic and durable responses [46]. Common immune-related adverse events (irAEs) of ipilimimub in this study were diarrhea (54%), colitis (32%) and pruritus (20%); grade 3/4 irAEs included colitis (16%) and hepatitis (10%). These results provided the impetus for a recently reported Phase III trial [47] for patients who progressed after docetaxel chemotherapy and were randomly assigned 1:1 to receive bone-directed radiotherapy followed by either ipilimumab at 10 mg/kg or placebo every 3 weeks for up to four doses. Nonprogressing patients could continue to receive ipilimumab or pla-

cebo as maintenance therapy every 3 months. There was no significant difference between the ipilimumab group and the placebo group (median OS: 11.2 vs 10.0 months; p = 0.053) in terms of OS as the primary endpoint. However, subgroup analyses suggested that ipilimumab might provide an OS benefit for patients with favorable prognostic features. For the subgroup of patients with an alkaline phosphatase <1.5-times the upper limit of normal, hemoglobin >11.0 mg/dl and no visceral metastases, median OS was 22.7 months with ipilimumab compared with 15.8 months with placebo (p = 0.0038).

Similarly, nivolumab (anti-PD-1) and anti-PDL-1 have shown robust activity in melanoma, renal cell, non-small-cell lung and bladder cancers, but minimal activity in prostate cancer [48,49]. The type of irAEs for nivolumab and anti-PDL-1 agents was similar to that seen with ipilimumab; dermatological adverse events, diarrhea and fatigue occurred in approximately 20, 20 and 30% of cases, respectively. Despite these results, there is still focus on investigating their effectiveness in prostate cancer via other combination approaches [50].

Biomarkers

It would be important to identify predictive biomarkers that could accurately assess antitumor immune responses and predict patient prognosis following the administration of cancer vaccines. Without clear markers to assess the clinical benefit in cancer vaccine treatments such as RECIST criteria or PSA decline, it might be difficult to integrate cancer vaccine treatments for prostate cancer [51]. In some clinical trials, several postvaccination biomarkers, including CTL responses, antibody responses, Th1 responses, delayed-type hypersensitivity and autoimmunity, have been reported to be associated with clinical responses [52–56]. Analysis of data with sipuleucel-T showed that markers of an antigen-specific immune response (APC numbers, APC activation and total nucleated cell numbers) correlated with OS, demonstrating immune activation as potential biomarkers [57]. Another analysis of PPV in 500 advanced cancer patients also showed that both lymphocyte counts before vaccination and increased IgG response to the vaccinated peptides after vaccination, along with performance status, were well correlated with OS [53]. Other trial using ipilimumab and GVAX showed that high pretreatment frequencies of $CD4^+$/CTLA-4^+ T cell, $CD4^+$/PD-1^+ T cell or differentiated $CD8^+$ T cells or low pretreatment frequencies of differentiated $CD4^+$ T cell or Tregs were associated with significantly prolonged OS [58]. However, there are currently no validated biomarkers for cancer vaccines in widespread use, and only survival benefit has been considered as a prominent endpoint in many cancer vaccine trials. Moreover, novel biomarkers for selecting patients who would benefit most from cancer vaccines remain to be addressed. Therefore, there is still an urgent need for definitive biomarkers to assess the benefits of immunotherapies.

Conclusion & future perspective

Improved understanding of the interactions between the immune system and prostate cancer has generated renewed interest in treating prostate cancer with immunotherapy. Immunotherapy will be most effective when disease burden is a minimal stage disease. While there are several promising immunotherapeutic agents under study, sipuleucel-T is clinically available as the first in class antigen-specific autologous immunotherapy approved for CRPC treatment. Ongoing Phase III trials of PROSTAVAC, PPV and the immune checkpoint inhibitor ipilimumab may soon broaden the scope of immunotherapies available to patients with CRPC. Current strategies are also exploring optimal combinations and sequencing of immunotherapies with other treatments. Moreover, novel biomarkers for selecting patients who would benefit most from immunotherapies remain to be addressed.

Financial & competing interests disclosure

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Executive summary

- Immunotherapy has emerged as a viable and attractive strategy for the treatment of prostate cancer.
- There are multiple ways to target the immune system.
- Data from studies support the activity and safety of therapeutic cancer vaccines and immune checkpoint inhibitors in prostate cancer.
- No surrogate biomarkers for clinical outcomes in patients treated with immunotherapy have been identified.

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Review

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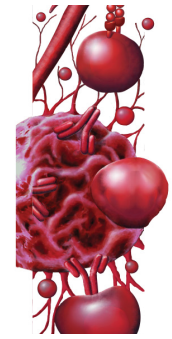
Immunotherapy



Vaccinia virus, a promising new therapeutic agent for pancreatic cancer

The poor prognosis of pancreatic cancer patients signifies a need for radically new therapeutic strategies. Tumor-targeted oncolytic viruses have emerged as attractive therapeutic candidates for cancer treatment due to their inherent ability to specifically target and lyse tumor cells as well as induce antitumor effects by multiple action mechanisms. Vaccinia virus has several inherent features that make it particularly suitable for use as an oncolytic agent. In this review, we will discuss the potential of vaccinia virus in the management of pancreatic cancer in light of our increased understanding of cellular and immunological mechanisms involved in the disease process as well as our extending knowledge in the biology of vaccinia virus.

Keywords: immunotherapy • oncolytic virus • pancreatic cancer • vaccinia virus

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Pancreatic cancer remains one of the most difficult cancers to diagnose and treat. It is the fifth most common cause of cancer death in the UK with 1 and 5 years survival of 20.8 and 3.3%, respectively. These figures have hardly improved since the early 1970s [1]. Complete surgical resection remains the only curative treatment. Unfortunately, less than 20% of pancreatic tumors are amenable to surgical excision at the time of diagnosis. However, even with complete surgical resection prognoses remains poor with 5 years survival around 20% [2,3]. Gemcitabine is the main chemotherapeutic agent approved for advanced pancreatic cancer. Despite being shown to improve life expectancy compared with 5-flurouracil, effect remains modest with median survival around 6 months [4]. Combining gemcitabin therapy with erlotinib led to minimal increase in life expectancy from 5.9 to 6.2 months [5]. Therefore, new treatment strategies are clearly imperative.

Vaccinia virus (VV) has played a prominent role in one of the greatest achievements in medical history: the eradication of smallpox (caused by Variola virus). Since then, VV has been developed as a vector for vaccines against infectious diseases such HIV, influenza, malaria and tuberculosis as well as in immunotherapies [6] and oncolytic therapies for cancer [7,8]. With regards to the latter, the earliest studies, which mainly used replication attenuated VV recombinants for fear of toxicity, were relatively disappointing in the clinic. Replication competent VVs retain their ability to lyse tumor cells and spread through tumor tissue. Recent advances in DNA recombinant technology enabling the rational manipulation of the viral backbone, coupled with the ever increasing knowledge gains in the fields of molecular virology and cancer cell biology have aided the development of safe and efficacious tumor-targeted oncolytic VVs. These are currently at the forefront of the most promising novel anticancer agents.

In this review, we will explore the potential of tumor-targeted oncolytic VV in the management of pancreatic cancer in light of our increased understanding of cellular and immunological mechanisms involved in the disease process as well as our extending knowledge in the biology of VV.

Future Medicine part of fsg

Tumor-targeted oncolytic viruses as a new class of cancer therapeutics

Targeted therapy of cancer using oncolytic viruses (OV) has generated much interest over the past decades in the light of the limited efficacy and the significant side effects of standard cancer therapeutics for advanced disease [9]. OVs have become an increasingly popular anticancer therapy platform due to their ability to selectively infect and lyse tumor cells (Figure 1). Cancer selectivity of OVs could be a result of natural tropism [10,11] or via genetic modification [9]. OVs can target multiple cellular pathways [12–14] minimizing the risk of tumor resistance and induce different modes of cell death [15–18]. In addition, OVs can break down the immunosuppressive tumor microenvironment and induce a long-lasting tumor-specific immunity [19,20] (Figure 2). OVs can specifically deliver therapeutic proteins into tumors at increasing levels following viral replication within the malignant cells. Furthermore, OVs can function in synergy with conventional cancer treatments of chemoradiotherapy [21–24]. Finally, OVs as a treatment platform are amenable to adjustment and development following our ever-increasing understanding of cancer cells, the virus and host immune responses to both tumor and virus.

H101, an adenovirus with *E1B 55K* gene deletion (Oncorine; Shanghai Sunway Biotech, Shanghai, China) was licensed in China in 2005 as the world's first OV for treatment of head and neck cancer when combined with chemotherapy [25]. The similar virus, *dl1520* (also known as, ONYX-015) has been administered by intratumoral injection under CT guidance into locally advanced primary tumors of pancreatic cancer patients in Phase I/II trials. The treatments were well tolerated, but no objective responses were seen in any of the patients with virus alone, and only 10% (2/21) patients showed objective response when gemcitabine was used in combination [26–28]. Another virus that entered clinical trials is HF10, a Herpes Simplex virus armed with granulocyte-macrophage colony-stimulating factor (GM-CSF). Phase I trial of intratumoral injection into nonresectable pancreatic tumors proved to be safe with some encouraging clinical results [29]. These early results warrant further investigation to seek more powerful agents for this cancer.

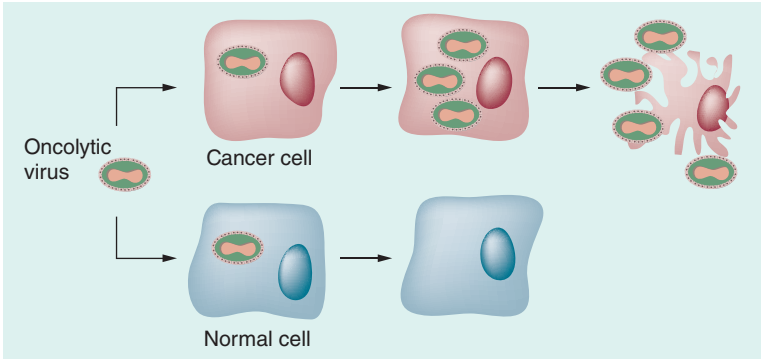

Figure 1. Tumor selectivity of oncolytic viruses. Tumor-targeted oncolytic viruses can exploit defective cellular pathways in cancer cells (top). oncolytic viruses can infect and replicate in cancer cells leading to cell lysis and release of viral particles. These in turn infect neighbor tumor cells and so forth. In normal cells (bottom) cellular defense mechanisms prevents viral replications.

Favorable features of vaccinia virus for cancer treatment

VV is a member the poxvirus family. It is a double-stranded DNA virus ~192 kbp in size. It can be stably accommodate up to 25 kbp of cloned exogenous DNA [30]. Structurally, it consists of a core region composed of viral DNA and a various viral enzymes including RNA polymerase and polyA polymerase encased in a lipoprotein core membrane. The outer layer of the virus consists of double lipid membrane envelope [31,32]. VV has two major forms of infectious virions; the intracellular mature virions, as described above, which is released upon cell lysis and the extracellular enveloped virion released from the cells via cell membrane fusion. The latter has an additional lipid bilayer membrane wrapped around the intracellular mature virion particle.

VV has many inherent characteristics that make it an ideal choice for oncolytic virotherapy. VV has a short life cycle of 8 h that takes place in its entirety in the cytoplasm eliminating the risk of genome integration. Replication usually starts 2 h after infection, at which time the host cell nucleic acid synthesis shuts down as all cellular resources are directed toward viral replication [33,34]. Cell lyses takes place between 12 and 48 h releasing packaged viral particles. Furthermore, the virus does not depend on host mechanisms for mRNA transcription making it less susceptible to biological changes of the host cell [33,35].

Unlike other OVs, VV does not have a specific surface receptor for cell entry allowing it to infect a wide range of cells unhindered by the lack of expression of said receptor. They depend on a number of membrane fusion pathways for cell entry [36,37].

The existence of various antigenically distinct forms of the mature virus allows it to evade host immune system. extracellular enveloped virion form of the virus is encapsulated in a host-derived envelope, with incorporated viral proteins, that contains several host complement control proteins [38–40]. In addition, VV infected cells secret Vaccinia complement control protein which binds an inactivate C4b and C3B inhibiting the classic and alternative complement activation pathways [41–43]. VV therefore can be disseminated relatively unharmed in the blood stream to reach distant tumors allowing

systemic delivery of the virus [44], which is more suitable for the treatment of the advanced pancreatic cancer.

The hypoxic nature of pancreatic cancer contributes to its aggressive and treatment-resistant phenotype. In contrast to adenovirus [45], we have found that hypoxic conditions did not affect replication, viral proteins production, cytotoxicity and transgene expression of the Lister strain of VV [46]. These results suggest that VV could be suitable for management of pancreatic cancers and potentially other hypoxic tumors.

Finally, VV has a good safety track record following its use as a vaccine for over a century. Minor and less severe side effects include fever, rash and inadvertent inoculation. Moderate-to-severe side effects include eeczema vaccinatum, generalized vaccinia, progressive vaccinia and postvaccinial encephalitis [47]. Sides effects are rare with an incident of less than 1:10,000 and severe side effects in particular are extremely rare [48]. Genetically modified recombinant VV could be potentially safer due to their tumor selectivity. Recent clinical trial of JX-594 virus in hepatocellular carcinoma showed the treatment to be well tolerated with mainly flu-like symptoms in all patients and a single severe side effect [8].

How Vaccinia virus selectively kills cancer cells by multiple action mechanisms

VV has a natural tropism to cancer cells [49,50]. The virus can utilize activated molecular pathways in tumor cells to aid its replication [51–53]. In fact, many of the hallmarks of cancer [54] make tumor cells susceptible to viral replication including immune escape, sustained cell proliferation and resisting cell death. In the case of VV, the EGFR family [55], potentially plays an important role in tumor selectivity. The viral SPGF, an EGF-like growth factor carried by VV, can activate host cellular pathways leading to increased viral replication [56]. In addition, Ras–GTP-activating protein S3H domain-binding protein, overexpressed in most human cancers [57], plays a role in VV replication by complementing the activity of the VITF-2 [58].

Various approaches can be utilized to enhance tumor selectivity of OVs. The virus depends for its replication in normal cells on a set of genes that prepare the cell resources for viral replication and block apoptotic pathways. Deleting these genes will limit the virus ability to replicate in normal cells. However, these pathways are often disrupted in cancer cells allowing the mutant virus to replicate despite the defective genes. One such example is the disruption of the vaccinia thymidine kinase gene (*TK* gene) affecting the virus ability to synthesize deoxyribonucleotides [59,60]. Normal cells have a much smaller reserve of deoxyribonucleotides, compared with tumor cells, limiting the ability of VV to replicate. Another example is the deletion of the *B18R* gene encoding the secreted IFN-binding protein that blocks IFNα signaling [61]. In normal cells, this gene deletion attenuates viral replication due to IFN antiviral effect while cancer cells remain permissive to VV replication as IFN signaling is often disrupted [62,63]. In addition, altering the expression of crucial vaccinia viral gene by microRNA also enables tumor-specific viral replication, which is a potentially novel and versatile platform for engineering VVs for cancer virotherapy [64].

GLV-1h68 is a replication-competent VV targeted at tumor cells by mutation of *J2R* (encoding thymidine kinase) and *A56R* (encoding hemagglutinin) loci. This virus was shown to be effective against human pancreatic cancer cell line *in vitro* and in nude mice xenografts. Importantly this efficacy was enhanced

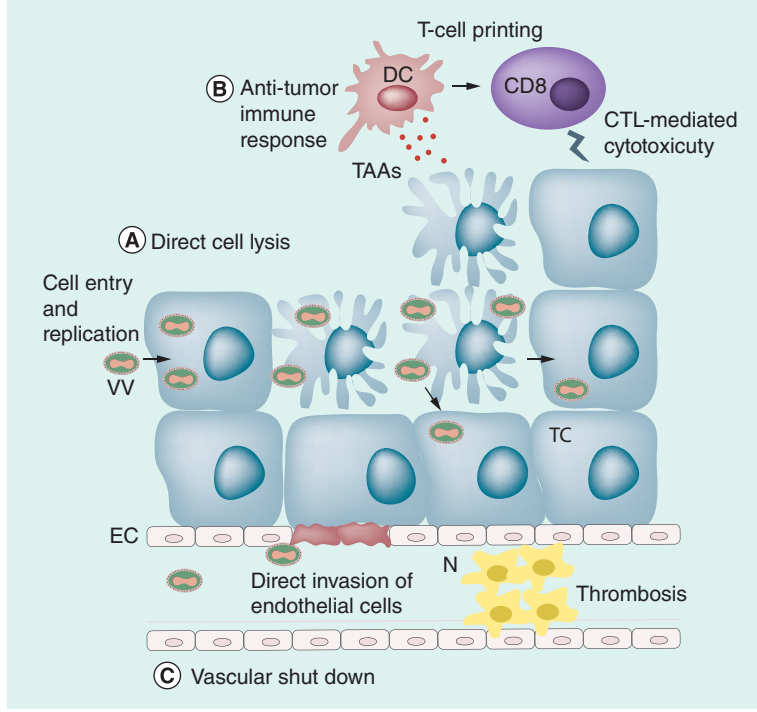

Figure 2. Multiple modes of actions of tumor-targeted oncolytic viruses.
Oncolytic viruses (OV) can kill cancer cells via a variety of mechanisms. First, they directly infect, replicate and lyse tumor cells sparing normal cells. Released virions can infect neighbor tumor cells and so forth. Second, OVs can induce immunogenic cell death associated with the release of pathogen-associated molecular patterns and damage-associated molecular patterns. In addition viral infection results in the release of cytokine and chemokines deviating the immune response toward a cytotoxic profile. Dendritic cells can pick tumor-associated antigens released from lysed tumor cells and prime $CD8^+$ T cells to induce a tumor-specific immune response. Third, OV infection can result in vascular shutdown caused by direct viral invasion of endothelial cells and thrombosis caused by cytokine-mediated neutrophils accumulation.
CD8: Cytotoxic T cell; DC: Dendritic cell; EC: Endothelial cell; N: Neutrophil; TAA: Tumor-associated antigen; TC: Tumor cell; VV: Vaccinia virus.

when virus therapy was combined with gemcitabine and cisplatin [65]. GLV-1h151, a virus with similar gene deletions but different marker proteins transgenes [66], was found to be effective *in vivo* and *in vitro* against human pancreatic cancer cell lines. Combining the virus with radiotherapy resulted in a synergistic antitumor effect [67].

In addition to direct cell lysis, VV can utilize vascular shut down to kill noninfected tumor cells [44,68–69]. This is believed to be caused by accumulation of neutrophils in blood vessels, mediated by cytokines and chemokines, leading to intravascular thrombosis [69]. In addition, VV can infect and destroy tumor-associated endothelial cells further contributing to vascular collapse [62]. Although this process has not been specifically shown in pancreatic tumors, we believe it to play an important role in the multimechanistic antitumor effect of VV, as pancreatic cancers are often well-vascularised and high microvascular density correlates with poor outcome after surgical excision [70]. To further capitalize on this process we have rationally armed Lister strain VV with endostatin–angiostaten fusion gene, a well-documented angiogenesis inhibitor [71]. The resultant VVhAE virus proved to be tumor selective *in vitro* and *in vivo*. It resulted in suppression of angiogenesis and prolonged survival of mice bearing human pancreatic cancer xenografts [50].

Vaccinia virus as immunomodulatory agent

The ability of OVs to alter the immune composition of the, ordinarily, immune-suppressive tumor microenvironment led to a new line of thinking of their mechanism of action. Large body of evidence suggests that antitumor immunity, where the virus is acting as an oncotropic immunomodulator, is the key determinant of a successful onclytic virotherapy [72–74].

VV kills cancer cells via a combination of necrosis and immunogenic apoptosis resulting in the release of damage associated molecular patterns [75–78] and pathogen associated molecular patterns [79–81] as well as the release of viral antigens into the tumor. This process leads to a strong inflammatory response that can overcome the immune suppression within the tumor microenvironment. In addition, tumor cell lysis releases tumor-associated antigens (TAA) into this inflammatory environment. Dendritic cells recruited by the virus can in turn pick up these exposed TAAs and cross-prime $CD8^+$ T cells resulting in a potent antitumor adaptive immune response. It has been demonstrated that an oncolytic VV (JX549) could induce tumor-specific immunity in human cancer patients [82] and preclinical study [20]. Therefore, oncolytic virotherapy may be considered as a method of vaccination *in situ*, enabling the adaptive immune response to clear residual disease as well remote metastatic cancer cells and provide long-term surveillance against relapse.

In the context of vaccination, heterologous prime-boost immunization regimen using recombinant adenovirus prime and VV boost has been shown to enhance $CD8^+$ T-cell immunogenicity with protective efficacy against malaria in a mouse model [83,84]. So, it seems logical that combining two different OVs for cancer treatment may induce a stronger tumor-specific immunity. We have, for the first time, combined the use of oncolytic adenovirus and VV, in a prime-boost strategy, for treatment of established tumors in the hope to harness the host immune response to the infected tumor cells. We found that sequential treatment via intratumoral injection with oncolytic adenovirus followed by oncolytic VV resulted in complete eradication of subcutaneous pancreatic cancer grafts in Syrian hamsters. More importantly, the surviving animals developed a long-lasting tumor-specific immune response that protected them against tumor rechallenge. This process was shown to be T-cell dependent [20].

Arming VV with various cytokines and chemokines can further enhance its antitumor activity. IL-10, a cytokine produced by Th2 T cells, is a potent inhibitor of antiviral immune response [85]. We have found that arming VV with IL-10 dampened antiviral immune response resulting in prolonged viral persistence in pancreatic tumors. This led to stronger antitumor immunity and improved survival in both subcutaneous and transgenic pancreatic cancer mouse models [86].

Vaccinia virus as vaccine vector

The first use of a recombinant virus armed with an antigen from a different organism as a vaccine vector was reported over 30 years ago. VV armed with hepatitis B surface antigen gene was able to induce a protective immunity against hepatitis in chimpanzees [87,88]. Since then there has been a great progress in recombinant VV vaccines in the veterinary field [89,90]. Unfortunately this success did not extend to human infectious diseases vaccines, mainly due to the lengthy and more stringent process for human licensing, with only a handful of recombinant VV vectors in current clinical trials [91–94].

One of the significant challenges for cancer vaccination lies in developing strategies to improve the delivery of antigens to antigen-presenting cells *in vivo*, allowing effective antigen processing and presentation and activation of a potent immune response against a unique background of immune tolerance toward 'self' TAAs. Viral vectors have become attractive antigen delivery systems as they mimic a natural viral infection, resulting in induction of cytokines and co-stimulatory molecules

that provide a powerful adjuvant effect and elicit potent cellular immunity [74,95].

Survivin is a member of the inhibitor of apoptosis family expressed in a variety of cancers. It plays a crucial role in tumor survival and drug resistance [96]. It is expressed during embryonic development but absent from differentiated cells [97]. Survivin is overexpressed in 70–80% of pancreatic cancers and is associated with resistance to chemoradiotherapy [98,99]. Vaccination with Vaccinia Ankara virus, a nonreplicating attenuated VV strain, armed with survivin induced survivin-specific CD8+ immune response resulting in a modest antitumor effect. When combined gemcitabine antitumor immunity and efficacy improved significantly. This is likely to be related to gemcitabine suppression of myeloid-derived suppressor cells [100].

The only VV-based cancer vaccine to enter clinical trials is PANVAC-V, a VV expressing carcinoembryonic antigen and mucin-1, both highly expressed in pancreatic cancers. The two antigens were packaged with three costimulatory molecules: B7.1 (cluster of differentiation 80), ICAM-1 (intracellular adhesion molecule one) and LFA-3 (leukocyte function-associated antigen-3) known collectively as TRICOM. To further enhance the immune response, the vaccination was delivered as a heterologous prime/boost regimen using a nonreplicating fowlpox vector expressing the same antigens and costimulatory molecules (PANVAC-F) [101]. GM-CSF was administered at the injection site as an adjuvant to enhance local antigen processing and presentation. In a Phase I clinical trial, the vaccine was found to be safe and well tolerable. It generated an antigen-specific immune response toward carcinoembryonic antigen and mucin-1 which correlated with increased survival [102]. However, Phase III trial (NCT00088660) targeting patients with metastatic pancreatic cancer who failed gemcitabine treatment failed to meet its therapeutic targets and was terminated [103]. The vaccine is currently under investigation for direct intratumoral injection under endoscopic ultrasound guidance with encouraging results of Phase I trial [104].

Future perspective

There has been a great interest in VV in recent years. Its safety, cancer tropism, amenability to genetic modification and ability to target solid tumors via a variety of mechanism of actions have made it a near-perfect onclytic virus to target pancreatic cancers. Nevertheless, as with any new therapeutic agents VV therapy need to overcome many hurdles and challenges before it enters routine clinical practice.

The first challenge is the selection of the right VV strain. The nonvaccine strain Western Reserve (WR) VV is widely used in the lab. JX-963, a GM-CSF armed mutant of WR VV with deletion of both the Thymidine Kinase and the Viral Growth Factor gene, has been reported as the most potent tumor-targeted oncolytic VV [52]. Other strains, such as the European vaccine Lister strain, are largely untested. We recently evaluated the antitumor potency and biodistribution of different VV strains using *in vitro* and *in vivo* models of cancer, including pancreatic cancer models. The Lister strain virus with Thymidine Kinase gene deletion (VVΔTK) demonstrated superior antitumor potency and cancer-selective replication *in vitro* and *in vivo*, compared with WRDD, especially in human cancer cell lines and immune-competent hosts. Further investigation of functional mechanisms revealed that Lister VVΔTK presented favorable viral biodistribution within the tumors, with lower levels of proinflammatory cytokines compared with WRDD, suggesting that Lister strain may induce a diminished host inflammatory response [105]. Our comprehensive study indicates that the Lister strain VV with TK deletion is a particularly promising VV strain for the development of the next generation of tumor-targeted oncolytic therapeutics. We anticipate that more and more people will use the Lister stain of VV as a backbone to develop new OVs for cancer treatment in the future.

Further genetic modifications of VV might enhance its oncolytic ability. Disruption of the *N1L* gene reduces virulence and inhibits VV replication in the brain reducing the risk postvaccinial encephalitis, a rare but significant complication of VV vaccination [106,107]. Our unpublished work on *N1L*-deleted VV suggests that *N1L*-deleted VV resulted in a superior antitumor efficacy compared with N1L-intact VV [Ahmed J *et al.*, Unpublished Data]. In addition, arming the new generation of VV with immune-modulatory genes or other therapeutic genes that enhance the antitumor immunity is a future for cancer treatment using tumor-targeted OVs.

Achieving the right immune response is of a paramount importance. Increasingly safer viruses permits the use of higher doses to maximize therapeutic effect [8], however higher viral load might deviate the immune response toward antiviral immunity resulting in rapid viral clearance and reduced antitumor immunity. Manipulating the immune system with cytokine-armed viruses is not without its risks including serious autoimmune side effects [108].

Systemic delivery of VV is particularly relevant in pancreatic cancer as most pancreatic tumors present with distant metastasis at the time of diagnosis. One such virus (JX594) has recently been shown to effectively target tumors after intravenous infusion, making it an ideal OV for treatment of inaccessible tumors such

as pancreatic cancer [7]. To date, the systemic delivery of OVs has been shown to be safe but not efficacious mainly due to the rapid clearance of these agents by the immune system [109]. When designing new strategy to enhance the systemic delivery of VV lessons can be learnt from other OVs. Serotype exchange [110,111], engineering new serotypes [112] and the use of chemical shielding [113] have been successfully used with other OVs. In fact the latter strategy have been used to modify the nonreplicating Vaccinia Ankara vaccine vector to circumvent pre-existing anti-VV immunity [114]. Other approaches include pharmacologically modifying the immune response to reduce the neutralization of the systemically delivered OVs [115–117].

Combining oncolytic virotherapy with traditional cancer treatments is an area of great promise. Gemcitabine can suppress myloid-derived suppressor cells in the tumor microenvironment resulting in a stronger antitumor immune response [118]. On the other hand, gemcitabine is a nucleoside analogue that inhibits DNA synthesis including that of double-stranded DNA viruses [119]. Using these agents in a sequential rather than combination manner might be the key to effective therapy [120]. Similarly, combining OVs with immune checkpoints inhibitors is an area that requires more investigation and optimization. PD-1 and CTLA-4 inhibitors might enhance the OV-induced antitumor immunity by creating a favorable immune profile in the tumor microenvironment [121,122].

Despite the challenges, the field of oncolytic virotherapy is generating a great interest of both researchers and pharmaceutical companies alike. The recent US FDA approval of talimogene laherparapvec (T-VEC, an engineered herpes simplex virus-1 expressing GM-CSF), for the treatment of melanoma has given the field a much needed boost. As safety and efficacy data start to accumulate the process of licensing new OVs will get easier. We anticipate other cytokine- and chemokine-armed viruses to enter clinical practice within the next few years. In addition, combining OVs with immune checkpoint therapies, monoclonal antibodies and CAR-T therapies will be an area of major research interest in the near future. Combining immune checkpoint antibodies with other immune-stimulating agents such as conventional drugs, targeted agents and most of all OVs, may increase the tumor types and individual patient profiles in which a durable clinical benefit can be achieved. OVs are finally being recognized for their ability to stimulate antitumor immunity, and with anti-CTLA-4 and anti-PD-1 agents on the market, OVs may finally have met their perfect match. It has never been a more promising era for cancer immunotherapy and personalized medicine.

We believe at the current rate of development it will not be long before OVs are part of routine clinical practice.

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Executive summary

- Pancreatic cancer is one of the most aggressive human cancers, without effective therapies.
- Tumor-targeted oncolytic viruses is a new class of cancer therapeutic agents.
- Oncolytic Vaccinia virus (VV) has distinctive features that make it ideal for treatment of pancreatic cancer.
- The antitumor efficacy of oncolytic VV can be further improved by modification of viral genes and arming the virus with therapeutic genes.
- Combination of oncolytic VV with other cancer therapies could be the future for treatment of pancreatic cancer.

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