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RADIOTHERAPY EFFECTS ON ANTI-TUMOR IMMUNITY: IMPLICATIONS FOR CANCER TREATMENT

Topic Editors
Silvia C. Formenti and Sandra Demaria

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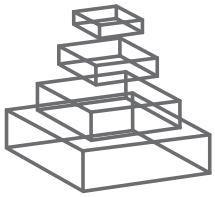

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RADIOTHERAPY EFFECTS ON ANTI-TUMOR IMMUNITY: IMPLICATIONS FOR CANCER TREATMENT

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Ionizing radiation is an important modifier of the tumor microenvironment that has been shown to have the potential for therapeutic synergy with immune response modifiers both in pre-clinical studies and some initial clinical studies. This emerging evidence suggests a new paradigm in the clinical use of radiotherapy whereby the goal transcends the local tumor control to evolve toward the induction of an immunogenic tumor cell death, resulting in an "in situ" form of individual vaccination. Ionizing radiation also induces key soluble cytokines and chemokines, as well as phenotypic changes in irradiated tumor and stroma further contributing to immune-mediated tumor rejection. Conversely, radiation induces expansion of immune-regulatory cells populations that may have relevant immune-suppressive effects, that could be counteracted with currently available drugs.

Overall, the ability of radiation to promote both, the priming and effector phase of the anti-tumor immune response provide a compelling argument for exploring these yet unexploited features in the clinic. However, many questions remain to be addressed before the use of radiation as an immunological adjuvant becomes a clinically available strategy. For instance, relatively limited information exists about which radiation regimens generate the optimal balance between induction of immune-stimulatory versus immune-suppressive pathways. Moreover, the choice of the immune therapies that best synergize with radiation, and their optimal sequencing are undefined.

The articles in this focused issue will illustrate examples of promising combinations between radiotherapy and immune response modifiers that have been tested in pre-clinical models, and early clinical trials.

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EDITORIAL
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Radiotherapy effects on anti-tumor immunity: implications for cancer treatment

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Ionizing radiation (IR) is a powerful therapeutic modality for cancer, commonly used for its capacity to kill cancer cells. In this Frontiers Research Topic Book radiotherapy effects are re-visited, from the point of view of the host's immune system (IS). An introductory article from Golden et al. (2012) examines the consequences of the many types of radiation-induced tumor cell death and how these coalesce to generate the key signals that define an immunogenic cell death (ICD). Cancer cells dying by ICD deliver a cascade of signals to the IS that culminates in the generation of anti-tumor T cells by providing a source of antigen for cross-presentation coupled with maturation signals to dendritic cells (DC). The ability of IR to induce an ICD is exploited by novel cancer therapies that have, for instance, shown the benefit of intra-tumoral injection of DC post-radiotherapy in preclinical models. Finkelstein and Fishman (2012) discuss this approach and the emergence of encouraging results from clinical pilot studies.

Burnette et al. (2012) provide an overview of the immunological environment existing in tumor-bearing hosts, emphasizing the challenge of overcoming tolerance and immunosuppression to achieve tumor rejection. To overcome this barrier, combinations of IR with specific immunotherapies have been tested by several labs and shown to be effective at eliciting robust anti-tumor immunity. One such strategy, discussed by Mason and Hunter (2012), is the combination of IR with intra-tumoral synthetic oligodeoxynucleotides such as CpG. The preclinical success of this combination was translated to the clinic where it has demonstrated to induce rejection of the irradiated tumor as well as tumors outside the radiation field (abscopal effect). Another strategy, which is in earlier stage of investigation but holds great potential, is nanovectorized radiotherapy discussed by Vanpouille-Box and Hindré (2012). Delivery of radionuclides using nanoparticles has the advantage of providing targeting specificity to the tumor as well as exploiting the intrinsic immunostimulatory properties of nanoparticles.

Importantly, IR effects exceed the classical cytocidal properties by also causing phenotypic changes in the fraction of surviving cells, markedly enhancing their susceptibility to T cell-mediated elimination. Kwilas et al. (2012) define these effects of IR as "immunogenic modulation" and illustrate the examples of IR-induced Major Histocompatibility Complex antigens and death receptors, which improve tumor rejection by T cells adoptively transferred or activated by vaccination.

However, not all IR-induced modifications of the tumor and its microenvironment favor immune rejection. Chiang et al. (2012) provide novel evidence for accumulation of pro-tumorigenic M2 macrophages in areas of hypoxia present in irradiated tumors. Schaue et al. (2012) discuss the increase of regulatory T cells post-radiotherapy, potentially hindering the development of effective anti-tumor T cell responses. Intriguingly, the dose and fractionation of radiotherapy may play a role in modulating the expansion of effector versus regulatory T cells. This aspect is critically addressed by Demaria and Formenti (2012). Since much of the available preclinical data come from experiments testing single IR doses, further exploration of fractionated regimens is warranted.

Overall, the book provides an overview of the available data and evolving concepts in support of a novel use of radiotherapy: that of an immune modulator and optimal partner for immunotherapy. While enthusiasm for the combination of IR and immunotherapy was enhanced by recent anecdotal reports in some cancer patients, much work remains to be done. Hopefully, the book will inspire more investigators to explore this new area, and encourage more discovery of the interaction of IR and immunity.

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REVIEW ARTICLE
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The convergence of radiation and immunogenic cell death signaling pathways

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Ionizing radiation (IR) triggers programmed cell death in tumor cells through a variety of highly regulated processes. Radiation-induced tumor cell death has been studied extensively *in vitro* and is widely attributed to multiple distinct mechanisms, including apoptosis, necrosis, mitotic catastrophe (MC), autophagy, and senescence, which may occur concurrently. When considering tumor cell death in the context of an organism, an emerging body of evidence suggests there is a reciprocal relationship in which radiation stimulates the immune system, which in turn contributes to tumor cell kill. As a result, traditional measurements of radiation-induced tumor cell death, *in vitro*, fail to represent the extent of clinically observed responses, including reductions in loco-regional failure rates and improvements in metastases free and overall survival. Hence, understanding the immunological responses to the type of radiation-induced cell death is critical. In this review, the mechanisms of radiation-induced tumor cell death are described, with particular focus on immunogenic cell death (ICD). Strategies combining radiotherapy with specific chemotherapies or immunotherapies capable of inducing a repertoire of cancer specific immunogens might potentiate tumor control not only by enhancing cell kill but also through the induction of a successful anti-tumor vaccination that improves patient survival.

Keywords: ionizing radiation, immunogenic cell death, apoptosis, necrosis, autophagy, mitotic catastrophe, senescence

INTRODUCTION

Radiation therapy (RT) is a well-established and effective form of cancer treatment. Since radiotherapy effects within an irradiated field are not tumor specific, the therapeutic ratio depends on the ability to localize ionizing radiation (IR) delivery to the tumor site and optimize dose and fractionation to preferentially kill tumor cells better than exposed normal cells.

IR can directly damage the atomic structures of nucleic acids, proteins, and lipids. In addition, molecular damage is indirectly mediated by byproducts of radiation exposure, consisting of free radicals produced from water radiolysis. Both the direct and indirect effects of IR initiate a series of downstream signaling events that result in either the repair of damaged macromolecules or evolve toward some form of cell death. The detrimental effects of radiation depend on both the dose and efficiency of damage repair of the irradiated target. In tumor cells, the most biologically sensitive and clinically relevant macromolecule influencing cell death is DNA, which is susceptible to single-strand breaks (SSBs) and double-strand breaks (DSBs) (Giusti et al., 1998). It is estimated that 1 Gy can produce 20–40 DSBs per cell, where unrepaired DSBs can result in cellular lethality (Jonathan et al., 1999; Schultz et al., 2000).

After radiation exposure, tumor cells undergo different types of tumor cell death, including: apoptosis, necrosis, mitotic catastrophe (MC), autophagy, and senescence (Gudkov and Komarova, 2003; Eriksson and Stigbrand, 2010). The type of cell death depends on several interrelated factors. These factors include the cell type, radiation dose and quality, oxygen tension, p53 mutation status, DNA repair capacity, redox state, and the cell cycle phase at the time of IR exposure (Stewart et al., 2011). In addition, different types of cell death pathways interact to contribute to the final outcome within the irradiated tumor.

The classical pathways of IR-induced cell death well described *in vitro* fail to adequately explain all *in vivo* experimental and clinical observations. An abscopal (*ab scopus*, away from the target) response is perhaps the most convincing evidence that direct DNA damage is not the only mechanism of tumor control. This is supported clinically when radiation is delivered focally and tumor response is systemic, for example when metastatic tumors outside of the treated field respond to treatment (Nobler, 1969; Ehlers and Fridman, 1973; Ohba et al., 1998; Takaya et al., 2007).

Evidence in experimental models suggests that radiation-induced promotion of anti-tumor immune responses can explain these abscopal effects (Chakravarty et al., 1999; Demaria et al., 2004; Shiraishi et al., 2008; Dewan et al., 2009). However, a gap exists in bridging the current understanding of principles in radiation biology and this effect of IR on immune activation. Immunogenic cell death (ICD) has become a topic of discussion to both explain initiating events and optimize the clinical benefit of the abscopal effect. This review will focus on the modes

of tumor cell death following IR, the methods used to interrogate cell death modalities, and the consequences of cell death on tumor-host interactions. Additional effects of RT on the tumor microenvironment have been reviewed elsewhere (Gudkov and Komarova, 2003; Barcellos-Hoff et al., 2005).

APOPTOSIS

Apoptosis is a highly regulated mechanism of programmed cell death that plays a fundamental role in embryonic development and tissue homeostasis to eliminate unwanted, damaged, or abnormal cells. Cells undergoing apoptosis are characterized by distinct cytoplasmic and nuclear morphologic changes, including membrane blebbing, DNA fragmentation and nuclear condensation. Dysregulation of apoptosis, however, is associated with unchecked cell proliferation and is thought to be essential for the development and progression of cancer (Fuchs and Steller, 2011). Thus, therapies that augment apoptosis have become a powerful tool in treating cancer.

The apoptotic pathway comprises a complex network of proteins that are cell type and spatiotemporally dependent. Genes involved in apoptosis may act in concert or show redundancy (Kuribayashi et al., 2011). Nonetheless, distinct apoptotic pathways have been clearly defined in IR exposed tumor cells.

Depending on dose and cell type, RT may cause apoptosis via the membrane stress pathway (ceramide production and subsequent second messenger signaling), the intrinsic pathway (mitochondrial release of cytochrome and subsequent apoptosome formation), and the extrinsic pathway (death receptor mediated caspase activation) (**Figure 1**) (Cain et al., 1999; Ogura et al., 2009). IR primarily acts through the intrinsic pathway, but it has also been shown to involve certain aspects of the membrane stress and extrinsic pathways (Takasawa et al., 2005).

IR-induced apoptosis through the intrinsic apoptotic pathway is mediated by DNA SSBs and DSBs (Gudkov and Komarova, 2003). This damage elicits subsequent downstream signaling to either block cell cycle progression, allowing for DNA repair, or progression to cell death when DNA damage is overwhelming. ATM and ATR activation mediate the early responses to IR that regulate cell cycle progression and DNA repair (Maltzman and Czyzyk, 1984). In the presence of DSBs, Mre11/Rad50/Nbs1 complexes form at DSB sites and recruit ATM to sites of repair. ATM undergoes autophosphorylation and phosphorylates checkpoint protein kinase 2 (Chk2) (Smith et al., 2010; Rodriguez-Rocha et al., 2011). At SSB sites, Rad1/Rad9/Hus1 and Rad17/RFC complexes form and recruit ATR. ATR undergoes autophosphorylation, and soon after phosphorylates checkpoint protein kinase 1 (Chk1) (Smith et al., 2010; Rodriguez-Rocha et al., 2011). Activated Chk1 and Chk2 block tumor cell cycle progression by regulating DNA repair and cell cycle proteins, including BRCA1, MDM2, and p53 (Cortez et al., 1999; Kim et al., 2002; Lukas et al., 2004; Shi et al., 2004).

The accumulation of p53 is critical to IR-induced apoptosis. Activated ATM phosphorylates nuclear p53 protein on serine 15, thereby preventing its ubiquitination by MDM2 and subsequent proteasomal degradation (Siliciano et al., 1997; Dumaz and Meek, 1999; Tichy et al., 2009). Additionally, ATM/ATR-activated Chk1 and Chk2 kinases phosphorylate the p53 transactivation domain on serine 20, thereby stimulating p53 activity (Dornan et al., 2003). These p53 phosphorylation events result in the nuclear accumulation of transcriptionally active p53, which in turn transactivates the pro-apoptotic genes PUMA, Bax, and Noxa (Oda et al., 2000; Dogu and Diaz, 2009; Kuribayashi et al., 2011).

A fine balance regulates this pathway. In the cytoplasm, p53 is associated with the anti-apoptotic protein Bcl-X_L. When PUMA is translocated into the cytoplasm it disrupts the Bcl-X_L/p53 complex and liberates p53 (Chipuk et al., 2005). Free cytoplasmic p53 disrupts the Bcl2/Bax complex by associating with the anti-apoptotic protein Bcl2 and releasing the pro-apoptotic protein Bax.

Bax induces permeabilization of the outer mitochondrial membrane to trigger cell death through the release of cytochrome c from the mitochondria (Marzo et al., 1998; Dejean et al., 2006; Dogu and Diaz, 2009). In the cytoplasm, cytochrome c, Apaf-1, and ATP form the apoptosome and activate caspase-9, thereby initiating the postmitochondrial-mediated caspase cascade by activating effector caspases 3 and 7 (Cain et al., 1999).

In addition to the intrinsic apoptotic pathway, IR is also involved in the canonical extrinsic apoptotic pathway. The classic apoptotic machinery of the extrinsic pathway involves signaling through death receptors (DRs), which belong to the tumor necrosis factor (TNF) receptor superfamily.

IR activation of p53, results in downstream transactivation of CD95/Fas, KILLER/DR5, and the CD95/Fas ligand (CD178) (Sheard, 2001; Harms et al., 2004). CD95/Fas ligand binding to CD95/Fas induces trimerization and clustering of the intracellular death domain (DD) region of the receptor. The DD recruits the adaptor protein, Fas-associated death domain (FADD) (Sheard, 2001). The death effector domain (DED) of FADD recruits pro-caspase-8, forming the death-inducing signaling complex (DISC). Activation of the initiator caspase-8 leads to activation of effector caspases 3 and 7, which act to disassemble cellular structures. Interestingly, IR associated up-regulation of CD95/Fas on tumor cells improves tumor cell kill by effector CD8+ cytotoxic T lymphocytes (CTLs) that express CD95/Fas ligand and may also play a role in delayed apoptosis associated MC (Luce et al., 2009).

Strategies aimed at augmenting apoptosis constitute a common research area in oncology. However, p53 is mutated in ∼50% of cancers and the apoptotic machinery is defective in most others, influencing responses to IR. In fact, tumors that are susceptible to p53 dependent apoptosis are quite radiosensitive, whereas, tumors that overexpress antiapoptotic proteins (BCL2, Bcl-X_L, and Survivin) or lose expression of proteins involved in the apoptotic machinery are radioresistant (Cuddihy and Bristow, 2004; Rodel et al., 2005).

In cancers with p53 mutations, unchecked cell proliferation occurs in spite of DNA damage by IR. When this happens, the tumor cells accumulate DNA mutations, become aneuploid, and develop micronuclei, leading to MC and subsequent cell death (Lane, 1992). Interestingly, MC is frequently followed by delayed apoptosis in apoptosis-competent cells (Gudkov and Komarova, 2003). Since p53 mutation is frequently seen in tumor cells,

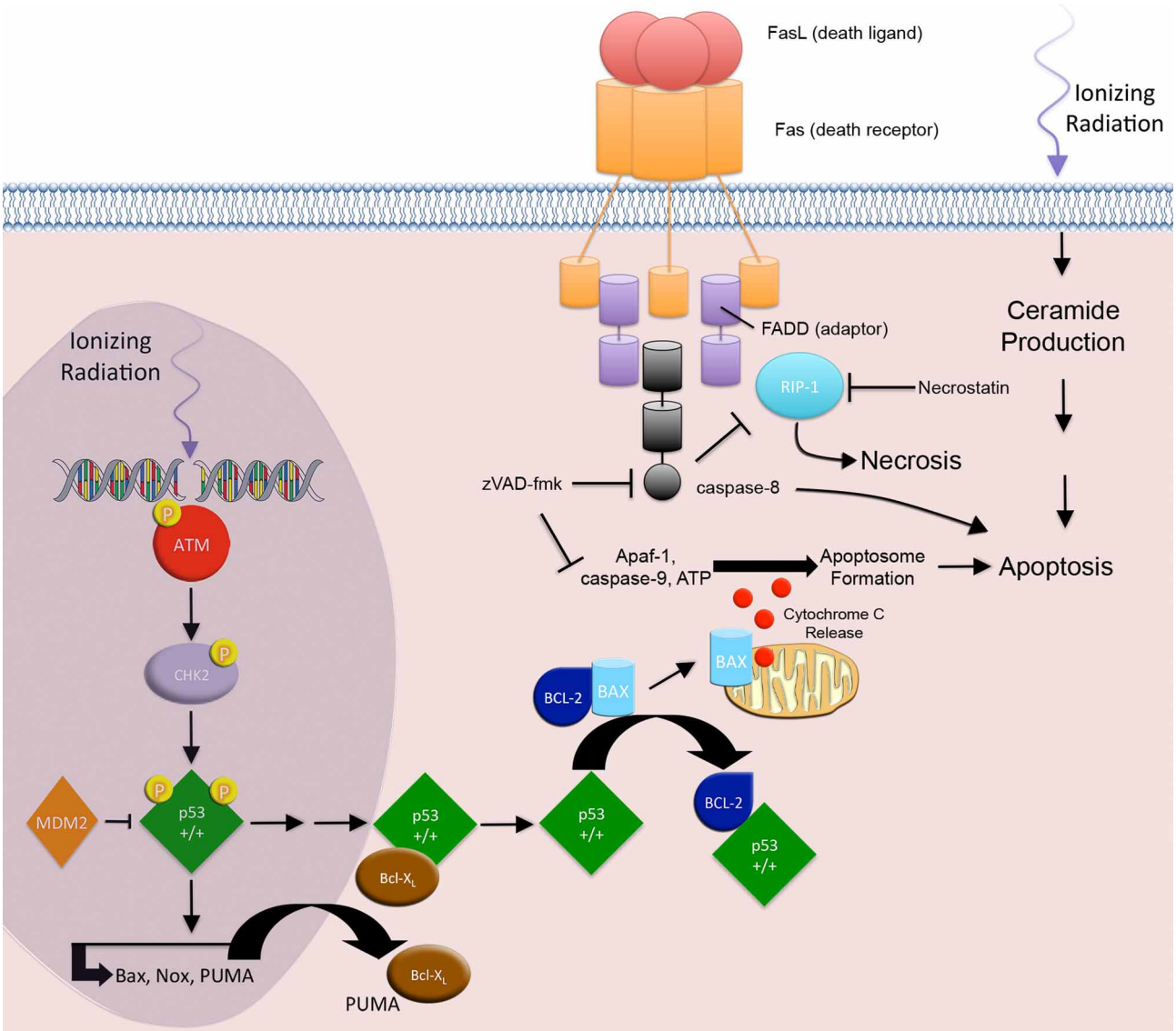

FIGURE 1 | Apoptosis and necrosis. IR-induced apoptosis through the intrinsic apoptotic pathway begins with the development of DNA DSBs. ATM and CHK2 associated p53 phosphorylation events result in the nuclear accumulation of transcriptionally active p53, which in turn transactivates the pro-apoptotic genes PUMA, Bax, and Noxa. Cytoplasmic PUMA disrupts the Bcl-X_L/p53 complex and liberates p53. Free cytoplasmic p53 disrupts the Bcl2/Bax complex by associating with the anti-apoptotic protein Bcl2 and releasing the pro-apoptotic protein Bax. Bax triggers cell death through the release of cytochrome c from the mitochondria, resulting in apoptosome formation and activation effector caspases and apoptosis. The extrinsic pathway involves signaling through death receptors. IR activation of p53, results in downstream transactivation of CD95/Fas and the CD95/Fas ligand. CD95/Fas ligand binding to CD95/Fas induces trimerization and clustering of the intracellular death domain (DD) region of the receptor. The DD recruits the adaptor protein, Fas-associated death domain (FADD). The death effector domain of FADD recruits pro-caspase-8, forming the death-inducing signaling complex. Activation of the initiator caspase-8 leads to activation of effector caspases and apoptosis. Additionally, IR-induced membrane stress leads to ceramide production, second messenger signaling, and apoptosis. RIP1 protein is associated with the FADD and is a key upstream kinase involved in the activation of regulated necrosis. Regulated necrosis is sustained in death receptor stimulated cells that are caspase-8 deficient or inhibited.

MC may be the predominant form of cell death following IR exposure to tumor cells, even though this effect is cell-type dependent.

NECROSIS

Necrosis is a tumor cell death pathway that predominates in response to very large doses of radiation. At lower doses it is often viewed as an accidental, unregulated event. In contrast to apoptosis, necrosis does not display signs of ordered DNA fragmentation. Necrotic morphology is evident in studies using light or electron microscopy. Cells display the morphological features of organelle swelling, mitochondrial dysfunction, and plasma membrane permeabilization with subsequent loss of intracellular contents, including immune stimulating

"danger signals" (Galluzzi and Kroemer, 2008; Hotchkiss et al., 2009).

IR-induced necrosis in tumor cells is not to be confused with the indirect effects of IR occurring as untoward toxic effects in the clinic, like delayed osteoradionecrosis or central nervous system (CNS) radiation necrosis after high dose IR exposure of normal bone or brain tissue, respectively (Chrcanovic et al., 2010; Fink et al., 2012; Siu et al., 2012). These indirect effects of IR are mediated by vascular dysfunction, thereby making normal cells susceptible to undergoing necrosis due to hypoxia and nutrient depletion (Garcia-Barros et al., 2003; Teng and Futran, 2005; Ruegg et al., 2011).

Recent literature suggests that IR can directly induce regulated tumor cell necrosis (Nehs et al., 2011). Programmed necrosis (necroptosis) displays some overlap with apoptosis. It is a cellular mechanism of necrotic cell death induced by apoptotic stimuli, i.e., ligand-DR engagement, under conditions where the apoptotic machinery is either deficient or blocked (**Figure 2**)

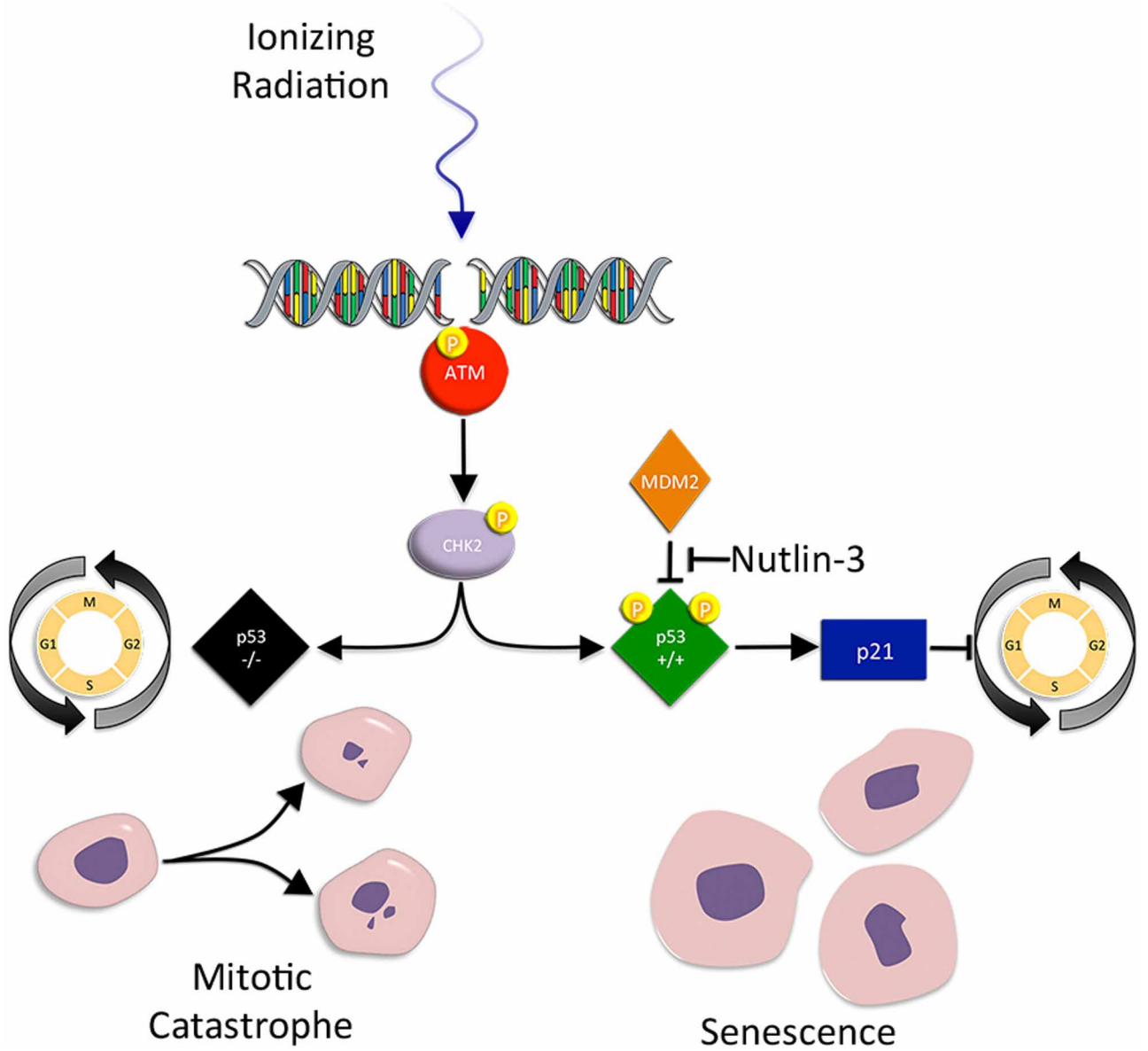

FIGURE 2 | Mitotic catastrophe and senescence. Mitotic catastrophe (MC) occurs after failed mitosis. Cells are characterized by an increased frequency of multiple nuclei and micronuclei. Checkpoint control is greatly reduced in mutant p53 tumor cells, making the cells susceptible to premature mitosis, chromosomal dysegregation, the generation of aneuploid progeny, and MC associated cell death. Senescence on the other hand, is a form of irreversible growth arrest that halts the proliferation of metabolically active cells. IR-induced accelerated senescence in tumor cells is centered around p53. When IR induced DSBs result in p53 activation, activated p53 promotes the activity of p21, an inhibitor of CDK/cyclin complexes and cell cycle progression. Senescent cells display the loss of replicative potential, cell cycle arrest, and enlarged and flattened morphology.

(Degterev et al., 2008). Both apoptosis and necrosis share components of the DR signaling apparatus, specifically at the level of the FADD (Stanger et al., 1995; Vanden Berghe et al., 2004). In both forms of cell death, the deciding factor of whether a cell commits apoptosis or necrosis depends on the FADD associated activities of caspase-8 and receptor interacting protein 1 (RIP1) (Lin et al., 1999; Holler et al., 2000).

RIP1 is a key upstream kinase involved in the activation of necroptosis (Degterev et al., 2008). It interacts with the DD of FADD and is regulated by its ubiquitination and cleavage states (Vanden Berghe et al., 2004; Declercq et al., 2009). When RIP1 is polyubiquitinated it functions as a pro-survival scaffold and promotes downstream activation of mitogen-activated protein kinases (MAPKs) and NFκ-B, which both govern the expression of pro-survival genes (Declercq et al., 2009). However, upon RIP1 polyubiquitin chain removal, RIP1 associated MAPK and NFκ-B activation is abolished, and RIP1 downstream necroptotic signaling is preferentially promoted through the mitochondrial permeability transition complex, as opposed to mitochondrial outer membrane permeabilization transition complex, seen in apoptosis (Declercq et al., 2009). This effect is sustained in DR stimulated cells that are caspase-8 deficient or inhibited (blocked by pan-caspase inhibitors, i.e., zVAD-fmk). Nevertheless, if caspase-8 is intact and active, it can cleave RIP1, thereby turning off necroptosis, and alter the balance of cell death in favor of apoptosis (Lin et al., 1999).

Recent work by Nehs et al. demonstrated that necroptosis contributed to IR-induced cell death of anaplastic thyroid and adrenocortical cancers (Nehs et al., 2011). They showed that IR-induced cell death could be abrogated with necrostatin-1, a small molecular inhibitor of RIP1, in RIP1 expressing tumor cells (Degterev et al., 2008; Nehs et al., 2011). They proposed that necroptosis augmentation, involving an activator of RIP1 kinase or its downstream effectors, might radiosensitize cells. However, further studies are required to clarify the role of necroptosis in IR-induced cell death and the subsequent spillage of immune stimulating "danger signals" (Nehs et al., 2011).

MITOTIC CATASTROPHE

MC occurs after failed mitosis. Cells are characterized by an increased frequency of multiple nuclei and micronuclei. MC acts as an oncosuppresive mechanism for the avoidance of genomic instability (Vitale et al., 2011). Tumor cells that undergo MC often have checkpoint deficiencies that result in incomplete DNA repair, replicative infidelity, and chromosomal dysegregation (Eriksson and Stigbrand, 2010). Thus, loss of checkpoint control in IR exposed tumor cells eventually leads to the generation of aneuploid progeny and MC associated cell death (Ianzini et al., 2006).

Mutant p53 tumor cells are susceptible to IR-induced MC (Ianzini et al., 2006; Eriksson and Stigbrand, 2010). Normally, p53 acts as a post-transcriptional negative regulator of cyclin B1 protein (a cell cycle regulated protein that abrogates the G2/M checkpoint) levels and centrosome amplification. However, in p53 mutant cells, cyclin B1 levels are elevated and centrosome frequency is amplified (Eriksson and Stigbrand, 2010). This contributes to both premature mitosis and chromosomal dysegregation, leading to MC. Not surprisingly, inhibition of other G2 checkpoint proteins (ATM, ATR, Chk1, Chk2, and p21) promotes DNA damage, aneuploidy, and MC (Castedo et al., 2004; Hirose et al., 2005; Vogel et al., 2007).

Interestingly, MC is associated with delayed apoptosis in irradiated tumor cells. An increase in CD95/Fas, TRAIL-R, and TNF-R expression and sensitization to early apoptosis heralds a delayed increase in FasL, TRAIL, and TNFα expression and results in the execution of delayed apoptosis linked to MC (Luce et al., 2009). Not only are the ligands expressed on the surface of tumor cells, but they are also produced in the soluble form, resulting in death of ligand sensitive bystander tumor cells (Luce et al., 2009).

Recently, caspase-2 has been identified as an initiator caspase following DNA damage and is activated during apoptosis following MC (Vitale et al., 2011). However, some researchers believe that caspase-2, at best, is an amplifier of the apoptotic cascade and may not be relevant to apoptosis at all (Krumschnabel et al., 2009). Moreover, some evidence suggests that that MC may promote necrosis (rather than apoptosis) (Vakifahmetoglu et al., 2008). Since the concept of regulated necrosis is gaining consensus, attempts at understanding its relationship to IR-induced MC may prove important.

SENESCENCE

Senescence is a form of irreversible growth arrest that halts the proliferation of metabolically active ageing and damaged cells. Similar to other forms of cell death, it is a process that prevents the transmission of damaged genetic material to daughter cells. Several key features distinguish senescent cells, including the loss of replicative potential, cell cycle arrest, enlarged and flattened morphology, and expression of senescence-associated markers (for example senescence-associated β-galactosidase, SA-β-gal) (Suzuki et al., 2001). IR has been reported to promote accelerated senescence in normal and cancer cells (Mendonca et al., 2011). Indeed, the progeny of irradiated cells accumulate structural chromosomal aberrations in a dose dependent fashion, which precedes senescence (Zahnreich et al., 2010). Similar to other forms of cell death, p53 plays a central role in IR-induced accelerated senescence in tumor cells (Jones et al., 2005; Quick and Gewirtz, 2006; Lehmann et al., 2007).

Senescence is an option exercised in normal epithelial cells during aging, where the process is well described. In brief, p53 activation promotes activity of p21, which acts to block CDK/cyclin complexes and cause G1 cell cycle arrest. This effect is paralleled by p53 suppression of cyclin B1 expression during IR-induced G2 cell cycle arrest. Subsequent to p21 induction, p16 expression is induced, while p21 levels decline. It is recognized that p21 is involved in the initial induction of G1 arrest and p16 is required for its extended maintenance, whereby p16 prevents CDK4 and CDK6 from phosphorylating Rb protein, which binds E2F and prevents transcription of genes required for cell cycle progression. As expected, inactivation of DNA damage checkpoint kinases prevents senescence and restores cell cycle progression (Fagagna et al., 2003).

The telomere also plays a crucial role in IR-induced senescence of cancer cells (Crompton, 1997). Telomeres consist of short, highly repetitive DNA sequences located at the ends of

chromosomes. Telomere length is maintained by telomerase (a complex consisting of a reverse transcriptase and RNA template). In tumor cells, IR produces chromosome end associated abnormalities, including end-to-end fusions (an indicator of telomere dysfunction) (Jones et al., 2005). Telomere dysfunction, rather than changes in telomerase activity or telomere length, induces senescence in a p53 dependent manner (Jones et al., 2005). In contrast, p53 mutant cells are unable to arrest and succumb to other forms of cell death, including apoptosis, necrosis, autophagy, and MC (Jones et al., 2005; Lehmann et al., 2007). Interestingly, nutlin-3, a small molecular p53 activator, was shown to be an effective radiosensitizer, and its effect was entirely attributable to an increased induction of p53 dependent cellular senescence in prostate cancer cells (Lehmann et al., 2007).

Senescent cells have a distinct secretory repertoire called senescence associated secretory phenotype or SASP (Coppe et al., 2010). Recent studies in liver cancer and sarcoma mouse models suggest that reactivation of p53 in p53-deficient tumors *in vivo* produces complete tumor regression predominately due to senescence induction (Ventura et al., 2007; Xue et al., 2007). These studies demonstrate that cellular senescence can limit tumor growth and may contribute to improved long-term survival. In fact, SASP mediated inflammatory cytokines may activate the innate immune system as a mediator of tumor regression (Xue et al., 2007). Again, the relationship between IR-induced senescence and an immune host response to tumor cells has not been established.

AUTOPHAGY

Autophagy is characterized by the segregation of damaged or unwanted ER and cytoplasmic constituents into autophagosomes, destined for lysosomal degradation. It is paradoxical as it is actually a survival mechanism that induces a particular type of death when overstimulated. Autophagy is noted for its role in maintaining metabolic homeostasis in tumor cells undergoing chronic hypoxia and nutrient depletion (Bursch et al., 2008; Munz, 2009; Orvedahl and Levine, 2009). Yet, its effects are twofold (Tsuchihara et al., 2009; Palumbo and Comincini, 2012; Wu et al., 2012). Low to moderate levels of autophagy enhance cell growth and repair by altering the cellular composition and generating building blocks available for the biosynthesis of complex molecules. Next to the proteasome, autophagy is an important catabolic pathway necessary for recycling amino acid, fatty acid, and energy (in the form of ATP) (Munz, 2009; Rodriguez-Rocha et al., 2011). In contrast, hyper-activation of autophagy promotes cell death, when degradation of cytoplasmic contents proceeds to completion (Huang and Klionsky, 2007; Chen and Karantza-Wadsworth, 2009).

While IR has been shown to induce autophagy in tumor cells, the literature is conflicting, regarding whether IR-induced autophagy promotes cell survival or cell death (Paglin et al., 2001; Yao et al., 2003; Ito et al., 2005; Chaachouay et al., 2011; Kim et al., 2011; Wu et al., 2012). Several studies demonstrate that blocking autophagy radiosensitizes, while promoting autophagy radioprotects. The authors argue that IR-induced autophagy is an adaptive response to sustain tumor growth and survival (Chaachouay et al., 2011; Kim et al., 2011). Conversely, other reports show that augmenting IR-induced autophagy increases cell death of radioresistant tumor cells, particularly when an overwhelming amount of autophagy is achieved (Fujiwara et al., 2007; Gewirtz, 2007; Gewirtz et al., 2009; Kuwahara et al., 2011). Undoubtedly, autophagy is a complex response and understanding its role in RT is evolving.

Specifically, the upstream molecular machinery involved in IR-induced autophagy remains unclear (Li et al., 2012). Although IR is known to damage proteins and lipids, IR-induced DNA damage is believed to be the initiating event responsible for autophagy. Recent reports indicate that p53 and PARP-1, a DNA repair enzyme activated by DNA damage, play important roles in autophagy initiation. Both proteins act to inhibit mTOR activity and regulate mTOR's downstream targets, including autophagy (Feng et al., 2005; Huang and Shen, 2009; Rodriguez-Rocha et al., 2011). Interestingly, PARP-1 activation has also been implicated in the necrotic pathway, whereas its caspase-dependent cleavage and inactivation is a downstream event of apoptosis (Huang and Shen, 2009).

Upon initiation of autophagy, the phagophore (a nidus for membrane production) is generated either *de novo* or from pre-existing ER membranes (Bernales et al., 2007; Li et al., 2008). A class III PI3K complex (Beclin, Class III PI3K, and p150) recruits LC3 and ATG proteins (ATG12-ATG-5-ATG16L complexes) to the membrane and facilitates membrane expansion. Complete sequestration by the elongating phagophore results in autophagosome formation. After formation, the autophagosome fuses with the lysosome to become an autophagolysosome, where lysosomal hydrolases digest the sequestered cytoplasmic derived contents (**Figure 3**) (Li et al., 2008, 2012).

Several key proteins regulate autophagy. The canonical class I PI3K/PKB/AKT/mTOR signaling pathway promotes protein synthesis and acts as a negative regulator of autophagy. The binding of insulin/IGF-1 to the insulin receptor has been shown to activate PI3K. Activated PI3K converts Ptdlns(4,5)P2 to yield Ptdlns(3,4,5)P3 at the plasma membrane, leading to PKB/AKT activation. Activated PKB/AKT further activates mTOR (an autophagy inhibitor) through inhibiting the TSC1/TSC2 complex, a repressor of the mTOR activating protein Rheb (Li et al., 2008, 2012; Vellai and Takacs-Vellai, 2010).

Autophagy can be manipulated at several nodes along its pathway. It can be blocked with chloroquine (a lysosomal enzyme inhibitor that reduces autophagosome clearance), Bafilomycin A (a lysosomal proton pump inhibitor that reduces lysosomal acidification and autophagy clearance), 3-MA (a class III PI3K inhibitor), and small interfering RNA to the autophagic machinery (Beclin and the ATG proteins) (Ito et al., 2005; Chen et al., 2011). Conversely, autophagy can be activated with AKT inhibitors and rapamycin, a small molecular inhibitor to mTOR (Fujiwara et al., 2007).

Recent evidence shows that blocking the autophagic machinery with small interfering RNA prevents the release of the immune stimulating "danger signal", ATP, in chemotherapy treated tumor cells undergoing ICD (Michaud et al., 2011). However, the connection between irradiated tumor cells and their release of ATP as part of an immune stimulating process is currently being defined (Ohshima et al., 2010; Zappasodi et al., 2010).

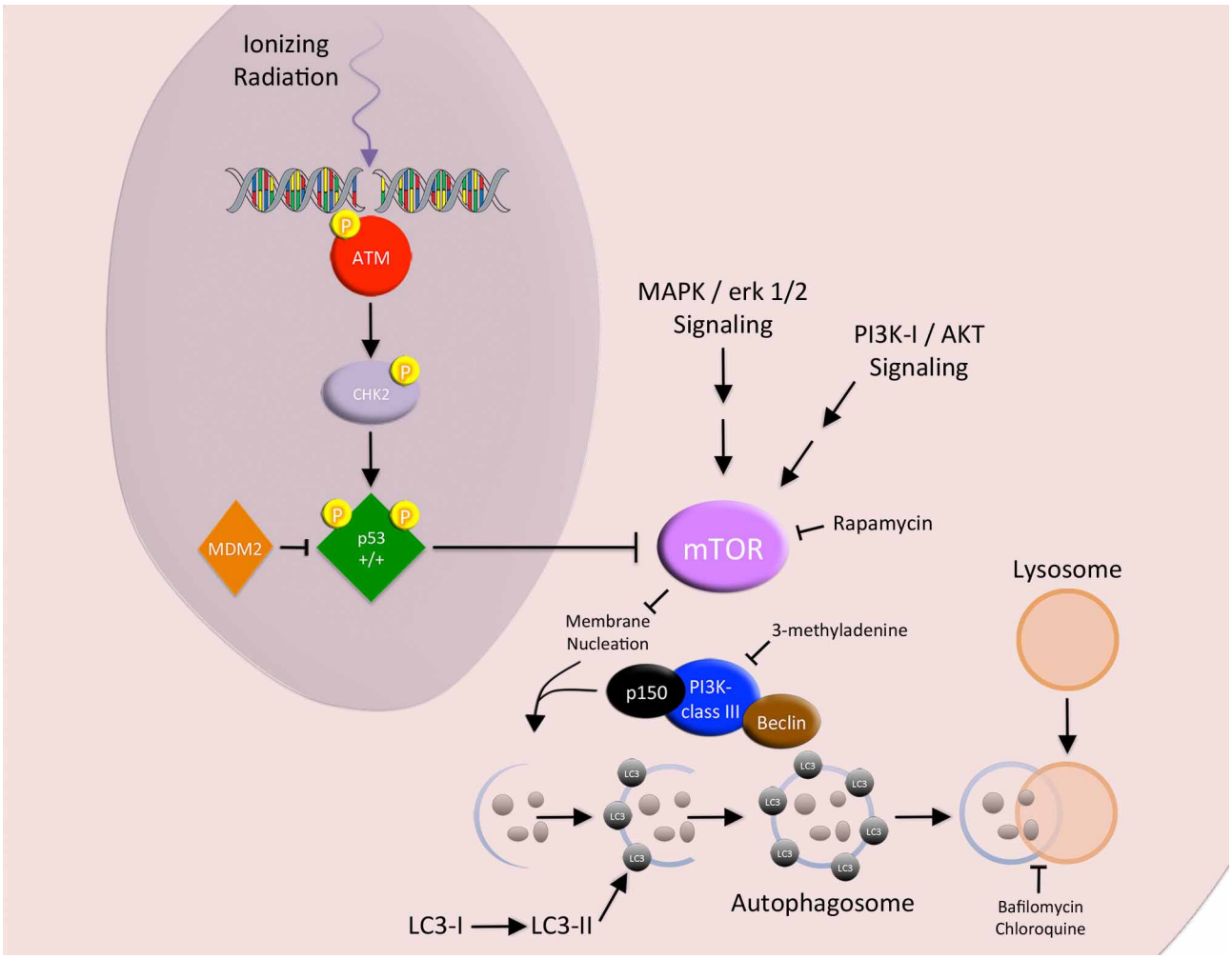

FIGURE 3 | Autophagy. The MAPK/erk 1/2 and PI3K-I/AKT signaling pathways block the promotion of autophagy via the activation of MTOR. IR-induced DNA damage activates p53, which acts as a negative regulator of MTOR. Upon initiation of autophagy, the phagophore is generated. A class III PI3K complex (Beclin, class III PI3K, and p150) recruits LC3 to the membrane and facilitates membrane expansion. Complete sequestration by the elongating phagophore results in autophagosome formation. After formation, the autophagosome fuses with the lysosome to become an autophagolysosome, where lysosomal hydrolases digest the sequestered cytoplasmic derived contents.

IMMUNOGENIC CELL DEATH

Three distinct arms orchestrate ICD in dying tumor cells and are required for immune priming and activation: (1) the cell surface translocation of calreticulin (CRT, an ER residing protein chaperone and potent DC "eat me" signal), and the extracellular release of (2) HMGB1 (a DNA binding protein and TLR-4 mediated DC activator) and (3) ATP (an activator of the DC P2X7 purinergic receptor that triggers DC inflammasome activation, secretion of IL-1β, and subsequent priming of IFNγ producing $CD8^+$ T cells) (**Figure 4**) (Ma et al., 2010). Whereby, the net effects of all three arms act to promote DC phagocytosis of tumor cells, processing of tumor-derived antigens, and DC-associated cross-priming of CD8 + CTLs. However, to date a direct causal link between radiation-induced ICD and an abscopal effect involving the immune system has not been established (Demaria et al., 2004; Dewan et al., 2009). Thus, the challenge remains in understanding the role of radiation-induced ICD and whether or not manipulation of this subroutine of cell death has any significant clinical implications.

CRT cell surface exposure, as described by Kroemer and Zitvogel, is a DC "eat me" signal that involves the coordinated activation of three specific modules: ER stress, apoptosis, and CRT/ERp57 translocation (Panaretakis et al., 2009). The ER stress module requires eIF2 phosphorylation (a marker for ER stress and translation inhibition). The apoptotic module requires caspase-8 activation, Bap31 cleavage, and Bax/Bak activation. Lastly, the translocation module requires anterograde ER-Golgi trafficking and extracellular exposure of CRT/ERp57. Recent studies show that cell surface CRT translocation occurs in IR exposed tumor cells (Obeid et al., 2007; Perez et al., 2009).

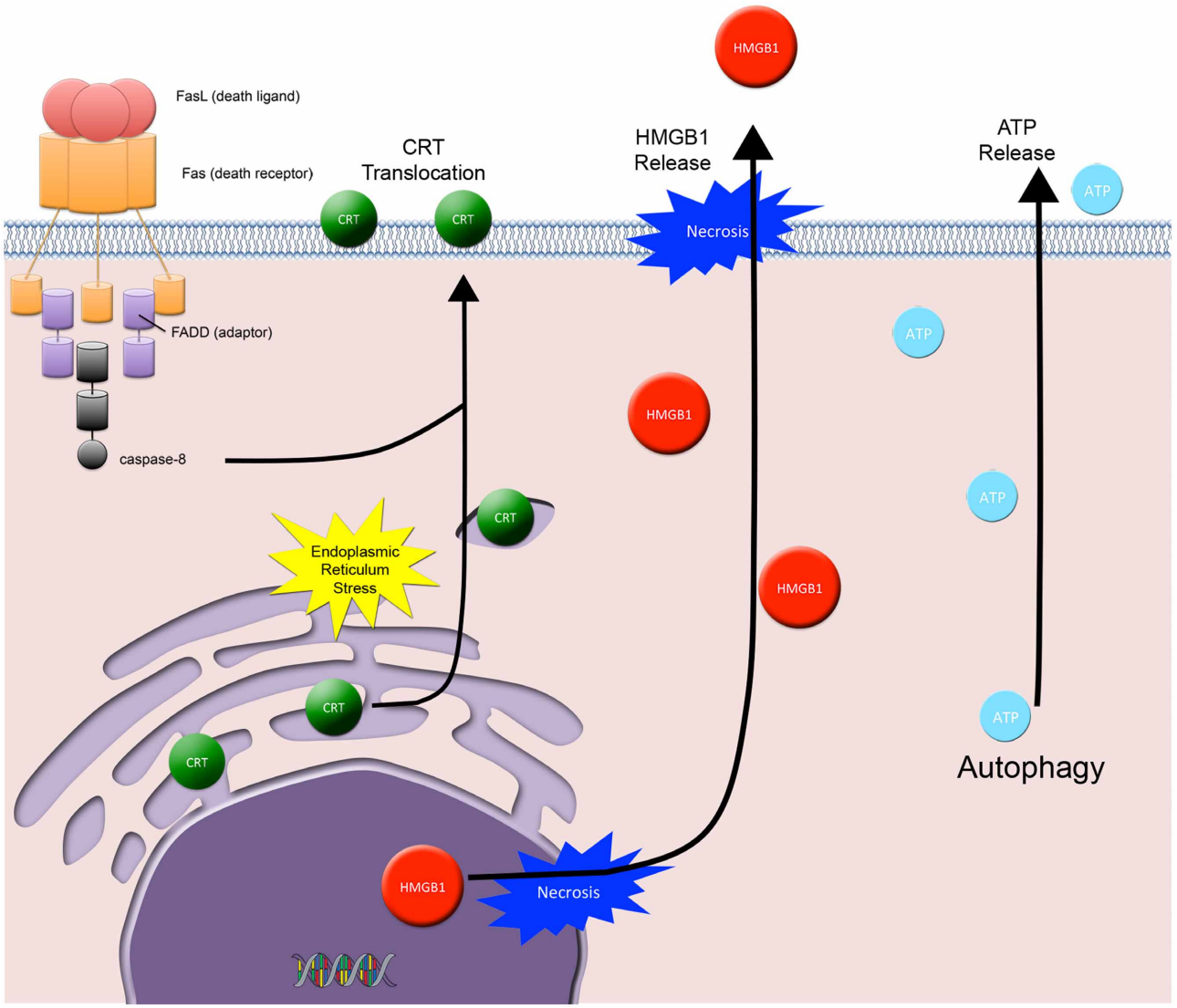

FIGURE 4 | Immunogenic cell death. Three distinct arms orchestrate ICD in dying tumor cells and are required for immune priming and activation: **(1)** the cell surface translocation of calreticulin (CRT) and the extracellular release of **(2)** HMGB1 and **(3)** ATP. CRT cell surface exposure acts as a dendritic cell "eat me signal" and involves the coordinated activation of 3 specific modules: ER stress, apoptosis, and ER-Golgi trafficking and extracellular exposure of CRT. HMGB1 is passively released from dying tumor cells and acts as a cytokine and danger associated molecular pattern protein that mediates responses to infection, injury, and inflammation. ATP is released from dying tumor cells and involves the autophagic machinery. Extracellular ATP activates the dendritic cell (DC) P2X7 receptor, which is involved in the upregulation and activation of the DC inflammasome.

In contrast to CRT, cell surface CD47 (a DC "don't eat me" signal) is widely expressed in solid and hematogenous tumor cells (Willingham et al., 2012). CD47 was discovered on newly formed circulating red blood cells (RBCs) and shown to prevent RBC clearance by the splenic reticuloendothelial system (Khandelwal et al., 2007). CD47 blockade of tumor cells and normal tissues is, respectively, associated with immune mediated tumor rejection and radioprotection (Maxhimer et al., 2009; Willingham et al., 2012). However, the role of CD47 in response to IR in tumor cells is yet to be determined.

HMGB1 is an evolutionary conserved nuclear protein that is expressed by almost all cells (cells with an intact nucleus) and is important for the regulation of transcription (Lotze and Tracey, 2005). When released from dying cells, it acts as a cytokine and danger associated molecular pattern (DAMP) protein that mediates responses to infection, injury, and inflammation; thus HMGB1 has been called by Lotze and Tracey the immune system's "nuclear weapon" (Lotze and Tracey, 2005). HMGB1 released from tumor cells binds to TLR4 on DCs, thus contributing to DC activation (Apetoh et al., 2007).

HMGB1 is released into the extracellular space from cells in one of two ways: either actively or passively. Active release involves HMGB1 hyperacetylation in the nucleus followed by vesicular secretion into the immunological synapse or into the extracellular

space. HMGB1 is actively secreted by activated macrophages, mature DCs, and activated NK cells (Lotze and Tracey, 2005). In contrast, tumor cells passively release HMGB1 when they undergo either sustained autophagy, late apoptosis, or necrosis (Lotze and Tracey, 2005). Passively released HMGB1 signals through RAGE, TLR2, and TLR4, where it promotes the transcription of pro-inflammatory genes in immune cells.

In addition to promoting an inflammatory response in immune cells, extracellular HMGB1 can trigger autophagy or apoptosis in bystander cancer cells, depending on its redox state. Reduced HMGB1 binds to RAGE, induces Beclin dependent autophagy and promotes resistance to IR and chemotherapy in pancreatic and colon cancer cells (Tang et al., 2010). In contrast, oxidized HMGB1 increases the cytotoxicity of these agents and induces apoptosis via the mitochondrial pathway (Tang et al., 2010). Currently, the redox state of HMGB1 released from IR exposed tumor cells has not been determined.

ATP release is yet another important ICD component. It involves the autophagic machinery, where knock down of ATG7 and ATG5 blocks ATP release (Michaud et al., 2011). Recently, IR has been shown in several models to cause the release of ATP from dying tumor cells and activation of immune cells via the P2X7 purinergic receptor pathway (Ohshima et al., 2010; Zappasodi et al., 2010). This pathway involves the ATP-P2X7 receptor stimulation followed by upregulation and activation of the DC inflammasome (a large multiprotein complex composed of NLRP3, Cardinal, the adaptor ASC, and pro-caspase-1). DC inflammasome activation results in the synthesis and secretion of IL-1β, where secreted IL-1β initiates further pro-inflammatory events (Petrovski et al., 2011).

ABSCOPAL RADIATION RESPONSES

RT is employed as a local treatment modality with the intent to kill tumor cells and reduce local recurrence. Considerable evidence demonstrates that RT effects extend beyond the treatment field (Formenti and Demaria, 2009). As mentioned earlier, the abscopal effect is a term used to describe tumor regression in lesions outside of the treatment field when one tumor site is irradiated. Known for almost 60 years as a rare unexplained phenomenon in patients receiving local RT (Mole, 1953), it could be the result of RT-induced ICD that generates an *in situ* vaccine (Ma et al., 2010). In support of this notion, interventions that promote the functionality of DCs or improve T cell activation induce the abscopal effect in an unfavorable tumor microenvironment, where the effect is otherwise unseen (Chakravarty et al., 1999; Demaria et al., 2004, 2005). This strongly suggests that, while RT may be efficient at releasing tumor antigens, the immunosuppressive tumor microenvironment may hamper the development of therapeutically effective anti-tumor immune responses.

Additional evidence supports the hypothesis that local RT induces immune-mediated systemic anti-tumor effects. For instance, the well documented association between optimal local control and survival in several breast cancer trials, at a time when occult systemic disease is often already present implies the induction of a systemic anti-tumor mechanism by local RT. In fact, two meta-analyses of prospective randomized trials on the effects of local radiotherapy for breast cancer determined that a 20% absolute reduction in 5-year local recurrence led to a 5% absolute reduction in 15-year breast cancer mortality (a four-to-one ratio of absolute effects) (Clarke et al., 2005; Darby et al., 2011).

Since some chemotherapy drugs can also induce ICD (e.g., Mitoxantrone, Adriamycin, and Oxaliplatin) (Garg et al., 2010; Zitvogel et al., 2010; Kepp et al., 2011; Kroemer et al., 2011), it is intriguing to consider if the superiority of concomitant versus sequential chemo-radiation is due to a synergistic induction of ICD (Glynne-Jones and Hoskin, 2007; Formenti and Demaria, 2008). Chemotherapy-induced ICD was found to be a non-mutually exclusive subroutine of tumor cell death that includes components of the apoptotic, autophagic, and necrotic machineries (Garg et al., 2010; Zitvogel et al., 2010; Kepp et al., 2011; Kroemer et al., 2011). Prior to dying, tumor cells exposed to ICD-inducing drugs were shown to release pro-inflammatory cytokines and alter their display of cell surface antigens, thereby becoming less tolerogenic and more immunogenic (Green et al., 2009). These dying tumor cells were able to prime the immune system of mice and prevent tumor reestablishment when the immunized mice were subsequently re-challenged (Tesniere et al., 2010; Michaud et al., 2011).

Interestingly, patients with breast cancer who are treated with chemotherapy and radiotherapy and carry a TLR4 loss-of-function allele relapse faster than those carrying the normal TLR4 allele (Apetoh et al., 2007). Thus, HMGB1-TLR4 DC signaling is a clinically relevant immunoadjuvant pathway triggered by tumor cell death (Apetoh et al., 2007).

FUTURE DIRECTIONS

Whether RT specifically and efficiently elicits ICD remains a critical research question. Most of the work of ICD has been described with the use of chemotherapeutic compounds. However, emerging clinical evidence has renewed interest in studying the mechanisms of IR-induced ICD.

Several reports have shown that IR and chemotherapeutic agents induce "danger signals" that may contribute to an immune-mediated response at the tumor site, thereby reverting the immunosuppressive microenvironment of established tumors (Ma et al., 2010). IR and chemotherapeutic agents act to promote an anti-tumor immune response in the tumor microenvironment via ICD pathways, triggering the cross-presentation of tumor-derived antigens by DCs (Ma et al., 2010). However, in the clinical setting each treatment alone may not quantitatively and/or qualitatively achieve tumor cell death in the manner that triggers immune-mediated tumor rejection. Thus, further studies are needed to determine the optimal IR and chemotherapeutic treatments that reposition each other to optimally elicit ICD.

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Clinical opportunities in combining immunotherapy with radiation therapy

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Preclinical work in murine models suggests that local radiotherapy plus intratumoral syngeneic dendritic cells (DC) injection can mediate immunologic tumor eradication. Radiotherapy affects the immune response to cancer, besides the direct impact on the tumor cells, and other ways to coordinate immune modulation with radiotherapy have been explored. We review here the potential for immune-mediated anticancer activity of radiation on tumors. This can be mediated by differential antigen acquisition and presentation by DC, through changes of lymphocytes' activation, and changes of tumor susceptibility to immune clearance. Recent work has implemented the combination of external beam radiation therapy (EBRT) with intratumoral injection of DC. This included a pilot study of coordinated intraprostatic, autologous DC injection together with radiation therapy with five HLA-A2(+) subjects with high-risk, localized prostate cancer; the protocol used androgen suppression, EBRT (25 fractions, 45 Gy), DC injections after fractions 5, 15, and 25, and then interstitial radioactive implant. Another was a phase II trial using neo-adjuvant apoptosis-inducing EBRT plus intra-tumoral DC in soft tissue sarcoma, to test if this would increase immune activity toward soft tissue sarcoma associated antigens. In the future, radiation therapy approaches designed to optimize immune stimulation at the level of DC, lymphocytes, tumor and stroma effects could be evaluated specifically in clinical trials.

Keywords: dendritic cells, immunotherapy, radiation effects, stereotactic radiosurgery, immune modulation

INTRODUCTION

RADIATION EFFECTS

A conventional view of radiation is an immune attenuator. In this perspective, damage, and destruction are the effects on living tissues – whether they are tumor, normal stroma, and parenchyma, or leukocytes. In the medical application of therapeutic radiation, this is a measured induction of apoptosis and other cell death within a carefully defined volume. The impact of radiation on leukocytes can be viewed in similarly detrimental terms, whether attenuating lymphocyte numbers as tolerable side effect (Johnke et al., 2005; Lissoni et al., 2005) a therapeutic effect, such as part of an allogeneic transplant protocol (Wei et al., 2004; Gupta et al., 2011), or precipitating a secondary malignancy (Brill et al., 1962). The measurement of accumulated radiation injuries, such as micronuclei and DNA breakage in circulating lymphocytes, has been proposed as a direct assay of individuals' relative radiosensitivity (Minicucci et al., 2005; Tang et al., 2008; Ishihara et al., 2012); that sensitivity can be relevant to either toxicity or to treatment efficacy.

We focus here on the effect of radiation on the bilateral relationship of tumor with the immune system, not just on the effects of radiation on the tumor or on the leukocytes, separately. Considered in isolation, radiation to any particular cell could be anticipated to have a detrimental impact. However, there is an opportunity in the interplay of tumor cell death, induced antigen expression on tumor cells, and inflammatory signals from the irradiated volume which affect lymphocyte and dendritic cell (DC) activation. **Figure 1** contrasts the perspectives of isolated versus system effects of irradiation. Immunotherapeutic impacts can be coordinated with therapeutic tumor irradiation. In this way, the whole therapeutic effect can exceed the sum of its parts.

PROCESSES OF CELLULAR IMMUNITY

Physiologic process of antigen presentation and lymphocyte activation are complex processes, and subject to modulation because of the tumor microenvironment (Fricke and Gabrilovich, 2006). Immature myeloid cells acquire antigen, whether by vaccination or through phagocytosis of material in the tumor microenvironment. These cells then mature, with acquisition of cell surface proteins such as MHC class I and II on which peptides derived from the antigen source can be presented, to interact with particular antigen-specific idiotypic receptors on T lymphocytes (discussed, for example, by Liao et al., 2004). Other maturational markers such as CD80, CD86 facilitate costimulation interactions, particularly the process of activation versus tolerogenic influence on those lymphocytes (these illustrated in Topalian et al., 2012, where the focus is on the PD-1/PDL-1 interaction, for example). The interaction of lymphocytes with the antigen-presenting cells, occurs in lymph nodes to which the DC migrate as part of the maturation process, and the subsequent potential anticancer effect of lymphocytes then is a consequence of lymphocytes' expansion within the lymph node, circulation, and penetration into the tumor mass. Other lymphocyte pathways, such as natural killer (NK) cells, may be influenced by T cell activation and the

tumor microenvironment, but do not require specific education and costimulation by DCs. Other antigen-presenting cells, such as macrophages, and inflammatory cells such as neutrophils may influence the tumor microenvironment (Fricke and Gabrilovich, 2006) in a way that indirectly, but overwhelmingly alters the polarization of macrophages, DC, or the activation state effector lymphocytes. Overall, the potential effect of radiation on the preponderance or phenotype of many cell types, some of which are discussed below, could influence availability of tumor antigens, the acquisition of the antigens by immature antigen-presenting cells, the migration of those cells to lymph nodes, the eventual polarization into tolerogenic or immunogenic phenotype, the efficiency of interaction with lymphocytes, the stimuli leading to intratumoral migration of lymphocytes, the extent of activation of the lymphocytes that are within the tumor, and the susceptibility of (still living) tumor cells to immune lysis. As for many anticancer pharmaceutical interventions, we are only beginning to understand the influences that irradiation can effect on this system.

RADIATION EFFECTS IN ISOLATION

RADIATION EFFECTS: THE TUMOR

The fundamental mechanism of tumor regression following radiotherapy is by induction of DNA damage in the neoplastic cells. This accumulation of DNA breaks and consequent insufficient repair is the trigger for pathways including Bcl2 family apoptotic and anti-apoptotic proteins, p53-dependent, and independent pathways, or TRAIL [tumor necrosis factor (TNF)-related apoptosis-inducing ligand) dependent mechanisms (Maduro et al., 2008; Roos and Kaina, 2012). However, this basic view is still not a complete picture of microenvironmental changes within tumor-associated endothelial cells, inflammatory infiltrates, or of systemic responses to the tumor. Areas of higher dose exposure, for example adjacent to brachytherapy seeds, or at hot-spots inside the bulk of the tumor may have markedly different pathways to cell death, emphasizing necrotic mechanisms not apoptotic ones (Nagorsen et al., 2003; Overwijk et al., 2003; Finkelstein et al., 2004; Klebanoff et al., 2004; Kakinuma et al., 2007). Additionally, the time course of changes of antigen expression by the irradiated cells may be relevant, with different patterns that are dependent on radiotherapy techniques' dose-rate and energy level (Finkelstein et al., 2011).

Besides the phenomenon of cells dying within an irradiated tumor, several processes have specific relevance to immunotherapy. Some relate to inflammation and clearance of antigens within the irradiated volume. Of the most interest are the processes that influence acquisition of a more activated general immune phenotype or of a more activated tumor-specific immune phenotype. The most dramatic clinical outcome is when a distant tumor mass regresses, the abscopal effect. Clinical examples described as case reports (Kingsley, 1975; Postow et al., 2012; Stamell et al., 2012) and preclinical examples are discussed in more detail below. Less apparent outcomes, still with major clinical impact, may occur as well. These include accelerating or completing definitive clearance of the tumor which was being irradiated. Another important impact can be clearance of other metastatic disease that was not clinically apparent because it was microscopic; this could lead to prevention of systemic recurrence as a consequence of radiation-triggered immune activation in the primary tumor.

Moravan et al. (2011) describe persistent inflammatory changes consisting of neutrophil and T cell infiltrates, within brains of C57BL/6 mice, as a specific and lasting effect of irradiation, in the absence of tumor. The protein CXCL16 (CXC motif ligand 16) is released from irradiated tumor. This binds the CXCR6 receptor, found on activated effector T cells (Matsumura and Demaria, 2010). A murine model, including use of a CXCR6 knockout control mouse, demonstrated this mechanism of T cell infiltration to the tumor (Matsumura et al., 2008). Another group, surveying 63 cytokines, found that CXCL16 levels went down after 30 Gy irradiation of skin (not tumor) in a murine model (Xiao et al., 2013). The specific relevance in clinical use remains to be elucidated.

High mobility group box 1 (HMGB1) is a protein which is released from some dying cells, including tumor cells killed by anthracyclines (Fucikova et al., 2011), and in a hyperthermia and radiation combination model (Schildkopf et al., 2010), and with radiation and chemotherapy combination treatments for colorectal cancer cell lines, particularly with the combination (Frey et al., 2012). The HMGB1 effect on DC can include maturation and a chronic inflammatory state (Fucikova et al., 2011). It is an important question whether a clinically relevant (adverse) changes of DC phenotype (Popovic et al., 2006) or of downstream T cell effector activity (Liu et al., 2011) occur from tumor therapy-derived HMGB1. It is not clear if irradiation protocols leading to higher or lower systemic HMGB1 levels would be better for induction of a general anticancer immunophenotype.

In a clinical report on patients receiving primary, curative-intent fractionated external beam radiation therapy (EBRT) for prostate cancer, Hurwitz et al. (2010) describe observation of consistent systemic changes. These were increases of (systemic) levels of tumor-derived protein Hsp72 (heat shock protein), and of inflammatory cytokines IL-6 and TNF-α. Circulating $CD8^+$ T cells and NK cells showed increases of 2.1- and 3.2-fold, respectively. While the changes of these particular proteins or leukocytes do not directly prove a functional augmentation of the systemic antitumor response, they are illustrative of impacts on the host's overall immunophenotype because of events within the tumor.

RADIATION EFFECTS: THE LYMPHOCYTES

There is not significant systemic lymphopenia from prostate cancer EBRT, our group has observed (Finkelstein et al., 2012d). Others suggest that hypofractionated radiation therapy can mediate a decrease in $CD4^+$ and $CD8^+$ lymphocyte number, but not of NK and of B lymphocytes. This effect was counterbalanced in those patients receiving combined androgen blockade, with goserelin and flutamide, suggesting a converse effect of testosterone suppression (Johnke et al., 2005). In a report describing serial flow cytometry analyses lymphocytes of cervical cancer patients (stage IIB through IVA) being treated with larger field external beam irradiation and concomitant intracavitary brachytherapy again it was observed that total lymphocyte count went down. In the patients without progressive disease, the $CD8^+$ T cell and NK cell percentages increased. The authors commented that these increases are consistent with a role of $CD8^+$ T cell and

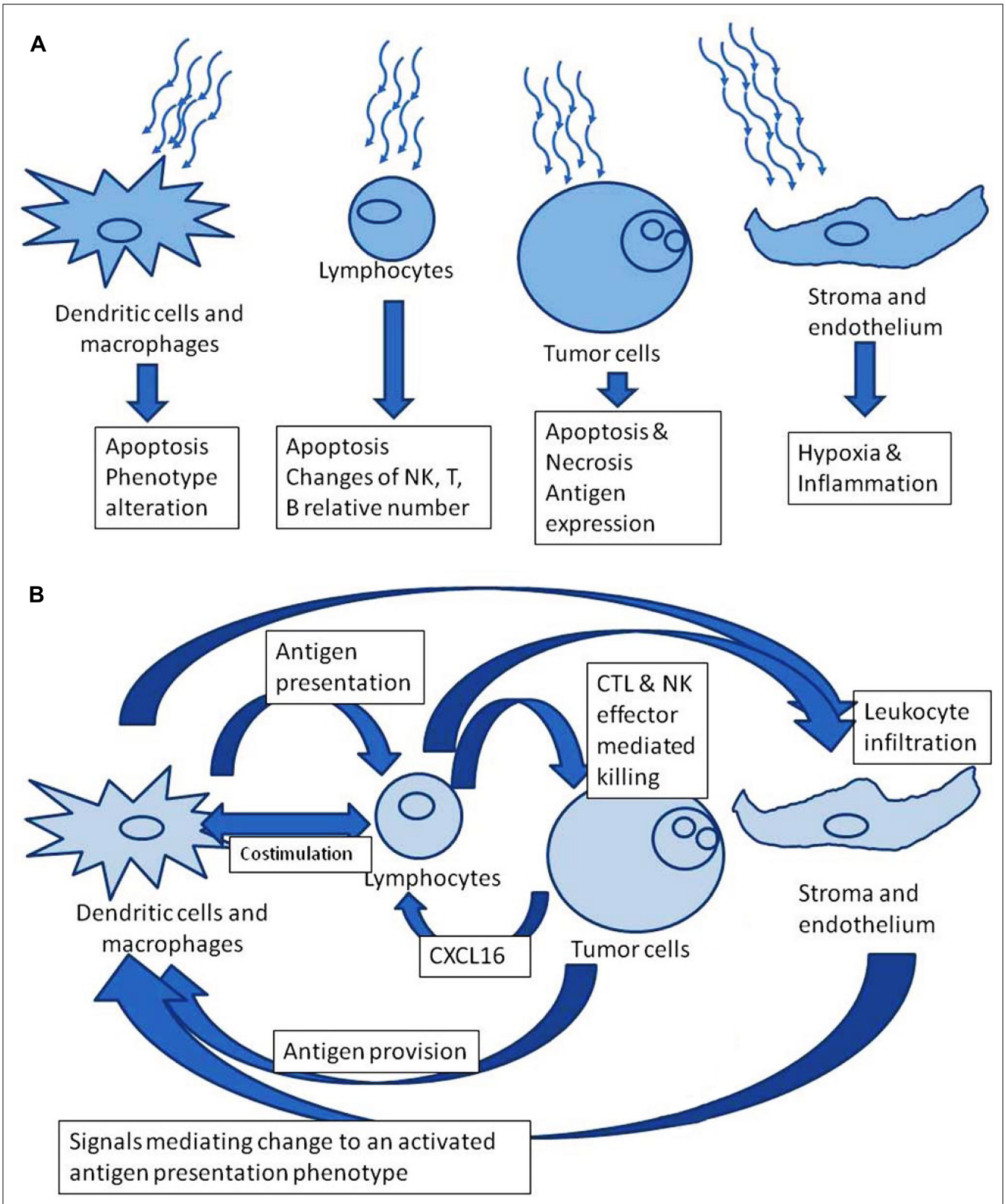

FIGURE 1 | (A) Radiation effects in isolation. **(B)** Downstream theoretically favorable immune modulation after irradiation.

NK cell in definitive tumor clearance (Lissoni et al., 2005). This is comparable with the CXCL16 mechanism discussed above (Matsumura et al., 2008).

Brachytherapy is a radiation therapy modality with markedly different kinetics of radiation exposure. In brachytherapy seed placement (Iodine 125 or Palladium 103) or radioembolization with Yttrium 90 microspheres (Carr and Metes, 2012), there is a longer exposure to radiation than with conventional external beam treatment, with potential for most of the circulating blood volume be transiently in very close proximity of the radioactive source. Carr and Metes (2012) evaluated the impact on lymphocytes of Yttrium 90 embolization of hepatocellular cancer, with finding that there was an early decrease on T cell number (both $CD4^+$ and $CD8^+$) and B cell number (assayed by CD19), but not on NK cells or neutrophils. Over time, the deficits persisted significantly for some patients; an impaired recovery was associated with worse prognosis. This could reflect a disease impact on the lymphocyte repopulation, more so than an ongoing radio-isotope mediated suppression (Lissoni et al., 2005).

RADIATION EFFECTS: THE DENDRITIC CELLS

The tumor microenvironment has potential to modulate the phenotype of DC to favor the pathologic tolerance of the tumor (Fricke and Gabrilovich, 2006). The focus of the therapeutic rationale for placing DC into the tumor microenvironment (discussed below) is that radiation will alter that effect, but the impact of radiation onto DC should be considered separately. Isolating the issue, higher doses of radiation (25–30 Gy) than would be used in a standard fractionated radiotherapy plan (generally less than about 2 Gy), were studied in an experimental setting assaying *ex vivo* priming of DC by Cao et al. (2004), in a report with a focus on multiple sclerosis patients. They report that the irradiated DC would still stimulate T cell proliferation in the MLR (mixed lymphocyte reaction) assay but at a lower level, and with higher T cell production of IL-2 and IL-4. Phenotypic changes related to maturational markers were observed, with lower levels of CD80 (B7.1), CD86 (B7.2), and HLA-DR on the DC.

On the other hand, Jahns et al. (2011) studied *ex vivo* preparations of leukocytes, focusing on quantitative functional impact on DC versus the impact onto lymphocytes. They found that DC are less sensitive to apoptosis than lymphocytes, and maintained the same functional level (in terms of cytokine profiles, surface markers, and maturation) after a radiation dose that impaired T cell function. In particular, there was lower expression of DC maturational markers (CD80, CD86, and HLA-DR) and the T cells had less activation. Bogdándi et al. (2010) tested splenocytes of mice (C57BL/6) exposed to increasing doses of radiation, up to 2 Gy, with the most sensitivity for B cells (at 2 Gy), but more resistance in the NK cells, DC and regulatory T cells, thus observing a similar pattern of relative sensitivity to irradiation. The specific impact of acquisition or suppression of these DC maturational markers on clinical outcomes must be studied empirically to address whether the net change was favorable.

Liao et al. (2004) isolated the issue of irradiation of DC, again in a model system with C57BL/6 mice, with B16 melanoma. The loading of the DC was by transfection with adenovirus engineered to express the MART-1 antigen, termed AdVMART1; the B16 melanoma expresses the MART-1 antigen, as do the majority of human melanoma specimens. Murine DC were obtained from bone marrow (femur and tibia), and cultured and transfected *in vitro*, after which they express the (full length, human) hMART-1 protein, and also the immunodominant MART-1_{27-35} peptide. The DC irradiation protocol consisted of 10 Gy, in a single fraction in just over 2 min. To assay the effect of irradiation of the DC on the class 1 antigen-presentation process, DC culture was irradiated (or not treated), then (immediately) transfected with AdvMART1, then injected into (non-tumor bearing) mice; this was repeated at a 7 days' interval. Then after an interval of 10–14 days, the T lymphocytes from the spleen were assayed with the finding that acquisition of elevated level of T lymphocytes with specificity for the test antigen (MART-1_{27-35} peptide) was eliminated by the radiation protocol. Similarly, subsequent challenge to test mice with B16 melanoma injection showed protection only for un-irradiated DC treatment, but not for mice not injected with DC, and not for mice injected with DC that had been treated on the irradiation protocol. Further, they investigated the potential maturation-related mechanisms for irradiation of DC affecting the capacity or tendency to present the class I epitopes of MART1; they observed that maturational markers of DC (particularly CD80, CD86, and MHC class I and II) were not changed. In testing the response to CD40L and interferon gamma (IFN-γ) stimulation (maturational signals), although there was (pretreatment) a decrease of some maturational markers (CD80, CD83, MHC class II), after treatment, the difference was not observed. Looking at those results, the effect of DC irradiation appears to be neutral or suppressive (Liao et al., 2004).

In a next set of investigations, to test for antigen-presentation effects isolated from antigen processing, a modified DC/tumor system was used. The HLA-A2.1/K^b transgenic mice bear human HLA-A2; the modified tumor B-16A2/K^b does as well. When DC from these mice were prepared and treated as above, but then instead of being transduced with the adenovirus, the DC were instead pulsed with the immunodominant MART-1_{27-35} peptide. These DC (or control DC that were pulsed but had not been irradiated) were use to vaccinate mice; 10 days after the last vaccination the mice were challenged with B-16A2/K^b tumor, it was found that mice in the group treated with the DC that had been irradiated had better survival, and a higher induced immunity as measured by IFN-γ production in an ELISPOT assay with the MART-1_{27-35} peptide (Liao et al., 2004). Thus, the irradiation of DC with 10 Gy in this model system, where antigen processing and maturation were not much changed or a little worse, showed a *better* anticancer effect, attributed to improved presentation.

THE TUMOR MICROENVIRONMENT

LOCAL IMMUNE SUPPRESSION

The immune system in the cancer-bearing host cancer has defects that allow the tumor cells to evade clearance. The way that immune privilege is maintained is heterogeneous across different disease stages and patients. Some characterizations can be in terms of DC phenotype; an excess of myeloid-derived suppressor cells (MDSC) that are not mature DC, but rather suppress DC function to impair anticancer immunity (Almand et al., 2000). Other

characterization can focus on the tumor microenvironment. That kind of suppression can be observed to operate through elaboration of particular proteins which have receptors on DC and MDSC, in some models and some clinical examples. Those microenvironment derived molecules include vascular endothelial growth factor (VEGF), tumor growth factor β (TGF-β), reactive oxygen species, the enzyme indoleamine-2,3-deoxygenase, granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-8, interleukin-10 (reviewed by Fricke and Gabrilovich, 2006). Specific inhibition of these pathways can have a favorable impact on DC phenotype and the capacity for meaningful immunologically mediated anticancer response, for example a murine tumor model was induced to be immunologically rejected by use of VEGF depleting antibody (Gabrilovich et al., 1999); a clinical trial using sequential bevacizumab (humanized anti-VEGF antibody, Roche USA, Indianapolis, IN) and then low dose subcutaneous IL-2 did not demonstrate a significant clinical impact nor impact on DC phenotype for VEGF depletion (Finkelstein et al., 2010). However, in a clinical trial utilizing another VEGF chelation strategy, with a similar testing scheme, found no functional improvement as a consequence of ziv-aflibercept treatment (formerly "aflibercept," also called VEGF-trap; Sanofi-Aventis, Bridgewater, NJ). Changes that were observable as flow cytometry defined phenotypic changes of DC from patients following treatment, however, were favorable (Fricke et al., 2007).

RATIONAL PLACEMENT OF DC VERSUS RADIATION THERAPY TIMING

Almost any radiation therapy protocol can be analyzed with respect to its theoretical immune impact, either on an anatomic or temporal perspective. From an anatomic perspective, regions of the treatment target volume with the highest doses could be anticipated to have higher and faster peaks of tumor cell death, and availability of antigenic material. Regions of lower dose could have radiation induced changes of antigen expression on the tumor cells. Leukocytes and stroma also would respond to irradiation, with variable amounts of induced regional inflammatory cytokines, or penetration with other inflammatory cells, such as macrophages and neutrophils. Since DC can be anticipated to potentially become activated when placed into this environment, that is a key rationale for intratumoral, versus intravenous or subcutaneous administration.

Considering a temporal perspective, the best time to introduce DC into an irradiated tumor is much less clearly defined. The onset of inflammatory changes may have a significant latency, particularly in conventionally fractionated treatment plans, with a high number of treatment fractions in the 180–200 cGy range. Placement of DC too early or too late could result in their exposure to a microenvironment more resembling an intact (immunosuppressive) tumor. The onset of apoptosis or other cell death, or changes of antigen expression on the tumors themselves is more difficult to predict in clinical tumors – when would DC have the richest supply? The potential that injected DC themselves would be irradiated, after acquiring antigen, but before migration out to lymph nodes also must be considered. The migration time appears relatively fast (on the order of a couple of days), but as Liao et al. (2004) found, the possibility of enhanced antigen presentation after DC irradiation is another theoretically favorable consideration.

INTRODUCTION OF DC INTO THE TUMOR LOCALE

Nikitina and Gabrilovich (2001) initially described the basic model of intratumoral DC injection coordinated with sub-curative irradiation of the primary tumor, in a model system using methA sarcoma (in Balb/C mice) and C3 tumor (in C57BL/6 female mice) tumors. Key findings for the combination treatment group (but not for the monotherapies or untreated controls) were longer survival of the mice, with higher T cell titer of tumor-specific tetramer peptides, and higher CD8 T cell response to tumor-specific peptides. Additionally DCs obtained from spleens of syngeneic mice and marked with fluorescent tracer that were injected subcutaneously were demonstrated to track into the irradiated tumor. Further, the T cell-mediated immunity was sufficient to reject tumor rechallenge. In sum, the unmanipulated DC that were placed into irradiated tumor-mediated systemic, lasting antitumor immunity, without any other systemic modulation (Nikitina and Gabrilovich, 2001).

In another murine tumor system (C57BL/6 female mice with the D5 tumor, which is a poorly immunogenic subclone the B16-BL6 melanoma, and with the MCA205 fibrosarcoma), Teitz-Tennenbaum et al. (2003) observed superior survival in mice treated with a combined radiation and intratumoral DC injection protocol. Further they found that loading of the DC with antigen *in situ* was superior to *ex vivo* loading with irradiated tumor lysate. This contributes to support the idea of particular microenvironmental attributes of the irradiated tumor that mediate the changes on DC function and the consequent antitumor immune effect (Teitz-Tennenbaum et al., 2003). In further work with the D5 tumor, they found that the loading and presentation of D5-associated antigens by DC was enhanced by D5 irradiation, independent of the low level of tumor cell death that was directly induced by radiation. Finally, trafficking of DC to regional tumors was better after tumor irradiation (Teitz-Tennenbaum et al., 2008), consistent with the findings of the earlier report discussed above (Nikitina and Gabrilovich, 2001). On the other hand, assays for several inflammatory cytokines (using cultures of tumor cells), including IL-12^{p70}, TNF-α, IFN-γ, IL-6, and IL-10 did not show changes following the tumor irradiation, and tumor-specific $CD8^+$ T cells did not accumulate in the tumor (Teitz-Tennenbaum et al., 2008).

CLINICAL TRIALS OF RADIATION PLUS DENDRITIC CELLS

INTRATUMORAL DC INJECTION

Several groups have developed clinical trials toward a goal of more effective anticancer immune response by tumor irradiation coordinated with intratumoral placement of DC. Primary radiation therapy for treatment of clinically localized prostate cancer was studied in a pilot trial, by our group (Finkelstein et al., 2012a). While the technique of intraprostatic injection was described generations ago, in a canine model addressing therapy of benign hypertrophy (O'Conor and Ladd, 1936), this is the initial trial of intraprostatic injection of apheresis derived autologous DC. There are several features of the clinical scenario that could be favorable. These include the expectation that the local therapy could be definitive, the accessibility for an injection technique that can be standardized, and simultaneous use of androgen suppression, which may favor an increased capacity for

immune response (Windmill and Lee, 1999; Johnke et al., 2005). Further, the bulk of residual (metastatic, extraprostatic) disease should be microscopic, at worst, in well-selected patients, and should have a multi-year latency until detectable recurrence, which could allowing time for immune clearance to go to completion. Disadvantages of this system, conversely, are that no immediate therapeutic effect is discernible. By limiting the inclusion to individuals with HLA-A*0201 haplotypes, it was hoped that it would thus be feasible to use an immunological endpoint to give a readout of an acquisition of a higher titer-specific $CD8^+$ CTL. To this end, serial assays of the titer of T lymphocytes by response to stimulation with class 1-associated peptides were used with the ELISpot (enzyme-linked immunosorbent spot-forming) IFN-γ assay. This endpoint tested for specificity to the peptides, derived from PSA, PSMA, PAP, Her2/neu, and p53, representing prostate-associated and prostate cancer-associated proteins (Finkelstein et al., 2012a).

Inclusion required localized cancers, without radiologically identified metastasis, but with high-risk features (T-stage, PSA, Gleason score) for eventual recurrence. The five patients were treated with a conventional therapy schedule of 28 months' androgen suppression, 45 cGy EBRT over 25 fractions, which was then followed by brachytherapy seed placement. Autologous DC were prepared from a single pretreatment apheresis, and injected after the 5th, 15th, and 25th radiation therapy fraction, in each case on a Friday, so as to give the injected DC about 72 h to potentially migrate out from the radiotherapy field, before the next (6th or 16th) fraction on the following Monday. Overall, the apheresis and injections were well tolerated. Some patients had detectable increases of titers for some of the peptides, but persisting elevations were not apparent. The low number of patients, and the heterogeneity of disease features, precludes a meaningful long-term efficacy assessment (Finkelstein et al., 2012a).

A second trial developed in our group addressed combined neo-adjuvant apoptosis-inducing EBRT plus intratumoral DC injection in larger group of patients, with soft tissue sarcoma (STS) diagnoses. The immunologic objective was to test for detectable increase of T lymphocyte titer on testing with autologous STS tumor cell lysate, using an ELISPOT assay (Finkelstein et al., 2012b). Patients with clinical stage T2N0M0 high-grade STS of the extremity, trunk, or chest wall were treated with standard neo-adjuvant EBRT 5040 cGy in 28 fractions of 180 cGy coordinated with additional DC injection, after weeks 2, 3, and 4. The DC were prepared from a pretreatment apheresis, *ex vivo* expansion and culture, and given as intratumoral injection of 10 million DC.

Secondary analyses included functional T cell activity, toxicity tabulation, primary tumor responses, and analysis of DC migration to lymph nodes, *in vivo*. Seventeen patients completed neo-adjuvant EBRT with and DC injection. Fifty-two per cent showed anti-autologous tumor cell immune responses, as determined using pre- and post-treatment ELISpot assays (Finkelstein et al., 2012b). This titer increased after the last DC injection.

Additionally, chromium release assays revealed that after the treatment there was a statistically significant improvement of the functional cell-killing response to autologous STS lysate. Examination of the tumor from the post-radiation, definitive-intent surgery showed that the combination treatment was associated with a dramatic accumulation of intratumoral T cells. Presence of $CD4^+$ T cells in the tumor positively correlated with tumor-specific immune responses that developed following combined therapy. Accumulation of MDSC but not of regulatory T cells negatively correlated with the development of tumor-specific immune responses.

The treatment was well tolerated, with no toxicity higher than grade 2 was observed during combined DC/EBRT. Post-operative wound complications were observed in five of the 17 patients (29%), applying the NCIC criteria of a secondary operation for wound repair or wound management without secondary operation. Twelve of 17 patients (71%) were progression free after 1 year.

Image-guided visualization of cellular-based vaccine migration was demonstrated for each patient. Experiments with ^{111}In labeled DCs demonstrated that these antigen-presenting cells need at least 48 h to start to migrate from tumor site (Finkelstein et al., 2012b). This experience led to a multi-institutional trial which is currently accruing (Finkelstein et al., 2012c).

CONCLUSION

The coming years offer opportunities to transform the phenomenon of radiotherapy-induced anticancer immune response from isolated case reports into a predictable therapeutic goal. To this end, several components and perspectives must be unified and coordinated. One is the understanding of how to use systemic therapies to make the host lymphocyte compartment and antigen-presenting cell compartments be primed for stimulation. Some examples of immune modulators with the potential to be having a significant impact on the phenotypes of the DC compartment include TLR9 agonists (Brody et al., 2010; Kim et al., 2012; Zhang et al., 2012) all *trans* retinoic acid (Mirza et al., 2006), inhibitors of VEGF, TGF-β, or use of other cytokines (Antony et al., 2005; Charo et al., 2005; Gattinoni et al., 2005; Klebanoff et al., 2005, 2011; Zeng et al., 2005; Seung et al., 2012). Comparably, stimulation of the lymphocyte compartment with checkpoint inhibitors and cytokines also appears poised to make a significant contribution to clinical practice. It will be of interest to see if radiation therapy can be systematically used to advantage in combinations with those new agents as well.

Another component will be the ways to provide tumor-associated antigen to the immune system. While recombinant vaccines and tumor lysates and synthetic peptides have attributes of convenience and definable antigen sets, they cannot be considered interchangeable with tumor irradiation as a source. Unique features of tumor irradiation include simultaneous elaboration of subtle microenvironmental changes with the capacity to improve antigen presentation, total tumor as a source of antigen, elaboration of radiation-induced antigens, and provision of antigen even before or independent of radiation-induced cell kill. Further, evolving flexibility of radiation technique, particularly in relation to conventional fractionation, hypofractionation, brachytherapy, stereotactic radiosurgery techniques, and high intratumoral dose exposure may be particularly of interest for optimization of antigen production and repolarization of the tumor microenvironment. The best way for radiation to trigger an abscopal

response may be related to tumor effect, DC effect, lymphocyte effects, or indirect modulation of the way the tumor is affecting leukocyte compartments.

A third component of interest is cellular therapy, particularly intratumoral DC injection – many questions about timing with respect to irradiation, details of *ex vivo* preparation remain to be addressed empirically. Optimal host preparation, patient selection, and antigen loading could improve outcomes as well. The best volume and number of injected DC merits empiric study. Finally, as a necessary part of clinical development, there must be some focus on specific diagnoses.

In summary, it is clear that radiation is a modulator of the interaction of the tumor and immune compartments, and their relationships with each other. Careful study of the microenvironment of the irradiated tumor should lead to exciting opportunities for putatively localized anticancer treatments to be leveraged to make the irradiated tumor a catalyst for systemic anticancer response.

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The confluence of radiotherapy and immunotherapy

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Radiotherapy (RT) has been considered a local modality and outcomes have emphasized local and regional control of tumors. Recent data suggests that RT may activate the immune system and the combination of radiation therapy and immune therapies may have the potential to improve both local and distant control of tumor deposits. Below we review principals underlying the concepts of combining both modalities.

Keywords: radiation, immunotherapy, radiotherapy, T cell, danger signal

INTRODUCTION

The utility of radiotherapy (RT) as an anti-tumor agent is usually based on the fact that radiation can induce irreparable DNA damage, and eventually cell death through a variety of mechanisms including; mitotic catastrophe, apoptosis, senescence, and autophagy (Rupnow and Knox, 1999; Eriksson and Stigbrand, 2010). Improvements in the clinical practice of RT were historically aimed at technically achieving maximal tumor cell killing while balancing damage to normal tissues. However, over the past decade RT has been the subject of a steady conceptual and experimental reinvention that has broadened both our understanding of the mechanisms by which RT mediates tumor eradication and possibilities for synergistic combinations with emerging anti-cancer therapies. Of particular relevance to this review is the finding that in a variety of preclinical animal models adaptive immunity plays a defining role in the efficacy of RT (Lugade et al., 2005; Lee et al., 2009). The mechanisms underlying the capacity of RT to engage the immune system are the subject of intense scientific inquiry. Published data demonstrate that RT can induce or augment all phases of the T cell response from T cell priming, trafficking, and effector responses within the tumor, which endorses a natural alignment of radiation and immunotherapy. The data from preclinical models may overemphasize the role of adaptive immunity in RT as a single modality, which may explain the paucity of supporting clinical data. Only relatively recently has there been a meaningful effort to assess immunological correlates in the course of traditional RT. Regardless of the overall contribution of adaptive immunity to RT, at the very least the immune system is poised to be a powerful ally with a demonstrated capacity to augment the anti-tumor effects of RT. Therefore, several aspects of clinical RT warrant reconsideration with respect to the role of endogenous anti-tumor immunity especially in light of combinatorial treatment strategies that incorporate immunotherapy. In this review, we will discuss these and other aspects of RT that could affect the proposed synergistic relationship between RT and immunotherapy and also highlight some novel strategies that aim to further exploit the immunogenicity of RT.

IMMUNE RECOGNITION OF TUMORS

The principals of tumor immunology were originally established by pioneering work of Burnet and Thomas when they proposed that nascent tumors can be recognized and eliminated by the host immune system in a process they termed "cancer immunosurveillance" (reviewed in Dunn et al., 2006). By inference, immunosurveillance governs the capacity of the immune system to "recognize" the tumor. From simplified viewpoint, this interaction can be divided into two processes whereby the immune system is first "alerted" to the presence of cells undergoing neoplastic transformation through stress or danger signals, and second, is equipped to directly interact with neoplastic cells to mediate destruction. Although considerable debate still exists regarding whether immunosurveillance exists in human and mouse tumors, the underlying principles that define the capacity of the immune system to specifically recognize tumors remain unchanged. Therefore, whether or not the emergence of clinically detectable tumors is reduced by immune-mediated mechanisms does not preclude subsequent immune recognition that could occur during the clinical treatment of tumors. A logical extension of the principles of cancer immunosurveillance, therefore, lies in the hypothesis that successful treatment of established tumors, as potential products of failed or blunted surveillance, could be achieved by rekindling immune recognition. This hypothesis is the foundation of the field of tumor immunology and its applied counterpart cancer immunotherapy. Cancer immunotherapy represents the use of agents proposed to amplify the host immune response to established tumors (Pardoll and Drake, 2012). Radiation therapy and immunotherapy may be natural partners given that radiation possesses immunomodulatory effects at multiple points in the processes of T cell priming and effector function. We will

review literature regarding the immunomodulatory properties of radiation and discuss available data dealing with the effect of dose and fractionation schedules on various aspects of the anti-tumor immune response.

EFFECTS ON TUMOR ANTIGENICITY

The first major requirement for tumor-specific adaptive immunity is the availability and immunogenicity of tumor antigens. A plethora of tumor antigens have been defined across a wide array of tumor types and they fall into three broad categories: (1) viral proteins, (2) mutated versions of self-proteins that include point mutations and oncogenic fusion proteins generated by recombinatorial events, or (3) non-mutated self-proteins enriched in tumor cells but with shared expression on non-tumor tissue (for review, see Jäger et al., 2001). Melanoma differentiation antigens and cancer testis (CT) antigens are the best characterized tumor-associated antigens (Engelhard et al., 2002; Scanlan et al., 2002). The etiology of tumor antigens has important implications on immunogenicity. Non-mutated tumor-associated antigens are self-antigens that are subject to immunological tolerance mechanism that drastically diminish the peripheral repertoire of high-affinity T cells capable of recognizing these antigens. However, tumor-associated antigens offer a convenient clinical target both for therapeutic vaccination and immunological assessment due to a high frequency of expression across many tumor types. Mutated tumor antigens represent the most unique antigens that, based on their extrathymic expression, would be excluded from central tolerance. Therefore, T cells expressing high-affinity T cell receptors (TCRs) specific for these antigens are likely to be present in the peripheral pool. Identification and vaccination against such antigens, however, requires sophisticated high-throughput screening methods to identify mutations and sift out the potential antigenic peptides with sufficient binding to major histocompatibility (MHC) antigens to mediate efficient presentation. Notable exceptions that can be readily identified are antigens generated by mutated oncogenic proteins that have high association with some cancers (Boon, 1996). Non-mutated tumor-associated antigens, on the other hand, are readily identified by established screening methods and are widely expressed across tumor types. Such antigens are often accompanied by some degree of T cell tolerance that dampens endogenous immunity (Engelhard et al., 2002). Nevertheless, clinically viable vaccination strategies have been developed that can induce durable T cell responses against tumor-associated antigens, and even low-avidity T cells that escape negative selection can mediate anti-tumor effects if properly activated (Uchi et al., 2006).

With regard to tumor-antigen expression, local high dose ablative (15–20 Gy) radiation has been shown to directly upregulate the expression of some tumor antigens including tumor antigens associated with viral transformation (Santin et al., 1998), and CT antigens (Sharma et al., 2011). A mechanistic basis for these changes was reported by Reits et al. (2006) who demonstrated that tumor cell irradiation leads to increased protein translation as a consequence of mTOR activation. Furthermore, radiation increased the degradation of cellular proteins as a result of direct free radical-mediated damage. The resulting increase in the intracellular pool of available peptides augments MHC loading and productive antigen presentation. Interestingly, dose-dependent effects of radiation were observed in terms of both the magnitude and duration of intracellular peptide availability. Single doses of higher than 4 Gy were required to dramatically enhance MHC class I surface expression, and a single dose of 25 Gy induced the most robust expression, which correlated with measurements of intracellular peptide levels. Together these mechanisms could overcome the poor antigenicity of some tumors in instances where availability of tumor antigens is a limiting factor to the induction of tumor antigen-specific T cells. Although, the mechanisms uncovered by Reits et al. (2006) provide an interesting mechanism by which local RT could enhance local T cell-mediated recognition, whether or not this mechanism plays any role in augmenting endogenous immunity remains unknown. It would be interesting to know whether the intermediate effect of local radiation alone on tumor growth in their model could be abrogated by systemic CD8 T cell depletion. Nevertheless, these studies suggest that larger doses of radiation are more potent at increasing tumor antigenicity, however, the effect of smaller daily fractionated doses was not investigated. It is possible that daily doses of less than 2 Gy, such as those used in traditional RT, might eventually result in a cumulative effect that could eventually approach the large single doses used by the authors given the kinetics of increased protein degradation. Notably, if the effect of radiation on MHC class I surface expression is indeed mediated through alleviation of the normally limiting pool of available intracellular peptides, then these effects would presumably be unrelated to intrinsic tumor cell radiosensitivity and therefore uniform across most tumor cells unless the efficiency of targeted protein degradation varies widely. In order to make these peptides available as substrates for T cell priming, however, transfer to professional antigen presenting cells (APCs) must occur in such a manner that stimulates efficient capture and intracellular processing within the APC to yield MHC:peptide complexes that are subsequently presented to T cells, a process termed antigen cross-presentation.

RADIATION-MEDIATED "DANGER SIGNALS" AND CROSS-PRESENTATION OF TUMOR ANTIGENS

The innate immune system is equipped with many molecular sensors that facilitate the recognition of unique molecular patterns found in the myriad pathogens present in the environment. These sensors are localized to subcellular locations including the plasma membrane, endosomes, and the cytoplasm poised to detect invading pathogens. Charles Janeway proposed a cellular recognition system, consisting of receptors that could exclusively recognize unique features of pathogens (pathogen-associated molecular patterns, PAMPs), that formed the central basis for "self" vs "non-self" discrimination. This pathogen recognition system was thought to explain why antigens derived from pathogens elicit potent adaptive immune responses, and self-antigens are "ignored." A highly provocative amendment to this hypothesis was proposed by Polly Matzinger, who hypothesized that tissue injury in the absence of pathogens could elicit innate immune recognition through stress signals that she collectively termed "Danger Signals" (Matzinger, 1994). It is now recognized that many of these same receptors do double duty and can recognize endogenous molecular signals emanating from stressed or dying cells in the

absence of pathogens (Matzinger, 2002). Thus, the host actually senses "danger" in the form of cellular stress and tissue injury rather than sensing the presence of a pathogen specifically. The endogenous ligands are termed molecular "alarmins" and together with PAMPs they are collectively termed "danger signals" (Bianchi, 2006). Alarmins function as endogenous adjuvants that form an essential bridge between innate inflammatory responses and the initiation of tumor-specific adaptive immunity following treatment of tumors with local radiation. The exposure or release of danger signals also depends on the type of cell death that occurs and many open questions remain regarding the relative contributions of each to the immunogenicity of radiation-mediated tumor cell death.

High mobility group box 1 (HMGB1) is a prototypical "alarmin" that was shown to be a central mediator in the immunogenicity of dying tumor cells following irradiation (Apetoh et al., 2007). Normally a nuclear protein associated with chromatin, extracellular release of HMGB1 from dying tumor cells was demonstrated to engage Toll-like receptor 4 (TLR4) expressed by dendritic cells (DCs) to facilitate their activation, maturation, and capacity to efficiently prime tumor antigen-specific $CD8^+$ T cells (T cell cross-priming). Since TLR4 binds ligands at the plasma membrane, HMGB1 must be released into the extracellular space in order to engage TLR4. Extracellular exposure could be mediated by direct necrotic cell death or secondary necrosis of lingering apoptotic bodies that are inefficiently cleared. Conceptually, apoptotic death of tumor cells is predicted to conceal HMGB1 from TLR4-mediated recognition (Bianchi and Manfredi, 2007). However radiation-mediated cell death of most solid tumors is thought to predominantly occur through induction of senescence, necrosis, or mitotic catastrophe. An exception is hematopoietic tumors that frequently undergo rapid induction of apoptosis following radiation exposure (Rupnow and Knox, 1999; Eriksson and Stigbrand, 2010). Radiation-mediated mitotic death shares features with both apoptosis and necrosis, however, the prevailing view places it more closely associated with necrosis. More recently, a specific receptor for necrotic cells was cloned and characterized. DNGR-1/CLEC9A, a c-type lectin, was shown to be essential for the induction of adaptive immunity to necrotic cells (Sancho et al., 2009). Importantly, interaction of DNGR-1 with necrotic cells did not affect the uptake of necrotic debris, but instead regulated the capacity of DCs to cross-present antigens contained therein. Furthermore, DNGR-1 expression was shown to specifically identify mouse and human DCs that express the transcription factor Batf3 and are specialized for cross-presentation of antigens to $CD8^+$ T cells (Poulin et al., 2010, 2012; Schreibelt et al., 2012). The ligand for DNGR-1 was recently identified to be filamentous actin (F-actin) that is exposed upon the loss of membrane integrity characteristic of necrotic cell death (Ahrens et al., 2012). These concepts may be related to radiation induction of antigen processing and remain to be studied.

In addition to ligands that promote DC activation and subsequent maturation, several other danger signals contribute to immunological recognition of dying tumor cells. In particular, nucleotides released by apoptotic cells function as a chemotactic signal for phagocytic myeloid cells including DCs by stimulating the P2RY2 purinergic receptor (Elliott et al., 2009). Extracellular ATP can also function through P2RX7 purinergic receptors to initiate NLRP3 inflammasome activation and subsequent IL-1β production that were all shown to be required for the induction of tumor antigen-specific $CD8^+$ T cells following challenge with dying tumor cells (Ghiringhelli et al., 2009). Finally, surface translocation of the ER resident protein calreticulin (together with ERP57) was shown to be an essential signal for efficient uptake of dying tumor cells by APCs and therefore a critical regulator of immunogenic cell death following tumor cell exposure to γ-irradiation (Obeid et al., 2006, 2007). Calreticulin exposure proceeds as a preapoptotic event that could be partially blocked by caspase inhibition, however, it is unclear whether translocation of calreticulin is a widely observed phenomenon across all modes of cell death induced by irradiation or if it is unique to cells destined to undergo apoptosis.

Taken together, the radiation-induced release of tumor antigens must be accompanied by coincident release and recognition of danger signals in order to efficiently generate tumor-specific CTLs. The ability of radiation to promote tumor antigen release has been demonstrated in several models, however, the type and magnitude of danger signal release is quite variable and may still provide suboptimal maturation signals to APCs. The coadministration of exogenous danger signals in the context of tumor irradiation has been shown to augment the immunogenicity of RT in both preclinical animal models and clinical trials. In particular, treatment of mice with a synthetic TLR9 agonist resulted in both enhanced local control and reduced distant metastasis when combined with single high dose RT (Zhang et al., 2012). Augmented tumor control was associated with enhanced activation and cytokine production by $CD8^+$ T cells and enhanced deposition of tumor-specific Ig in the tumor bed. Furthermore, a phase I/II clinical trial demonstrated that lymphoma patients that received combined radiation and TLR9 agonist had improved clinical responses suggesting that supplemental danger signals in the context of tumor irradiation my drive more potent host T cell responses (Brody et al., 2010). Experiments that further elucidate both the unique and overlapping aspects of radiation-induced danger signals and exogenous adjuvants on host T cell activation and priming are needed.

T CELL PRIMING FOLLOWING TREATMENT OF ESTABLISHED TUMORS WITH LOCAL RT

Data in preclinical models have demonstrated increased priming of tumor antigen-specific $CD8^+$ T cells in the draining lymph node (dLN) several days following treatment of established tumors with ablative single dose local RT (Lugade et al., 2005; Lee et al., 2009). Particularly, our group demonstrated enhanced cross-presentation of tumor antigen by $CD11c^+$ DCs present in the dLN following migration from the tumor (Lee et al., 2009). Recently, this mechanism was expanded to incorporate proximal events in the tumor microenvironment. Local radiation has been shown to induced rapid recruitment and infiltration of leukocytes (Shiao and Coussens, 2010; Burnette et al., 2011). Among the recruited cells, circulating monocytes can give rise to $CD11c^+$ DCs. Within the irradiated tumor microenvironment these DCs encounter myriad danger signals and capture antigens from dying tumor cells through phagocytic receptors (discussed above). Our

group demonstrated that treatment of tumors with local ablative RT could greatly enhance the cross-priming capacity of tumor-infiltrating DCs (TIDCs), and this effect was shown to be critically dependent on type I interferon (IFN) signaling in bone marrow-derived hematopoietic cells (Burnette et al., 2011). Importantly, the enhanced cross-priming capacity of TIDC was not simply dependent on availability of newly liberated tumor antigen, but rather was dependent on signals unique to the irradiated tumor microenvironment. The development of DCs in the irradiated tumor microenvironment that are competent to prime tumor antigen-specific T cells precedes the enhanced T cell priming that we and others have observed in the dLN. These sequential observations suggest that migration of functional TIDCs to the dLN drives T cell priming following local RT (Lugade et al., 2008; Meng et al., 2010; Gupta et al., 2012). Interestingly, type I IFN was shown to be a critical mediator of spontaneous tumor antigen-specific $CD8^+$ T cell priming (Fuertes et al., 2011). More specifically, DCs were shown to be the essential targets of type I IFN signaling to mediated T cell priming and drive tumor immunoediting (Diamond et al., 2011). Together these results paint a compelling picture that local radiation may, in fact, rekindle central aspects of innate and adaptive immunity to induce subsequent rounds of immunoediting, and in some cases, complete regression.

T CELL MIGRATION AND EFFECTOR FUNCTION IN THE TUMOR MICROENVIRONMENT

The tumor microenvironment represents a formidable challenge to immune-mediated recognition and killing of tumor cells. In the absence of local RT, strategies aimed at increasing the pool of tumor antigen-specific T cells, such as therapeutic vaccination or adoptive transfer of large numbers of specific T cells, fails to exert significant effects on tumor outgrowth. However, combining these strategies with local RT can yield impressive results in preclinical models (Harris et al., 2008; Takeshima et al., 2010). Therefore, the capacity of local RT to support immune-mediated tumor regression extends far beyond the effects of local RT on T cell priming, and involves local changes that reinforce immunity subsequent to priming. Local radiation has been shown to facilitate the recruitment of activated T cells to the tumor and induce changes in the local microenvironment and on tumor cell themselves that can greatly enhance T cell effector function. RT can upregulate expression of adhesion molecules, such as VCAM-1, E-selectin, and ICAM-1, by vascular endothelial cells within the tumor and induce expression of T cell chemokines that promote T cell adhesion and extravasation into the tumor microenvironment (Handschel et al., 1999; Lugade et al., 2008). Both the expression of adhesion molecules and the production of T cell attractive chemokines are likely a product of a feedforward mechanism induced by production of IFNs in the tumor microenvironment (Lugade et al., 2008; Meng et al., 2010). In addition, tumor cells can directly produce CXCL16 following irradiation leading to the recruitment of activated $CD8^+$ $CXCR6^+$ effector cells (Matsumura et al., 2008; Matsumura and Demaria, 2010). Furthermore, RT has been shown to induce several changes that directly affect the ability of effector T cells to efficiently recognize and kill tumor cells.

As previously noted, radiation increases surface expression of MHC on tumor cells, which increases the likelihood of a productive interaction with cognate antigen-specific T cells. Upregulation of MHC likely occurs through several mechanisms that coordinately drive robust expression. In addition to the mechanism proposed by Reits et al. (2006; discussed above), local radiation has been shown to induce MHC expression through induction of IFN-β that can signal to tumor cells in an autocrine/paracrine fashion (Wan et al., 2012). IFN-γ secretion by infiltrating effector cells can also further augment MHC expression and promote T cell-mediated recognition of tumor cells through cognate TCR:peptide/MHC interactions. Bolstering the direct TCR-mediated recognition of tumor cells, is the local expression of ligands for the NKG2D activating receptor. NKG2D ligands have been shown to be expressed as a consequence of cellular transformation, are upregulated by cellular stress, and directly induced by irradiation through activation of the DNA damage pathway (Gasser et al., 2005). NKG2D is an activating receptor expressed by NK cells and activated $CD8^+$ T cells that, upon engagement, can significantly increase cytolytic potential (Markiewicz et al., 2005; González et al., 2008; Champsaur and Lanier, 2010). Upregulation of NKG2D ligands could be a robust mechanism for local restimulation of CTL and enhanced cytokine production, however, the mechanisms regulating expression are complex and expression among tumors is variable (Nausch and Cerwenka, 2008). Finally, radiation can upregulate expression of the FAS death receptor on tumor cells to induce sensitivity to T cell expressed FAS ligand (Chakraborty et al., 2004). The induced sensitivity of tumor cells to FAS-mediated killing represents a TCR-independent mechanism for tumor cell killing and can function as a more potent cytotoxic modality especially in instances where TCR affinity is low and perforin-mediated cytotoxicity is less efficient (Kessler et al., 1998). The combination of these local effects likely accounts for a significant portion of the interaction with immunotherapy.

SYNERGY WITH IMMUNOTHERAPY

The immune modulating capacity of RT is clearly multifaceted and can, in some preclinical models, lead to robust anti-tumor immunity that can mediate complete tumor regression as a single modality. However, it has been reported that radiation could increase some immunosuppressive aspects of the tumor microenvironment such as regulatory T cell (Treg) accumulation depending on the dose and timing (Kachikwu et al., 2011; Schaue et al., 2011). Therefore, in order to maximize the immunostimulatory effects of RT, strategies that combine local RT with immunotherapy are required to generate durable T cells responses in patients. Among the prospects for targeted therapies that can directly enhance T cell responses, monoclonal antibodies that modulate T cell coactivating and coinhibitory receptors, or their ligands, are the most accessible. Productive T cell priming and the induction of tolerance are determined by a complex integration of many stimulatory and inhibitory receptors that reinforce and dampen the primary TCR:peptide/MHC interaction, respectively. Recently, a monoclonal antibody targeting the T cell negative regulator, CTLA-4, received FDA approval following a proven survival benefit in a randomized clinical trial of

patients with metastatic melanoma (Hodi et al., 2010). CTLA-4 is expressed by activated T cells and functions as a natural regulatory mechanism to dampen T cell activation and prevent autoimmunity by competitively inhibiting the interaction of CD28 on T cells with B7-1/B7-2 on APCs (Rudd et al., 2009). CD28 cosignaling is required for optimal induction of CD25, which together with IL-2Rβ and common gamma chain, form the high-affinity IL-2 receptor. IL-2 signaling induces both the differentiation and survival of effector T cells, and the inability to upregulate CD25, and therefore respond to IL-2, is associated with T cell anergy and tolerance. In addition, Tregs constitutively express CTLA-4, which has been shown to directly control DC maturation and the induction of T cell tolerance by downregulating B7-1/B7-2 expression on DCs to block the CD28:B7-1/B7-2 signal (Wing et al., 2008; Qureshi et al., 2011).

An accepted hypothesis is that anti-CTLA-4 blocking antibodies promote enhanced T cell activation and proliferation to promote effector cell priming (Chambers et al., 2001; Pardoll and Drake, 2012). In preclinical models, local RT and CTLA-4 blockade was shown to mediate synergistic effects (Dewan et al., 2009). Furthermore, in mice concurrently challenged with two tumors, treatment of one tumor with local RT in combination with systemic administration of anti-CTLA-4 could induce significant growth delay in the second tumor that did not receive local RT; a process referred to as the abscopal effect (Dewan et al., 2009). The precise mechanism underlying the abscopal regression of unirradiated tumors was not investigated, but the results are consistent with increased priming of tumor antigen-specific T cells that subsequently infiltrate the tumor. Such an effect would likely be mediated by blocking the engagement of CTLA-4 on effector T cells in the context of heightened cross-priming capacity of DCs in the dLN (discussed above). Interestingly, data from Dewan et al. (2009) also reported that a fractionated dose of 8 Gy × 3 was optimal for induction of an abscopal effect when combined with anti-CTLA-4, whereas and abscopal effect was not observed when tumors were treated with 20 Gy × 1 or 6 Gy × 5 alone or in combination with anti-CTLA-4. Although the authors refer to 8 Gy × 3 as a fractionated schedule, this treatment scheme is probably more accurately described as hypofractionation. The precise mechanistic basis for the ability of 8 Gy × 3 to properly synergize with anti-CTLA-4 was not explored, however, the authors did note that this dose scheme did result in the highest level of infiltration and IFN-γ production by T cells. The synergy between local RT and CTLA-4 blockade observed in preclinical models appears to translate well into the clinic. Several reports in melanoma patients have demonstrated abscopal regression following treatment with local RT and anti-CTLA-4 (ipilimumab) that was associated with elevated immunity to tumor-associated antigens (Postow et al., 2012; Stamell et al., 2012). At present, there is no clear role for CTLA-4 blockade in the tumor microenvironment. It is reasonable to suspect that Tregs expressing CTLA-4 in the tumor microenvironment could similarly modulate DCs that infiltrate the tumor, however, definitive evidence that CTLA-4 participates in Treg-mediated suppression in the tumor microenvironment is lacking. Strategies that enhance T cell activation by engaging costimulatory receptors expressed on T cells represent a complimentary approach to blockade of negative regulators.

Enhancement of effector cell priming is a shared mechanism between anti-CTLA-4 and other targeted therapies employing agonistic antibodies against the costimulatory receptors OX40, 4-1BB (CD137), and CD27 (for a detailed review, see Redmond et al., 2009). Briefly, stimulation of T cells through OX40 results in enhanced T cell activation and effector cell differentiation, in part, through enhancing the expression of CD25 and promoting T cell sensitivity to IL-2. A recent report demonstrated that agonistic OX40 antibodies in combination with systemic IL-2 administration could generate potent anti-tumor immunity, and the synergistic nature of the combination resulted from the ability of systemic IL-2 to upregulate OX40 expression on activated T cells (Redmond et al., 2012).

Results from a phase I study of stereotactic body radiation therapy (SBRT) and systemic IL-2 in melanoma and renal cell carcinoma demonstrated that this combination could result in impressive responses in both tumor types (Seung et al., 2012). Addition of OX40 agonistic antibody to this clinical protocol would be predicted to further enhance responses and perhaps increase the rate of complete response. Based on these results, it seems likely that a natural synergy might exist between agonistic OX40 antibodies and anti-CTLA-4 to induce optimal expression of CD25 and OX40 and maximize effector T cell differentiation. Agonistic OX40 antibody has also been shown to synergize with high dose local RT (20 Gy × 3) and was associated with enhanced expression of CD25 by tumor-infiltrating $CD8^+$ T cells (Gough et al., 2010). Importantly, OX40 stimulation possesses no inherent ability to polarize T cells toward one particular effector subset, but rather, drives T cell polarization in the context of the inflammatory milieu. Considering the nature of most tumor-associated antigens, it is important to note that costimulation through OX40 can rescue priming of low avidity T cells, and can also reverse T cell tolerance against self-antigens. Taken together, the mixed preclinical and clinical data employing local ablative RT with OX40 agonistic antibody, systemic IL-2, or anti-CTLA-4 antibody demonstrate that signaling through CD25 and OX40 reciprocally reinforce each other to augment effector cell priming initiated by local RT and improve the quality and magnitude of T cell responses against tumor-associated antigens. Future clinical trials that employ local RT, anti-CTLA-4, and agonistic OX86 are likely to yield impressive results.

In addition to the goal of improving T cell activation and effector cell generation, strategies that target immune suppressive mechanism in the tumor microenvironment are equally important. Local RT does have the ability to modify the tumor microenvironment, however, many tumors exploit natural immune regulatory mechanisms to subvert induced T cells responses. Expression of programmed death ligand-1 (PD-L1) in the tumor microenvironment can deliver an inhibitory signal through it's receptor PD-1 that is expressed on a majority of activated effector T cells. PD-L1 expression has been observed across many tumor types (Zou and Chen, 2008) where it mediates apoptosis of infiltrating T cells leading to tumor immune evasion (Dong et al., 2002). Interestingly, PD-L1 expression can be directly induced by IFNs indicating that effector T cell activity within the tumor microenvironment can initiate PD-L1 expression as a negative feedback loop to squelch T cell effector function (Lee et al., 2006). Corroborating

the role of effector T cell-mediated PD-L1 upregulation, a recent study in human melanoma demonstrated a strong correlation between PD-L1 expression and intratumoral T cell infiltration and IFN-γ (Taube et al., 2012). Data in preclinical models suggest that PD-L1 blockade is necessary in some circumstances to fully uncover anti-tumor immunity that is induced by local RT in combination with costimulatory receptor engagement. Verbrugge et al. (2012) demonstrated that local RT combined with anti-OX40 could mediate significant growth delay of orthotopic AT-3 mammary tumors, however, the addition of anti-PD-L1 was required to mediate complete tumor regression. Future studies will likely continue to uncover optimal combinatorial strategies that enhance the effects of local RT during each phase of the T cell response.

ADDITIONAL CONSIDERATIONS AND CONCLUDING REMARKS

From the data discussed above it is clear that combination strategies employing high dose ablative RT and immunotherapy hold a lot of promise for improving anti-tumor immunity and mediating complete tumor regression. There are many important outstanding questions before RT and immunotherapy can reliably be combined in cancer therapy. Amongst the most important are what is (are) the optimal fractionation (dose delivery of radiation) schemes to increase anti-tumor immunity? Does daily fractionation continuously kill infiltrating T cells and/or reduce their function, or are tumor-infiltrating activated T cells functionally resistant to the low doses employed in traditional fractionation or hyperfractionated schedules? Does inclusion of the dLNs suppress or enhance the immunogenic effects of radiation, and does the timing of dLN irradiation change the response to treatment or therapeutic vaccination. What is the optimal "immune activating" strategy, e.g., high dose cytokines, vaccination, etc.? Answers to these and other questions may improve the local effects of RT and help to understand the basis of activating and improving the local tumor response to RT and the abscopal effect against distant metastasis.

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CpG plus radiotherapy: a review of preclinical works leading to clinical trial

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Studies performed three decades ago in our laboratory supported the hypothesis that radiation efficacy may be augmented by bacterial extracts that stimulate non-specific systemic antitumor immune responses. Application to the clinic was halted by unacceptable side effects and toxicities resulting from exposure to whole bacterial pathogens. Later scientific advances demonstrated that DNA isolated from bacteria was immunostimulatory and could be reproduced with synthetic oligodeoxynucleotides (ODNs), thus fueling the transition from bugs to drugs. Unmethylated CpG motifs within bacterial DNA induce activation of Toll-like receptor 9 and subsequently activate antigen-specific cellular immune responses. CpG ODNs have demonstrated favorable toxicity profiles in phase I clinical trials. We showed that this potent immunoadjuvant can be used in combination with radiation therapy to enhance local and systemic responses of several murine tumors. Studies demonstrated that enhanced tumor response is mediated in part by the host immune system. Antitumor efficacy was diminished in immunocompromised mice. Animals cured by combination of radiation and CpG ODN were resistant to subsequent tumor rechallenge. This body of work contributes to our understanding of the dynamic interplay between tumor irradiation and the host immune system and may facilitate translation to clinical trials.

Keywords: CpG, oligodeoxynucleotides, radiotherapy, immunotherapy

INTRODUCTION

The immune system can influence growth of malignant tumors and responses to therapy with radiation or cytotoxic drugs. Immune deficiency can lower tumor response to conventional treatments, whereas stimulation of the immune system may enhance therapeutic responses (Dunn et al., 2002). This understanding led to the use of immunologic approaches for cancer treatments as monotherapy or in combination with chemotherapy or radiotherapy. In early developmental stages of cancer immunotherapy, bacteria or bacterial extracts, such as *Bacillus Calmette–Guérin* and *Corynebacterium parvum* were used to stimulate antitumor immunity (Yron et al., 1973; Milas and Scott, 1978). These bacteria or their extracts elicited or augmented many facets of immunological reactions, including macrophage and natural killer cell activation, induction of antibody-dependent cell cytotoxicity, and production of cytokines with antitumor activity. They were shown to be potent antitumor agents in a variety of rodent tumors, and they improved the efficacy of chemotherapy and radiotherapy (Yron et al., 1973; Milas and Scott, 1978). In contrast with promising preclinical results, however, these first-generation bacterial immunotherapeutics provided only modest clinical benefits (Mihich and Fefer, 1983). In addition, patients given multiple treatments of whole bacteria and their crude extracts showed symptoms of toxicity, including fever, nausea, vomiting, and pain at the injection site (Milas and Scott, 1978; Mihich and Fefer, 1983).

Recent advances in immunotherapy led to the discovery that immunostimulatory activity of bacteria resides in their DNA (Tokunaga et al., 1999), notably in unmethylated CpG motifs (Krieg et al., 1995) prevalent in bacterial but not in vertebrate genomic DNA. This led to chemical synthesis of oligodeoxynucleotides (ODNs) containing unmethylated CpG motifs that are recognized by immune cells expressing Toll-like receptor 9 (TLR9) in plasmacytoid dendritic cells and B cells (Hemmi et al., 2000). By stimulating TLR9, CpG ODNs induce a cascade of cellular and molecular responses leading to secretion of antigen-specific antibodies and cytokines and chemokines that trigger a wide range of secondary effects such as natural killer cell and monocyte activation (Uhlmann and Vollmer, 2003). Importantly, this receptor-mediated signaling pathway activates both innate and adaptive immunological reactions with less toxicity than do whole bacteria or their extracts (Hemmi et al., 2000). Early studies using CpG in experimental animals showed that these ODNs slowed tumor growth and prolonged tumor–host survival (Blazar et al., 2001; Kawarada et al., 2001; Heckelsmiller et al., 2002; Baines and Celis, 2003; Lonsdorf et al., 2003; Weigel et al., 2003; Krieg, 2004). In addition, CpG ODN treatment improved the outcome of surgery and chemotherapy (Weigel et al., 2003; Krieg, 2004). Our group pioneered work showing that this potent immunoadjuvant can be used in combination with radiation therapy to enhance local and systemic responses in murine tumors (Milas et al., 2004; Mason et al., 2005).

EARLY STUDIES: COMBINATION OF CORYNEBACTERIA AND RADIOTHERAPY

Earliest studies with systemic injections (iv) of *Corynebacterium granulosum* or *C. parvum* in mice showed that these agents could induce complete regression of established s.c. immunogenic

fibrosarcomas (Milas et al., 1974a,b). The response of individual tumors was extremely variable: some regressed permanently and others grew only slightly more slowly than controls. *C. parvum* and *C. granulosum* also reduced the number of metastatic lung tumor nodules when mice were treated within a few days of i.v. injection of fibrosarcoma cells, and many mice were cured of metastatic disease (Milas et al., 1974a; Milas and Scott, 1978).

These results led to studies to determine whether non-specific immunotherapy with *C. parvum* was an effective adjunct to radiotherapy, since treatment response depends not only on radiobiological factors but also on the immune response of the tumor-bearing host (Milas et al., 1975a; Milas and Scott, 1978; Milas, 1980). *C. parvum* increased radiosensitivity of well-established (8 mm diameter) immunogenic murine fibrosarcomas when local irradiation was given as a single-dose or in multiple fractions (Milas et al., 1975a,b; Milas, 1980). Combination treatment prolonged survival of mice more than radiotherapy or immunotherapy alone, and *C. parvum* significantly improved radiocurability. Tumors not cured by combination treatment grew more slowly and produced fewer metastases than tumors exposed to the individual treatments (Milas and Scott, 1978; Milas, 1980). In one study, local irradiation of a highly metastatic immunogenic mammary carcinoma with 60 Gy caused complete tumor regression but greatly increased the number of spontaneous lung metastases compared with mice whose primary tumors were surgically removed (Milas et al., 1976; Milas, 1980). *C. parvum* given before irradiation protected mice against this effect and reduced the frequency of lung metastases below that in mice whose tumor was surgically removed.

Therapeutic efficacy of immunotherapy plus radiotherapy was shown to depend on a number of factors including tumor size and immunogenicity, dose and route of *C. parvum* administration, and sequence of administration (Milas and Scott, 1978; Milas, 1980). Higher doses of local irradiation were required to cure immunogenic tumors in mice immunocompromised by whole-body irradiation (Stone and Milas, 1978; Milas, 1980), and *C. parvum* was less effective in augmenting radiocurability of weakly immunogenic tumors (Suit et al., 1976).

BUGS TO DRUGS: CpG OLIGODEOXYNUCLEOTIDE AND RADIOTHERAPY

The discovery that immunostimulatory activity of bacteria resides in their DNA (Tokunaga et al., 1999), notably in unmethylated CpG motifs (Krieg et al., 1995), led to explorations of CpG ODN's immunotherapeutic and immunomodulatory effects. Our recent studies demonstrated that synthetic CpG ODNs can be used as potent immunoadjuvants in combination with radiotherapy to enhance radioresponse of murine tumors (Milas et al., 2004; Mason et al., 2005). Experiments were performed using murine immunogenic fibrosarcomas growing in the leg of C3Hf/Kam mice. CpG ODN 1826 was administered one, three, or seven times s.c. peritumoral starting when tumors were 6 mm in diameter. CpG ODN 1826 monotherapy had minimal effect on tumor growth. Primary tumors were irradiated when they reached 8 mm in diameter. Response to radiotherapy was assessed by tumor growth delay and TCD50 (radiation dose yielding 50% tumor cures). The ODN dramatically enhanced tumor growth delay in response to single-dose irradiation by 2.58–2.65 and improved radiocurability, reducing TCD50 by a factor of 1.93, from 39.6 (36.1–43.1) Gy to 20.5 (14.3–25.7) Gy (Milas et al., 2004). Multiple administrations of the ODN were more effective than single administration. Importantly, improvement in radioresponse was also observed when CpG ODN 1826 was combined with conventional daily fractional doses of 2 Gy (Mason et al., 2005). A total dose of 83.1 (79.2–90.0) Gy was needed to achieve 50% tumor cure in mice treated with radiation plus the inactive ODN control and only 23.0 (11.5–32.7) Gy was needed when CpG ODN 1826 plus radiation was given. Tumor response to fractionated radiotherapy at the TCD50 level was potentiated by a radiation enhancement factor (EF) of 3.61, substantially higher than that observed for single-dose radiotherapy (EF 1.93). The superiority of CpG ODN treatment in combination with fractionated radiotherapy bodes well for translation of this treatment approach to the clinic.

Fractionated radiation cure probability curves are shown in **Figure 1**. The shallower slope of the CpG ODN 1826 plus radiation group most likely reflects heterogeneity of antitumor responses in mice treated with CpG ODN 1826. Variability in tumor response to combined treatment was also observed when tumor growth delay was the treatment endpoint. Since this fibrosarcoma grows rapidly, treatment with clinically relevant 2-Gy fractions twice a day for 5 days caused only a small delay in tumor growth. The effect of CpG ODN 1826 on radioresponse was initially observed several days after the start of irradiation in the fractionated protocol, when tumors had grown considerably. For example, some tumors began to regress after they grew as large as 9–14 mm, demonstrating

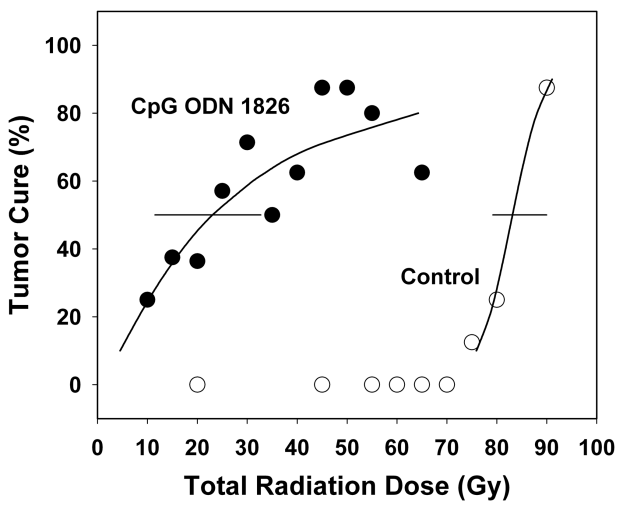

FIGURE 1 | Effect of CpG ODN 1826 on tumor radiocurability. Percentage of tumor cures was plotted as a function of radiation dose. Mice bearing FSa tumors in the leg were exposed to a range of fractionated doses when tumors reached 8 mm in diameter and treated seven times with the active CpG ODN 1826 (●) or the inactive ODN 2138 (○), at a dose of 100 μg per mouse given s.c. peritumorally, when tumor diameters were 6 and 8 mm and once weekly for five additional weeks. The TCD50 was determined at 100 days after irradiation. *Horizontal bars*, 95% confidence intervals. Reprinted by permission from the American Association for Cancer Research (Mason et al., 2005).

that once elicited, the brisk antitumor response was capable of eliminating many cells in the large bulky tumors.

Mice cured of their fibrosarcoma by CpG ODN 1826 plus local irradiation were tested for resistance to tumor rechallenge (Mason et al., 2005). Mice cured of their tumor by treatment with either radiation alone or CpG ODN 1826 plus irradiation were resistant to subsequent s.c. tumor cell inoculation compared with previously untreated age-matched non-tumor-bearing mice (**Figure 2**). In normal mice, 100% tumor take was achieved with inoculations as low as 2.5×10^5 tumor cells. At 100–120 days after treatment, mice cured by radiation alone required 2×10^5 tumor cells to produce 50% tumor take, whereas mice treated with CpG ODN 1826 plus irradiation were totally resistant to tumor rechallenge with cell numbers as high as 8×10^5. Like the animals rechallenged by the s.c. route, mice locally cured by CpG ODN 1826 plus irradiation were much more resistant to development of artificial metastases in the lung than were those cured by radiation alone. These results showed that the systemic antitumor rejection response generated by CpG ODN 1826 plus radiotherapy exerted antitumor effects long after exposure to the agents. Secondary tumor rejection was most likely due to development of a memory response and possibly specific T cell-mediated immunity (Koski and Czerniecki, 2005; Mason et al., 2005). A similar memory response was reported recently using a tumor vaccine composed of C-class CpG ODNs and irradiated melanoma tumor cells that induced long-term antitumor immunity against B16F1 tumors in mice (Cerkovnik et al., 2010).

The mechanisms of action of CpG ODNs for cancer immunotherapy have been reviewed in detail elsewhere (Krieg, 2001, 2006; Jahrsdorfer and Weiner, 2008; Krieg, 2008). We observed histological changes in fibrosarcomas treated with CpG ODN and radiation characterized by increased necrosis and heavy-infiltration of host inflammatory cells, primarily lymphocytes, and granulocytes (Milas et al., 2004). The specific nature of the antitumor rejection response at the primary tumor site and on metastases outside the irradiated field was subsequently investigated (Hart et al., 2008). An abscopal-like tumor model was used in which bilateral tumors in mice were left untreated in one hind leg and treated with radiation, CpG, or the combination in the contralateral leg. CpG ODN elevated systemic cytokine levels of IL-12p40, known to induce activation of NK cells and cytolytic $CD8^+$ T cells, and IL-10, suggesting induction of antitumor antibody production. Compared to radiation alone, increased numbers of $CD11c^+$ and $CD8^+$ cytolytic T cells were found within the tumor draining lymph nodes following combined treatment with CpG ODN 1826 and local tumor irradiation. Enhanced local tumor control was accompanied by a measurable decrease in tumor burden at distant sites. A more recent study showed that fractionated (but not single-dose) radiotherapy induced an immune-mediated abscopal effect when combined with anti-CTLA-4 antibody in two preclinical rodent tumor models (Dewan et al., 2009).

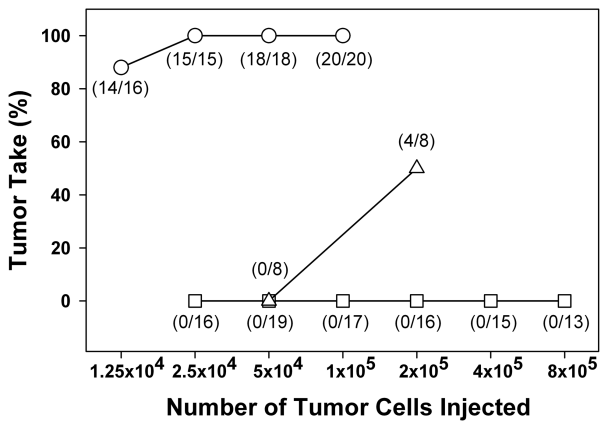

FIGURE 2 | Resistance of cured mice to reinoculation of tumor cells. Mice cured of their primary tumor after irradiation alone (Δ) or after treatment with CpG ODN 1826 plus irradiation (□) were reinoculated with FSa tumor cells 100–120 days after local tumor irradiation. Age-matched untreated mice were used as controls (○). Mice were injected s.c. on the abdomen with graded doses of FSa tumor cells and tumor takes observed for up to 2 months after inoculation. *Numbers in parentheses,* tumor takes over total injection sites. Reprinted by permission from the American Association for Cancer Research (Mason et al., 2005).

Studies by other investigators suggested that CpG ODN induces antigen-specific antitumor T cell responses and activation of dendritic cells promoting strong immune memory responses (Shah et al., 2003). We hypothesized that when radiotherapy is given after CpG ODN injection, tumor antigens released from dying cells are taken up by activated dendritic cells, leading to induction of a tumor-specific T cell response. Others proposed that *in situ* tumor destruction by combination therapy may create a unique "*in situ* dendritic cell vaccine" (den Brok et al., 2006; Jahrsdorfer and Weiner, 2008). Radiotherapy has been reported to potentiate therapeutic efficacy of intratumoral dendritic cell vaccination (Teitz-Tennenbaum et al., 2008). Other possible mechanisms underlying the therapeutic efficacy of radiation with CpG ODNs include altered expression of critical molecules involved in immune recognition and killing by T cells; direct radiation damage to and killing of tumor cells, increased vulnerability of surviving cells to immune attack; or radiation-induced suppression of mechanisms inhibiting antitumor responses (Koski and Czerniecki, 2005). Subsequent investigations supported the theory that an immunoadjuvant effect of tumor cell death is an important aspect of radiotherapy response (Apetoh et al., 2007a). Radiation can promote changes in the tumor microenvironment that may enhance infiltration and activation of immune cells that have potential to influence tumor responses (Shiao and Coussens, 2010). Radiation was shown to up-regulate expression of CXL16 in tumors and to enhance recruitment and activation of $CD8^+$ T cells (Matsumura et al., 2008; Matsumura and Demaria, 2010). Expression of MHC 1, important in antitumor T cell responses, was increased in a murine melanoma after irradiation (Lugade et al., 2005). Secretion of HMGB1 protein by lethally irradiated tumor cells and its effect on danger signaling was important in promoting antigen presentation (Apetoh et al., 2007b). Calreticulin exposure on the cell surface was shown to be required for the immunogenicity of radiation-induced apoptosis (Obeid et al., 2007; Formenti and Demaria, 2008).

Previously, we observed that enhancement of tumor radioresponse induced by CpG ODN 1826 was largely dependent on

host immunocompetence (Milas et al., 2004). CpG ODN 1826 treatment of mice immunocompromised by sublethal whole-body irradiation caused only modest radiation-induced tumor growth delay of immunogenic fibrosarcoma, and the curative effect was lost. Since human tumors are generally considered to be weakly immunogenic, we tested the effect of CpG ODN 1826 on radioresponse of a non-immunogenic murine fibrosarcoma (Mason et al., 2005). CpG ODN enhanced radiation-induced tumor growth delay of non-immunogenic tumors when the ODN was injected s.c. (EF 1.41) or intratumorally (EF 1.73). Thus, in addition to being effective against the highly immunogenic fibrosarcoma, CpG ODN 1826 improved the radioresponse of a non-immunogenic tumor.

Several other animal tumor models have since shown response to combined therapy with CpG ODN and radiation. Treatment with CpG ODN and radiation-induced tumor remission in two-thirds of rats inoculated with 9L glioma (Meng et al., 2005). The combination treatment also enhanced tumor growth delay of s.c. B16F1 tumors (Cerkovnik et al., 2009). CpG ODN 1826 enhanced radiation-induced growth delay of Lewis lung cancer in mice and enhanced the apoptotic index in tumors given combined treatments compared to either treatment alone (Yuan et al., 2011). The combination of radiation with a CpG-based tumor vaccine significantly inhibited established LLC-OVA-carcinomas and cured about 60% of treated mice (Chamoto et al., 2009).

CLINICAL TRIALS WITH CpG ODNs AND RADIOTHERAPY

Results with preclinical models suggested that CpG ODN would be more useful when combined with other therapeutic approaches in the treatment of cancer rather than as monotherapy (Krieg, 2006; Jahrsdorfer and Weiner, 2008). Although positive preclinical results are not necessarily predictive of clinical outcome, our findings provide compelling evidence that CpG ODN in combination with conventional radiotherapy is a strong candidate for clinical testing. Mice and humans have different TLR9 expression patterns, and so exposure to CpG motifs stimulates a narrower profile of cytokines/chemokines in humans than in mice (Krieg, 2008). Clinical trials are necessary to confirm the synergy between CpG ODNs and radiotherapy that was evident in preclinical testing.

Early clinical reports showed CpG 7909 was an effective and well-tolerated adjuvant for improving vaccine responses (Cooper et al., 2004a,b). Minor side effects were mild to moderate injection-site reactions and transient flu-like symptoms (Cooper et al., 2004a,b; Krieg, 2006). Key preclinical studies by Levy and colleagues led to development of therapeutic vaccination strategies for clinical treatment of lymphoma (Li et al., 2007; Houot and Levy, 2009; Brody et al., 2011; Goldstein et al., 2011). Combination of intratumoral CpG with cytotoxic therapy induced tumor-reactive CD8 T cells and cured primary subcutaneous and widely metastatic murine lymphomas (Li et al., 2007). Combination of intratumoral CpG and immunomodulatory T cell antibodies increased antitumor efficacy of CpG without the need for chemotherapy (Houot and Levy, 2009). A CpG-loaded tumor cell vaccine induced CD4 T cell-mediated antitumor immunity leading to regression of established murine lymphoma (Goldstein et al., 2011). A recent phase I/II clinical trial of low grade B cell lymphoma was based on the rationale that intratumoral CpG given with localized low dose radiation could be effective therapy for the primary tumor and produce immune-mediated abscopal effects (Brody et al., 2010). The *in situ* vaccination strategy with CpG ODN (PF-3512676) was well-tolerated and induced systemic antitumor responses even in patients with significant tumor burden (Brody et al., 2010). Encouraging preliminary results were also achieved in a parallel phase I/II study using a similar *in situ* vaccination strategy combined with radiation in patients with T cell lymphoma mycosis fungoides skin lesions (Kim et al., 2012).

CONCLUSION

Treatment of mice bearing established immunogenic or non-immunogenic tumors with CpG ODN 1826 markedly enhanced response to single-dose and fractionated radiotherapy, likely through immune-mediated mechanisms. CpG ODN also induced a durable systemic immune memory response against subsequent rechallenge with tumor cells. These observations suggest CpG ODN could be used not only as an "immunosensitizer" in combination with radiotherapy but also as an adjuvant to prevent or reduce metastatic disease at sites distant from the primary irradiated tumor. These findings and others have demonstrated that CpG ODNs can be given in combination with conventional radiotherapy to improve therapeutic efficacy. Further studies are warranted to elucidate the dynamic interplay between tumor irradiation and the host immune system to facilitate translation to clinical trials. Our studies using CpG ODNs as radiation enhancing agents are being supplemented by new integrated approaches proposing a partnership between radiotherapy and immunotherapy designed to capitalize on radiation's ability to enhance immunogenicity of the primary tumor and its microenvironment (Demaria et al., 2005; Formenti, 2010; Shiao and Coussens, 2010; Haynes and Smyth, 2012).

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Nanovectorized radiotherapy: a new strategy to induce anti-tumor immunity

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Recent experimental findings show that activation of the host immune system is required for the success of chemo- and radiotherapy. However, clinically apparent tumors have already developed multiple mechanisms to escape anti-tumor immunity. The fact that tumors are able to induce a state of tolerance and immunosuppression is a major obstacle in immunotherapy. Hence, there is an overwhelming need to develop new strategies that overcome this state of immune tolerance and induce an anti-tumor immune response both at primary and metastatic sites. Nanovectorized radiotherapy that combines ionizing radiation and nanodevices, is one strategy that could boost the quality and magnitude of an immune response in a predictable and designable fashion. The potential benefits of this emerging treatment may be based on the unique combination of immunostimulatory properties of nanoparticles with the ability of ionizing radiation to induce immunogenic tumor cell death. In this review, we will discuss available data and propose that the nanovectorized radiotherapy could be a powerful new strategy to induce anti-tumor immunity required for positive patient outcome.

Keywords: anti-tumor immunity, nanoparticle, radionuclides, biomaterials, active targeting

INTRODUCTION

The Janus face of the immune system in carcinogenesis has long been controversial and one of the most challenging in immunology. With progress in biological tools such as transgenic mouse technologies, it is now recognized that the immune system plays a dual role in cancer. For instance, it suppress tumor progression by identifying and destroying neoplastic cells (Dunn et al., 2002; Schreiber et al., 2011) but also promotes tumor growth by selecting tumor cells more adept at evading immune-mediated destruction (Khong and Restifo, 2002; Smyth et al., 2006; Zitvogel et al., 2006; Vesely et al., 2011) leading to the establishment of an immunosuppressive microenvironment that fosters carcinogenesis (Radoja et al., 2000; Whiteside, 2008). However, the host immune system not only impacts on cancer development but also on response to treatment. Experimental evidence strongly supports the concept that the activation of the immune system is essential for successful chemo- and radiotherapy (Casares et al., 2005; Apetoh et al., 2007b; Obeid et al., 2007a,b; Zitvogel et al., 2008). By improving the quality of released signals, some conventional treatments trigger a peculiar type of cell death that elicits a potent anti-tumor immune response required for positive patient outcome (Zitvogel et al., 2008). Called "immunogenic cell death" (ICD), this type of tumor cell death is defined by at least three signals: calreticulin (CRT) exposure (Obeid et al., 2007b; Zitvogel et al., 2010), release of high mobility group box-1 (HMGB-1; Apetoh et al., 2007a,b), and ATP (Ghiringhelli et al., 2009; Martins et al., 2012). Among all current available treatments, only radiotherapy (Chakraborty et al., 2004), anthracyclines (Casares et al., 2005; Mattarollo et al., 2011), oxaliplatin (Panaretakis et al., 2009; Tesniere et al., 2010), and cyclophosphamide (Schiavoni et al., 2011) have been shown to generate these signals in the proper spatiotemporal order leading to an *in situ* tumor vaccine (Ma et al., 2010; Hannani et al., 2011).

Therefore, conventional treatments could be used not only for their cytocidal effects but also for their ability to induce anti-tumor immunity. This idea extends far beyond treatments that already exhibit pro-immunogenic effects since envisioning the use of immune response modifiers (IRM) to optimize the synergy with the immune system offers great opportunities to provide alternative ways of tumor-specific immunity (Schiller et al., 2006; Cheever et al., 2008). For instance, Demaria and colleagues demonstrated significant increase in treatment efficiency when radiotherapy is combined with anti-cytotoxic T lymphocyte antigen-4 (CTLA-4; Demaria et al., 2005; Matsumura et al., 2008; Dewan et al., 2009; Pilones et al., 2009), a monoclonal antibody that blocks CTLA-4 receptor well-known to be implicated in immune tolerance (Peggs et al., 2006; O'Day et al., 2007).

In consideration of this emerging vision, the ability of anti-cancer strategies to induce anti-tumor immunity has to be investigated. Among new treatment approaches, internal radiotherapy using nanoparticles (NPs) holds great promise for the management of refractory tumors (Allard et al., 2008; Vanpouille-Box et al., 2011b). Primarily designed to focus radiation to a specific target while protecting healthy tissues from radiation, nanovectorized radiotherapy has been shown to elicit anti-tumor immunity in a preclinical model of glioblastoma (Vanpouille-Box et al., 2011a). This new treatment concept is based on the use of NPs as reservoir for radionuclides enabling

the entrapment of alpha (α) and beta (β) emitters conferring them different ways to directly kill tumor cells as well as distinct interactions with the microenvironment (Ting et al., 2010). The NP itself can also be designed to have properties of an IRM able to modify and improve the immune response through the use of peculiar biomaterials and/or surface ligands. Therefore, nanovectorized radiotherapy that combines ionizing radiation and nanodevices, is one therapy that could boost the quality and magnitude of an immune response in a predictable and designable fashion. Given the novelty of nanomedicines application, only a few studies analyzed NP's adjuvant effect on the host's innate and adaptive immune response. In this review, we will discuss available data and propose that the nanovectorized radiotherapy could be a powerful new strategy to induce anti-tumor immunity required for successful anti-cancer treatment.

NANOPARTICLE: A NEW KIND OF IMMUNE RESPONSE MODIFIER

The ideal anti-cancer treatment would be the one capable of reducing and eliminating tumors without causing any damage to surrounding healthy tissues. In that context, over the past two decades, nanotechnology-based approaches have emerged as a promising field that aims at overcoming limitations encountered in conventional anti-cancer treatments. Numerous nanodevices have been engineered using top-down or bottom-up approaches, generally ranging in dimensions from one to a few hundred nanometers in at least one dimension (Perry et al., 2011). NPs can be designed to carry therapeutics drugs (chemo- or radio-therapeutics) loaded on or within the nanocarriers by chemical conjugation or simply by encapsulation (**Figure 1**; Sengupta et al., 2005; Vanpouille-Box et al., 2011b; Vrignaud et al., 2011). Therefore, NPs have the ability to improve stability of encapsulated drug as compared to free entities and release in a more controlled manner over time to maintain anti-cancer agents within a therapeutic window (Amstad and Reimhult, 2012). Additionally, their flexible chemical properties allow NP surface modifications to increase their blood circulation half-life and improve their biodistribution profile. For instance, NP can be functionalized with polyethylene glycol (PEG) in order to generate a steric barrier on the surface preventing adherence of opsonins to the NP and therefore reducing their clearance by the reticuloendothelial system (RES; Otsuka et al., 2003; Yoncheva et al., 2005).

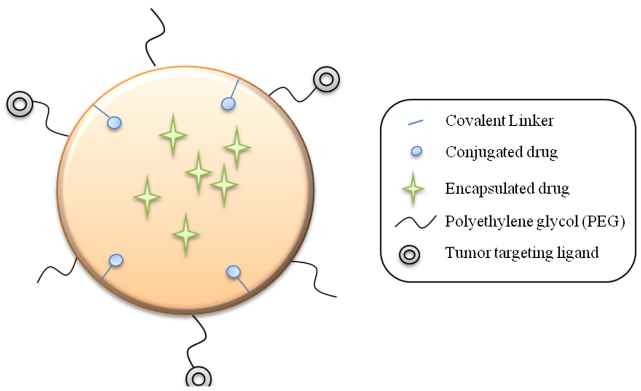

FIGURE 1 | Schematic nanoparticle.

A wide range of nanodelivery systems are currently in development. NPs can be composed of natural (Liu et al., 2011; Tavangar et al., 2011) or synthetic (Powell et al., 2011), and degradable (Huynh et al., 2009) or non-degradable polymers (Peek et al., 2008). The choice of components that constitute the nanodevice is critical as it considerably influence the NPs properties. For instance, the drug release profile can be tuned by the size and material composition of the NP (Paillard et al., 2010). Additionally, the NP is amenable to surface modifications (Brannon-Peppas and Blanchette, 2004; Fahmy et al., 2005; Weiss et al., 2007; Beduneau et al., 2008; Byrne et al., 2008; Hirsjarvi et al., 2011; Talekar et al., 2011) providing them targeting properties to reach specifically an organ or even a specific cell (Weissleder et al., 2005; Beduneau et al., 2008; Gu et al., 2008; Talekar et al., 2011). With this unique ability, NPs can easily be engineered to precisely synergize with the immune system and be considered as a powerful "smart" IRM designed to reach a specific location and to interact with specific cells.

As a result, we will discuss each steps that could be harnessed in NP's designing to interact with the immune system in a predictable fashion, that are (1) the choice of biomaterials that composed the NPs, (2) the proper size and charge of NPs to better synergize with the host, and (3) the possible use of ligand on NPs surface to specifically target immune or tumor cells.

IMMUNE ADJUVANT PROPERTIES OF NANOPARTICLES COMPONENTS

The main goal of immunotherapy-based strategy is to harness immune system not only to fight cancer by targeting and killing tumor cells in a specific manner, but also to alert the immune system so that the residual tumor cells are kept in check. Active forms of immunotherapy, including cancer vaccines, represent one of the promising strategies. These approaches aims at inducing the activation and expansion of tumor-specific T cells, which have proven to be the most powerful immune mechanism to clear tumors (Porter et al., 2011).

Many efforts have focused on enhancing cross-presentation, a process mediated by antigen presenting cells (APCs) that are defined as cells that can process antigens of both endogenous and exogenous origin (Trombetta and Mellman, 2005). Endogenous antigen (such as normal cell proteins, tumor or viral antigens) are processed in the cytosol and presented in the context of major histocompatibility complex (MHC) class I molecules to be recognized by $CD8^+$ T cells (**Figure 2**; Itano and Jenkins, 2003) leading to strong cytolytic and Th1 inflammatory responses. APCs are also capable to internalize exogenous antigens. The latter are processed in specialized compartments called endocytic vesicles or endosome, and presented through MHC class II molecules to be recognized by $CD4^+$ T cells (**Figure 3**; Watts, 2004). APCs include B cells, macrophages, and dendritic cells (DC). Because of their wide distribution, location at critical sentinel sites (skin and mucosal surfaces), intrinsic migratory capacity, and ability to activate naïve T cells, DCs are considered as the most powerful professional APCs (Itano and Jenkins, 2003; Trombetta and Mellman, 2005; Delamarre and Mellman, 2011). DCs are

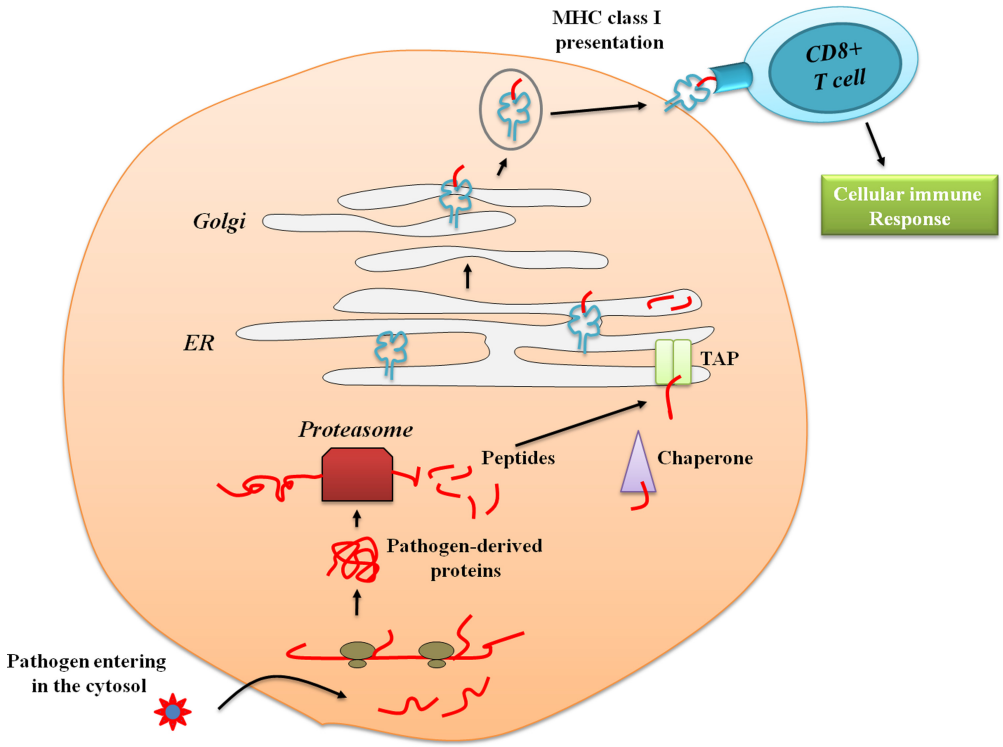

FIGURE 2 | Antigen presentation via major histocompatibility complex class I. Pathogen-derived or self-proteins within the cytosol of antigen presenting cells (APCs) are enzymatically digested into peptides, mainly by cytosolic proteases (proteasomes), and are then transported by transporter associated with antigen processing molecules (TAP) into the endoplasmic reticulum (ER). In the ER lumen, peptides bind to MHC class I molecules, which are subsequently transported via the Golgi to the plasma membrane. The endogenous antigen presented by MHC class I will then be recognized by the $CD8^+$ T cells leading to adaptive cellular immune responses.

indeed capable of processing both exogenous and endogenous antigens and present peptide in the context of either MHC class I or II molecules. As DCs mature, they acquire the properties necessary to form and transport peptide-loaded MHC class II complexes to the cell surface (Cella et al., 1997). Antigen transport to the cell surface is correlated with increased expression of co-stimulatory molecules, such as CD80, CD86, and CD40, molecules well-known to amplify T cell receptor (TCR) signaling and promote T cell activation (Ni and O'Neill, 1997). Given the critical role of DC in eliciting adaptive immune response, efforts have been made to develop new strategies that target and stimulate DCs.

Nanomedicine-based treatments represent one of the main promising approaches since nanoscale drug delivery system could be thought and designed from the beginning to properly interact with the host immune system. For instance, some NPs are able to entrap drug already known to induce ICD (i.e., radionuclide; Sun and Xie, 2011; Tang et al., 2011; Vanpouille-Box et al., 2011a), oxaliplatin (Jain et al., 2010; Paraskar et al., 2012), and cyclophosphamide (Salgueiro et al., 1999), and exhibit biological effects such as endolysosomal escape (Panyam et al., 2002; Paillard et al., 2010) and biological barrier crossing (De Jong and Borm, 2008; Paillard et al., 2010). Among them, NPs of poly(D,L-lactide-co-glycolide) (PLGA) hold great promise and have been extensively studied for their ability to activate DCs for priming antigen-specific T cell responses (reviewed in Hamdy et al., 2011). PLGA is a Food and Drug Administration (FDA)-approved biodegradable polymer that had been widely used in several controlled release drug products for human use (Jain, 2000; Dinarvand et al., 2011; Jain et al., 2011). One of the main characteristic of PLGA relies with its flexibility that allows manipulating its physico-chemical properties. Therefore, PLGA can shift the delivery of encapsulated drugs to either cytoplasm (for MHC class I presentation and $CD8^+$ T cell activation) or to the endosome (for MHC class II and $CD4^+$ T cells activation; Hamdy et al., 2007, 2008; Heit et al., 2007). More recently, PLGA has been shown to activate the NOD-like receptor family pyrin domain containing 3 [NLRP3 also known as cryopyrin, cold-induced autoinflammatory syndrome 1 (CIAS1) or NALP3] inflammasome (Demento et al., 2009; Sharp et al., 2009). It has indeed been demonstrated that cellular internalization of PLGA and polystyrene microparticles activate of the NLRP3 inflammasome through lysosomal damage and caspase-1 activation leading to the production of large amount of IL-1β by DCs (Sharp et al., 2009). The ability of NP's components to directly influence NLRP3 inflammasome is very important since it has been described that NLRP3 inflammasome and subsequent IL-1β secretion is critical for stimulation of anti-tumor T cells responses following chemotherapy (Ghiringhelli et al., 2009; Menu and Vince, 2011).

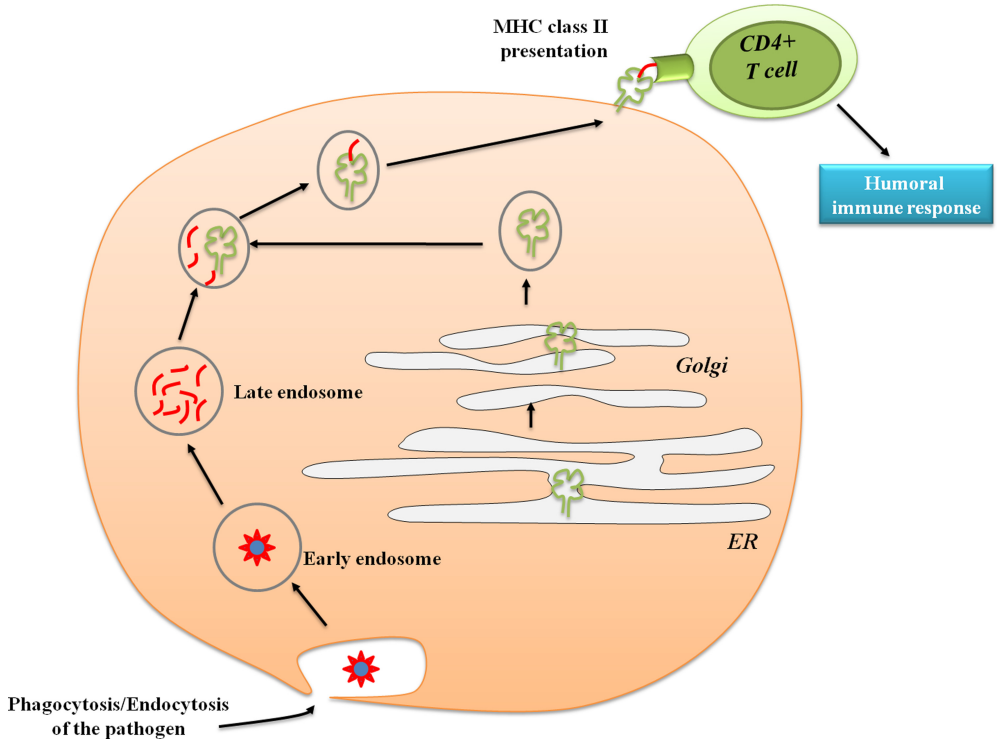

FIGURE 3 | Antigen presentation via major histocompatibility complex class II. Exogenous antigens are derived from proteins that are endocytosed and processed by proteases. Peptides bind to newly synthesized MHC class II molecules in specialized antigen-processing vesicles (MHC class II-enriched compartment), and the complexes are externalized to the plasma membrane. $CD4^+$ T cells will then recognize the exogenous antigen presented by MHC class II leading to the stimulation of CD4 T cells humoral responses.

Poly(D,L-lactide-co-glycolide) is not the only strategy that has been investigated to achieve DC cross-presentation. The use of pH-responsive materials that naturally foster antigen escape from the endosome into the cytosol where MHC class I antigen processing begins has emerged. For instance, Murthy et al. (1999) and Jones et al. (2003) have developed synthetic polymer containing alkyl(acrylic acid) monomers that become protonated at endosomal pH levels (5.5–6.5). Once protonated, the polymers destabilize the endosomal membrane and allow antigen to escape into the cytoplasm (Jones et al., 2003).

Other particle materials can stimulate signaling pathways that lead to cellular activation. Baba and colleagues have shown that poly(gamma-glutamic acid) NPs can be used as a vaccine adjuvant. These NPs induced DC maturation through MyD88-mediated nuclear factor kappa B (NF-κB) activation and the p38 mitogen-activated protein kinase (MAPK) pathways, in a manner somewhat similar to lipopolysaccharide (LPS)-induced maturation of DC (Uto et al., 2007, 2011a,b; Hamasaki et al., 2010). Therefore, NPs components act as immune adjuvant simply by inducing maturation of DC. This concept was also supported by Babensee and colleagues and Elamanchili and colleagues work, showing that exposure of bone marrow derived DC to polymers, notably PLGA, results in DC maturation as measured by the up-regulation of cell surface stimulatory markers such as MHC class II, CD40, CD80 and CD86 (Diwan et al., 2003; Elamanchili et al., 2004; Yoshida and Babensee, 2004, 2006; Yoshida et al., 2007; Babensee, 2008).

Taken together, evidences clearly indicate that nanodevices for targeted delivery of drugs or radionuclides can be composed of biomaterials that possess different immune adjuvant properties. Therefore, the choice of biomaterials to design NPs could provide a potent tool to induce anti-tumor immunity.

INFLUENCE OF NANOPARTICLE SIZE AND CHARGE ON IMMUNE SYSTEM

Another parameter to consider for immunogenic NP designing is the size and the charge of the NP. DCs and macrophages are both phagocytic cells. Hence, particles with dimension similar to pathogens ($\geq$10 μm) are generally readily phagocytosed. Studies have shown that DCs preferentially phagocyte smaller particles in the viral range, while macrophages more efficiently ingest bacterial size particle (Gamvrellis et al., 2004). It has also been reported that NP with a diameter <500 nm were more effective in stimulating cytotoxic T lymphocytes (CTL) responses *in vivo* (Allsopp et al., 1996; Nixon et al., 1996). Possible explanation relies with the interactions of NPs with opsonins. Indeed, larger surface area of the NP allows more opsonins bounding and therefore, a faster degradation and rapid release of the encapsulated drug inside the phagosome (Owens and Peppas, 2006).

Additionally, physico-chemical properties of particle surface, particularly surface charge and surface chemistry, are known to affect both DC uptake and maturation. For instance, positively charged cationic particles in general have greater initial

affinity toward cell surface than negatively charged or neutral particles (Josephson et al., 1999; Foged et al., 2005; Perez-Martinez et al., 2011).

SURFACE MODIFICATION OF NANOPARTICLES

To promote and enhance specific interactions between NP and the microenvironment, the surface of particles can be decorated with targeting moieties that are recognized specifically by targeted cells. Two main strategies can be envisioning: the one that target immune cells and the other one that target tumor cells to kills them and therefore, to provide proper "danger signal" required for immune system activation.

Immune cells targeting

In order to specifically enhance the maturation of DC, Palumbo et al. (2011) bound CD40 ligand (CD40L) on NP's surface. CD40L is indeed transiently expressed on activated $CD4^+$ T helper cells and its binding with the CD40 receptor on DCs is important for their complete maturation and transformation into competent APC (Loskog and Totterman, 2007). However, no significant results have been reported in their studies, suggesting the complexity of conferring immunogenic properties to NPs (Palumbo et al., 2011).

In another study, Dominguez and Lustgarten (2010) engineered immunogenic NPs to induce anti-tumor immune response. They indeed succeeded in binding not only one ligand but two to further stimulate the immune system. By linking anti-neu mAb directed against a tumor antigen and anti-CD40 mAb on NP's surface, they generated an anti-tumor response resulting in tumor rejection with high production of Th1-proinflammatory cytokines, a stark reduction of regulatory T cells within the tumor and activation of specific cytotoxic immune response (Dominguez and Lustgarten, 2010). These recent results strongly support the potential use of biodegradable NPs to stimulate a tumor-specific immune response.

Tumor cell targeting

Specific tumor targeting could indirectly stimulate the immune system if the quantity and the quality of released signal in a specific location (i.e., the tumor) can be achieved. Many active targeting of NPs to tumor has been extensively studied and led notably to the development of NP conjugated with specific ligands that recognize a tumor-surface marker.

Over the past three decades, the generation of murine mAbs against tumor-associated antigens became a focal point of research illustrated by numerous studies being reported during the 1980s that dealt with NPs and mAb binding to their surface (Leserman et al., 1980; Barbet et al., 1981; Harsch et al., 1981; Hashimoto et al., 1983; Guidoni et al., 1984). Since then, a number of clinical trials have demonstrated the feasibility of antibody-based targeting (Bernard-Marty et al., 2006; Yoong et al., 2011; Foran, 2012; Smyth and Cunningham, 2012). Among mAb that were studied, Trastuzumab (or Herceptin®), a mAb that binds to the human epithelial growth factor receptor 2 (HER2), has been bound on NP's surface to specifically target breast cancer cells (Hayes et al., 2006; Kirpotin et al., 2006). This targeting strategy has improved therapeutic efficiency of an HER2-targeted NPs formulation in comparison to its non-target one (Park et al., 2002).

Although antibodies have proven to be effective targeting agents, there are inherent issues such as decreased receptor affinity due to inadequate conjugation methods, insufficient tumor cell penetration, and non-specific binding of antibodies to cellular receptors. In that context, new technologies are currently being explored to enhance the selectivity and efficacy of ligands while attempting to overcome the shortcomings associated with existing targeting moiety. For example, peptides have recently emerged as targeting agent owing to the relative simplicity of synthesis and purification. The integrin family, particularly the αvβ3 integrins, has been widely studied to target cancer cells with NPs. For instance, a synthetic peptide of arginine–glycine–aspartic acid (RGD) residues has been used as a ligand conjugated to NPs for targeting αvβ3 integrins expressed on endothelial cells. Recent studies are further optimizing integrin targeting by engineering novel peptide moieties which bind with better affinity to integrins that current RGD tags (Ji et al., 2012; Xu et al., 2012; Zhan et al., 2012).

Binding bombesin (BBN) synthetic peptides on NP's surface is another targeting strategy in development. BBN peptides are composed with 14 amino acids and present high affinity toward gastrin-releasing peptide (GRP) receptors (Smith et al., 2005) that are overexpressed in many cancer such as prostate (Markwalder and Reubi, 1999; Nagasaki et al., 2012), breast (Chao et al., 2009), and small-cell lung carcinoma (Moody et al., 1985; Oremek and Sapoutzis, 2003). Promising results were reported, notably by Chanda et al. (2009, 2010), which demonstrated that the conjugation of BBN peptides on gold NPs' surface lead to selective uptake of NP-BBN conjugates in prostate tumor sites.

However, NPs targeting strategies are not limited to those two approaches. Conferring targeting properties to NPs was indeed one of the main focuses of nanomedicine (Katsogiannou et al., 2011; Kolhatkar et al., 2011; Talekar et al., 2011). Therefore, a plethora of ways to generate "smart" NPs targeting a specific cell is currently in development which highlights the extreme flexibility of this new technology.

RADIONUCLIDES FOR NANOVECTORIZED RADIOTHERAPY

Conventional radiotherapy (X-rays) is the mainstay adjuvant treatment of cancer. However, the radiation dose to surrounding normal tissues often limits its use and therefore, opened a very challenging research area in radiation oncology: the one that aims at reducing and destroying tumors without causing any damage to healthy tissues.

In that context, new external photon beam radiation therapy modalities have recently been emerged with the development of three-dimensional conformal radiotherapy (3D-CRT)/volumetric-modulated arc therapy (VMAT), helical tomotherapy, intensity-modulated radiotherapy (IMRT), γ-rays (^{60}Co)-knife-therapy, cyber-knife-radiotherapy–radiosurgery with 4D-image-guided tracking and 6D-image-guided stereotactic-radiotherapy, that dynamically synchronize imaging, patient positioning and treatment delivery with a dose escalation. These new approaches allow obtaining more conformal "radio-ablative" treatment of tumor lesions while minimizing the damage to the nearby normal

tissues (Deb and Fielding, 2009; Teoh et al., 2011; Yu and Tang, 2011; Wen et al., 2012).

Another increasing successful radiation technique is the hadron therapy that uses a focus beam of quark-constituted of proton (H^+), carbon ion or neutrons, allowing more precise ionizing radiation delivery. Compared to photons (X-rays and γ-emissions), proton beams are characterized by a low entrance dose while a maximal at a user-defined depth ("Bragg peak") and almost no damage on the exit path. As a result, the chief advantage of proton therapy relies with its ability to precisely localize the radiation dosage compared to other form of external beam radiotherapy (DeLaney, 2011; Liu and Chang, 2011).

These newly developed external either photon- or especially hadron-therapy technologies are becoming more and more competitive, as for precisely target locally confined tumors, with brachytherapy modalities as alternatives options to anyhow carried out surgical approaches.

Radiation brachytherapy with either permanent interstitial implantation or temporary implant has also gained large acceptance in the last decades particularly for the management of prostate cancer (Alberti, 2011; Gomez-Veiga et al., 2012) and cervical cancer (Beddy et al., 2011; Walsh et al., 2011). This internal radiation approach is highly linked to the tumor type and size. For instance, brachytherapy is usually initiated toward the ends of external beam radiation after tumor regression has occurred and allows high doses to be delivered to the residual disease with relative sparing of surrounding normal tissues (Monk et al., 2007).

Another arm of brachytherapy consists in harnessing nanomedicines, such as radiolabeled monoclonal antibodies and/or biomaterial vectors, to generate a localized radiation (Allard et al., 2008; Barbet et al., 2012; Memon et al., 2013). As a result, with the identification of biological target overexpressed in cancer, brachytherapy is no longer limited to a specific tumor. In that context, nanovectorized radiotherapy that combines NPs and ionizing radiation is becoming a potent new radiotherapy approach that also overcomes non-specific radiation. Radioactive NPs have indeed been shown to modify the radiation distribution profile of a radionuclide by avoiding its fast elimination (Vanpouille-Box et al., 2011b) but also by maintaining radiation to a specific location for 96 h after their injection (Vanpouille-Box et al., 2011a). Even if few data regarding radioactive particle loading capacity, specific radioactivity has been shown to be compatible with clinic application (Salem et al., 2002, 2005).

Compared to the newly developed radiotherapy strategies, nanovectorized radiotherapy presents the main advantage of being a low-cost technology by the use of radionuclides eluted from generators easily available, such as the $^{188}W/^{188}Re$ generator (Lepareur et al., 2011). More importantly, radioactive NPs' formulation is simple providing them high availability and accessibility to patient. As a consequence, a spread of this new technology in most of clinical institutions, including those of developing countries, can be envisioned.

Radionuclides that decay by the following three general categories of decay have been studied for therapeutic potential of nanovectorized radiotherapy: beta (β)-particles emitters (yttrium-90, rhenium-188; Li et al., 2004; Tsai et al., 2011), alpha (α)-particles emitters (bismuth 213, astatine-211; Sofou et al., 2004; Couturier et al., 2005; Boskovitz et al., 2009) and auger electron-emitters (iodine-125, gallium-67; Snelling et al., 1995). However, the extreme toxicity of auger particles as well as concerns regarding radioprotection limited their use (Bodei et al., 2003; Milenic et al., 2004). Therefore, we will focus on α- and β-emitters and discuss their main characteristics that may lead to different interactions with the microenvironment.

ALPHA (α) vs. BETA (β) EMITTERS

Particles emitted during atomic decay can be classified as low or high linear energy transfer (LET) radiation. The LET corresponds to the energy released by the radiation over a certain distance (expressed in keV/μm). At absorbed doses that are equivalent to those of low-LET radiation, high-LET particles are more cytotoxic. This phenomenon is called "radiation quality." Most of the radionuclides used in internal radiotherapy; such as iodine-131 (Grunwald and Ezziddin, 2010; Leahy and Turner, 2011), yttrium-90 (Kulik et al., 2008; Menda et al., 2010; Kunikowska et al., 2011), lutetium-177 (Gains et al., 2011; Kunikowska et al., 2011), ^{188}Re (Kumar et al., 2007; Torres-Garcia et al., 2008), or rhenium-186 (Syed et al., 2006; van Dodewaard-de Jong et al., 2011); emit low-LET radiation of 0.2 keV/μm in the form of β-particles as well as internal conversion electrons (Milenic et al., 2004). High-LET particle emitters used in internal radiotherapy only include the α-emitters bismuth-213, bismuth-212, and astatine-211, as well as lead-212 and actinium-225, which generate bismuth-212 and bismuth-213, respectively. These radioisotopes emit high-LET radiation (60–230 keV/μm) that produces clusters of DNA damage that are difficult to repair.

Linear energy transfer is intimately linked to the energy carried by a particle and the depth it penetrates into the biological tissue. Therefore, β-particles carry intermediate energy (0.50–2.30 MeV) but have a long range in tissues (1–12 mm of tissue penetration). This lengthy range reduces the need for cellular internalization and so targeting close to or at the cell membrane is sufficient. Additionally, the range of β-particles, as compared to the diameter of cells, allows them to traverse clusters of cells (from 10 to 1,000 cells; O'Donoghue et al., 1995).

Alpha-particles have a high energy (5–8 MeV) and an intermediate path length (50–80 μm) in biological tissues that corresponds to the diameter of several cells (2–10 cells).

Beta-emitters and alpha-emitters are produced either by cyclotron irradiation or by reactor irradiations, incorporated into a generator, and subsequently eluted (Haddad et al., 2008; Halime et al., 2009; Bakht and Sadeghi, 2011; Pillai et al., 2012). For therapeutic application, numerous criteria have to be considered while selecting a radionuclide. Therefore, regarding the tumor size, the advantage of a type of radiation decay will be preferably used in a specific application. For instance, β-particles will be more suitable radionuclides for solid tumors because of their ability to deposit a large amount of energy at a high dose rate. However, other criteria have to be considered for clinical applications: (1) availability of the radionuclide at a reasonable cost, (2) proper nuclear decay properties and absence of hindering daughter nuclides, and (3) a physical half-life long enough to allow internal radiotherapy. As a consequence, among all radionuclide available, only a few are currently developed for nanovectorized radiotherapy

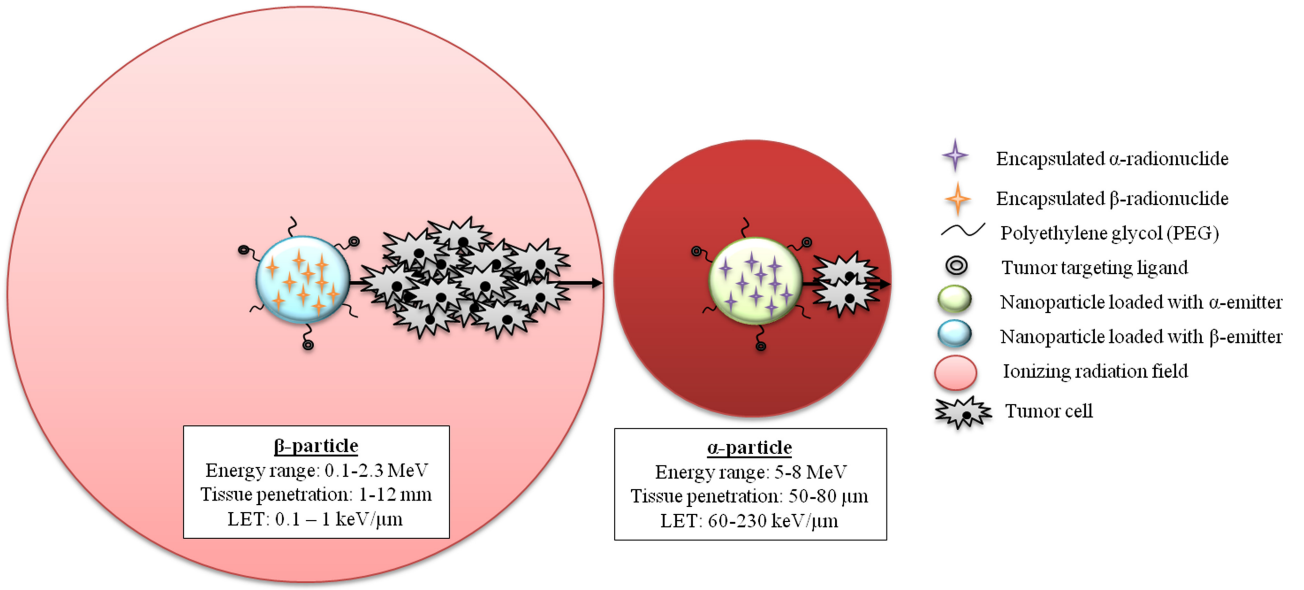

FIGURE 4 | Schematic depiction of nanoparticles loaded with beta- and alpha-emitters.

(Sofou et al., 2004; Allard et al., 2008; Hamoudeh et al., 2008; Bult et al., 2010; Vanpouille-Box et al., 2011a,b). Explanations can mainly be ascribed to the variable pertaining to their physico-chemical properties and to their chemistry that could be somewhat complex according to the NP used.

It is well-established that the radiobiology of high-LET radiation differs greatly from that of low-LET radiation (Goodhead et al., 1993). For instance, increase mRNA expression of inflammatory mediators and cytokines [e.g., interferon-γ (IFNγ)] that prompt immune responses has been identified in lymphocytes after their exposure to low-LET radiation (Amundson et al., 2000, 2004; Kang et al., 2003). In this respect, we can suppose that APCs are able to detect radiolytic products that lead to the production of cytokines such as IFNγ, well-known to be implicated in adaptive immune response (Schoenborn and Wilson, 2007). An increased expression of genes coding for CD1C, CD1D, CD40, CD69, and IFNγ in lymphocytes after α-radiation exposure has been reported (Turtoi et al., 2010). Turtoi and Schneeweiss (2009) and Turtoi et al. (2010) indeed showed that a number of rapidly modulated early response genes in α-particle-irradiated lymphocytes that are associated with DNA repair and immune response mechanisms. However, the current knowledge of the biology of high-LET radiation is insufficient to make definite conclusions.

EFFECT OF THE NANOVECTORIZED RADIOTHERAPY ON IMMUNE SYSTEM ACTIVATION

Immunotherapies are rarely effective as monotherapy but growing evidence supports a synergy between radiotherapy and IRM (Demaria et al., 2005; Dewan et al., 2009; Formenti and Demaria, 2009; Pilones et al., 2009; Newcomb et al., 2010). Among emerging new approaches, nanovectorized radiotherapy holds great promises as a new powerful anti-cancer treatment that could harness immunogenic properties of both NPs and ionizing radiations. Supporting this concept, we recently demonstrated that NPs loaded with rhenium-188, a β-emitter, are potent stimulators of tumor-specific immune response resulting in tumor rejection with high production of IFNγ cytokine, increase recruitment of immune effector T cells within the tumor and memory response in long-term survivor animals (Vanpouille-Box et al., 2011a). Intriguingly, remarkable survival benefit was only seen when two different types of stereotactic injections were used suggesting that the distribution of NP loaded with rhenium-188 within the tumor has a direct impact on the treatment efficiency. Therefore, the use of radionuclide within NP could provide additional advantages as compared to conventional radiotherapy where the distribution of ionizing radiation is homogenous.

Much work remains to be done to determine the effects of both low-LET (β-emitters) and high-LET (α-emitters) emitters on the host immune system. Nevertheless, the capability of NPs to entrap α- and β-radionuclides potentially provides additional means to fine tune the microenvironment interactions (**Figure 4**). Further investigations are required to better understand the interactions between ionizing radiations and the host immune system. Nevertheless, the potential benefits of nanovectorized radiotherapy may be based on the unique combination of immune-stimulatory NP with the ionizing radiation ability to induce an immunogenic tumor cell death.

CONCLUSION

In summary, NPs represent a potent immune adjuvant able to mimic, enhance, stimulate, and interact with the host immune system especially at the level of DCs. Although PLGA's immune effects have been studied in some details, other biomaterials used to produce NP may have different chemical properties that affect immune cells. Given the considerable variety of biomaterials that

can be used to design NPs, further investigations that aim at identifying the immune stimulant abilities of NP's components are required. This could be very critical to develop personalized nanomedicine that aims to induce anti-tumor immunity in a predictable and desirable fashion. Similar to the immune system itself, nanodevices present tremendous flexibility and plasticity and could be therefore considered as an IRM platform capable to be tailored according to the desired application. Their unique abilities to encapsulate a high payload of radionuclide; notably high-LET α-particles and low-LET β-emitters; and to undergo surface modifications, further support their strong potential as a new anti-cancer strategy enable to induce effective anti-tumor immunity.

Much remains to be learned about the effect of nanovectorized radiotherapy but initial data showing that the delivery of ionizing radiation via NPs can be effective at inducing anti-tumor immunity suggest that this new approach warrants further investigations.

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In the field: exploiting the untapped potential of immunogenic modulation by radiation in combination with immunotherapy for the treatment of cancer

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Radiation has long been the standard of care for many types of cancer. It is employed to locally eradicate tumor cells as well as alter tumor stroma with either curative or palliative intent. Radiation-induced cell damage is an immunologically active process in which danger signals are released that stimulate immune cells to phagocytose and present locally released tumor-associated antigens (TAAs). Recent studies have indicated that radiotherapy can also alter the phenotype of cancer cells that remain after treatment. These cells upregulate TAAs as well as markers, including major histocompatibility complex and costimulatory molecules, that make them much more immunostimulatory. As our understanding of the immunomodulatory effects of radiation has improved, interest in combining this type of therapy with immune-based therapies for the treatment of cancer has grown. Therapeutic cancer vaccines have been shown to initiate the dynamic process of host immune system activation, culminating in the recognition of host cancer cells as foreign. The environment created after radiotherapy can be exploited by active therapeutic cancer vaccines in order to achieve further, more robust immune system activation. This review highlights preclinical studies that have examined the alteration of the tumor microenvironment with regard to immunostimulatory molecules following different types of radiotherapy, including external beam radiation, radiolabeled monoclonal antibodies, bone-seeking radionuclides, and brachytherapy. We also emphasize how combination therapy with a cancer vaccine can exploit these changes to achieve improved therapeutic benefit. Lastly, we describe how these laboratory findings are translating into clinical benefit for patients undergoing combined radiotherapy and cancer vaccination.

Keywords: radiation therapy, cancer immunotherapy, cancer vaccine, abscopal effect

RATIONALE FOR COMBINING RADIATION AND IMMUNOTHERAPY

Radiation therapy (RT) is an integral component of cancer care. A recent article in the *Journal of Clinical Oncology* reported that the demand for RT during the initial course of cancer treatment is expected to increase by 22% (from 470,000 patients receiving RT in 2010 to 575,000 in 2020) as a result of the aging and diversification of the U.S. population (Smith et al., 2010). Depending on the presentation and site of disease, RT can have either a curative or palliative intent. In the traditional view, ionizing radiation causes cancer cell death through irreparable DNA damage, which results in apoptosis or failure to progress through the cell cycle. An additional consequence of RT that has sparked significant interest is its effects on cells not killed by RT and the resulting impact on the immune system. Here, we review the immunogenic nature of radiation in preclinical models as well as in the clinic. We also provide a rationale for combining RT with immunotherapeutic approaches.

Several studies have shown the various mechanisms by which RT stimulates the immune system. One vital by-product of radiation damage to tumors is the exposure of a large amount of tumor antigens, in the form of necrotic and apoptotic tumor cells and cellular debris, to the immune system (Melcher et al., 1999; Chen et al., 2001; Kotera et al., 2001). The increased availability of released tumor-associated antigens (TAAs) for uptake by circulating dendritic cells (DCs) and other antigen-presenting cells (APCs) can result in tumor-specific immune attack. One report confirmed that irradiating tumors expressing low levels of antigen caused sufficient release of antigen to sensitize tumor stromal cells to destruction by cytotoxic T lymphocytes (CTLs; Zhang et al., 2007). In addition to causing the release of TAAs, RT also creates an inflammatory milieu by inducing the expression of several proinflammatory cytokines, including IL-1β and TNF-α (Hallahan et al., 1989; Ishihara et al., 1993; Hong et al., 1999; Demaria et al., 2005). Increased expression of these cytokines has been linked to tumor regression, growth inhibition, and tumor-cell death. Furthermore, upregulation of major histocompatibility complex (MHC) molecules, costimulatory molecules, adhesion molecules, and death receptors in tumor cells, surrounding stroma, and vascular endothelium following irradiation can also potentiate $CD8^+$ cytolytic responses (Friedman, 2002; McBride et al., 2004; Demaria et al.,

2005; Nesslinger et al., 2007). Similarly, radiation-induced damage can upregulate expression of vascular cell adhesion molecule 1 (VCAM-1) on tumor vessels, thus facilitating T cell migration (Lugade et al., 2005). Cytokine release by irradiated tumor cells can also increase T cell infiltration into the tumor microenvironment (Matsumura et al., 2008). Other reports have focused on the release of "danger" signals in response to ionizing radiation, which may link initial non-specific immune responses to the development of specific adaptive immunity (McBride et al., 2004). Two such signals that can promote antitumor immune responses after irradiation include the translocation of calreticulin to the cell surface (Obeid et al., 2007) and the release of high-mobility group box 1 (HMGB1) by dying tumor cells, which can activate DCs through Toll-like receptor 4 (Apetoh et al., 2007).

Although the most common form of RT, external beam radiation therapy (EBRT), is conventionally administered in fractionated doses, it is unclear what the optimal dose schedule for EBRT should be when it is combined with immunotherapy. Recent studies have focused on the importance of the dose and fractionation of EBRT in modulating the immune system in order to answer this question. As opposed to conventional RT, stereotactic body radiotherapy takes advantage of technological advances to allow for highly precise administration of ablative doses of RT to tumors, while avoiding damage to the surrounding organs. Lee et al. (2009) used a murine model to show that doses of RT (15–25 Gy $\times 1$ fraction) alone generated robust $CD8^+$ T cell-dependent immunity that led to tumor reduction, reduced relapse of primary tumor, and eradication of metastasis in some settings. This group concluded that the fractionation and dose schedule examined successfully disrupted physical and immunologic barriers, introduced danger signals, increased DC cross-presentation of tumor antigen, and possibly reversed T cell unresponsiveness in tumor-bearing hosts, leading to the rejection of local and distal tumors. In a similar study, mice bearing OVA-expressing B16-F0 tumors that were treated with a total dose of 15 Gy of localized RT delivered in a single fraction had enhanced APC trafficking to draining lymph nodes and greater capability to present tumor antigens compared to non-irradiated mice. This led to increased numbers of tumor-specific T cells that secreted IFN-γ upon peptide stimulation within tumor-draining lymph nodes and improved lysis of tumor-cell targets (Lugade et al., 2005). A report by Schaue et al. (2011) not only reinforced the importance of dose and fractionation, but also highlighted the delicate balance between the immunostimulatory and immunosuppressive effects of radiation. In this study, mice bearing B16-OVA murine melanoma were treated with up to 15 Gy of radiation, given in various size fractions. Subsequent observation of tumor growth revealed that after single doses, tumor control increased with the size of radiation dose, as did the number of tumor-reactive T cells. However, this was offset at the highest dose by an increase in regulatory T cells (Tregs), which are known to suppress tumor-specific immunity (Nishikawa and Sakaguchi, 2010). Fractionated treatment with medium-size radiation doses of 7.5 Gy/fraction resulted in the best tumor control and tumor immunity, while maintaining low Treg numbers (Schaue et al., 2011). Taken together, these results indicated that greater doses of RT delivered in fewer fractions can generate tumor-specific immune responses similar to that of lower doses given more frequently, although a threshold level above which the balance shifts toward immunosuppression may exist. Interestingly, preclinical studies suggest that modalities of RT other than EBRT are able to modulate tumor phenotype and enhance T cell-mediated killing. These modalities include bone-seeking radionuclides, radiolabeled monoclonal antibodies (mAbs), and brachytherapy, all of which will be discussed later in this review.

In addition to the preclinical data presented above, there is substantial clinical evidence of radiation-induced immune activation. Nesslinger et al. (2007) evaluated pre- and post-treatment serum samples from 73 men with non-metastatic prostate cancer and described the development of treatment-associated autoantibody responses in nearly 14% of patients treated with EBRT and 25% who received brachytherapy, compared with 0 of 14 patients who underwent radical prostatectomy. In agreement with their preclinical findings, Schaue et al. (2008) observed that tumor-specific T cells clearly increase in most colorectal cancer patients after completion of chemoradiation therapy and in most prostate cancer patients after RT. Of note, levels of Tregs increased in colorectal cancer patients following treatment, again suggesting a potential threshold above which immunosuppressive effects may dominate. In a recent case report published in *The New England Journal of Medicine*, a patient suffering from metastatic melanoma with disease progression on ipilimumab (IPI, Yervoy; Bristol-Myers Squibb), a mAb that inhibits CTL-associated antigen 4 (CTLA-4), an immunologic checkpoint on T cells, showed a favorable response only after receiving local RT for a metastatic spinal lesion (Postow et al., 2012). The patient experienced out-of-field tumor shrinkage, with antibody responses to tumor-specific antigens, changes in peripheral-blood immune cells, and increases in antibody responses to other antigens. These findings highlight a rare but important phenomenon known as the abscopal effect, where local RT elicits a systemic response and causes tumor regression at a site distant from the irradiated field. The abscopal effect has also been reported in tumors other than melanoma, such as lymphomas, hepatocellular carcinoma, and certain adenocarcinomas (Ehlers and Fridman, 1973; Antoniades et al., 1977; Rees and Ross, 1983; Ohba et al., 1998).

Taken together, these data indicate that RT effectively stimulates immune responses by increasing the production of inflammatory cytokines, causing the release of large amounts of tumor antigen, enhancing antigen processing and presentation, improving T cell migration to sites of disease, and activating tumor-specific CTLs (**Figure 1**). As described above, this activation may translate into both local and systemic clinical benefit. Nevertheless, a large tumor burden often creates enough immune suppression to prevent successful immune intervention. In this case, studies have proposed that local RT can also sufficiently reduce tumor burden to allow for further therapeutic intervention by immunotherapy, such as vaccination or blockade of inhibitory molecules, and, in some cases, may synergize with such therapy (Kamrava et al., 2009). By enhancing the frequency, magnitude, and character of the immune responses induced by RT with immunomodulatory agents, cancer patients could experience further improved outcomes.

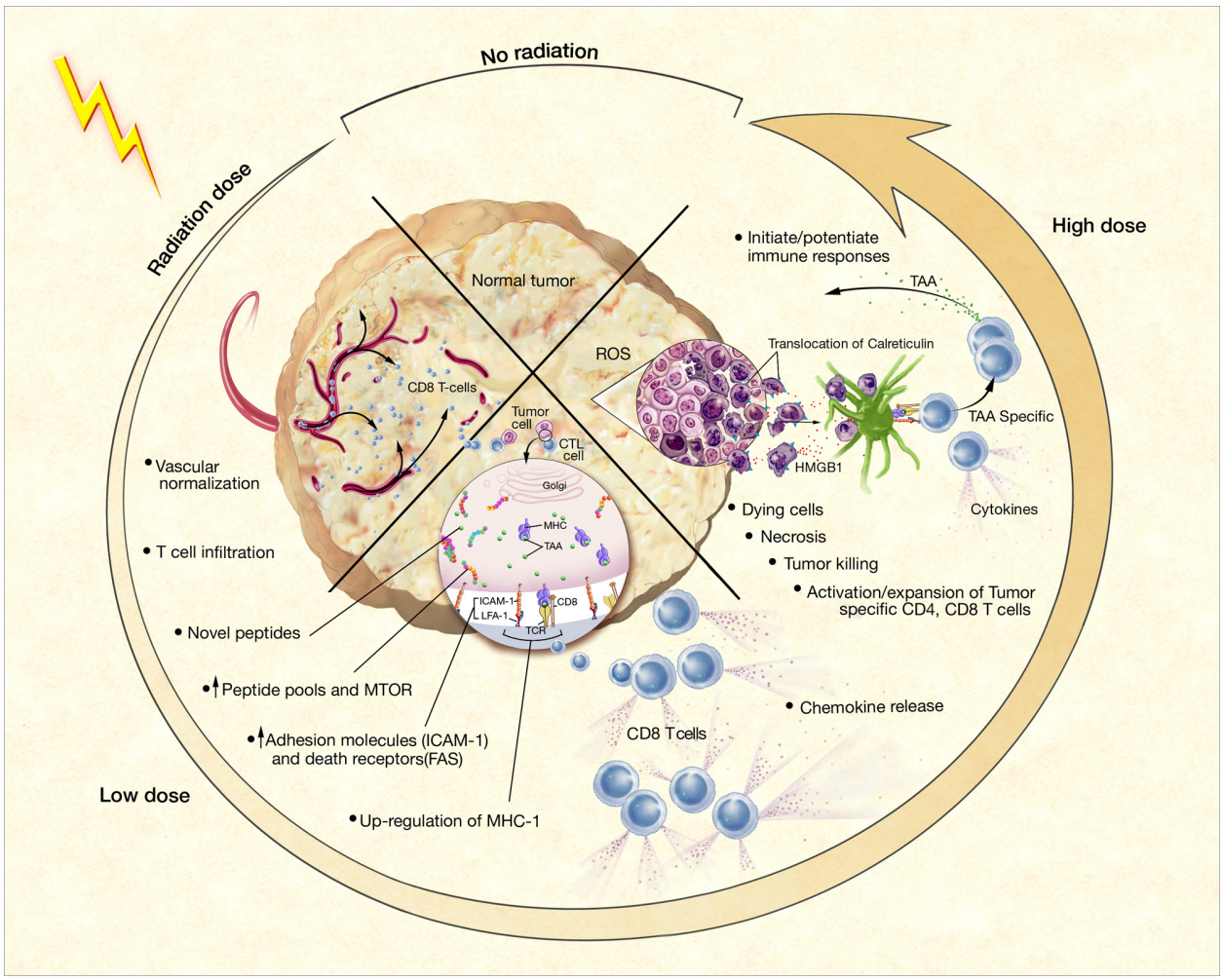

FIGURE 1 | Phenotypic and microenvironmental changes in tumors elicited by RT that can be exploited by immunotherapy (Hodge et al., 2008). Each of the potential mechanisms of RT-induced immunogenic modulation shown here is discussed further and referenced in the text.

PRECLINICAL EVIDENCE OF SYNERGY WHEN RADIATION AND IMMUNOTHERAPY ARE COMBINED

Several recent studies have indicated that radiation-induced cell death is an immunologically active process. This is demonstrated in one way because radiation-induced cell death causes the release of TAAs that can potentially be exploited to stimulate robust tumor-specific immune responses (Hannani et al., 2011). On their own, tumor cells typically do not generate potent anti-tumor immune responses due to their inefficient expression of molecules that are critical for antigen processing and presentation, such as the antigen transporter gene product TAP-2, MHC class I molecules, and T cell costimulatory molecules such as B7-1 (CD80; Sanda et al., 1995). However, radiation-induced cell death results in the release of novel TAAs that can be taken up, processed, and presented by APCs in the tumor microenvironment and draining lymph nodes. Reits et al. (2006) demonstrated that RT increases the peptide repertoire available for MHC class I molecules to present to CTLs, not only by increasing the degradation of existing proteins, but by activating the mammalian target of rapamycin pathway, leading to increased protein translation and creation of a novel peptide repertoire. Irradiation has additionally been shown to induce the expression of membrane-bound calreticulin on tumor cells, which acts as a recognition and phagocytosis signal for DCs. It can also induce the release of "danger signals" for DC activation, such as various heat shock proteins and HMGB1 (Demaria et al., 2005; Tesniere et al., 2008). Friedman (2002) has previously described a "danger model" of immunity, wherein ionizing radiation generates an inflammatory microenvironment filled with apoptotic and necrotic cells, chemokines, cytokines, and other inflammatory mediators. This inflammatory milieu is believed to activate APCs and support their processing of newly exposed TAAs.

Although RT is traditionally employed to destroy tumor cells, some of the cells within a given tumor mass receive doses of radiation that do not result in cell death because of the need to limit damage to normal tissues. A number of preclinical studies have shown that these lower doses of radiation are capable of inducing phenotypic changes within tumor cells that ultimately facilitate

immune-cell recognition and immune-mediated tumor killing. Molecules reported to be altered by such doses of radiation include TAAs, MHC class I, Fas/CD95, and the costimulatory molecules B7-1, lymphocyte function-associated antigen 3 (LFA-3), and intercellular adhesion molecule 1 (ICAM-1; Vereecque et al., 2000; Vondracek et al., 2001; Chakraborty et al., 2003; Garnett et al., 2004; Reits et al., 2006; Ifeadi and Garnett-Benson, 2012). These molecules are well known to play a role in CTL-mediated killing. MHC class I is responsible for direct presentation of tumor antigen peptides to CTLs, while increased numbers of adhesion molecules improve cell-to-cell attachment, enhancing the ability of a T cell to kill its target (Zamai et al., 1994; Baluna et al., 2006; Reits et al., 2006). Fas-mediated apoptosis plays an important role in CTL-mediated tumor-cell destruction, with interaction of the Fas ligand on activated CTLs with the Fas receptor on the target cell, inducing apoptosis of the target cell.

Using a murine adenocarcinoma cell line transfected to express carcinoembryonic antigen (CEA, MC38-CEA), Chakraborty et al. (2003) demonstrated *in vitro* that irradiation enhanced the surface expression of two molecules involved in T cell-mediated immune attack, Fas/CD95 and ICAM-1, in a dose-dependent manner. Moreover, they reported that exposure to radiation (20 Gy) enhanced the sensitivity of this murine cell line to antigen-specific CTL killing by up to fourfold, and that this increase in CTL sensitivity was shown to be via the Fas/Fas ligand pathway (Chakraborty et al., 2003). A follow-up study examined whether this phenomenon similarly occurs in human cancer cells. Utilizing a variety of human carcinoma cell lines (12 colon, 7 lung, and 4 prostate), Garnett et al. (2004) investigated whether 10 or 20 Gy of gamma radiation could alter the cell surface expression of a variety of molecules involved in T cell-mediated immune attack, including Fas/CD95, adhesion molecules, MHC class I, and TAAs such as CEA and mucin-1 (MUC-1). They found that at least one of these molecules was upregulated in 91% of the cell lines post-irradiation (Garnett et al., 2004). Moreover, five of five irradiated CEA^+, $HLA\text{-}A2^+$ colon cancer cell lines demonstrated significantly enhanced killing by CEA-specific HLA-A2-restricted $CD8^+$ CTLs compared to non-irradiated controls (Garnett et al., 2004). Modrak et al. (2003) also showed an increase in TAA expression among irradiated colon cancer cell lines. These *in vitro* studies collectively demonstrated that RT can make both mouse and human tumor cells more amenable to immune recognition and attack.

Another clinically relevant form of radiation, bone-seeking chelated radionuclide, is similarly capable of inducing phenotypic changes within tumor cells, thereby enabling immune-cell recognition and enhancing CTL killing. Chakraborty et al. (2008b) evaluated the FDA-approved bone-seeking radionuclide samarium-153 (^{153}Sm-EDTMP; Quadramet®, Cytogen), used as palliation for pain caused by metastatic bone lesions, for its ability to change the phenotype of tumor cells. The calculated dose of radiation delivered to bone metastases by this agent is between 18 and 80 Gy (Eary et al., 1993; Maini et al., 2004). In this study, 10 human tumor cell lines representing classes of tumors that metastasize to bone (four prostate, two breast, four lung) were exposed to clinically relevant levels of ^{153}Sm-EDTMP for 4 days, then examined by flow cytometry for modulation of several cell surface molecules. Of the 10 cell lines, 100% upregulated Fas and CEA, 70% upregulated MUC-1, 40% upregulated MHC class I, and 30% upregulated ICAM-1. Exposure of the prostate cancer cell line LNCaP to ^{153}Sm-EDTMP also resulted in upregulation of prostate-specific antigen (PSA), prostate-specific membrane antigen (PSMA), and prostatic acid phosphatase (PAP). Additionally, treatment of LNCaP cells with ^{153}Sm-EDTMP rendered them more susceptible to killing by a variety of antigen-specific CTLs. These preclinical data suggest that ^{153}Sm-EDTMP may work synergistically with immunotherapy to increase the susceptibility of tumor cells to CTL killing, and have formed the basis for an ongoing clinical trial.

These and other preclinical studies have collectively demonstrated that radiation can be utilized to make tumor cells more amenable to immune recognition and attack, and form the rational basis for the combinatorial use of local tumor irradiation and immunotherapy. A number of preclinical studies have demonstrated that localized treatment of tumors with lower doses of EBRT acts synergistically with immunotherapy to enhance antitumor immune responses. Chakraborty et al. (2003) demonstrated that EBRT (8 Gy) of subcutaneous MC38-CEA tumors markedly enhanced the efficacy of immunotherapy in the form of CTL adoptive transfer. In this study, C57B6 mice were implanted subcutaneously on the hind leg with MC38-CEA cells. Nine days later, mice were randomized to receive no treatment, EBRT of the tumor alone, adoptive transfer of CEA-specific CTLs alone, or the combination of both EBRT and adoptive transfer. EBRT alone and adoptive transfer alone ultimately failed to significantly impact tumor growth in these mice relative to untreated controls (Chakraborty et al., 2003). However, treatment of tumors with the combination of EBRT and CTL adoptive transfer resulted in a significant reduction in tumor growth rate and volume relative to mice receiving either no treatment or EBRT or CTL adoptive transfer alone. Moreover, 50% of mice receiving the combination treatment remained tumor-free for the duration of the experiment (40 days; Chakraborty et al., 2003). In a similar study by Reits et al. (2006), mice were implanted with MC38 tumor cells. When tumors became established, mice received EBRT (10 Gy) and/or adoptive transfer of gp70-specific CTLs (Reits et al., 2006). Neither radiation nor adoptive transfer alone was curative; however, the combination of local irradiation of the tumor and adoptive transfer of CTLs significantly reduced tumor burden and, in most mice, completely eradicated the tumor mass.

A number of preclinical studies have revealed that RT acts synergistically with active therapeutic vaccination to enhance antitumor immune responses. Chakraborty et al. (2004) focused on the combination of 8 Gy EBRT delivered directly to the tumor in combination with a vaccine composed of vaccinia and fowlpox vectors that express CEA and a triad of costimulatory molecules: B7-1, ICAM-1, and LFA-3 (rV/F-CEA/TRICOM). Although either treatment alone was ineffective at reducing tumor burden, the combination of EBRT and vaccine was not only curative in 50% of mice bearing CEA-expressing tumors, but also imparted protection from subsequent tumor challenge (**Figure 2**; Chakraborty et al., 2004). Notably, mice cured of tumors demonstrated antigen cascade, developing CD4 and CD8 T cell responses not only to CEA, but also to other tumor antigens not encoded in the vaccine, such as gp70 (Chakraborty et al., 2004). They reported that the

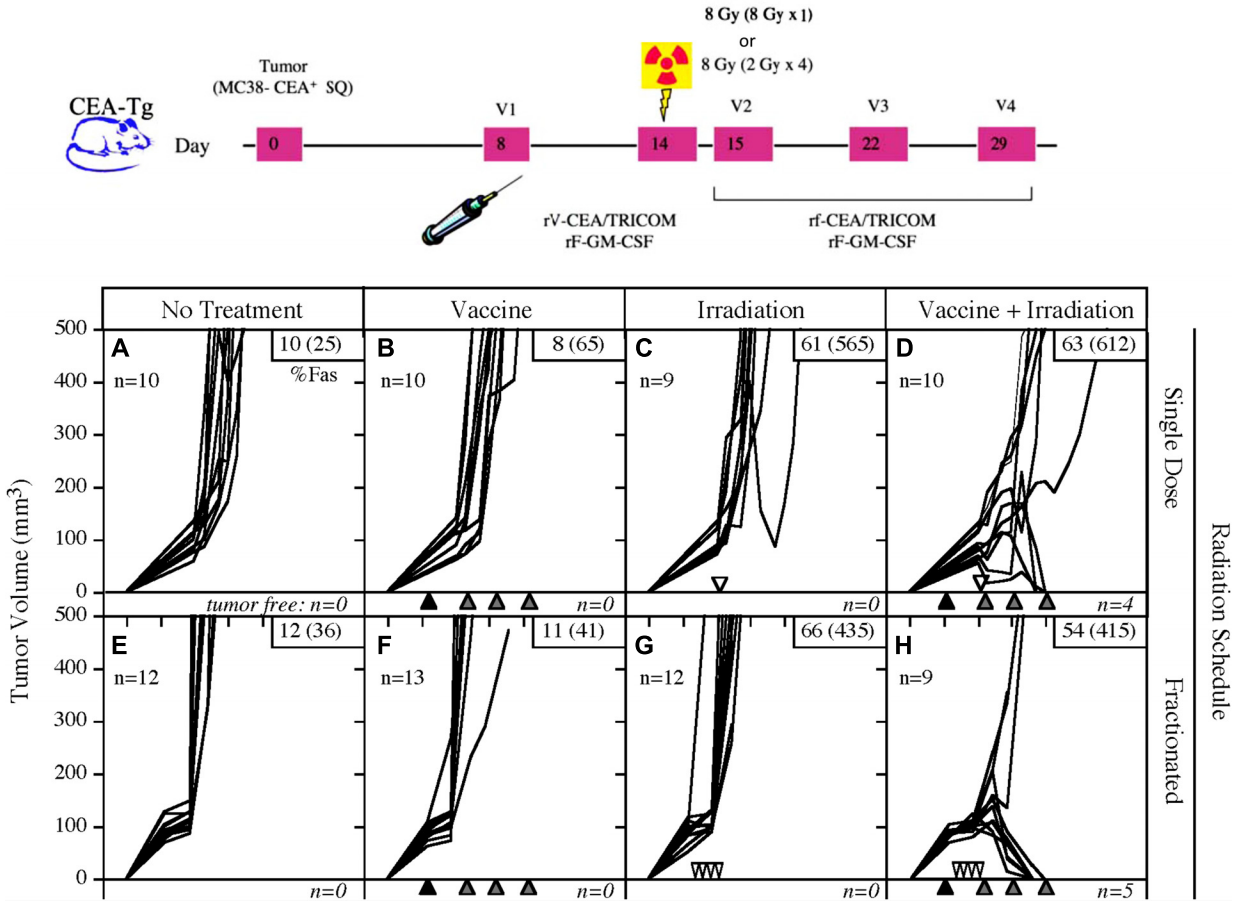

FIGURE 2 | Combination of single-dose or fractionated RT with vaccine therapy. Mice transgenic for CEA were implanted subcutaneously on day 0 with the MC38-CEA tumor cell line, then randomized to receive either no treatment, vaccine alone, EBRT alone, or the combination of vaccine and EBRT. The vaccine consisted of poxviral vectors expressing CEA and TRICOM (rF/V-CEA/TRICOM). All vaccines were coadministered with a poxviral vector expressing GM-CSF. RT was administered either as a single dose (8 Gy on day 14) or fractionated (2 Gy on days 11, 12, 13, and 14). Neither modality was effective alone, but the combination of vaccine with single-dose or fractionated RT was curative in 40 and 55% of mice, respectively. (Adapted from Chakraborty et al., 2004.)

immune response to gp70 was markedly greater than that seen to the antigen encoded in the vaccine, suggesting that the immune response to the cascade antigens may play an important role in the observed antitumor activity. Results from this preclinical study provided the rationale to evaluate the use of EBRT and therapeutic cancer vaccines in the clinic.

Therapeutic synergy has also been reported utilizing vaccine-mediated immunotherapy combined with radiolabeled mAb. mAbs can guide radionuclides to cancer cells, precisely and preferentially target tumor cells, and seek out micrometastases that are unobservable by current imaging technology and cannot be targeted by EBRT. A recent study cited the ability of radiolabeled mAb to alter tumor-cell phenotype and enhance immunologic targeting of tumor cells (Chakraborty et al., 2008a). In that study, mice transgenic for CEA were transplanted with MC38-CEA tumor cells, then treated with yttrium-90-labeled anti-CEA mAb alone or in combination with CEA-targeted vaccine therapy. A single dose of yttrium-90-labeled anti-CEA mAb, in combination with vaccine, statistically increased survival in tumor-bearing mice relative to vaccine or mAb therapy alone (**Figure 3**; Chakraborty et al., 2008a). Of note, mice receiving the combination therapy also had a marked increase in the percentage of viable tumor-infiltrating CEA-specific CD8 T cells relative to vaccine alone, demonstrating that these cells were unaffected by the residential radiation source. Similar to what was noted with EBRT, mice cured of tumors demonstrated an antigen cascade, resulting in CD4 and CD8 T cell responses not only for CEA, but also for tumor antigens not encoded in the vaccine.

Brachytherapy, yet another form of clinically relevant RT, has also been evaluated in combination with vaccine-mediated immunotherapy. Brachytherapy, which involves implanting a radiation source such as iodine-125 into or near the site of a malignant tumor to target tumor cells with continuous high-dose radiation, has also been shown to alter the phenotype of tumor cells. A single study demonstrated the ability of iodine-125 to increase the expression of Fas >2-fold in tumors relative to sham-treated mice (Hodge et al., 2012). In this study, CEA-transgenic mice were implanted with a Lewis lung carcinoma

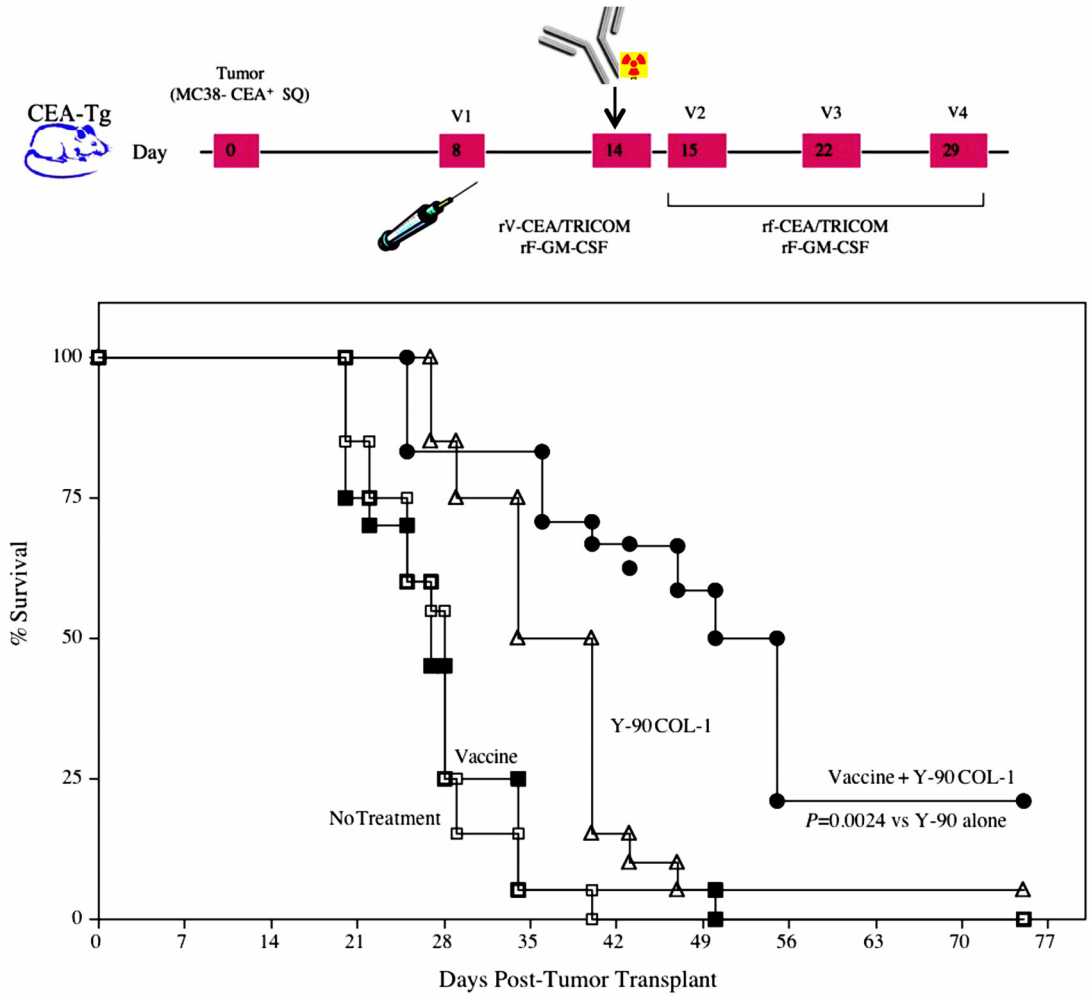

FIGURE 3 | Combination of a radiolabeled mAb with vaccine increased survival in tumor-bearing mice. Mice transgenic for CEA were implanted subcutaneously on day 0 with the MC38-CEA tumor cell line. A control group (open squares) received HBSS buffer only. A second group (closed squares) received a vaccine consisting of poxviral vectors expressing CEA and TRICOM (rV/F-CEA/TRICOM). All vaccines were coadministered with a poxviral vector expressing GM-CSF. A third group (open triangles) received RT consisting of 100 μCi yttrium-90-labeled anti-CEA mAb (Y-90-labeled COL-1) intravenously on day 14. A fourth group (closed circles) received the combination of vaccine plus radiolabeled mAb. Mice were monitored weekly for tumor size and survival. (Adapted from Chakraborty et al., 2008a.)

cell line expressing CEA (LL2-CEA) both subcutaneously and intravenously. Mice received either no treatment, brachytherapy alone in which iodine-125 seeds were implanted near the subcutaneous tumor, vaccine alone, in this case a diversified prime and boost of poxviral vectors expressing gp70 and TRICOM, or the combination of brachytherapy and vaccine. The only therapeutic regimen that suppressed the number of pulmonary metastases in this model was the combination of brachytherapy (directed at the primary subcutaneous tumor alone) with vaccination (Hodge et al., 2012). Thus, the abscopal effect only occurred in mice treated with the combination of brachytherapy and vaccine. A recent study by Dewan et al. (2009) similarly noted that RT induced an abscopal effect only when used in combination with immunotherapy. In their study, they noted that fractionated local radiotherapy to one palpable tumor synergized with CTLA-4 blockade to induce antitumor T cell immunity and inhibit the growth of a second palpable tumor outside the radiation field.

CLINICAL EVIDENCE OF THE EFFICACY OF COMBINED RADIATION AND IMMUNOTHERAPY

Results from the preclinical studies described above and from additional reports as well have provided the rationale for clinical evaluation of the combination of RT and cancer immunotherapy. In a phase I study of patients with advanced hepatoma, participants were given 8 Gy of radiation, followed 2 days later by an intratumoral injection of autologous immature DCs. Of 10 patients evaluated for immune response, six showed increased natural killer cell activity, eight had increases in alpha-fetoprotein (AFP)-specific immune responses by cytokine-release assay, and seven showed increased AFP-specific immune responses by ELISPOT. Of the 14 patients who entered the trial, four

had minor responses and two had partial responses, including a patient who had a decrease in AFP from 128 to 1.6 ng/mL (Chi et al., 2005).

A randomized phase II study in men with localized prostate cancer evaluated the use of a recombinant poxviral-based vaccine expressing PSA combined with standard definitive radiotherapy (Gulley et al., 2005). Patients in the combination arm received a priming vaccine of recombinant vaccinia (rV) expressing PSA (rV-PSA) admixed with rV expressing the costimulatory molecule B7-1. This was followed by monthly boosts with recombinant fowlpox (rF)-PSA. The vaccines were administered with local granulocyte–macrophage colony-stimulating factor and low-dose systemic IL-2 (4 million IU/M^2). Two courses of EBRT were given daily for 5 days, with a 2-day holiday between the fourth and sixth vaccinations. Results from this clinical trial indicated that the combination was safe, well tolerated, and, more importantly, effective at generating PSA-specific immune responses. Approximately 76.5% of patients (13 of 17) in the combination therapy arm showed a $\geq$3-fold increase in PSA-specific T cells vs. 0% (0 of 8) in the radiation-alone arm ($P < 0.0005$). In addition, six of eight patients developed post-treatment T cell responses specific for at least one additional endogenous TAA not encoded by the vaccine, indicating the presence of antigen cascade. These included the generation of T cells against PSMA, PAP, prostate stem cell antigen (PSCA), and/or MUC-1 (**Table 1**). In some cases the immune response to a cascade antigen was even greater than the response to PSA. There were no significant changes in the patients' responses to flu peptide, and all patients remained negative for responses to HIV. Only grade 2 toxicities were related to the vaccine itself; however, some grade 3 toxicities were attributed to IL-2. A follow-up study was conducted to evaluate the use of a metronomic dose of IL-2 (0.6 million IU/M^2) in order to reduce some of the toxicity seen in the previous trial (Lechleider et al., 2008). This study used the same vaccination schedule as the previous trial, except that RT was administered following the third booster vaccination instead of the fourth. Patients in this trial experienced less toxicity attributable to IL-2 and developed similar immune responses (**Table 2**). A third trial was conducted evaluating the combination of the rV/F-CEA/TRICOM vaccine with EBRT delivered directly to liver metastases in patients with CEA^+ solid tumors (Gulley et al., 2011). Twelve patients, 11 with CEA^+ colon cancer and 1 with CEA^+ rectal cancer, received a priming vaccination with rV-CEA/TRICOM on day 1, with biweekly booster vaccinations with rF-CEA/TRICOM. Four 8-Gy courses of EBRT were delivered to sites of liver metastasis 1 day following booster vaccinations. Unfortunately, the design of this study was not optimal for assessing the ability of radiation to enhance the clinical benefit of vaccine treatment strategies. Of the two evaluable patients, neither showed an increase in CEA-specific T cells above baseline after therapy.

The combination of ^{153}Sm-EDTMP and vaccine is also currently being studied in a randomized phase II trial in patients with castration-resistant prostate cancer (CRPC) metastatic to bone (Heery et al., 2012). The primary endpoint of the trial is to determine if ^{153}Sm-EDTMP combined with vaccine can improve time to progression over ^{153}Sm-EDTMP alone. Patients will receive 1 mCi/kg ^{153}Sm-EDTMP alone or in combination with an rV/F-PSA/TRICOM vaccine (PROSTVAC®, Bavarian Nordic) administered in a biweekly diversified prime/boost regimen for the first three vaccinations starting on day 1, then monthly thereafter. ^{153}Sm-EDTMP will be administered on day 8, then every 12 weeks thereafter. Currently, 37 of a projected 68 patients have been enrolled. Interim analysis determined that at 4 months, 5 of 17 patients (29.4%) receiving combination therapy remained progression-free, while only 2 of 17 (11.8%) remained progression-free on ^{153}Sm-EDTMP alone. The median time to progression was 60 days in the ^{153}Sm-EDTMP-alone group and 117 days in the combination group. This early indication of improved time to progression supports the continuation of this trial, allowing for the evaluation of secondary endpoints of immunogenic stimulation and overall survival.

In addition to vaccines, RT has also been evaluated clinically in combination with additional types of immunotherapy. Three trials have been undertaken to determine if RT can enhance the antitumor efficacy of IPI in patients with metastatic CRPC. In all three trials, single-fraction RT was given just prior to the start of IPI therapy which was given at doses of either 3 or 10 mg/kg once every 3 weeks for four cycles. All three trials determined that the combination was well tolerated, but similar reductions in PSA were observed in the IPI treatment groups regardless of the addition of RT (Beer et al., 2008; Slovin et al., 2009, 2012). Additional trials examining the timing of RT with respect to IPI treatment

Table 1 | Immune responses following treatment with poxviral vaccines expressing PSA and B7-1 in combination with EBRT and low-dose IL-2 (Gulley et al., 2005).

Patient	Sample	PSA	PSMA	PAP	PSCA	MUC-1
3	pre	ND	ND	ND	ND	ND
	post 3	1/50,000	ND	1/85,714	1/85,714	1/23,077
	post 8	1/46,154				
6	pre	ND	ND	ND	ND	ND
	post 3	1/54,545	1/85,714	ND	ND	1/60,000
	post 8	1/22,222				
7	pre	ND	ND	ND	ND	ND
	post 3	1/42,857	1/200,000	1/85,714	ND	ND
	post 8	1/15,000				
8	pre	ND	ND	ND	–	1/80,000
	post 3	ND	1/62,500	ND	–	1/46,154
	post 8	1/66,667				
11	pre	1/100,000	ND	ND	ND	ND
	post 3	1/85,714	ND	ND	ND	1/40,000
	post 8	ND				
12	pre	1/100,000	ND	1/200,000	1/200,000	ND
	post 3	1/150,000	ND	ND	ND	1/35,294
	post 8	1/200,000				

ND, none detected (< 1/200,000).
Samples obtained after indicated vaccine cycle.

Table 2 | Immune responses following vaccination with poxviral vaccines expressing PSA and B7-1 in combination with EBRT and metronomic IL-2 (Lechleider et al., 2008).

Patient	Sample	PSA	MUC-1	PAGE-4	XAGE-1
31	pre	ND	1/85,714	ND	ND
	post 3	ND	ND	ND	ND
	post 3 + 2	1/45,455	–	–	–
	post 5 + 2	–	ND	ND	ND
	post 8	1/60,000	1/37,500	ND	1/27,273
32	pre	1/120,000	ND	1/100,000	1/23,077
	post 3	1/17,391	ND	1/80,000	1/28,571
	post 3 + 2	ND	–	–	–
	post 5 + 2	–	ND	1/22,222	1/46,154
	post 8	ND	ND	1/100,000	1/50,000
33	pre	–	ND	ND	ND
	post 3	–	ND	1/200,000	1/54,545
	post 5	–	ND	ND	ND
	post 8	–	1/46,154	ND	1/24,000
34	pre	ND	–	–	–
	post 3	1/46,154	–	–	–
	post 5 + 3	ND	–	–	–
	post 8	ND	–	–	–
37	pre	1/150,000	–	–	–
	post 2	ND	–	–	–
	post 5	1/12,000	–	–	–
38	pre	ND	–	–	–
	post 3	1/85,714	–	–	–
	post 8	1/28,462	–	–	–

PAGE-4 and XAGE-1 denote members of the PAGE/GAGE family of prostate cancer TAAs.
ND, none detected (< 1/200,000).
Samples obtained after indicated vaccine cycle (i.e., post 3 + 2 = 2 months after cycle 3).

may lead to a combination treatment that acts synergistically similar to that reported in metastatic melanoma (Postow et al., 2012). A recent single arm phase I/II trial examined the efficacy of combining low-dose RT (4 Gy over 2 days) with administration of the TLR9 agonist PF-3512676 to 15 patients with low-grade B cell lymphoma (Brody et al., 2010). In this trial, PF-3512676 was administered via intratumoral injection to the same site as local RT. PF-3512676 was administered immediately prior to the first dose of radiation, immediately following the second and then weekly for 8 weeks. The combination was well tolerated and resulted in one complete response, three partial responses, and two patients having stable/regressing disease. Responding patients displayed increases in tumor-reactive $CD8^+$ T cells and a reduction in Tregs. Another recent phase I trial examined the combination of stereotactic body RT with systemic IL-2 therapy for the treatment of metastatic melanoma and renal cell carcinoma (RCC; Seung et al., 2012). Twelve patients (seven with melanoma, five with RCC) received one, two, or three doses of 20 Gy stereotactic body RT with bolus IL-2 (600,000 IU/kg) beginning 3 days following the final dose of RT. IL-2 was given every 8 h for a maximum of 14 doses with a second cycle of treatment occurring 2 weeks later. By positron emission tomography, five patients with melanoma and one with RCC achieved a complete response while two additional RCC patients achieved a partial response. Responding patients exhibited a higher frequency of early-activated effector memory $CD4^+$ T cells in the peripheral blood. Both of these studies support the immunomodulatory activity of RT and its combination with additional forms of immunotherapy. Additional trials, however, still need to be performed to determine the extent of the increased efficacy of these combinations.

PERSPECTIVES ON THE FUTURE OF COMBINED RADIATION AND IMMUNOTHERAPY FOR THE TREATMENT OF CANCER

The goal of cancer immunotherapy is to overcome tolerance to weakly immunogenic TAAs and to stimulate an immune response to tumor cells. Ionizing radiation induces tumor-cell death, thereby releasing the multiple novel tumor antigens required to overcome tolerance and igniting the "danger signals" needed to stimulate an immune response. RT may be able to overcome the ability of cancer cells to escape immune recognition and therefore act synergistically with immunotherapy to enhance immune responses, inhibit immunosuppression, and/or alter the phenotype of tumor cells, rendering them more susceptible to immune-mediated killing. Preclinical studies have shown that RT from a variety of different sources cannot only induce tumor-cell death in a manner consistent with antitumor immune activation, but can also phenotypically modify tumor cells not killed by RT in a way that facilitates both immune recognition and immune-mediated killing. Capitalizing on the immunologic effects induced by RT by adding potent antitumor immunotherapy agents may lead to synergistic approaches to cancer management that offer feasible, well-tolerated therapeutic options for cancer patients.

Questions remain, however, as to how best to exploit the largely untapped resource of radiation and immunostimulatory combination therapy. First, although many modes of RT have been shown to induce similar alterations in tumor phenotype and microenvironment, there may be subtle variations in the induction of these responses brought about by a given type of RT. These variations may be better exploited by a specific type of cancer immunotherapy, including those discussed herein or other emerging immunotherapies such as the vaccine sipuleucel-T (Provenge®, Dendreon Corp.). Second, as discussed here, combining a specific dosage and course of RT with immunotherapy may be more efficacious at enhancing clinical benefit; this concept, however, needs further investigation. Along these same lines, the timing of administration of RT and immunotherapy during combination treatment also needs further investigation. As discussed, administering immunotherapy prior to RT allows for the generation of a memory immune response that is less susceptible to immunodepletion brought about by RT. Although it was not designed for definitive determination, one may infer from the trial evaluating rV/F-CEA/TRICOM with EBRT delivered directly to liver metastases in patients with CEA^+ solid tumors that 1 day post-vaccination is too soon for RT. On the other hand, administering immunotherapy following RT may take advantage of the

homeostatic peripheral expansion of the immune compartment that occurs following some types of RT. As with phenotypic and microenvironmental changes, the timing of each therapy may depend on the specific therapies being combined. The final consideration concerning the combination of RT and immunotherapy for the treatment of cancer is identifying the most appropriate patient population. EBRT and vaccine combination trials in prostate cancer may have yielded more positive results because definitive RT for localized prostate cancer does not involve extensive lymph node irradiation, thus sparing much of the patients' lymphocyte population. This could suggest that combination therapy should be examined further in the setting of RT that avoids extensive lymph node irradiation. However, in the prostate cancer trials discussed here, some patients developed more stable immune responses that were less susceptible to blunting by RT than others. The reason for this result is unclear, however, indicating that further studies are required to determine which patient population would benefit most from this combination therapy. In addition, it will also be important to determine the stage of disease at which this combination will be most beneficial to the patient. As monotherapies, both immunotherapy and radiation may be insufficient to eliminate bulky tumor masses or an entire metastatic burden. Even though combination therapy may be more effective in this advanced state, patients with smaller primary tumors and lower metastatic burdens may derive greater clinical benefit due to the lower tumor burden needed to be overcome. Additional clinical trials in earlier disease settings will be needed to confirm this approach.

Substantial preclinical evidence has revealed a synergistic relationship between RT and immunotherapy. Anecdotal evidence and prospective clinical data also support the efficacy of this treatment regimen. As most of the studies reviewed here have focused on an immunological response as the primary endpoint, further clinical trials are needed to determine if adding active immunotherapy to definitive RT can affect clinical outcomes. Learning how best to exploit radiation-induced immunogenic changes in cancer patients with the addition of active immunotherapy is an exciting frontier in cancer therapy research, and has the potential to greatly improve patient care in the future.

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Irradiation promotes an M2 macrophage phenotype in tumor hypoxia

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Macrophages display different phenotypes with distinct functions and can rapidly respond to environmental changes. Previous studies on TRAMP-C1 tumor model have shown that irradiation has a strong impact on tumor microenvironments. The major changes include the decrease of microvascular density, the increase of avascular hypoxia, and the aggregation of tumor-associated macrophages in avascular hypoxic regions. Similar changes were observed no matter the irradiation was given to tissue bed before tumor implantation (pre-IR tumors), or to established tumors (IR tumors). Recent results on three murine tumors, TRAMP-C1 prostate adenocarcinoma, ALTS1C1 astrocytoma, and GL261 glioma, further demonstrate that different phenotypes of inflammatory cells are spatially distributed into different microenvironments in both IR and pre-IR tumors. Regions with avascular hypoxia and central necrosis have $CD11b^{high}$/Gr-1+ neutrophils in the center of the necrotic area. Next to them are $CD11b^{low}$/F4/80+ macrophages that sit at the junctions between central necrotic and surrounding hypoxic regions. The majority of cells in the hypoxic regions are $CD11b^{low}$/CD68+ macrophages. These inflammatory cell populations express different levels of Arg I. This distribution pattern, except for neutrophils, is not observed in tumors receiving chemotherapy or an anti-angiogenesis agent which also lead to avascular hypoxia. This unique distribution pattern of inflammatory cells in IR tumor sites is interfered with by targeting the expression of a chemokine protein, SDF-1α, by tumor cells, and this also increases radiation-induced tumor growth delay. This indicates that irradiated-hypoxia tissues have distinct tumor microenvironments that favor the development of M2 macrophages and that is affected by the levels of tumor-secreted SDF-1α.

Keywords: radiation, tumor-associated macrophages, tumor microenvironment

INTRODUCTION

A major obstacle in cancer radiation therapy (RT) or chemotherapy is the presence of hypoxic tumors, and this could be an even more serious issue in recurrent tumors in which the hypoxia can shift from transient to chronic hypoxia (Chen et al., 2011). The recurrent tumors are not only less responsive to salvage RT or chemotherapy, but also have a higher risk of metastasis (Vicini et al., 2003). Although the effects of hypo-perfusion and low oxygen contents on tumor cells are often blamed for poor treatment response, distinct tumor microenvironments within hypoxic regions such as where there are more acidic or contain high numbers or distinct populations of macrophages, also play significant roles in tumor resistance to therapy (Jiang et al., 2010; Zhang et al., 2010; Denardo et al., 2011). Several new treatment protocols to target the tumor microenvironments have been suggested, such as pH responsive drug delivery (Chiu et al., 1999; Benoit et al., 2010) and macrophage-targeted (Ahn et al., 2010; Jiang et al., 2010) or -assisted (Alizadeh et al., 2010; Muthana et al., 2011) cancer therapy. However, the improvement of cancer therapy by these approaches has still to be realized. One critical issue in targeting the tumor microenvironment is that its changes during or after the therapy. This continuous and dynamic process is crucial for the right timing of intervention. We have previously shown that there are temporal and spatial changes in the subcomponents within tumor microenvironments following single or fractionated radiation (Chen et al., 2009). Better understanding of the dynamic features of hypoxic microenvironments following RT may provide new strategies to improve the efficacy of cancer treatment.

One remarkable feature in hypoxic tumor microenvironments is the large amount of infiltrated macrophages, so-called tumor-associated macrophages (TAMs). TAMs represent the largest population of infiltrating inflammatory cells in malignant tumors. They were originally thought of as one host defense mechanism against the developing cancer. However, evidence has accumulated indicating that TAMs may assist tumors to survive hazardous environments in various ways (Nishie et al., 1999; Bingle et al., 2002; Murdoch and Lewis, 2005; Lewis and Pollard, 2006; Li et al., 2007; Ahn and Brown, 2008; Qian and Pollard, 2010; Chen et al., 2011) and even promote tumor resistance to chemotherapy (Zhang et al., 2010; Denardo et al., 2011). Two distinct TAM phenotypes, M1 or M2, have been described with the abilities to inhibit or promote tumor growth, respectively. The M1 phenotype

is proinflammatory and has high levels of iNOS production; the M2 phenotype (Mantovani et al., 2002) is anti-inflammatory, pro-angiogenic (Dirkx et al., 2006; Lin et al., 2006), metastasis-promoting (Leek et al., 1996; Hanada et al., 2000), and has high levels of Arg I production. However, TAMs may change their functions under different microenvironments (Stout et al., 2005; Chiang et al., 2008; Redente et al., 2010). It has been hypothesized that initial TAMs are predisposed to have M1 function, but are gradually changed to M2 function as tumor grow (Weigert and Brune, 2008). This is associated with factors, such as IL-4, IL-10, TGF-β, PGE2, and chemokines, released by tumor cells in response to the changes in the microenvironments, in particular the development of hypoxia (Mantovani et al., 1986; Lewis et al., 1999). Furthermore, TAMs within different subcomponents of the same tumor may also have different functions. Ohno et al. (2003) had shown that TAMs in different niches of gastric carcinoma have different influences on patients' survival, stressing the heterogeneity of TAMs and the effects of tumor microenvironment on TAM function. It has been proposed that M1 and M2 TAMs will segregate to different areas of the tumor, with M2 TAMs migrating to and aggregating in avascular or hypoxia regions (Lewis and Murdoch, 2005; Murdoch and Lewis, 2005). This may explain the variation of M1/M2 ratio and inconsistency in the correlation of the number of TAMs with the prognosis in different types of tumor (Bingle et al., 2002). For example, a positive correlation has been reported in breast and prostate cancer, but a negative correlation in colon caner. Contrary conclusions from the same type of tumor had also been reported in brain tumors when different types of surface markers were used (Bingle et al., 2002). These findings indicate that host or tumor factors may be critical for assessment of the effects of tumor microenvironments on TAM function, in particular for tumor re-growth after RT because our previous studies showed TAMs are actively involved in the remodeling of post-radiation microenvironments (Chen et al., 2011). However, systematic studies on this area are lacking.

The potential of RT to alter TAM phenotype and function has rarely been studied. In our previous study, TAMs isolated from irradiated tumors expressed higher Arg I, COX-2, and iNOS levels than those from un-irradiated tumors and were more effective at promoting tumor growth (Tsai et al., 2007), indicating more M2 TAMs in irradiated-TRAMP-C1 tumors. In this study, we used three different murine tumors, TRAMP-C1 prostate adenocarcinoma, ALTS1C1 astrocytoma, and GL261 glioma, to explore how irradiation affects the relationship between hypoxia and TAMs and whether those changes in irradiated tumor microenvironments are affected by tumor or host factors.

MATERIALS AND METHODS

TUMOR MODEL AND TUMOR IRRADIATION

All experiments were performed using 7- to 8-week-old male C57BL/6J mice obtained from National Laboratory Animal Center, Taiwan. The TRAMP-C1 prostate cancer cell line was derived from transgenic mice with adenocarcinoma of the mouse prostate (Foster et al., 1997) and was purchased from the ATCC (CRL-2730). ALTS1C1 was derived from primary astrocytes transformed by SV40 large T antigen and serial *in vivo* passage (Wang et al., 2012) and is deposited in Bioresource Collection and Research Center (BCRC-60582), Taiwan. GL261 was a generous gift from Prof. Newcomb, E. W., Departments of Pathology, New York University School of Medicine (Newcomb et al., 2010). For intramuscular model, tumors were generated by intramuscular inoculation of 3×10^6 viable cells into the thigh. Mice with tumors of 4 mm in diameter were selected and randomly allocated to groups for experimentation (tumor diameter was defined by $(a + b)/2$, where a and b are the width of two dimensions of mouse thigh) that contained at least five mice per time point. To implant ALTS1C1 or GL261 cells into the brain, 2 μl containing 1×10^5 cells were inoculated intracranially (i.c.) into 6- to 8-week-old C57BL/6 mice as described (Wang et al., 2012). Prior to sacrifice, the animals were anesthetized and then perfused transcardially with PBS followed by 4% paraformaldehyde. The maximum tumor cross sectional area was used to compare the tumor growth for i.c. tumor model and defined by $[(a + b)/2]^2 \times \pi$, where a and b are the width of two dimensions of maximum cross section.

The irradiation protocol was as previously described (Tsai et al., 2007). Tumors were irradiated with either a single dose of 25 Gy to the intramuscular tumor or 8 Gy to intracranial tumors. The tumors were removed at indicated times following irradiation. During the experiments, all mice were cared for in accordance with the approved guide by the Institutional Animal Care and Use Committee (IACUC), National Tsing Hua University, Taiwan (approved number: IACUC:09705).

cDNA MICROARRAY

Total RNA was isolated by PureLink RNA purification system (Invitrogen) according to the manufacturer's instructions to generate cRNA targets. The samples of primary astrocytes and two cell lines, ALTS1C1, and GL261, were hybridized using Affymetrix Mouse Genome 430A 2.0 Oligonucleotide Microarrays in the Genomic Medicine Research Core Laboratory (GMRCL) of Chang Gung Memorial Hospital (Wang et al., 2004). After scanning, hybridization signals were collected and the signals that were differentially expressed twice as compared with the normal astrocyte were selected for further analysis.

RT-PCR

Total RNA was extracted with TRIzol (Invitrogen). Two micrograms of total RNA was reverse-transcribed using Super Script III RNase H reverse transcriptase (Invitrogen, CA, USA) and random hexamer primers (Invitrogen) at 25°C for 10 min and 42°C for 1 h. Two microliters of the reverse transcription product was used as a template for PCR amplification. PCR was performed using Taq polymerase (Invitrogen) and 150 nmol/L of primers. The PCR conditions consisted of 3 min of an initial denaturation step (95°C followed by 30 cycles of denaturation (95°C, 30 s), annealing (57°C, 30 s), and extension (72°C, 30 s) followed by a final elongation step of 10 min at 72°C. Ten microliters of PCR product was analyzed on 2% agarose gels stained with ethidium bromide. Quantitation of bands was done with the Bio-Rad Fluor-S apparatus (Bio-Rad, Hercules, CA, USA) with Quantity One (version 4.2.1) software.

IMMUNOHISTOCHEMISTRY

Tumor hypoxia was studied by i.v. injection of 4 mg pimonidazole hydrochloride (Hypoxyprobe™-1 Kit, Hypoxyprobe, Burlington,

MA, USA) in 0.1 ml solution 1 h before tumor harvest. Tissues were removed and placed in cold 4% paraformaldehyde overnight then processing and embedding in paraffin or OCT. Ten micrometers cryostat sections were fixed in methanol at −20°C for 10 min, and then rehydrated in PBS. Non-specific binding was blocked by incubating sections in 1% of bovine serum albumin (BSA) in PBS for 30 min. Tumors sections were double-stained for pimonidazole in combination with CD31 or CD68. Pimonidazole (POMO) was detected with mouse antibody (Hypoxyprobe) and goat anti-mouse $IgG_{\gamma 1}$ Alexa 488 (Invitrogen). For endothelial cells, rat anti-CD31 antibody (BD biosciences, San Jose, CA, USA) was used, followed by goat anti-rat Alexa 594 (Invitrogen). For macrophages, rat anti-CD68 (Serotec, Raleigh, NC, USA), anti-F4/80 (Serotec), or anti-CD206 (Biolegend) was used, followed by goat anti-rat Alexa 594 (Invitrogen). Slides were rinsed in PBS and mounted with ProLong® Gold anti-fade reagent (P-36931, Invitrogen).

IMAGE ACQUISITION, PROCESSING, AND ANALYSIS

Immunofluorescent images from each tumor section were captured using an external digital camera (Dxm 1200C, Nikon, Tokyo, Japan) on a Nikon fluorescence microscope (Nikon Eclipse TE 2000-S) or an AxioCam MRC-5 camera on an Axiovert40 fluorescence microscope (Carl Zeiss, Göttingen, Germany) and processed using Image-pro plus 6.0 software (MediaCybernetics, Bethesda, MD, USA). Microvascular density (MVD) was determined as the number of pixels positive for CD31 divided by the total tumor area. The hypoxia fraction was defined as the area positive for pimonidazole divided by the total tumor area (necrosis excluded). The density of macrophages in the hypoxic region was defined as the fraction of pixels positive for CD68 in the pimonidazole positive tumor area divided by the fraction of total CD68 positive pixels within the selected field. The mean intensity of Arg-1 of CD68 positive TAMs was calculated as the sum of intensity of Arg-1 that are double positive for Arg-1 and CD68 divided by the Arg-1 staining color pixels in selected area. The mean intensity of CD11b positive cells in control tumor was calculated as a reference. Regions with relative CD11b intensity >125 or <75% of reference CD11b mean intensity were arbitrarily defined as $CD11b^{high}$ or $CD11b^{low}$ regions, respectively.

STATISTICS

Statistical analyses used GraphPad Prism version 3 (GraphPad Software, San Diego, CA, USA). For all comparisons, assessment of statistical significance was by unpaired *t*-tests with significance set at $P = 0.05$.

RESULTS

THE ASSOCIATION OF CD68+ TAMs WITH HYPOXIA IS TUMOR AND TISSUE DEPENDENT

Murdoch et al. (2004) have shown that CD68+ TAMs were preferably situated at hypoxic regions in a xenograft model of human cervix cancer. However, our previous study in TRAMP-C1 model has shown that CD68+ TAMs do not have any specific preference for hypoxic or non-hypoxic regions (Chen et al., 2009). To further clarify this issue, we injected murine astrocytoma, ALTS1C1, or murine glioma, GL261, murine astrocytoma and glioma, into the muscle to mimic TRAMP-C1 tumor model. The result (**Figures 1A,B**) clearly shows that CD68+ TAMs are randomly distributed within the tumor without any preference for the hypoxia. However, when ALTS1C1 was implanted into the brain, a degree of preference for CD68+ TAM to accumulate in hypoxic regions was found (**Figure 1C**), but this preference is not seen for GL261 tumors grown in the brain (**Figure 1D**). This result indicates that the association of CD68+ TAMs with hypoxia is tumor dependent. The differences in distribution pattern for ALTS1C1 growing in intramuscular versus intracranial sites indicates that the association of TAMs with hypoxia also depends on local environmental cues.

To further explore whether the association of TAMs with hypoxia is associated with factors released by tumors, a gene microarray approach was used to compare the expression profiles between ALTS1C1 and GL261 because both display different TAMs-hypoxia association pattern in the brain (**Figure 2A**). Following RT-PCR confirmation, the expression levels of, at least three monocyte-associated factors, SDF-1α, VEGF, and MMP-2, were identified as different between ALTS1C1 and GL261 tumor cells (**Figure 2B**). When the expression of SDF-1α by ALTS1C1 cells was suppressed by the transfection of lentiviral siRNA particles, the association of CD68+ TAMs with hypoxia disappeared (Wang et al., 2012). This supports the notion that factors released by tumor cells can affect the function of TAMs.

SPATIAL DISTRIBUTION OF DIFFERENT SUBSETS OF INFLAMMATORY CELLS IN IR OR PRE-IR TUMORS

An important finding from our previous study in the TRAMP-C1 tumor model was aggregation of CD68+ TAMs into chronic hypoxic regions after RT (Chen et al., 2009). These CD68+ TAMs expressed weaker CD11 staining by IHC but another subtype cells with stronger CD11 expression was found within the necrotic region (**Figure 3A**). These cells were further demonstrated to be Gr-1+. Based on the Gr-1 staining and intensity of CD11b expression by IHC, the CD11b+ cells can be further divided into two sub-populations, $CD11b^{high}$/Gr-1+ neutrophils and $CD11b^{low}$/Gr-1− TAMs. The $CD11b^{low}$ TAMs could be further divided into $CD11b^{low}$/CD68+ and $CD11b^{low}$/F4/80+ TAMs. The $CD11b^{low}$/CD68+ TAMs were highly centered in PIMO+ regions, and $CD11b^{low}$/F4/80+ TAMs were on the edge of PIMO+ regions next to necrotic regions. In other words, this study shows that randomly distributed CD11b cells in un-irradiated control tumors re-distributed into distinct spatial location after RT. The flow cytometry assay (**Figure 3B**) found that the total number of CD11b cells increased after RT, but this increase was mainly the result of infiltration of Gr-1+ cells. The number of CD68+ TAMs during this period showed no significant change over a 3-week period.

The association of TAMs with hypoxic tumor before and after RT was further examined using the ALTS1C1 and GL261 brain tumor models. **Figure 4** shows that RT induced the aggregation of CD68+ TAMs into PIMO+ regions in both ALTS1C1 and GL261 tumors, which was not seen in control tumors as shown in **Figure 1C** versus **Figure 1D**. This indicates that radiation-induced hypoxic regions have factors to attract or trap CD68+ TAMs. Actually, the association of CD68+ TAMs with hypoxia is

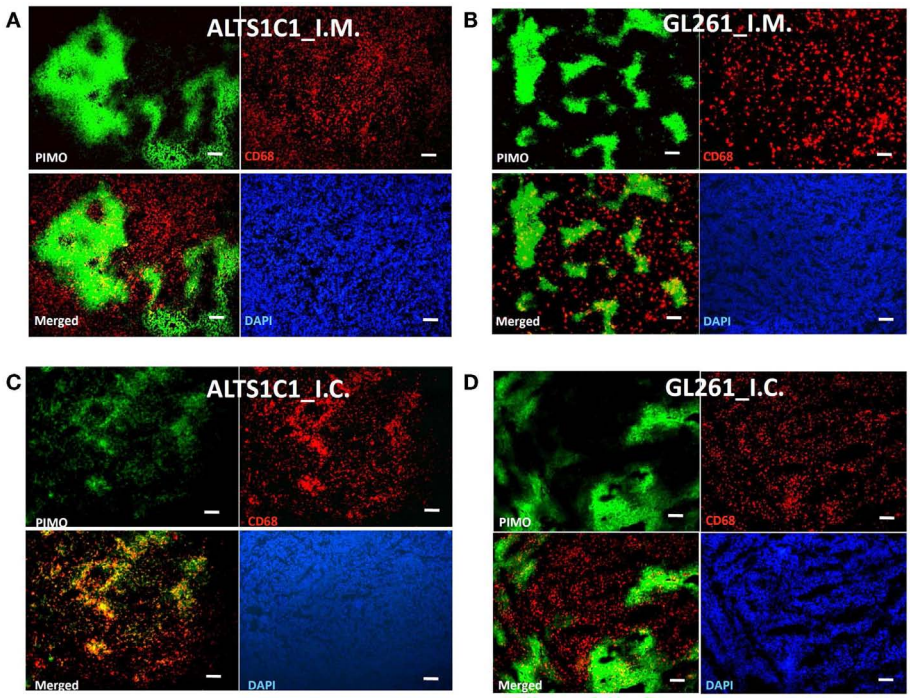

FIGURE 1 | The association of CD68+ TAMs with hypoxia is tumor and tissue dependent. The distribution of CD68+ TAMs and PIMO+ hypoxia in ALTS1C1 astrocytoma **(A,C)** and GL261 glioma **(B,D)** grown in the thigh (i.m.) **(A,B)** or in the brain (i.c.) **(C,D)**. Green: anti-PIMO stain for hypoxic region; red: anti-CD68 antibody for TAMs. Merged images: the colocalization of hypoxia and TAMs. Scale bar = 100 μm.

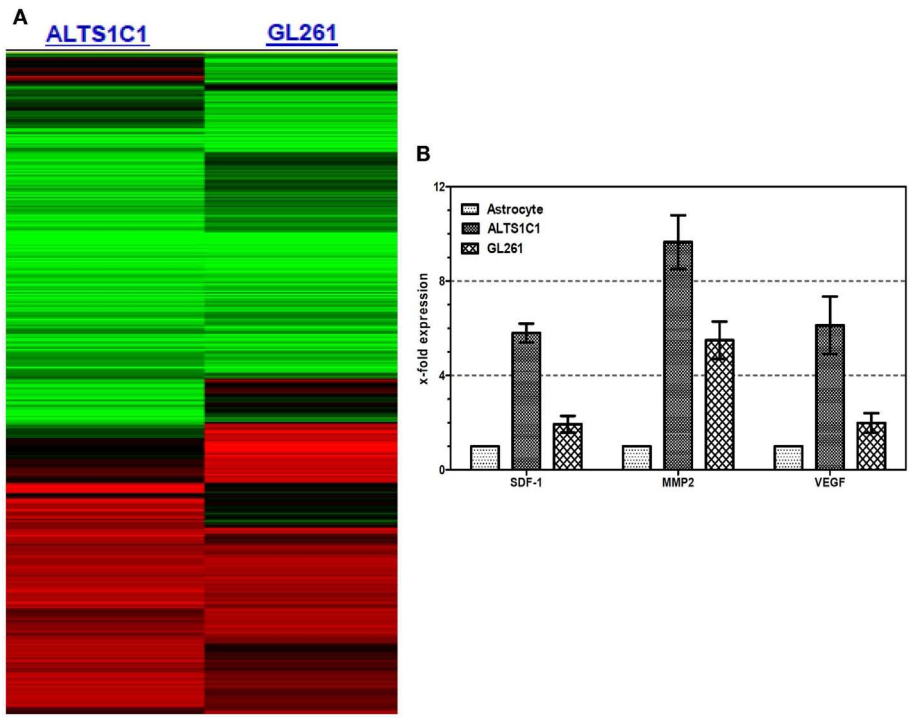

FIGURE 2 | The gene expression profiles between ALTS1C1 and GL261 cells by (A) cDNA microarray and (B) RT-PCR analysis. The mRNA level of primary astrocyte was used as reference.

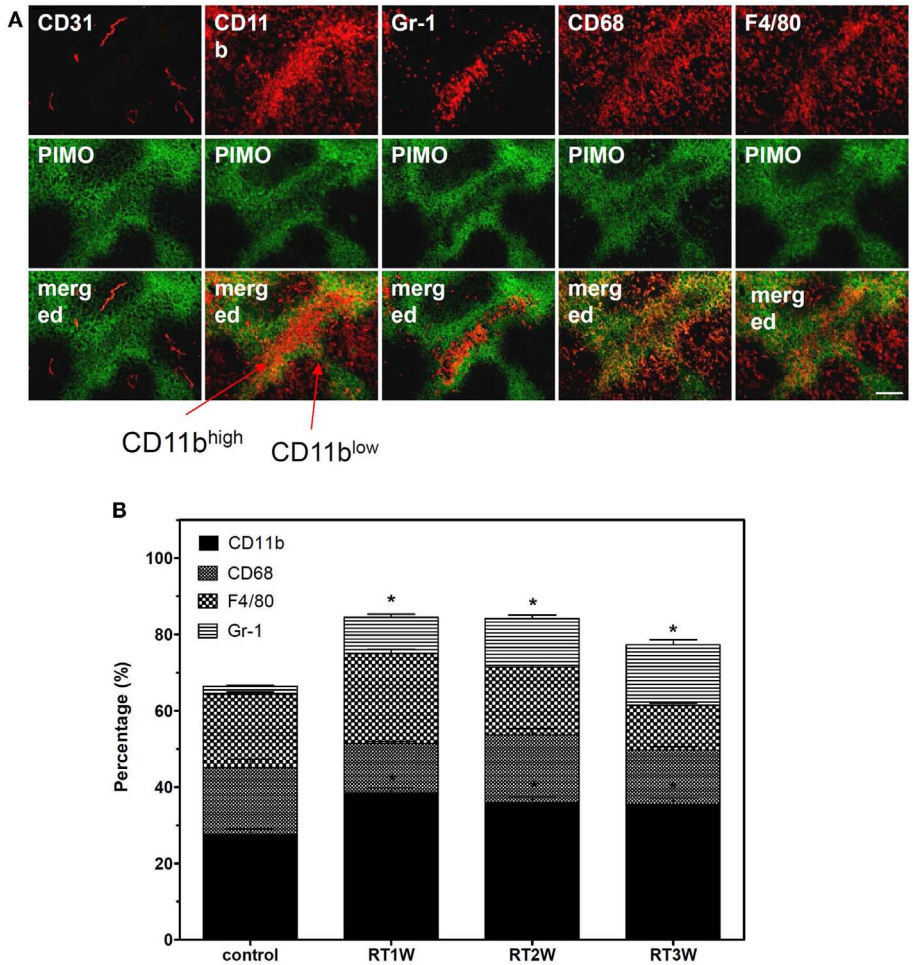

FIGURE 3 | Irradiation redistributes the localization of subtypes of inflammatory cells. (A) The distribution of CD31, CD11b, Gr-1, CD68, and F4/80 staining with PIMO+ hypoxia in series 25 Gy-irradiated-TRAMP-C1 tumor sections. Green: anti-PIMO stain for hypoxic region; red: anti-CD31, anti-CD11b, anti-Gr-1, anti-CD68, or anti-F4/80 antibody. Merged images: the colocalization of hypoxia and inflammatory cells or vessels. Scale bar = 100 μm. **(B)** Percentage of CD11b, CD68, F4/80, or Gr-1 positive cells within control or 25 Gy-irradiated TRAMP-C1 tumors as assayed by flow cytometry.

not only in irradiated tumors, but also occurs in tumors growing from pre-irradiated tumor bed, so-called pre-IR tumor. This is not only seen in TRAMP-C1 tumor model (Chen et al., 2011), but also occurs in ALTS1C1 astrocytoma tumor growing in either pre-irradiated brain or muscle tissues (**Figure 5**).

TAM AGGREGATION INTO AVASCULAR HYPOXIC REGIONS IS A SPECIFIC EFFECT OF IRRADIATION

Using the pre-IR TRAMP-C1 tumor bed model, we have reported that the accumulation of TAMs is only seen in PIMO+ hypoxic areas with low MVD, but not in areas with high MVD regions (Chen et al., 2011). This challenges us to wonder whether the decrease of MVD is the prime factor for TAM aggregation. In fact, the nature of PIMO+ region in control and irradiated tissues is different (**Figure 6**). In control ALTS1C1 tumor, there are vessels intervening within the PIMO+ areas (**Figures 6A,C**) and the hypoxia is likely to be the result of vessel malfunction. On the other hand, RT destroys most vessels or alters the way they are formed within tumors (Chen et al., 2011) and the PIMO+ regions (**Figures 6B,D**). ALTS1C1 tumors do not contain vessels whether the tumor or the tumor bed had been irradiated. In other words, the hypoxia in irradiated tissues is likely the result of vascular insufficiency, so-called avascular chronic hypoxia. The avascular chronic hypoxia could be found in tumors receiving 25 Gy of irradiation before or after tumor implantation in both TRAMP-C1 prostate (Chen et al., 2011) and ALTS1C1 astrocytoma models (**Figure 6**).

To further address the vascular issue, we used the anti-angiogenic agent, sunitinib, to treat TRAMP-C1 tumor grown in C57BL/6J mice. Anti-angiogenic agents, such as sunitinib, have been proposed as potential candidates for clinical use in recurrent tumors expressing high levels of angiogenic factors (Rauh-Hain and Penson, 2008). Our previous study in TRAMP-C1 tumors has shown that the administration of sunitinib could generate a 3-day tumor growth delay, which is less than the effect of 25 Gy of IR. Although the effects of sunitinib on growth delay

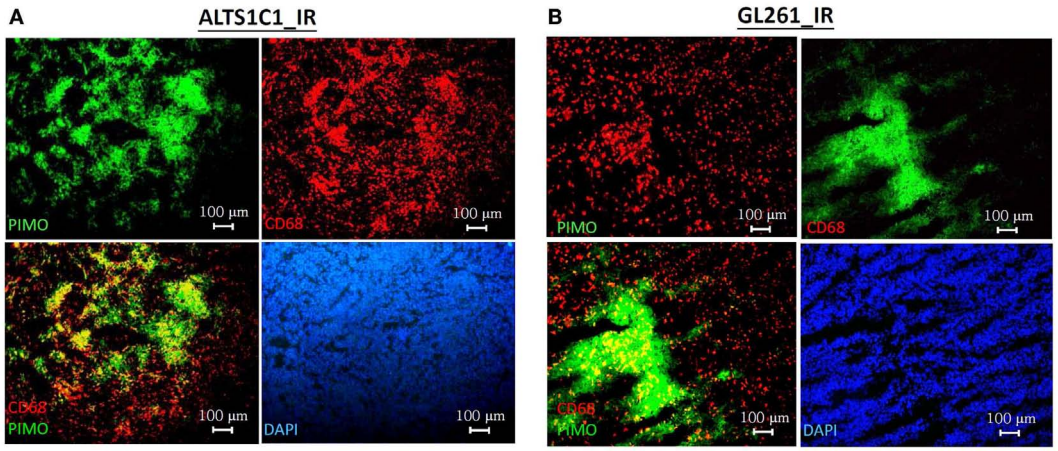

FIGURE 4 | Irradiation induces CD68+ TAMs aggregation in hypoxic regions in both ALTS1C1 and GL261 i.c. tumor models. The distribution of CD68+ TAMs and PIMO+ hypoxia in 8 Gy-irradiated ALTS1C1 astrocytoma **(A)** and GL261 glioma **(B)** grown in the brain. Green: anti-PIMO stain for hypoxic region; red: anti-CD68 antibody for TAMs. Merged images: the colocalization of hypoxia and TAMs. Scale bar = 100 μm.

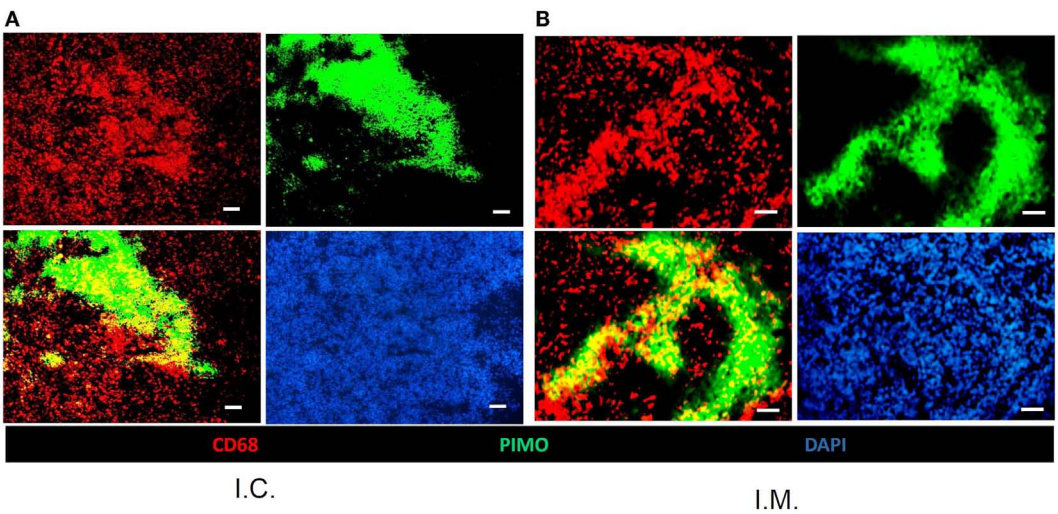

FIGURE 5 | Pre-irradiation induces CD68+ TAMs aggregation in hypoxic regions in ALTS1C1 tumors grown in both the brain (i.c.) and thigh (i.m.). The correlation of CD68+ TAMs with PIMO+ hypoxia in pre-irradiated ALTS1C1 astrocytoma grown in the brain (i.c.) **(A)** or in the thigh (i.m.) **(B)**. Green: anti-PIMO stain for hypoxic region; red: anti-CD68 antibody for TAMs. Merged images: the colocalization of hypoxia and TAMs. Bar: 100 μm.

were modest, dramatic changes in tumor hypoxia and MVD were found following sunitinib administration (Chen et al., 2011). Many hypoxic regions in sunitinib-treated tumors did not have vessels and were chronically hypoxic due to vascular insufficiency (**Figure 7**), as seen in IR tumors. The center of the avascular chronic hypoxic areas contained CD11b+ Gr-1+ cells, as was seen in IR or pre-IR tumors. However, the aggregation of CD68+ TAMs into avascular hypoxic areas that was seen in IR or pre-IR tumors did not exist in sunitinib-treated tumors (**Figure 7**). In fact, most CD68+ TAMs accumulated in PIMO negative regions. This demonstrates that the accumulation of CD68+ TAMs in avascular chronic hypoxia is a specific effect of RT and has less to do with the decrease in MVD or the development of avascular chronic hypoxia.

HYPOXIA-ASSOCIATED TAMs EXPRESS HIGHER LEVEL OF ARG I

Our early study using whole tumors has shown that CD11b+ TAMs isolated from irradiated tumors express higher level of Arg I and had tumor-promoting activity (Tsai et al., 2007). However, that approach cannot identify local environmental effects. In this study, the expression of Arg-1 by randomly distributed-CD68+ TAMs (i.e., at PIMO− non-hypoxic regions) versus aggregated CD68+ TAMs (i.e., at avascular PIMO+ hypoxic regions) was further verified in the ALTS1C1 intracranial model by IHC. **Figure 8** shows CD68 and Arg-1 double staining in fluorescent imaging taken from control, IR, or pre-IR tumor samples with the same exposure time. It shows a higher percentage and intensity of Arg-1 staining in hypoxia-aggregated CD68+ TAMs than CD68+ TAMs that are not aggregated in hypoxic area (**Figure 8B**). This indicates

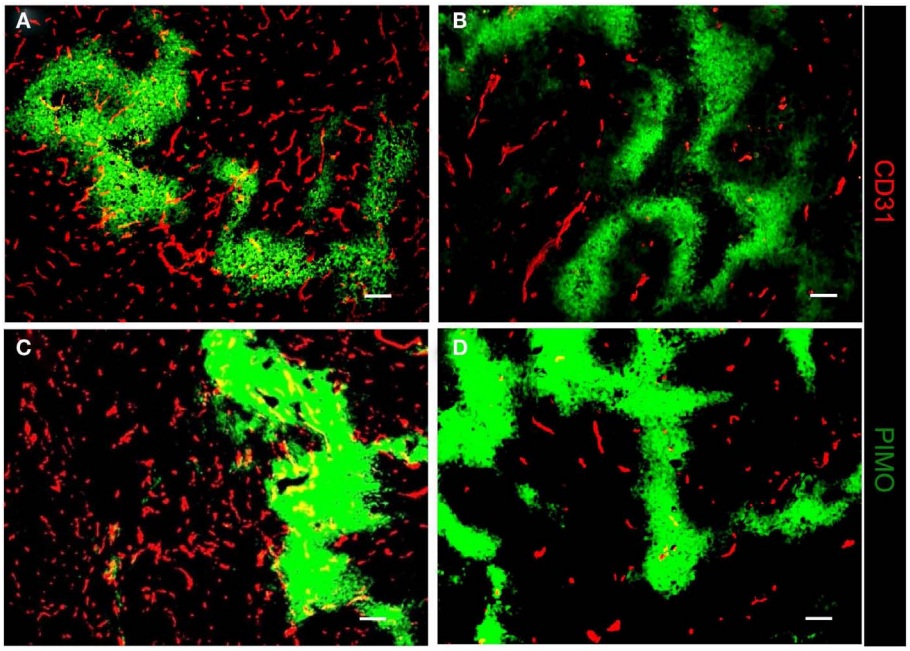

FIGURE 6 | The nature of PIMO+ hypoxic region in control and irradiated tissues is different. The IHC staining for the distribution of hypoxia and vascular in control **(A,C)**, irradiated **(B)**, or pre-irradiated **(D)** ALTS1C1 tumors. The hypoxic regions in control tumors were vascularized. There was almost no vasculature in the hypoxic regions of irradiated **(B)** or pre-irradiated tumors **(D)**. Green: anti-PIMO stain for hypoxic region; red: anti-CD31 antibody for vessels. Merged images: the colocalization of hypoxia and vessels. Scale bar = 100 μm.

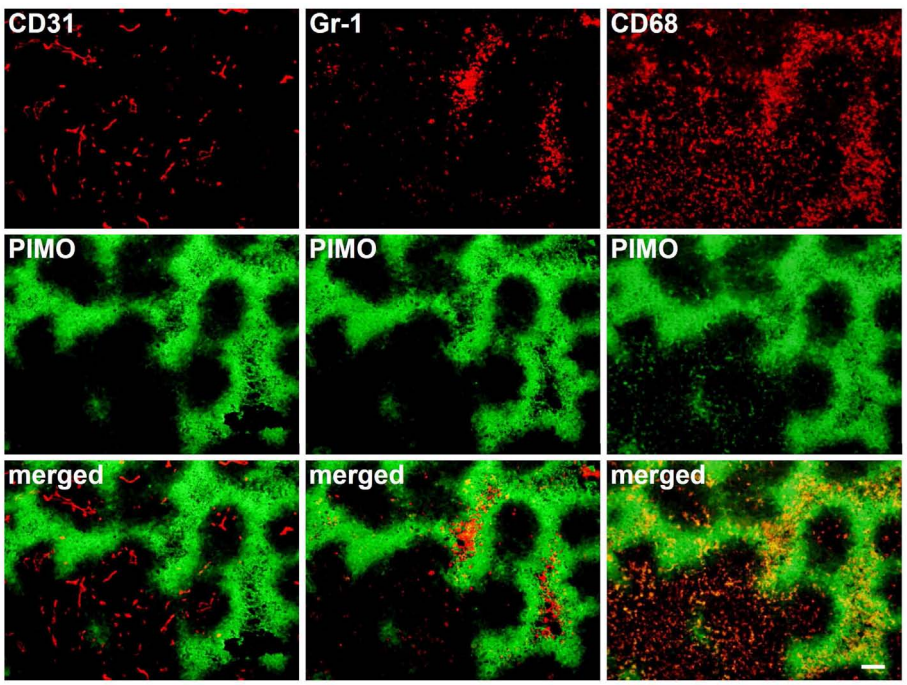

FIGURE 7 | IHC staining for CD31,Gr-1, and CD68 in series sutent-treated tumor sections. Administration of sutent decreased vascular density and accumulated Gr-1+ cells at central necrosis in chronic hypoxia. However, CD68+ TAM does not aggregate at chronic hypoxia. Scale bar = 100 μm.

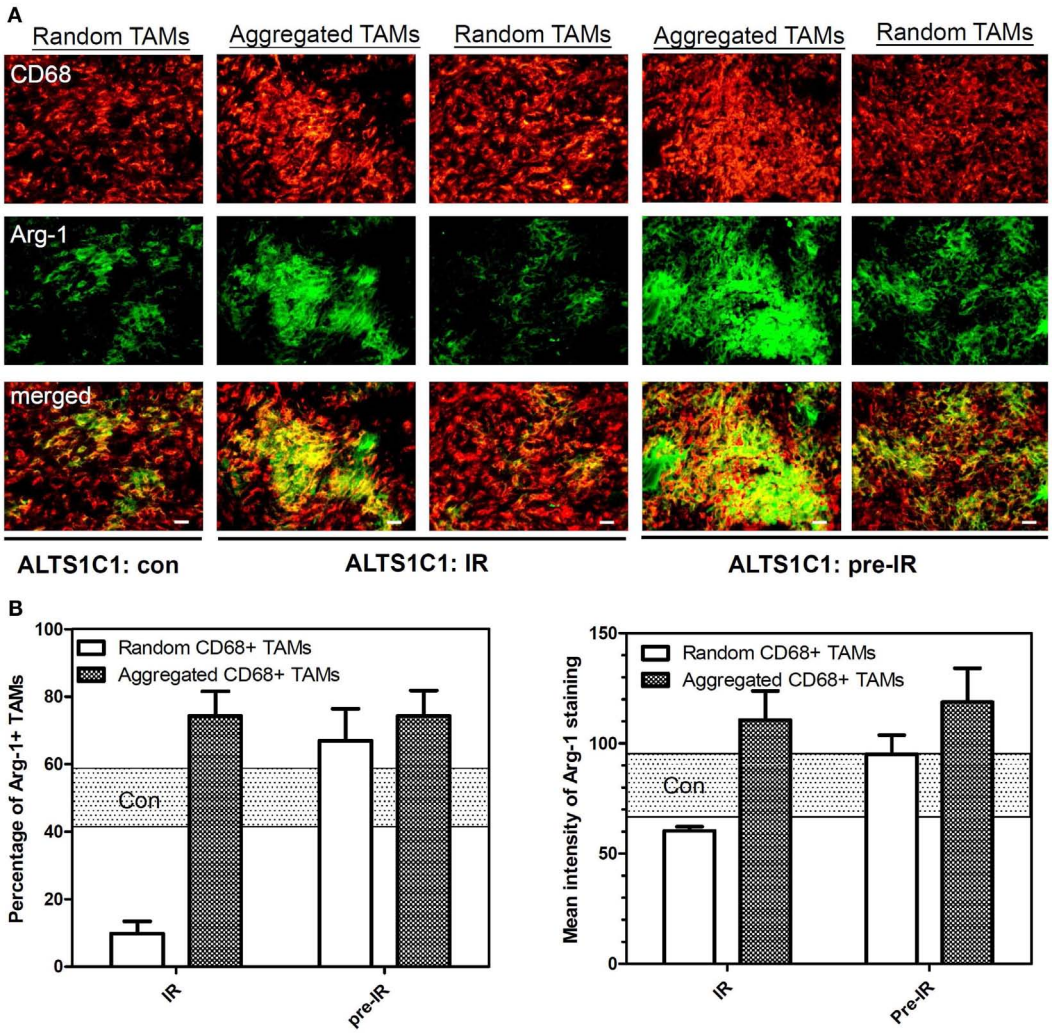

FIGURE 8 | Aggregated TAMs express higher level of Arg-1 than random TAMs. (A) The IHC staining for the expression of Arg-1 by random or aggregated CD68+ TAMs in control, irradiated (IR) or pre-irradiated (pre-IR) ALTS1C1 tumor Green: anti-Arg-1 antibody; red: anti-CD68 antibody. Merged images: the colocalization of Arg-1 and TAMs. Scale bar = 100 μm. **(B)** The percentage of Arg-1+ TAMs (left graph) and the mean intensity of Arg-1 staining (right graph) by random or aggregated CD68+ TAMs in control (rectangular dot region), irradiated (IR) (white bar) or pre-irradiated (pre-IR) (dark dot bar) ALTS1C1 tumors. The rectangular dot region represents the average value ± SD in control ALTS1C1 tumor for the purpose of clarity.

that hypoxia-aggregated TAMs could be more polarized toward an M2 phenotype.

SDF-1α PLAYS A CRITICAL ROLE IN TAM AGGREGATION AND TUMOR RE-GROWTH AFTER RT

SDF-1α production by ALTS1C1 is a critical factor for the accumulation of TAMs in hypoxia (Wang et al., 2012). Knock down of SDF-1(SDF^{kd}) in ALTS1C1 tumors growing in a pre-irradiated tissue also inhibited CD68+ TAM aggregation whether they were grown i.c. (**Figure 9A**) or i.m. (**Figure 9B**). Tumor growth delay was also further enhanced in both i.c. (**Figure 9C**) and i.m. (**Figure 9D**) models when the expression of SDF-1α by ALTS1C1 was suppressed by siRNA. This is further indirect evidence to support the view that radiation-induced TAM aggregation in hypoxic areas stimulates tumor growth through SDF-1 production.

DISCUSSION

The interplay between TAMs and hypoxia is thought to be bi-directional. Hypoxia-induced HIF-1α stabilization leads to the expression of various angiogenic factors, such as VEGF, and chemotactic factors, such as SDF-1α and CSF-1, by hypoxic tumor cells. These factors further recruit peripheral macrophages to the hypoxic regions to restore blood delivery and nourish the hypoxic cells. The association of TAMs with hypoxia was originally thought to be a natural link as is indicated by several renowned publications (Leek et al., 1999; Lewis et al., 1999, 2000; Crowther et al., 2001; Burke et al., 2002; Murdoch and Lewis, 2005; Murdoch et al., 2005; Degrossoli and Giorgio, 2007; Corzo et al., 2010). However, our previous study in murine TRAMP-C1 prostate adenocarcinoma demonstrated that the CD68+ TAMs have no preference for PIMO+ hypoxia region in the control, untreated tumors (Chen et al., 2009). On the other hand, CD68+, but not F4/80+, TAMs

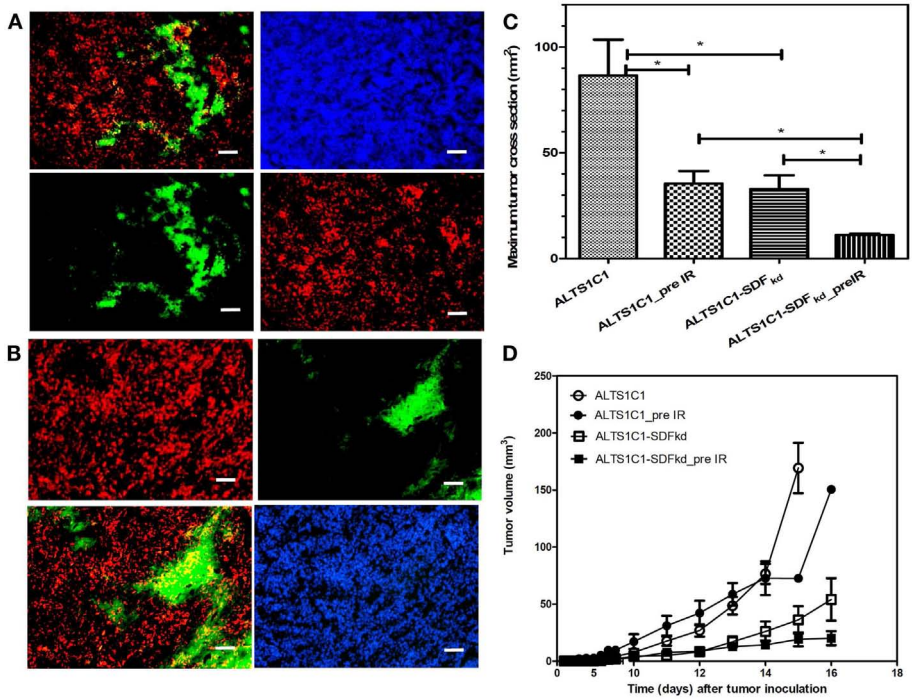

FIGURE 9 | Suppression of SDF-1 expression by ALTS1C1 tumor disrupts pre-IR-induced TAM association with hypoxia in both i.c. (A) and i.m. (B) models and prolong pre-IR-induced growth delay in both i.c. (C) and i.m. (D) models. Green: anti-PIMO stain for hypoxic region; red: anti-CD68 antibody for TAMs. Merged images: the colocalization of hypoxia and TAMs. Scale bar = 100 μm.

prefer to accumulate in PIMO+ hypoxia in intracranial ALTS1C1 astrocytoma. It appears that the association of CD68+ TAMs with hypoxia is tumor dependent. This concept is further supported here by another murine brain glioma, GL261, in which CD68+ TAMs do not have a preference for PIMO+ hypoxic regions. It becomes more interesting when ALTS1C1 astrocytomas were inoculated in the muscle, where the association of TAMs with hypoxia seen in intracranial model disappeared. This indicates that the association of TAMs with hypoxia is not only tumor type dependent, but also stroma dependent. In other words, both tumor-released factors and environmental cues determine TAM function.

In this study, we used ALTS1C1 and GL262 tumors models to demonstrate that the hypoxic regions in irradiated tumors or tumors growing in pre-irradiated tissues had more CD68+ TAM accumulation than control tumors. These results are in agreement with our previous studies in TRAMP-C1 tumor model and demonstrate their reproducibility in several tumors. Most PIMO+ hypoxic regions in control tumors contain CD31+ vessels, suggesting that hypoxia resulted from abnormal vessel perfusion and these may be transiently hypoxic. On the other hand, most radiation-induced hypoxic regions did not contain CD31+ vessels, indicating that the hypoxia is caused by insufficient blood vessels and may be avascular chronic hypoxia. These radiation-induced hypoxic regions frequently develop central necrosis and are filled by Gr-1+ neutrophils. In fact, avascular chronic hypoxia could be occasionally found in larger control tumors, but no CD68+ TAM aggregation were found (Fu, S. Y. manuscript in preparation). This indicates that radiation-induced hypoxic environments have specific factors that cause CD68+ TAM aggregation. This was further supported by the use of the anti-angiogenic agent sunitinib, a tyrosine kinase inhibitor that interrupts the signaling pathways of endothelial growth factor receptor 1-3 and PDGF receptors α and β. The sunitinib-treated tumors display the decrease of MVD and the increase of avascular chronic hypoxia filled with Gr-1+ neutrophia as was seen in irradiated tumors. However, CD68+ TAMs do not accumulate in the avascular chronic hypoxic region of sunitinib-treated tumors. Instead, even more CD68+ TAMs were found in PIMO negative regions. This supports the notion that irradiated hypoxic regions have factors to cause CD68+ TAM aggregation. Since the ALTS1C1 and GL261 tumors have different CD68+ TAMs-hypoxia association patterns, microarray and RT-PCR techniques were used to isolate the genes responsible. At least three monocytes-associated factors, SDF-1α, VEGF, and MMP-2, were candidates. These factors have been separately reported to be chemoattractant for macrophages (Lewis et al., 2000; Gazitt and Akay, 2004; Kang et al., 2010) and induced by hypoxia (Burke et al., 2003; Ide et al., 2006; Williams et al., 2006; Zagzag et al., 2006; Chen et al., 2009; Wang et al., 2012). Previous studies have shown that RT can induce SDF-1α production to promote the homing of hematopoietic progenitor cells toward gliomas and enhance vessel formation (Tabatabai et al., 2006; Kioi et al., 2010). We have also found that SDF-1α in the conditioned medium produced by ALTS1C1 astrocytoma not only enhance macrophage migration toward hypoxia, but also prolong their survival in hypoxic condition (Wang et al., 2012). The current

study further demonstrates that SDF-1α production by tumor cells is one of factors that are responsible for the accumulation of TAMs in radiation-induced hypoxic regions as its knock down in ALTS1C1 tumor growing in intramuscular or intracranial pre-irradiated sites prevented TAM accumulation in hypoxia. What was more interesting was that the tumor growth delay was further enhanced in SDF-1α-suppressed tumors. This implies: (1) that SDF-1α promotes tumor growth in an irradiated microenvironment or (2) that the association of TAMs with hypoxia enhances tumor growth rate. If the latter is the case, it also indicates that hypoxia-aggregated TAMs have an M2 phenotype, which is supported by Arg I staining being greater (both number and intensity) in TAMs aggregated in hypoxic than non-hypoxic regions. This further enhances the general view that TAMs in radiation-induced hypoxia are of the M2 type and have better tumor-promoting function.

In addition, this study also demonstrates that RT, no matter given before or after tumor implantation, alters tumor microenvironments so that not TAM aggregate in hypoxic regions, and Gr-1 positive neutrophils, CD68+ TAMs, and F4/80+ TAMs resegregate into different microenvironments. We believe that this is the first report to show that three subtypes of monocytic cells have their own niches in the irradiated tumor microenvironment; these cells probably play different roles based on their different locations, although we do not clearly know these roles at the present. It is reasonable to speculate that the role of Gr-1 positive neutrophils in the central necrotic regions is to clean the debris in this area. The CD68+ TAMs within avascular chronic hypoxia are likely associated with their M2 functions. The roles or functions of F4/80+ TAMs at the junction of avascular hypoxia and central necrosis need better understanding. However, it needs to caution that these changes following RT are stage dependent because the vascular damage and chronic hypoxia following RT are dynamic, which depends on radiation dose, tissues, and factors released by the tumors. For example, the maximum hypoxia-induced TAM re-segregation for TRAMP-C1 prostate tumor or ALTS1C1 astrocytoma grown in the thigh following 25 Gy of radiation was at 3 week after RT. The maximum effect for ALTS1C1 astrocytoma grown intracranially following 8 or 15 Gy of radiations was at 3 or 2 week, respectively, after RT (Wang, S. C. manuscript in preparation).

At the end, we have sorted out the relationship among tumor cells, tumor microenvironments, and tumor response to RT. We conclude that factors released from tumor cells are prime factors for the formation of specific type of tumor microenvironments such as the association of TAMs with hypoxia. Among the tumor microenvironments, MVD is the prime factor determining the nature of hypoxia and the distribution of TAMs. Following irradiation, radiation-induced tissue damage may release factors, such as SDF-1α, that dominate the effects of original tumor- or stroma-released factors. More importantly, this study shows that the aggregation of CD68+ TAMs into hypoxic regions is associated with the re-growth rate. This is further evidence to support the view that CD68+ TAMs associated with chronic hypoxia are likely M2 TAMs and a target for enhancing the efficacy of RT. Tumor-secreted SDF-1α may not be the only factor responsible for the TAM accumulation in hypoxic regions. Several studies have also shown that hypoxia-induced iNOS expression can also promote TAM migration (Weigert and Brune, 2008; Zhou et al., 2009). However, the story for iNOS may be more complex than SDF-1α because the hypoxic regions have limited supply for oxygen, the iNOS substrate (Robinson et al., 2011).

ACKNOWLEDGMENTS

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Regulatory T cells in radiotherapeutic responses

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Radiation therapy (RT) can extend its influence in cancer therapy beyond what can be attributed to in-field cytotoxicity by modulating the immune system. While complex, these systemic effects can help tip the therapeutic balance in favor of treatment success or failure. Engagement of the immune system is generally through recognition of damage-associated molecules expressed or released as a result of tumor and normal tissue radiation damage. This system has evolved to discriminate pathological from physiological forms of cell death by signaling "danger." The multiple mechanisms that can be evoked include a shift toward a pro-inflammatory, pro-oxidant microenvironment that can promote maturation of dendritic cells and, in cancer treatment, the development of effector T cell responses to tumor-associated antigens. Control over these processes is exerted by regulatory T cells (Tregs), suppressor macrophages, and immunosuppressive cytokines that act in consort to maintain tolerance to self, limit tissue damage, and re-establish tissue homeostasis. Unfortunately, by the time RT for cancer is initiated the tumor-host relationship has already been sculpted in favor of tumor growth and against immune-mediated mechanisms for tumor regression. Reversing this situation is a major challenge. However, recent data show that removal of Tregs can tip the balance in favor of the generation of radiation-induced anti-tumor immunity. The clinical challenge is to do so without excessive depletion that might precipitate serious autoimmune reactions and increase the likelihood of normal tissue complications. The selective modulation of Treg biology to maintain immune tolerance and control of normal tissue damage, while releasing the "brakes" on anti-tumor immune responses, is a worthy aim with promise for enhancing the therapeutic benefit of RT for cancer.

Keywords: radiation, danger, Tregs

RADIATION AND "DANGER" SIGNALING

Local RT has complex, systemic consequences (Formenti and Demaria, 2009) that, if harnessed properly have the power to significantly shape host-tumor relationships and ultimately affect treatment outcome. This review will focus on those aspects of RT that could translate into anti-tumor immunity, and their immune regulation.

Tissues that have been damaged by radiation display various "danger" signals to the immune system that can be secreted and/or released into extracellular spaces. The so-called Damage-Associated Molecular Pattern molecules (DAMPs; Shi et al., 2003; Lotze et al., 2007; Curtin et al., 2009; Sato et al., 2009). What characterizes DAMPs is that they are endogenous molecules that signal through a set of common pattern recognition receptors (PRRs; Matzinger, 2002; Lotze et al., 2007; Kawai and Akira, 2011), such as the Toll-like receptor (TLR) family (Medzhitov et al., 1997; Beutler, 2009), nucleotide binding oligomerization domain (NOD)-like, and retinoic acid inducible gene (Rig)-like receptors (Meylan et al., 2006), and C-type lectins (Robinson et al., 2006). Once engaged, PPRs initiate signaling cascades to establish communications between immune cells through generally pro-inflammatory cytokine and chemokine networks. The system has evolved to recognize and deal with dangerous pathological situations, restore homeostasis, and to regenerate and heal tissues (Schaue and McBride, 2010; Schaue et al., in press).

Within tumors, DAMPs are generated by cell stress and death during progressive growth and increasing vascular abnormalities, and by oxidative damage and hypoxia (Ullrich et al., 2008; Sato et al., 2009). DAMP signaling and the cytokines they generate not only affect the content and function of innate immune cells within tumors, but also can play critical roles in the generation of adaptive immunity. This is because dendritic cells (DCs) have to mature to be competent at antigen-presentation, which requires pro-inflammatory "danger" signals (Banchereau and Steinman, 1998; Gallucci et al., 1999). Mature DCs are crucial for providing signal 2, the verification co-stimulatory signal that is needed to translate signal 1 (antigen) into a T cell-mediated immune response. Conversely, antigen-presentation in the absence of co-accessory signaling leads to immune tolerance (Steinman et al., 2003). In cancer treatment, the potential role of DAMP recognition and the initiation of adaptive anti-tumor immunity is seen in breast cancer patients with defective TLR-4 signaling who are less able to respond to standard therapy presumably because of a lack in tumor immune eradication (Apetoh et al., 2007). There is however a possible negative side to this equation as all cells, including

tumor cells, express DAMP receptors of varying types which can drive tumor progression (Sato et al., 2009).

Tumor RT certainly will increase the amount of DAMPs released, but the extent to which it qualitatively and quantitatively changes DAMPs levels is not known, nor how such changes will affect the immune responses that are made. Exacerbation of the level of "danger" signaling in the tumor microenvironment by RT has however the potential to activate innate immune cells and link to the development of tumor antigen-specific, adaptive immunity. In support, we, and others, have observed that radiation can mature DCs, enhancing expression of numerous molecules that further aid immune recognition, such as MHC class I and II molecules, co-stimulatory CD80, cell adhesion molecules such as ICAM-1, integrins, and selectins, and damage recognition molecules such as phosphatidyl serine (Santin et al., 1996; Morel et al., 1998; Seo et al., 1999; Garnett et al., 2004; Reits et al., 2006; Tyurina et al., 2011), in addition to creating a pro-oxidant, pro-inflammatory milieu that encourages infiltration by immune cells (Lorimore et al., 2001; Lugade et al., 2005, 2008; Matsumura et al., 2008; Burnette et al., 2011). Overall, these responses seem to be a deliberate attempt by the tissue to improve immune cell access and to encourage immunogenicity and susceptibility to attack by T lymphocytes and other immune cells (Garnett et al., 2004). For example, irradiated tumor cells can show enhanced expression of the death receptor Fas *in vitro* and *in vivo*, consequently sensitizing tumors to antigen-specific cytotoxic T cells and, ultimately, rejection (Chakraborty et al., 2003, 2004).

A case can therefore be made for cancer therapies like RT being able to act as immune adjuvants, in addition to having direct anti-tumor action (Roses et al., 2008). Such responses must be carefully controlled. Optimization of anti-tumor immune responses following RT is not trivial and requires consideration of many additional contributing factors.

RADIATION AS AN IMMUNE ADJUVANT

If RT can induce a pro-oxidant, pro-inflammatory microenvironment, one would expect that irradiated tumors often induce measurable systemic immune responses that can lead to tumor regression in preclinical models (Lugade et al., 2005; Lee et al., 2009; Perez et al., 2009; Spanos et al., 2009). There are a few encouraging reports indicating that humans receiving RT may make increased immune responses when combined with other immunostimulatory therapies (Nesslinger et al., 2007; Ferrara et al., 2009; Stamell et al., 2012), with chemotherapy or even alone (Schaue et al., 2008). In the last example, we showed that circulating tumor-specific CD8+ T cells can rise in colorectal cancer patients toward completion of chemo-radiation with 45 Gy and continuous 5-fluorouracil infusion (Debucquoy et al., 2006, 2009; Schaue et al., 2008). More general support for the view that the immune system can be a powerful and independent prognostic indicator of a good response to cancer therapies comes from studies on T cells infiltration in solid tumors (Galon et al., 2006; Pages et al., 2010) and from abscopal effects that can be attributed to the systemic development of immunity (Formenti and Demaria, 2009; Stamell et al., 2012). Questions however remain as to why tumor-specific responses are not always generated by therapies, even within one tumor type, why some types of tumors generate such responses only rarely, and the ultimate question of why tumors continue to grow even in the presence of an immune response that appears effective *in vitro*.

One issue that must be considered is that by the time therapy is initiated tumors have already escaped the attentions of the immune system. Multiple mechanisms have been described by which this is achieved (Zitvogel et al., 2006; Whiteside, 2009). The nature of the immune escape mechanism strongly influences the tumor-host relationship, the tumor antigens that are expressed, and probably the outcome of any therapeutic approach. For example, even highly immunogenic tumors can grow progressively and maintain strong tumor antigen expression if they generate powerful suppressor T cells and macrophages (Howie and McBride, 1982; McBride and Howie, 1986; Iwai et al., 2002). On the other hand, tumors may undergo immunoediting that selects for cells lacking antigen expression during tumor development. In the former situation, tumors are more likely to respond to removal of immune suppressor cells than in the latter. In some tumors, the rate of tumor cell death and turnover could be critical in balancing the immune system so as to favor tumor growth. In this case, simply changing this equation through aggressive therapies may have a positive effect. In each of these scenarios, the tumor antigens that are expressed are likely to differ in potency for stimulating immunity and the suppressor mechanisms that have to be overcome will vary in strength and type. This indicates that different strategies for potentiating tumor immunity may need to be tailored to the existing state of the tumor-host relationship. Additional factors that might limit the generation of the "dangerous" microenvironment and the extent of adaptive immunity to the tumor include the nature of the vasculature, the degree of oxidative stress, and the extent of hypoxia in a tumor (Conejo-Garcia et al., 2004; Rius et al., 2008; Sitkovsky, 2009; Facciabene et al., 2011; Kandalaft et al., 2011). RT has been shown to change the tumor microenvironment by causing vascular damage, inhibiting angiogenesis, and enhancing chronic hypoxia at the expense of transient hypoxia, with the newly generated hypoxic areas becoming infiltrated with tumor-promoting macrophages (Dewhirst et al., 1990; Garcia-Barros et al., 2003; Chen et al., 2009; Ahn et al., 2010; Kioi et al., 2010). These crucial variables may shape the tumor response to RT and vary with the tumor and its location (Chiang et al., 2012).

The dose and delivery schedule for RT also influences the development of anti-tumor immunity. For RT to be an immune adjuvant there seems to be an optimal size of dose and dose per fraction, with moderate dose fractions of around 5–6 Gy being superior to 2 Gy fractions (Dewan et al., 2009; Schaue et al., 2012). And in the case of the murine melanoma model, tumor-specific immune responses following RT were found to inversely correlate with tumor size illustrating an interesting dichotomy in the tumor-host relationship (Schaue et al., 2012). These findings generally support the belief that therapy-induced tumor damage can translate into measurable immune activation.

LIMITING THE IMMUNE RESPONSE TO PROTECT SELF

The transition from the rapidly generated, innate immune response to activation of the slower, more sophisticated adaptive immune system is a critical step in the development of tumor

immunity. Importantly, adaptive immunity tends to be polarized, especially with respect to antigen-specific helper and regulatory T cell subsets (Th/Tregs; Fernandez-Botran et al., 1988) that can ultimately dictate immune-mediated regression or progression, most often mediated through $CD8^+$ T cell activation. $CD4^+$ naïve cells (Th0) recognize antigenic peptides on DCs through their T cell receptor-$CD3^+$ complexes and, based on the signals received, can differentiate along one of at least four pathways to form Th1, Th2, Th17, or iTregs. This dramatic cellular polarization is orchestrated by the prevailing cellular microenvironment through a network of transcription factors and microRNAs; T bet for Th1, GATA-3 for Th2, RORgammat for Th17 cells, and Foxp3, miR-10a, miR-155 for Tregs (Zhu and Paul, 2010; Dang et al., 2011; Gao et al., 2012; Takahashi et al., 2012).

The important result is the emergence of T cell subsets that, while they are antigen-specific, exert much of their influence through distinctive effector cytokine profiles that influence bystander non-immune and immune cells alike, depending upon their cytokine receptor patterns. Th1 cells respond primarily to IL-12 to produce IFN-γ, GM-CSF, and TNF-α and are important for assisting cytotoxic $CD8^+$ T cell-mediated responses that can eliminate tumors. They also activate macrophages to express a pro-inflammatory phenotype that can be cytotoxic to tumors. Th2 cells, in contrast, are stimulated primarily by IL-4 to produce IL-4, IL-5, IL-6, IL-13, and IL-25. They assist B cells in the generation of antibodies that form allergic responses. Th17 cells differentiate in response to IL-6 or IL-22 to produce IL-17, IL-21, IL-22, IL-23, and GM-CSF. Th17 cells have been implicated in the pathogenesis of many chronic inflammatory and autoimmune diseases (Waite and Skokos, 2012). The concept that distinct functional T cell subsets exist as balanced forces to maintain homeostasis has established validity and has been extended to $CD8^+$ T cells, "classically" activated M1, and "alternatively" activated M2 macrophages and DC1/DC2 DCs (Czerniecki et al., 2001; Van Ginderachter et al., 2006), although there is some controversy as to the degree of reprogramming that is possible within these other immune cell types.

As crucial for tumor immunity and as life-saving as any of the above immune players are, the mutual antagonism that exists between different Th subsets in itself is insufficient to control the immune system, which can cause extensive tissue damage if left unrestrained, as in chronic inflammation, autoimmune, and allergic reactions. Tregs (also known as suppressor T cells) are the major players in preventing excessive damage to self (Peterson, 2012) and they represent that other side of the immunological coin from Th cells. The presence of T cells that could suppress antigen-specific inflammatory T cell activity was first recognized by Gershon and Kondo (1971), who called the phenomenon "infectious immunological tolerance." Plagued by lack of appropriate markers for T cell subpopulations, the Treg field fell into disrepute for many years, but re-emerged with the discovery of Tregs that are now known to fall into two major subsets of natural (nTregs) and induced (iTregs). These have largely non-overlapping distinct antigen recognition repertoires (Haribhai et al., 2009, 2011). Unlike Th cells, both Treg subsets focus on recognition of "self" antigens to maintain peripheral immunological tolerance and exert homeostatic control over inflammation through release of immunosuppressive cytokines (Bluestone and Abbas, 2003; Curotto de Lafaille and Lafaille, 2009).

TREGS MAKE US TOLERANT OF OUR SELF AND OF OTHERS

The importance of Tregs in maintaining peripheral self-tolerance, preventing autoimmune disease, and limiting inflammation and immunity (Sakaguchi, 2004; Shevach, 2004) is exemplified by the havoc caused in their absence, ranging from excessive lymphoproliferation, immune, and inflammatory tissue damage, to death. For example, a loss-of-function mutation in the essential regulator of Tregs, the forkhead box transcription factor Foxp3, leads to a lethal autoimmune and inflammatory disorder in the "scurfy" mouse and the IPEX syndrome (Immune dysregulation Polyendocrinopathy Enteropathy X-linked Syndrome) in humans (Fontenot and Rudensky, 2005; Chatila, 2009). Interesting in this context is the fact that high fractionated doses of radiation delivered to the lymphoid system of mice also generates autoimmunity (Sakaguchi et al., 1994).

Tregs function in widely diverse scenarios to control other T and B lymphocyte subsets, DCs, and macrophages, as well as non-immune cells. Although T cell receptor recognition and activation is through cognate antigen, suppression in their immediate environment can be rather indiscriminate, at least *in vitro* (Shevach, 2009). They use various immunosuppressive effector mechanisms, any one of which may be favored under specific conditions (Pillai et al., 2011). These include cell-to-cell contact, the release of cytokines such as IL-10, IL-4, IL-35, and/or TGF-β, and the production of adenosine that drives cAMP elevation and inhibition of T effector cells (Chen et al., 2005; von Boehmer, 2005; Deaglio et al., 2007; Shevach, 2009; Efimova et al., 2011). By generating an anti-oxidant/adenosinergic microenvironment, Tregs are tissue protective and the antithesis of pro-oxidant acute inflammation.

Most Tregs are naturally occurring, functionally mature $CD4^+CD25^{hi}Foxp3^+$ Tregs (nTregs) that are "hard-wired" with respect to their immune repertoire through thymic development and are already primed for suppressive function. In contrast, $CD4^+CD25^-$ naïve T cells can be converted outside the thymus into $CD4^+CD25^{hi}Foxp3^+$ Tregs, and are therefore called inducible or adaptive, iTregs. Induction can be a result of exposure to low doses of antigen, IL-2, and TGF-β (Apostolou and von Boehmer, 2004; Curotto de Lafaille et al., 2004). Given these differences in origin, it is not surprising that recombinase-deficient mice can generate iTregs but have no nTregs (Curotto de Lafaille et al., 2001; Mucida et al., 2005).

The functional distinction between iTregs and nTregs has still to be fully established, but they do not share the same workload in controlling the adaptive immune response. Overall, the regulatory phenotype of iTregs and their Foxp3 expression is less stable than that of nTregs possibly due to differences in epigenetic regulation and microRNA miR-10a availability (Floess et al., 2007; Takahashi et al., 2012). Their gene expression profiles are not identical (Feuerer et al., 2010). Molecular studies indicate that nTregs, but not iTregs, express Helios, an Ikaros family transcription factor (Thornton et al., 2010) and are activated by TNF-α (Housley et al., 2011) and by IL-6, the latter converting them to Th17 cells that can mediate potentially pathogenic autoimmunity (Xu et al., 2007). iTregs resist such Th17 conversion (Zheng et al.,

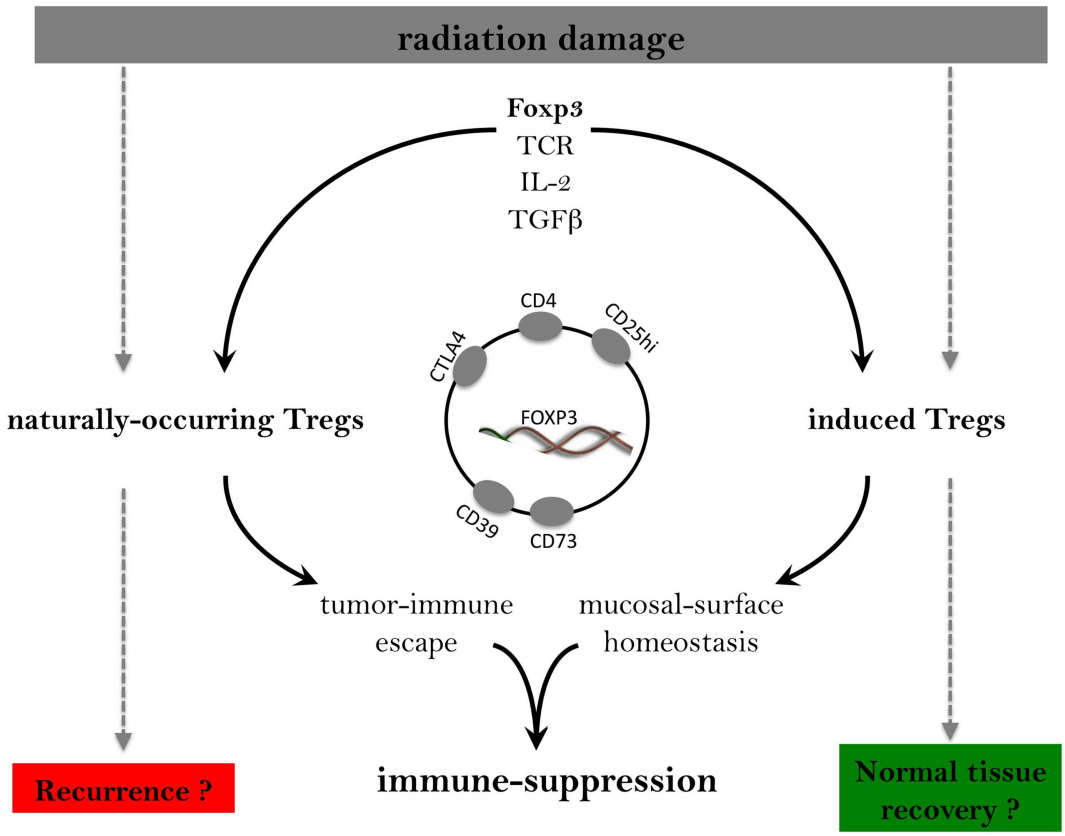

FIGURE 1 | Systemic immune control is exerted through the combined effort of thymically derived, naturally occurring nTregs, and peripherally induced iTregs that have specificity for "self" antigens but with distinct, minimally overlapping TCR repertoires. Both Treg pools depend heavily on the transcription factor FoxP3 and on IL-2 with TGF-β providing additional stimulation. While both Treg subsets contribute to immune suppression, iTregs seem to be selectively involved in mucosal surfaces. Radiation therapy drives an increase in Tregs that may limit potential anti-tumor immunity and aid tumor escape on one side but that may also nurture normal tissue recovery on the other.

2008). These differences may be important in that there is some evidence that iTregs exert control of inflammatory responses at normal mucosal surfaces while nTregs appear more important for mediating self-tolerance and tumor immune escape (Sakaguchi, 2004, 2005; Curotto de Lafaille and Lafaille, 2009; Haribhai et al., 2011; Rosenblum et al., 2011; Josefowicz et al., 2012; **Figure 1**). There is a distinct possibility that RT might differentially affect these Treg subpopulations, but this has yet to be established.

RADIATION EFFECTS ON IMMUNITY *IN VIVO*

The concept that RT is purely immunosuppressive because lymphocytes are very radiation sensitive is out-moded. While scientific wisdom indicates that lymphocytes are very radiosensitive, subsets differ in this regard and because all immune cells can be induced by radiation itself, as well as by DAMPs, cytokines, and other stimuli to respond at the molecular level, RT is clearly better regarded as being immunomodulatory. In very general terms, a spectrum of radiosensitivity exists from B cells through naive Th cells, NK cells, T memory cells (Belka et al., 1999), Tregs, and DCs to radioresistant macrophages, with a tendency toward apoptosis denoting a more radiosensitive phenotype and non-proliferative cells and activated lymphocytes being more radioresistant (McBride et al., 2004). As a result of blood flow through the field, even local RT will have a purely physical cytotoxic effect of the circulating immunocyte pool, which will vary with the tissue, and the delivery time and dose. Induced responses in tumor and normal tissues, and in the immune cells themselves add considerable additional complexity to the immune equation. The usual radiobiological parameters such as dose, dose rate, fraction size, and radiation quality are pertinent in all cases. Further, if chemotherapy is also given, different drugs are expected to target different immune cell populations, again with dose and scheduling being important parameters.

The ability of radiation to differentially modulate T cell subsets was in fact observed by North, Hellstroem, and others more than 30 years ago. They showed that sublethal, whole-body irradiation eliminated suppressor T cells leading to partial or complete tumor regression in immuno-competent, but not in immuno-incompetent, mice (Hellstrom et al., 1978; Tilkin et al., 1981; North, 1986). The same subset appeared sensitive to low dose cyclophosphamide (Bonavida et al., 1979; Awwad and North, 1989). This introduced the concept of metronomic low dose chemotherapy treatment that might assist elimination of immune suppressor cells, but angiogenesis and other cells are also possible targets (Penel et al., 2012). In contrast to these studies, we

and others have shown that Tregs are relatively radioresistant (Kusunoki et al., 2010; Nakatsukasa et al., 2010; Qu et al., 2010; Weng et al., 2010; Kachikwu et al., 2011). A possible explanation for this discrepancy lies in the fact that the timing of the radiation exposure post-tumor implantation was critical in North's experiments and that a Treg subpopulation may have been induced that became sensitive to radiation. Although Tregs have often been considered inherently anergic, robust Treg proliferation has been observed after stimulation (Walker, 2004). The sensitivity of Tregs to chemo- and radiotherapy in cancer patients is of great clinical interest but largely unknown. The suggestion is that there are immune mechanisms of action as an alternative to direct cytotoxicity, although at present there are no definitive data. In fact, there may be other immune targets such as the myeloid cells that can be induced following RT and whose elimination enhances radiation-induced tumor regression (Ahn et al., 2010).

What we do know is that the tumor-specific immune responses made by cancer patients receiving RT appear to be held in check by increases in the systemic Treg pool (Schaue et al., 2008). We have seen this phenomenon also in murine tumor models mice treated with radiation (Schaue et al., 2012). Interestingly, radiation can increase Treg representation even in the absence of a tumor (Cao et al., 2009; Kusunoki et al., 2010; Nakatsukasa et al., 2010; Qu et al., 2010; Billiard et al., 2011; Kachikwu et al., 2011). This can be interpreted as a response to control radiation-induced inflammation and normal tissue damage. One possible mechanism is through induction and activation of the powerful immune-suppressive cytokine TGF-β by RT (Martin et al., 2000), which is known to boost Tregs (Chen et al., 2003; Beal et al., 2012; Takahashi et al., 2012). In addition, we were able to detect radiation-enhanced expression of the ectonucleotidase CD39 on the Treg population, which has also been observed in treated cancer patients (Mandapathil et al., 2009). Adenosine production through nucleotide catabolism by CD39 and CD73 is probably the most primitive immunosuppressive response to "danger." Adenosine has long been known to play a critical, non-redundant role in the protection of normal tissues from collateral damage during inflammation (Cronstein, 1994), including radiation-induced tissue damage (Hosek et al., 1992;Pospisil et al., 1993, 1998; Hou et al., 2007), where it plays a protective role (Hofer et al., 2002). Support for this scenario comes from the observation that tissue derived adenosine acting through its receptor $A_{2A}R$ drives Tregs and limits autoimmune tissue destruction (Zarek et al., 2008).

INHIBIT THE INHIBITORS TO WIDEN THE RADIOTHERAPEUTIC WINDOW?

The existence of tumor-induced immunosuppressive T cells and myeloid cells has been known for decades (Howie and McBride, 1982) and Tregs may influence the development of suppressor macrophages through cytokine release. It has taken longer for the concept that the immune system is under continuous negative regulation to be recognized and that loss of these important control mechanisms under steady state conditions can augment inflammation and autoimmunity. Importantly, tools are now available for investigating the role of these subsets in RT settings and for modifying their influence.

There are numerous reports that myeloid-derived suppressor cells (MDSC) and Treg levels are elevated in the peripheral circulation of cancer patients. They are also increased in lymphoid organs and tumors of tumor-bearing mice (Howie and McBride, 1982; Chen et al., 2009). Further, systemic depletion of $Foxp3^+$ Tregs enhances natural as well as vaccine-induced anti-tumor T cell responses (Liyanage et al., 2002; Curiel et al., 2004; Dannull et al., 2005; Miller et al., 2006), as does targeting $CD11b^+$ myeloid cells (Ahn et al., 2010). It is now generally accepted that a rise in MDSC or Tregs in a patient's blood or tumor is often associated with poor outcome and that this can be attributed to their immunosuppressive and/or tumor growth promoting effects. The possible exceptions are colorectal and head and neck cancers (Ladoire et al., 2011; Deleeuw et al., 2012), which may indicate greater microbial involvement in these sites. Also, it is difficult to reliably conclude that a rise in Tregs is a negative prognostic indicator if simultaneous measurements are not made in cytotoxic immune cells, the reason being that any pro-inflammatory response is likely to solicit an adaptive compensatory response (Litjens et al., 2012; Tang et al., 2012). In this sense, Tregs may be considered as another immunological readout that mirrors the development of cytotoxic effector T cells, further supporting the general thesis that radiation can be an immune adjuvant (Schaue et al., 2008). Both Tregs and myeloid suppressor cells may be viewed as wound healing responses to tissue damage, only in this case the damage is caused by tumor growth.

From an immunological perspective, the challenge for cancer RT is to create an immunologically permissive environment. This is complex with many pre-existing and induced negative regulatory barriers to be overcome. The size of the challenge will vary with the pre-existing tumor-host environment, the clinical stage and type of tumor, the condition of the patient, and many other variables. These hurdles will vary in height and it may not be possible to generate observable responses in all cases. However, some approaches to unmasking the adjuvanticity of RT show considerable promise.

One of the most effective ways to overcome such barriers is through broad Treg targeting with anti-CD25 antibody and/or immunotoxin or anti-CTLA-4 antibody (Leach et al., 1996; Rasku et al., 2008; Hodi et al., 2010; Byrne et al., 2011; Mellman et al., 2011). Enhanced anti-tumor immunity in general and the effectiveness of RT in particular have been shown (Demaria et al., 2005; Kachikwu et al., 2011; Postow et al., 2012). Currently, the extent of any Treg subset selectivity in these approaches is not known, nor whether radiation-induced normal tissue complications are increased. The use of anti-CTLA-4 as a monotherapy (Phan et al., 2003; O'Day et al., 2007; Yang et al., 2007; Weber et al., 2009), for example, is associated with some toxicity and should be used with caution when combined with other therapies. Furthermore, there are suggestions that Foxp3 may not always be a desirable target in every cancer setting because $Foxp3^+$ T cell infiltration does not always predict poor prognosis, for example in colorectal cancer, and because Foxp3 appears to act as a tumor suppressor gene when expressed in non-immune tissues (Deleeuw et al., 2012; McInnes et al., 2012). The influence of myeloid cells may be decreased by colony stimulating pathways on which they depend (Ahn et al., 2010; Vincent et al., 2010), but once RT or

chemotherapy is over, both are likely to rebound, which may be the best time to target these brakes on the development of anti-tumor immunity. The potential power of these immunological approaches is very appealing and they may be enhanced even more in the future by more selective targeting of tumor-specific Treg TCRs with antibodies to eliminate those driving immune suppression or with cytokines that could enhance macrophage anti-tumor action or drive Tregs into an effector mode (Byrne et al., 2011).

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Radiation as an immunological adjuvant: current evidence on dose and fractionation

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Ionizing radiation to a cancer site has the ability to convert the irradiated tumor in an immunogenic hub. However, radiation is a complex modifier of the tumor microenvironment and, by itself, is seldom sufficient to induce a therapeutically significant anti-tumor immune response, since it can also activate immune suppressive pathways. While several combinations of local radiation and immunotherapy have been shown in pre-clinical models to induce powerful anti-tumor immunity, the optimal strategy to achieve this effect remains to be defined. When used *in vivo*, radiation effects on tumors depend on the dose per fraction applied, the number of fractions used, and the total dose. Moreover, the interplay of these three variables is contingent upon the tumor setting studied, both in pre-clinical and clinical applications. To enable repair of the collateral damage to the normal tissue, radiation is usually given in multiple fractions, usually of 2 Gy. Generally, the use of larger fractions is limited to stereotactic applications, whereby optimal immobilization reduces inter- and intrafraction movement and permits a very conformal delivery of dose to the target, with optimal exclusion of normal tissue. Translation of the partnership of radiation and immunotherapy to the clinic requires a careful consideration of the radiation regimens used. To date, little is known on whether different dose/fractionation regimens have a specific impact on the anti-tumor immune response. Most experiments combining the two modalities were conducted with single fractions of radiotherapy. However, there is at least some evidence that when combined with some specific immunotherapy approaches, the ability of radiation to promote anti-tumor immunity is dependent on the dose and fractionation employed. We critically review the available *in vitro* and *in vivo* data on this subject and discuss the potential impact of fractionation on the ability of radiation to synergize with immunotherapy.

Keywords: abscopal effect, fractionation, immunogenic cell death, immunotherapy, inflammation, *in situ* vaccine, radiation regimen, T cells

INTRODUCTION

The therapeutic use of local ionizing radiation has been largely guided by a strategy designed to achieve the goal of effectively eliminating cancer cells while causing the least toxicity to normal adjacent tissues. The mechanisms underlying this strategy were defined by Withers as "The 4 R's of Radiotherapy" (Withers, 1975), an helpful mnemonic reference to the factors thought to determine the response of tissues to fractionated radiation: repair, reassortment, repopulation, and reoxygenation. Steel added a fifth factor, radiosensitivity, in recognition of the fact that the intrinsic vulnerability of different cancer cells differs markedly (Steel et al., 1989).

The choice of fractionating (i.e., delivering the prescribed dose in multiple fractions during separate radiation sessions, usually once/day) derives from the necessity of enabling normal tissue to repair during and after the course of radiation, to exploit the fact that the tumor is at a disadvantage, since its repair machinery is generally damaged. Based on extensive empirical experience, the use of multiple daily doses of about 2 Gy to a total dose of approximately 45–50 Gy, has evolved as a "standard" approach to control microscopic disease for most tumor types, after surgical resection. Generally, higher doses are required when the tumor is in place. However, while total doses as low as 35–45 Gy are sufficient to control most lymphomas, in tumors considered relatively radioresistant such as melanoma or sarcomas, higher total doses are necessary (Khan et al., 2011).

With the development of image-guided radiotherapy (IGRT) the uncertainty about the target volume is markedly reduced, permitting smaller and more conformal fields that reduce the inclusion of normal tissue and allow the delivery of larger single radiation doses with acceptable complications (Verellen et al., 2007). Particularly when immobilization is assured, and the inter- and intrafraction movement is minimized, single fractions have shown to be both safe and effective. For brain metastases, for example, stereotactic radiosurgery (SRS) utilizes single doses of radiation in the order of 20 Gy, generally achieving control of the lesion for the rest of the patient's life (Frazier et al., 2010).

These considerations also apply to pre-clinical *in vivo* models. Conversely, studies in cell lines inevitably avoid the issue of normal tissue tolerance, and are generally conducted using a single dose

approach. *In vitro*, the radiosensitivity of tumor cells is usually determined by the clonogenic survival assay, which measure the proliferative impairment of cells exposed to various single doses of ionizing radiation, expressed as "surviving fraction". At least in the first 24 h, there is generally little or no apoptotic response in cancer cells of non-hematopoietic origin (Amundson et al., 2008). In fact, depending on the cell cycle phase at the time of irradiation, carcinoma cells that have lost clonogenic ability have been shown to die by necrosis or by other forms of cell death and after a few or several divisions, up to at least 10 generations post-irradiation (Chu et al., 2002).

Similar to the clinical setting, when radiation is applied in experimental tumors, neoplastic cells, tumor stroma and some adjacent normal tissue are also exposed. *In vivo*, the radiosensitivity of a tumor depends on the complex interaction between the intrinsic sensitivity of the cancer cells and that of the tumor microenvironment, with hypoxia representing a major modulator of radiosensitivity (Vaupel, 2004). Importantly, the results depend on the integrity of the animal immune system. Stone et al. (1979) showed that the radiation dose required to cure 50% of the tumors (TCD_{50}) was more than twice as high in mice lacking T cells, providing the first evidence that radiotherapy induces anti-tumor T cell responses that contribute to tumor control.

RADIATION-INDUCED CELL DEATH, IMPLICATIONS FOR IMMUNOTHERAPY COMBINATIONS

There are at least two important implications of the kinetics of cell death post-radiation. The first is that most irradiated cells survive at least for a limited time, during which they undergo a stress response, transmitted through multiple signal transduction pathways to the surrounding tissue. This process is associated with changes in specific gene expression that depend on the tissue of origin, the genetic background of the host, the p53 status of the tumor and the radiation type and regimen used (Amundson et al., 1999, 2008; Tsai et al., 2007b; John-Aryankalayil et al., 2010). Among genes up-regulated following radiation are those controlling expression of growth factors, cytokines, chemokines, and cell surface receptors that modulate the interaction of the tumor with the immune system (Demaria and Formenti, 2007; Formenti and Demaria, 2008). As an important modifier of the tumor microenvironment, radiation can change tumor immunogenicity with consequences outside of the irradiated field (Formenti and Demaria, 2009).

The second implication is that, after radiation exposure, among the cells programmed to die, the type of death is highly variable, spanning from apoptosis and necrosis to autophagy and mitotic catastrophe. Importantly, radiation has been shown to induce an immunogenic cell death (ICD), characterized by three molecular signals that promote uptake of dying cells by dendritic cells, cross-presentation of the tumor-derived antigens to T cells, and activation of anti-tumor T cells: exposure of calreticulin on the tumor cell surface, release of high-mobility group protein B1 (HMGB1), and release of ATP (Apetoh et al., 2007; Obeid et al., 2007a,b; Ghiringhelli et al., 2009). *In vitro* calreticulin exposure in a mouse colon carcinoma was shown to occur after a single 75 Gy dose (Obeid et al., 2007a), an unrealistic dose to translate to the clinic. However, HMGB-1 release in the EL4 lymphoma occurred after a single 10 Gy dose (Apetoh et al., 2007): currently, little is known about the exact dose-dependency of these effects.

Overall, available evidence suggests that local radiation at clinically therapeutic doses always elicits some activation of the innate and adaptive immune system (McBride et al., 2004). However, the proportion of tumor cells undergoing ICD is variable. Similarly, variable is the type of remodeling of the tumor microenvironment after radiation, for example, in terms of recruiting more functional DC rather than immunosuppressive myeloid cells and regulatory T cells. The results of this balance are likely to determine the ability of radiation to convert the cancer in an effective *in situ* vaccine (Formenti and Demaria, 2012). Understanding this balance has relevant clinical implications, with the potential to expand the application of ionizing radiation.

This review will discuss the available, albeit limited data in support of an effect of dose and fractionation of local radiotherapy in determining successful anti-tumor immunity. We will deliberately exclude discussing the immunosuppression caused by total body radiation, which is largely due to different mechanisms (i.e., deletion of the more sensitive naïve T cells and other cells; McFarland et al., 2012).

EXPERIMENTS TESTING SINGLE FRACTIONS OF RADIATION

Several studies have shown that ionizing radiation induces or up-regulates cell surface molecules involved in recognition and/or killing of tumor cells by cytolytic T cells (CTL). These include major histocompatibility class I molecules (MHC-I), Fas/CD95, intercellular adhesion molecules-1 (ICAM-1), and NKG2D ligands (Hareyama et al., 1991; Gaugler et al., 1997; Chakraborty et al., 2003, 2004; Garnett et al., 2004; Gasser et al., 2005; Kim et al., 2006; Newcomb et al., 2006; Reits et al., 2006). In most studies, escalating doses of radiation, delivered in a single fraction, were tested on one or a few cell lines of mouse or human origin.

In one of the most comprehensive studies, Garnett et al. (2004) analyzed a panel of 23 human tumor cell lines of colon, lung, and prostate origin for the effect of radiation on expression of MHC-I, Fas, ICAM-1, and two tumor-associated antigens, carcinoembryonic antigen (CEA) and mucin-1 (MUC-1). Exposure to a single dose of 10 or 20 Gy induced the up-regulation of at least one molecule in 91% of the cell lines (Garnett et al., 2004). These data indicate that phenotypic changes, which may impact tumor immunogenicity, are common post-radiation. They also highlight the variability among tumors in terms of which molecules are up-regulated. In this respect, the frequent loss or alterations of genes encoding one or more of the molecules required for the generation and assembly of MHC-I in cancer cells, precludes an effective MHC-I up-regulation in response to radiation (Chang and Ferrone, 2007) .

Reits et al. (2006) demonstrated *in vitro* and *in vivo* up-regulation of MHC-I, and provided important insight in the regulation of these molecules by radiation. Human melanoma cells exposed to a single dose of radiation increased MHC-I expression in a dose- and time-dependent manner: while 1 Gy did not cause any significant increase above baseline, 4 Gy slightly increased MHC-I expression. Ten and 25 Gy caused a larger, over

twofold, increase at 72 h. A slightly faster kinetic was observed after 25 Gy (Reits et al., 2006). They demonstrated that surface MHC-I expression is enhanced in response to increased availability of antigenic peptides for loading on MHC-I. Within the first 4 h after radiation, an increased degradation of cellular proteins damaged by radiation-induced radicals occurs, and the process evolves to activate mTOR. mTOR activation results in increased protein synthesis and enhances defective ribosomal products. Interestingly, a change in the repertoire of peptides displayed on surface MHC-I molecules was noticeable, with appearance of new peptides not present in non-irradiated tumor cells, reflecting protein synthesis in response to DNA damage (Reits et al., 2006). Employing the mouse MC38 colon carcinoma, Reits et al. (2006) showed that single doses of 8, 10, or 20 Gy enhanced MHC-I expression for up to 11 days: despite the fact that most cells survived irradiation, a larger proportion was eventually eliminated by tumor-specific CTL than those observed in non-irradiated controls. Importantly, *in vivo* there was a synergy between tumor irradiation with a single dose of 10 Gy and adoptive transfer of CTL. The majority of tumors receiving the combination therapy regressed. In contrast, radiation alone only slightly inhibited tumor growth, and CTL transfer by itself had no effect.

Overall, the data suggest that even a radiation regimen relatively ineffective at killing tumor cells and inhibiting tumor growth may still sensitize the tumor to rejection by CTL, if sufficient signaling to repair, triggering mTOR activation, is produced.

Many pro-inflammatory cytokines and chemokines are induced by irradiation of tumors and/or normal tissues. *In vivo*, this is often a reflection of the type of inflammation that develops as an acute or chronic response to the radiation-induced tissue damage (Hong et al., 1999; Johnston et al., 2002; Lugade et al., 2008). *In vitro*, induction of interleukin (IL)-1β was detected as a rapid response to irradiation with a single dose of 20 Gy in normal mouse spleen cells and leukemia cells (Ishihara et al., 1993). IL-1β was also induced in human alveolar macrophages by a single dose of 2 Gy (Degenhardt et al., 2006). IL-1β secretion requires processing by caspase-1, which is activated by the NLRP3 inflammasome (Barker et al., 2011). Death of normal or tumor cells that occurs by apoptosis associated with autophagy is required to activate the inflammasome in macrophages and dendritic cells (Michaud et al., 2011; Petrovski et al., 2011). Activation of the inflammasome and production of IL-1β were identified as essential events for the optimal activation of anti-tumor T cells following treatment-induced ICD (Ghiringhelli et al., 2009). Given that radiation can induce macrophages to release IL-1β in the absence of tumor cells *in vitro* (Degenhardt et al., 2006), it is intriguing to consider whether, *in vivo*, this effect may contribute to the development of anti-tumor immunity after radiotherapy. This could be especially relevant among cancer cells with impaired autophagy pathways that are unable to generate all necessary signals for ICD (Michaud et al., 2011).

Radiation can also directly stimulate the production of some cytokines and chemokines from cancer and/or tumor stromal cells. For example, tumor necrosis factor-alpha (TNF-α) was produced by human sarcoma cells exposed to a single dose of 5 Gy (Hallahan et al., 1989). CXCL16 was induced *in vitro* in human breast cancer cells and murine mammary, prostate, and colon carcinoma cells by a single dose of 12 Gy (Matsumura et al., 2008; Matsumura and Demaria, 2010). Induction of CXCL16 in mouse breast cancer cells was dose-dependent, starting at 2 Gy and reaching a plateau between 6 and 12 Gy. Interestingly, maximal secretion was reached after 6 Gy in one tumor but it required $\geq$12 Gy in another, suggesting inter-tumor variability in the response (Matsumura et al., 2008). Importantly, CXCL16 induction by local radiotherapy with two doses of 12 Gy was also seen *in vivo* in the mouse 4T1 mammary carcinoma, and shown to enhance tumor infiltration by $CXCR6^+$ effector CD8 T cells (Matsumura et al., 2008).

Radiation can also induce the activation of anti-inflammatory pathways. For instance, the pleiotropic immunosuppressive cytokine transforming growth factor-beta (TGF-β) was activated by radiation from its latent form after a single dose of 5 and 10 Gy (Barcellos-Hoff, 1993; Barcellos-Hoff et al., 1994; Jobling et al., 2006). TGF-β suppresses the function of dendritic cells and effector CD8 T cells, while promoting the conversion of CD4 T cells into regulatory (Treg) cells (Wrzesinski et al., 2007). Therefore, increased activated TGF-β post-radiation could hinder the development of anti-tumor T cells and their function in the tumor. Strategies to inhibit TGF-β post-radiation are currently investigated in the clinic.

EXPERIMENTS TESTING DOSE FRACTIONATION

Fewer studies have addressed the effects of dose fractionation. *In vitro*, when mouse B16 melanoma cells were exposed to multiple daily doses of 2 Gy, 5 days/week up to a total dose of 50 Gy, mimicking clinical protocols, MHC-I expression was increased after the second week, when the total dose amounted to 20 Gy (Hauser et al., 1993). The increased expression was stable for at least 5 weeks after the last radiation fraction, and was associated with increased expression of MHC-I heavy chain mRNA, suggesting the possibility that different mechanisms than activation of mTOR (Reits et al., 2006) are responsible for MHC-I up-regulation induced by different radiation regimens.

The contribution of the different mechanisms of MHC-I up-regulation by radiation described *in vitro* remains to be demonstrated *in vivo*. In the B16 murine melanoma model Lugade et al. (2008) found that MHC-I up-regulation after *in vivo* irradiation with a single dose of 15 Gy required host-produced interferon-gamma (IFN-γ) since it was not seen in IFN-γ deficient mice. This suggests that signaling by host cells may dominate *in vivo*, shifting the focus from tumor cell-intrinsic responses to the cross-talk between irradiated tumor cells and the local immunological microenvironment. Consistently, we found that the tumor microenvironment *in vivo* alters the phenotype of cancer cells, as well as their response to radiation. 4T1 mouse breast cancer cells had increased baseline expression of MHC-I *in vivo* as compared to cells cultured *in vitro*, but they lost expression of NKG2D ligands. Radiation increased expression of MHC-I on 4T1 cells *in vitro* but not *in vivo*, while ICAM-1 and Rae-1, one of the NKG2D ligands, were increased by radiation *in vivo* (Ruocco et al., 2012).

Radiation can also up-regulate or induce other molecules that enhance the efficiency of cancer cell killing by CTL. For example, Fas was induced in an *in vivo* mouse model of colon carcinoma

after a single dose of 8 Gy or after four fractions of 2 Gy given in consecutive days (Chakraborty et al., 2003, 2004). The two radiation regimens combined with vaccines (vaccinia and avipox recombinant vaccines expressing CEA and three T cell costimulatory molecules) achieved comparable tumor regression, whereas either vaccine or radiation as monotherapy failed to significantly affect tumor growth (Chakraborty et al., 2004). Therefore, Fas expression appears to occur with either single or fractionated RT and results in a clinically detectable effect.

It is tempting to speculate whether this radiation-induced increase in the expression of MHC-I, Fas, or other molecules contributes to better tumor regression particularly in patients with pre-existing higher levels of natural anti-tumor T cells (Galon et al., 2006; Vesely et al., 2011; Wang et al., 2012). In other words, a pre-existing anti-tumor immunity may result to be a predictor for response to radiotherapy.

Using the B16 mouse melanoma, Lee et al. (2009) showed that tumor growth delays obtained with a single dose of 20 Gy or three fractions of 15 Gy were comparable, and almost abrogated by CD8 T cell depletion, suggesting that both regimens can promote cross-priming of anti-tumor T cells. In contrast, 5 Gy × 4 given over a 2-week interval, showed inferior tumor growth inhibition, although the contribution of CD8 T cells to this effect was not investigated. In the same model, Lugade et al. (2005) had previously shown that cross-priming of T cells against tumor antigens was induced in the draining lymph nodes after irradiation with a single dose of 15 Gy or 3 Gy × 5. While both regimens induced the activation and expansion of anti-tumor T cells, and increased VCAM-1 expression on tumor endothelium and T cell infiltration in the tumor, the regimen of 3 Gy × 5 failed to cause a significant inhibition of tumor growth (Lugade et al., 2005).

These results are in contrast with our findings with radiation and anti-CTLA-4. Comparing three radiation regimens, 20 Gy × 1, 8 Gy × 3, and 6 Gy × 5, we demonstrated a marked difference between single dose and fractionated regimens, in the ability to synergize with anti-CTLA-4 antibody treatment and induce an anti-tumor immune response able to inhibit tumor locally, at the irradiated site, and systemically (Dewan et al., 2009). Two poorly immunogenic tumor models not expressing model antigens, a mammary and a colorectal carcinoma, respectively syngeneic to mice of different genetic background were studied. All three regimens had similar ability to cause growth delay of the irradiated tumor, without affecting the growth of a tumor outside of the radiation field. While anti-CTLA-4 by itself or in combination with a single 20 Gy dose was ineffective, when combined with the two fractionated regimens it significantly improved inhibition of both the irradiated and tumors outside the irradiated field (abscopal response, from *ab-scopus*, i.e., outside the target). The effectiveness of the generated anti-tumor response was highest with 8 Gy × 3, with 80% of the irradiated tumors and 40% of the tumors outside the field regressing completely. Since anti-CTLA-4 antibody is known to be ineffective against poorly immunogenic tumors but to synergize with vaccination in inducing anti-tumor immunity (Peggs et al., 2008), these data imply that radiation used as single dose of 20 Gy failed to convert the tumor into an *in situ* vaccine. These results suggest that, for the combination with anti-CTLA-4, there may be an optimal window for the pro-immunogenic effects of radiation, with a hypofractionated regimen providing the best results. We are currently performing genome-wide gene expression analyses to investigate the changes that distinguish the two regimens and may be responsible for the interaction of the irradiated tumor with the immune system.

Overall, while some degree of immunization against the tumor may be always promoted by radiotherapy, the magnitude of this effect and the overall changes in the tumor toward a more or less immunosuppressive environment are likely to be the determinants of treatment success. Pre-clinically, a specific dose and fractionation may be superior to another, and it appears to be model-dependent. In this regard, Schaue et al. (2012) recently proposed that the relative ability of a given radiation regimen to increase cross-priming while not increasing Treg cell numbers will determine its pro-immunogenic effect. They identified a hypofractionated regimen with two fractions of 7.5 Gy as providing the best compromise between promotion of T cell cross-priming to tumor antigens versus relative induction of Treg (measured as increased Treg cell numbers) in the mouse B16 melanoma model. The mechanisms underlying Treg cells increase by radiation as well as their suppressive function in this setting remain to be clarified (Qu et al., 2010; Billiard et al., 2011; Kachikwu et al., 2011).

LEARNING FROM CLINICAL EXPERIENCE

Local radiation by itself may occasionally be able to elicit the development of a sustained anti-tumor immune response able to control tumor locally and systemically as suggested from reported cases of abscopal effects, i.e., tumor responses outside of the field of radiation in cancer patients receiving radiation to one site (Mole, 1953; Ehlers and Fridman, 1973; Rees and Ross, 1983; Ohba et al., 1998; Wersall et al., 2006). In one report, 4 of 28 patients with metastatic renal cell carcinoma showed abscopal effects: in two patients after receiving 8 Gy × 4 to the primary tumor by SBRT, and in another two patients after two doses of 15 Gy to a metastatic site (Wersall et al., 2006). The lack of data about the immune response in these patients makes it difficult to reach any definite conclusion about the involvement of the immune system.

However, immunological changes associated with an abscopal response were recently reported in a melanoma patient treated with local radiotherapy and ipilimumab, the anti-CTLA-4 antibody approved for clinical use (Postow et al., 2012). The patient received radiotherapy in three fractions of 9.5 Gy, a regimen comparable to the regimen (8 Gy × 3) showing optimal synergy with anti-CTLA-4 therapy in mouse tumor models (Dewan et al., 2009). The patient displayed a complete clinical remission of the primary and metastatic sites, despite previous progression with ipilimumab therapy when given alone.

Abscopal responses were also reported in a clinical study of patients with low-grade B cell lymphoma treated with 2 Gy × 2 to a single tumor site that was injected with a Toll-like receptor 9 (TLR9) agonist PF-3512676, an activator of B cells and antigen-presenting cells (Brody et al., 2010).

It has been speculated that conventional fractionated radiotherapy with multiple fractions of about 2 Gy is immunosuppressive (Lee et al., 2009). However, clinical data disproves this contention: Gulley et al. (2005) administered a poxviral vaccine

encoding prostate-specific antigen (PSA) to 17 prostate cancer patients undergoing radiotherapy with total external beam dose ≥70 Gy given in 1.8 to 2.0 Gy per fraction. PSA-specific T cell responses were analyzed before radiotherapy, immediately after and 3 months later. Eight patients showed blunted immune responses to PSA following radiotherapy, six had stable responses, and in two patients the response was increased after radiotherapy (Gulley et al., 2005). Moreover, six out of eight patients evaluated showed the development of T cell responses against tumor antigens not present in the vaccine, suggesting that radiation promoted the activation of T cells against other tumor antigens (Gulley et al., 2005).

Finally, a recent report in patients with metastatic melanoma and renal cell carcinoma treated with SBRT given in one, two, or three doses of 20 Gy, in combination with high dose IL-2 showed a response rate in non-irradiated lesions (abscopal responses) higher than expected with IL-2 alone (Seung et al., 2012).

Overall, while emerging clinical data confirm at least some of the observations in experimental models, they fail to provide indications about the best radiation regimen to be used to elicit anti-tumor immune responses. One limitation in interpreting these results is the lack of randomized studies comparing radiation regimens for their ability to synergize with immunotherapy.

CHALLENGES AND FUTURE DIRECTIONS

While data about the effects of radiation on various immune parameters is rapidly accumulating in experimental model, potential pitfalls exist in correctly interpreting the results. For instance, tumors which are more immunogenic, for example, engineered to express a "model" antigen such as ovalbumin (OVA), tend be more susceptible to immune-mediated rejection than poorly immunogenic tumors. The latter, however, much better mimic the reality of clinical cancer patients, who generally are diagnosed after immune-selection had edited out more immunogenic antigens (Vesely et al., 2011).

Tumor size and intrinsic radiosensitivity also affect the degree of response to a tested intervention. Importantly, pre-clinical evidence of an induced immune response based on re-challenging with the same experimental tumor initially used to immunize remains only an indirect predictor for a successful therapeutic paradigm. In fact, in the clinic the endpoint is control of pre-existing micro- or macrometastatic foci and impact on survival, a much harder goal to achieve. Additionally, different immunosuppressive mechanisms (e.g., Treg cells versus myeloid-derived suppressor cells) may dominate the models compared. This variability is likely to also exist in different clinical tumor types.

For example, there is some evidence that radiation promotes the immunosuppressive function of macrophages (Tsai et al., 2007a). On the other hand, a recent report shows that radiotherapy induces the production of type I IFN by myeloid cells infiltrating the tumor and promotes the development of anti-tumor immunity (Burnette et al., 2011). Whether these contrasting effects are dependent on the radiation regimen or the tumor model employed remains to be established.

Therefore, conclusions about the relative efficacy of different radiation regimens can only be made by direct comparison using the same, unmodified tumor model and testing the combination with the same immunotherapy strategy.

Finally, since we are witnessing a paradigm change in the use of radiotherapy that promises to revolutionize patient treatment (Formenti and Demaria, 2012), the development of a common language is essential. A cautious choice of terminology is warranted. For instance, terms as "ablative" should be reserved to settings where a complete elimination of tumors is achieved. In addition, attributes like "conventional" and "standard" need to be carefully justified. This is especially needed since radiation biology is becoming a point of encounter for other specialties.

CONCLUSIONS

Tumor rejection by the immune system involves a common final pathway mediated by the activation of a specific set of genes (Wang et al., 2008). These include the coordinate activation of IFN-stimulated genes and immune effector functions. However, multiple factors serve as checkpoints in the pathway toward this canonical response (Wang et al., 2012). In order to identify the radiation regimens that can best overcome the checkpoints toward immune-mediated tumor rejection, it should be possible to identify a gene signature that defines the pro-immunogenic effects of radiation. Such signature is the result of the interaction between the pre-existing tumor microenvironment, the genetic predisposition of the host, and the radiation regimen used.

In support of this concept, Tsai et al. (2007b) reported distinct molecular responses of human breast, prostate, and glioma tumor cells exposed to single dose (10 Gy) versus fractionated (2 Gy × 5) radiation *in vitro* and *in vivo*. Importantly, selective up-regulation of IFN-related genes by fractionated but not single dose radiation was seen in all three cell lines (Tsai et al., 2007a). In addition, a comparison of the response of the prostate carcinoma cell line to single dose and fractionated radiation *in vitro* versus *in vivo* showed that there was no overlap between the four conditions, indicating that the gene response to radiation is highly dependent on the microenvironment and the regimen (Tsai et al., 2007a). Although in this study the recipient mice were T cell deficient, they had an intact innate immunity, which is likely to play a key role in the initial triage of tissue damage. John-Aryankalayil et al. (2010) showed in human prostate cancer cells that genes regulating immune and stress response, cell cycle, and apoptosis were significantly up-regulated by multi-fractionated radiation compared to single-dose radiation.

Although the optimal pro-immunogenic radiation regimen may not necessarily be the same for all tumor types or settings, a signature that defines the pro-immunogenic effects of radiation could be used to optimize protocols of radiation and immunotherapy in different tumor types, as well as to predict response to treatment in different patients.

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