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RESEARCH TOPICS

RADIATION-INDUCED EFFECTS AND THE IMMUNE SYSTEM

Topic Editors
Gabriele Multhoff, Alan G. Pockley,
Udo S. Gaipl and Franz Rödel

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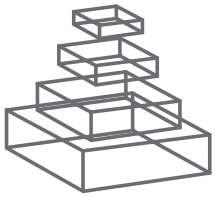

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RADIATION-INDUCED EFFECTS AND THE IMMUNE SYSTEM

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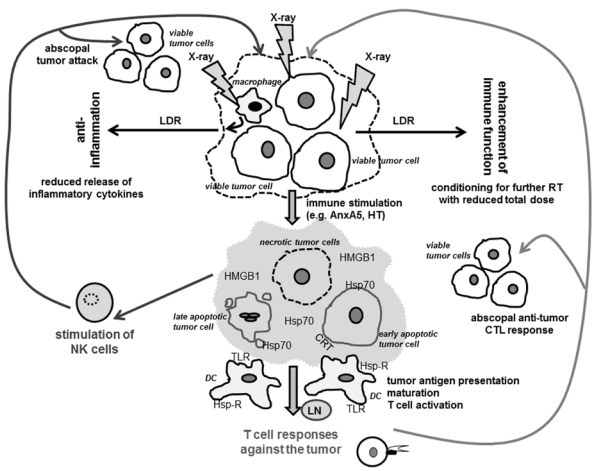

Ionizing radiation modifies the tumor cell phenotype and induces a tumor microenvironment that fosters innate and adaptive immune responses against the tumor. Figure taken from Rubner Y, Wunderlich R, Rühle P-F, Kulzer L, Werthmöller N, Frey B, Weiss E-M, Keilholz L, Fietkau R and Gaipl US (2012) How does ionizing irradiation contribute to the induction of anti-tumor immunity? *Front. Oncol.* 2:75. doi: 10.3389/fonc.2012.00075

Numerous developments in molecular biology have led to an explosive growth in the knowledge underlying mechanisms of carcinogenesis, cell signalling, tumor progression and development of metastasis. However, cure of cancer is still hampered by the inherited capacity of tumors to become resistant to standard therapies, to metastasize from their initial location and to proliferate in other tissue compartments. Radiotherapy is one of the main treatment modalities to achieve locoregional tumor control. However, the treatment of distant metastases further remains to be a challenge. In this special topic we are interested to elucidate immunological aspects which are initiated and affected by radiotherapy. We also aim to describe the development of innovative immunological strategies from a preclinical stage to clinical application which could be combined with standard radiotherapeutic approaches. A special interest will also deal with the effects of radiotherapy on tumor initiating cells as well as on the tumor microenvironment. Last but not least the effects of different irradiation sources and qualities such as photones, protones and heavy ions will be analyzed with respect to immunological outcome.

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EDITORIAL
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Frontiers research topic: radiation-induced effects and the immune system

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The development of novel, more precise instruments for the delivery of ionizing radiations, the accretive utilization of protons and heavy ions, and the emphasis on hypofractionated irradiation schemes have resulted in major improvements in locoregional tumor control. However, radiation therapy alone is still insufficient to cure locally advanced metastatic tumors. The heterogeneity of neoplasms and their microenvironment also affect the radiosensitivity of cancers. Thus, there is an urgent clinical need for innovative therapeutic concepts that may be translated into novel radio(chemo)therapeutic regimens. In order to develop new therapeutic strategies, a profound knowledge of tumors and their microenvironment is essential.

In this e-book, the authors deal with various aspects of interrelationship between neoplasms and their microenvironments, including how high and low dose irradiation affects the immune system, the effects of targeted and non-targeted photon, and particle irradiation on tumor and normal tissues, as well as the impact of epigenetics on irradiation therapy. Furthermore, the authors report on the effects of irradiation on inflammatory pathways and on the microcirculation, two aspects that are highly relevant for the delivery of macromolecules and other therapeutics to irradiated tumors. A major focus of this e-book is to elucidate immunological facets of ionizing irradiation. It is indeed becoming clear that high- and low-dose irradiation elicit distinct immunological effects. Furthermore, the mode of irradiation-induced cell death (e.g., apoptosis, necrosis) has a major impact on anticancer immune responses. Overall, the e-book aims at review the current knowledge on the role of ionizing irradiation in antitumor immunity. This knowledge is instrumental for the development of innovative therapeutic modalities involving the combination of radio(chemo)therapy and immunotherapy in patients with locally advanced tumors.

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REVIEW ARTICLE
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Radiation, inflammation, and immune responses in cancer

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Chronic inflammation has emerged as one of the hallmarks of cancer. Inflammation also plays a pivotal role in modulating radiation responsiveness of tumors. As discussed in this review, ionizing radiation (IR) leads to activation of several transcription factors modulating the expression of numerous mediators in tumor cells and cells of the microenvironment promoting cancer development. Novel therapeutic approaches thus aim to interfere with the activity or expression of these factors, either in single-agent or combinatorial treatment or as supplements of the existing therapeutic concepts. Among them, NF-κB, STAT-3, and HIF-1 play a crucial role in radiation-induced inflammatory responses embedded in a complex inflammatory network. A great variety of classical or novel drugs including nutraceuticals such as plant phytochemicals have the capacity to interfere with the inflammatory network in cancer and are considered as putative radiosensitizers. Thus, targeting the inflammatory signaling pathways induced by IR offers the opportunity to improve the clinical outcome of radiation therapy by enhancing radiosensitivity and decreasing putative metabolic effects. Since inflammation and sex steroids also impact tumorigenesis, a therapeutic approach targeting glucocorticoid receptors and radiation-induced production of tumorigenic factors might be effective in sensitizing certain tumors to IR.

Keywords: radiation, inflammation, radioresistance, PGHS-2, heat shock proteins, NF-κB, STAT-3, HIF-1

INTRODUCTION

Chronic inflammation has emerged as one of the hallmarks of cancer impacting any stage of tumorigenesis (Colotta et al., 2009; Grivennikov and Karin, 2010). The persistent expression of inflammatory mediators exert pleiotropic effects on the malignant process. On the one hand, they affect carcinogenesis and malignant transformation, tumor growth, invasion, and metastasis, on the other hand they activate immune effector mechanisms limiting tumor growth. The link between cancer and inflammation can be viewed as consisting of two pathways: an intrinsic and an extrinsic pathway (Mantovani et al., 2008). At the level of the tumor cell, both pathways converge and induce the activation of several transcription factors culminating in the formation of numerous pro-inflammatory molecules that recruit and activate various leukocyte populations into the tumor microenvironment (for a review, see Multhoff et al., 2012). The tumor cell-derived pro-inflammatory molecules now activate the same transcription factors within the cells of the microenvironment and the tumor cells themselves resulting in a more pronounced generation of inflammatory mediators driving a tumor-promoting amplification loop. This amplification mechanism further enhances the impact of inflammatory stimuli within the tumor environment and triggers the manifestation of a cancer-related inflammatory milieu contributing to tumor growth and invasiveness.

NF-κB provides a mechanistic link between inflammation, carcinogenesis, and tumor radioresistance (Magne et al., 2006; Ben-Neriah and Karin, 2011). NF-κB is regarded as the key orchestrator controlling the ability of both, preneoplastic and malignant cells, to resist apoptosis-based tumor surveillance mechanisms activated by DNA damage and chromosomal rearrangement or anti-cancer drugs and radiation, respectively. NF-κB might also regulate tumor angiogenesis and invasiveness (Karin, 2006), and may contribute to the characteristic radio-/chemoresistance of tumor cells (Fahy et al., 2004; Singh and Khar, 2006; Antoon et al., 2011; Chaturvedi et al., 2011). In conjunction with STAT-3 and HIF-1, NF-κB serves as a modulator of the expression of several factors promoting cancer development. Novel therapeutic approaches thus aim to interfere with the activity or expression of these factors, either in single-agent or combinatorial treatment or as supplements of the existing therapeutic concepts. Noteworthy, targeting the pro-inflammatory signaling pathways for tumor radiosensitization represents a promising novel therapeutical approach in cancer. A great variety of classical or novel drugs including nutraceuticals have the capacity to interfere with the inflammatory network in cancer and are progressively tested for tumor radiosensitization. Accumulating evidence over the last few years indicate that most chemotherapeutic agents and radiation therapy activate NF-κB (Wang et al., 1996; Sandur et al., 2009; Li and Sethi, 2010). Thus, NF-κB blockage has been recognized as a promising tool in increasing radiosensitivity of tumors. Apart from NF-κB, STAT-3, HIF-1, and PGHS-2 are further inflammatory factors crucially involved in radioresistance of tumors. Thus, interrupting the inflammatory network in cancer by targeting these molecules may be a promising radiosensitization approach in cancer therapy. **Table 1** provides an overview on natural and (semi-)synthetic compounds that are considered as putative radiosensitizers.

Table 1 | Natural and (semi-)synthetic compounds as putative radiosensitizers und their targets.

Compound	Source/systematic name	Target
Anacardic acid	*Anacardium occidentale* (cashew nuts)	IKK, NF-κB
Berberine	*Berberis aristata* (Indian barberry, tree turmeric)	NF-κB
Butein	*Rhus verniciflua* (Chinese lacquer tree)	NF-κB
Caffeic acid phenethyl ester	Honeybee propoplis	GSH, NF-κB
Celecoxib	4-[5-(4-methylphenyl)-3-(trifluoromethyl) pyrazol-1-yl]benzenesulfonamide	PGHS-2, NF-κB
Cepharanthine	*Stephania cepharantha* Hayata	NF-κB, STAT-3
Crotepoxide	*Kaempferia pulchra* (peacock ginger)	TAK-1
Curcumin	*Curcuma longa* (turmeric)	Akt, IKK, NF-κB
Daidzein, genistein	*Glycine max* (soy bean)	STAT-3, HIF-1α
Deguelin	*Derris trifoliata* (threeleaf derris)	Hsp90, HIF-1α
EGCG	*Camellia sinensis* (green tea)	NF-κB
Emodin	*Rheum rhabarbarum* (Rhubarb), *Aloe vera*	HIF-1
Erufosine	Alkylphosphocholine (synthetic phospholipid analog)	Akt
Ethaselen	1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)] ethane; BBSKE	Thioredoxin reductase, NF-κB
Flavopiridol	Semi-synthetic flavonoid based on an extract from the Indian plant, *Dysoxylum binectariferum*	CDKs, cyclin D1, Rb, Bcl-2
Geldanamycin	Naturally occurring ansamycin antibiotic from *Streptomyces hygroscopicus*	Hsp90
KNK437	Benzylidene lactam compound	Hsp27, Hsp70
Nitidine chloride	*Zanthoxylum nitidum* (Tez-mui, Tejamool in Assamese; locally called "liangmianzhen")	STAT-3
Oleandrin	*Nerium oleander* (nerium)	Caspase-3
Parthenolide	*Tanacetum parthenium* (feverfew)	NF-κB, p53
Piceatannol	Hydroxylated resveratrol analog found in various plants, e.g., *Vitis spec.*	NF-κB
Picroliv	*Picrorhiza kurroa* (katuka)	NF-κB
Piperine	*Piper nigrum* (black pepper)	CYP450 enzymes
Plumbagin	*Plumbago rosea* (Scarlet leadwort)	NF-κB
Resveratrol	*Vitis spec.* (grape, red wine)	STAT-3, NF-κB
Silymarin	*Silybum marianum* (milk thistle)	NF-κB
Xanthohumol	*Humulus lupulus* (common hop)	NF-κB

GENETIC INSTABILITY

Recent observations allow a deeper insight into the molecular and cellular mechanisms linking inflammation and tumorigenesis. Emerging data suggest that genetic destabilization of tumor cells is regarded as a further hallmark of most human cancers contributing to tumor initiation and progression (Colotta et al., 2009). Apart from the production of cytokines, chemokines, proteases and prostanoids, inflammatory cells are able to produce reactive oxygen species (ROS) and reactive nitrogen species (RNS). Leukocytes are the main source of RNS and ROS acting as chemical effectors in inflammation-driven carcinogenesis (Kundu and Surh, 2008). All of these mediators act together in perpetuating and amplifying the inflammatory cascade. As outlined in **Figure 1**, they suppress DNA repair mechanisms leading to an increase in genetic instability termed microsatellite instability (MSI) as a result of mutations or epigenetic alterations of members of the mismatch repair (MMR) family (Hakem, 2008). The MMR system is strongly affected by inflammatory conditions. It has been shown previously that the transcription factor HIF-1 is induced in tumor cells not only by different cytokines and prostaglandins (Jung et al., 2003) but also by ROS and RNS (Sandau et al., 2000). HIF-1 is a heterodimeric transcription factor consisting of a constitutively expressed β-subunit and an oxygen-regulated α-subunit (Kaelin Jr. and Ratcliffe, 2008). It was proved that HIF-1 plays a pivotal role in hypoxia-induced tumor radioresistance (Moeller and Dewhirst, 2006; Harada, 2011). In this context, our own investigations revealed no correlation between basal HIF-1α levels and the survival fraction in irradiated tumor cell lines implying that basal HIF-1α levels in human tumor cell lines obviously do not predict their radiosensitivity under normoxia (Schilling et al., 2012a). Moreover, HIF-1α has been found as being responsible for genetic instability to down-regulated MMR proteins by inhibiting the MMR proteins MSH-2 and MSH-6, thereby decreasing levels of the MSH-2/MSH-6 complex, MutSα, which recognizes base mismatches. HIF-1α displaces the transcriptional activator c-Myc from Sp1 binding to repress MutSα expression in a p53-dependent manner (Koshiji et al., 2005). Chang et al. (2002) observed that hydrogen peroxide inactivates members of the MMR family at the protein level. From this observation the authors speculate that inactivation of the MMR function in response to oxidative stress may be responsible for the low-frequency MSI (MSI-L) seen in non-neoplastic and cancer tissues associated with chronic inflammation.

Chromosomal instability can also be the result of the deleterious action of inflammatory mediators culminating in abnormal chromosomal segregation and aneuploidy. The deleterious actions

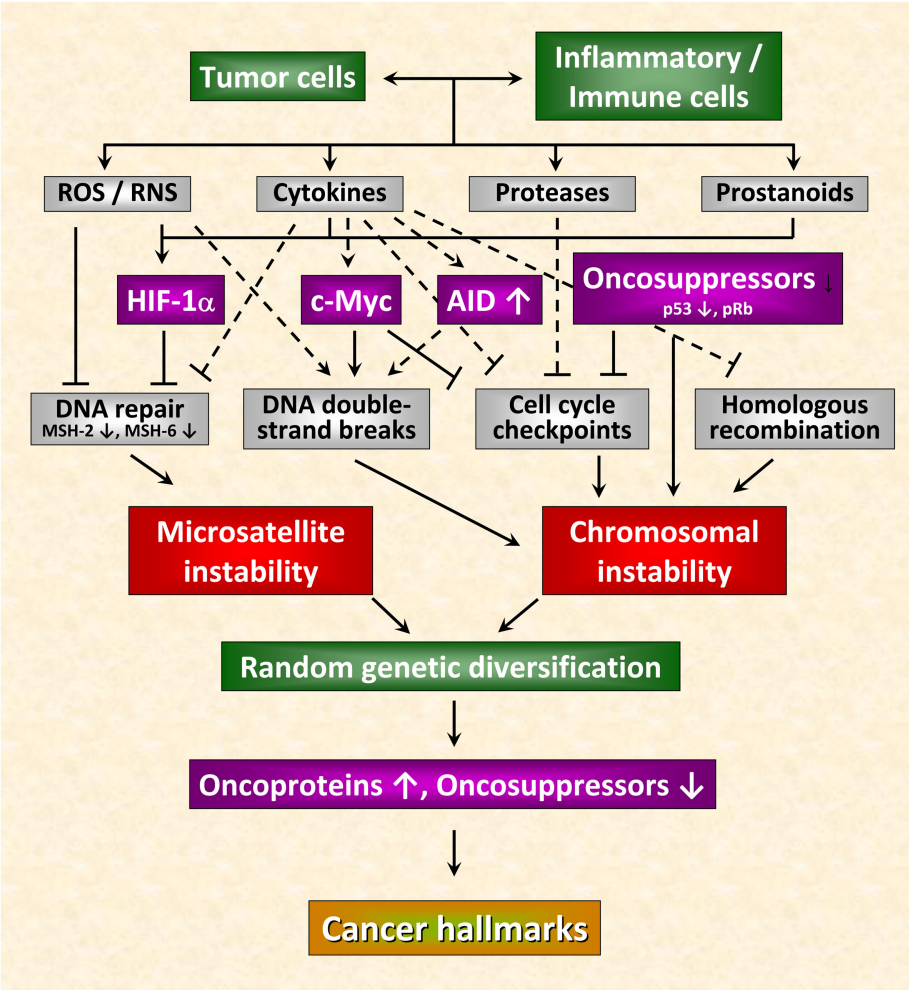

FIGURE 1 | Inflammation-induced molecular pathways causing genetic instability in cancer cells. Genetic destabilization of tumor cells is regarded as a further hallmark of most human cancers contributing to tumor initiation and progression. Apart from the production of cytokines, chemokines, proteases, and prostanoids, inflammatory cells are able to produce reactive oxygen (ROS) and nitrogen species (RNS). All of these mediators act together in perpetuating and amplifying the inflammatory cascade. On the one hand, they suppress DNA repair mechanisms leading to microsatellite instability. On the other hand, they can cause chromosomal instability culminating in abnormal chromosomal segregation and aneuploidy. These inflammatory mediators induce DNA double-strand breaks, affect function of mitotic checkpoint molecules and dysregulate homologous recombination of DNA double-strand break repair leading to random genetic diversification of tumor cells. Cancer cells harboring the optimal combination of activated oncoproteins and inactivated oncosuppressor proteins will develop the malignant phenotype (figure adapted from Colotta et al., 2009; for details see text).

of inflammatory mediators include direct or indirect induction of DNA double-strand breaks (Karanjawala et al., 2002; Mills et al., 2003), defective mitotic checkpoints (Rajagopalan et al., 2003; Menssen et al., 2007), and dysregulated homologous recombination of DNA double-strand break repair (Saintigny et al., 2001; Hakem, 2008; Plo et al., 2008). Further critical molecules that affect genetic stability comprise activation-induced cytidine deaminase (AID; Endo et al., 2008), c-Myc (Vafa et al., 2002), phosphoretinoblastoma protein pRb (Pickering and Kowalik, 2006), and p53 (Tomasini et al., 2008). By causing microsatellite as well as chromosomal instability these molecules induce random genetic diversification of tumor cells. As already discussed by Colotta et al. (2009), cancer clones harboring the optimal combination of activated oncoproteins and inactivated oncosuppressors will develop the malignant phenotype (**Figure 1**).

CELL–CELL INTERACTIONS

In the tumor microenvironment, an intensive interaction between tumor cells and infiltrating immune cells occurs. The latter comprise macrophages, dendritic cells (DC), T cells as well as NK cells with macrophages and T cells as being the most frequent ones (Luster et al., 2005). Inflammatory mediators secreted by tumor and immune cells have been found to play a dual role in tumor development. On the one hand, they promote tumor development and survival of tumor cells, on the other hand they exert surveillance mechanisms against tumor cells (Ben Baruch, 2006; Kim et al.,

2006b). In case of a predominance in anti-tumor immunity, tumor cells are eradicated whereas a predominance in surveillance mechanisms provides cancer cells with an immunosuppressive network extending the immune evasion and promoting tumor progression and metastasis (Hadden, 2003). Among the inflammatory mediators secreted by tumor and immune cells TNF, IL-6, and IL-17 act as crucial players in developing chronic inflammation resulting in immune escape and acceleration of tumor progression and metastasis. Upon activation, myeloid cells produce pro- and anti-inflammatory mediators not only affecting growth, survival, and invasiveness of tumor cells but also controlling functional activities of Th1, NK, Treg, and Th17 cells (Lin and Karin, 2007). TRAIL, a member of the TNF superfamily and the product of activated T and NK cells, directly induces apoptosis in numerous cancer cells (LeBlanc and Ashkenazi, 2003) thus playing a crucial role in tumor surveillance mechanisms. Regulatory T cells (Treg) function as key components for regulating anti-tumor immunity (Yamaguchi and Sakaguchi, 2006). Treg specifically suppress the cytotoxicity of expanded $CD8^+$ cytotoxic T cells (Chen et al., 2005) and induce release of IL-17 from Th17 cells acting as key players in chronic inflammation (Mangan et al., 2006). IL-17-mediated effects on the inflammatory response involve recruitment of immune cells (Park et al., 2005), induction of pro-inflammatory factors (IL-1, IL-6, TNF) as well as promotion of angiogenesis and tumor growth (Numasaki et al., 2003). Development of Th17 cells is stimulated by IL-6, IL-23, TGF-β, and TNF released from activated myeloid cells. Anti-inflammatory IL-10 inhibits tumor progression and development by blocking synthesis of IL-6, IL-12, and TNF via NF-κB inhibition (Moore et al., 2001). Furthermore, the anti-tumor activity of Treg is mediated by IL-10 released from Treg themselves (Erdman et al., 2003). IL-23 belonging to the IL-12 family of cytokines enhances the production of IFN-γ and IL-12 by activated T cells, induces IL-17 release from Th17 cells and promotes inflammation at its final stage (Cho et al., 2006). Moreover, IL-23 has been found to up-regulate expression of MMP-9, to increase angiogenesis and to decrease $CD8^+$ T cell recruitment to tumors possibly providing the basis for the development of a tumor-promoting environment (Langowski et al., 2006). IL-12, also released from antigen-presenting cells (APC) after stimulation with IL-23, is a further member of the IL-12 family of cytokines and harbors anti-tumor activities in particular via stimulation of Th1- and CTL-mediated immune responses (Trinchieri, 2003; Langowski et al., 2006). **Table 2** summarizes the cellular and molecular outcomes based on interactions between various cell types in the tumor microenvironment.

SEX STEROIDS

An increasing number of data currently reveal the close relationship between the two classical pathways in tumor progression: inflammation and gonadal hormones. Since the discovery of the hormone dependency of mammary carcinoma in the 1890s, it has become clear that gonadal steroids play a crucial role in the pathogenesis of breast and prostate cancer. The Scottish surgeon George Thomas Beatson was about the first who showed that oophorectomy in a premenopausal woman with breast cancer led to a complete remission (Beatson, 1896a,b) highlighting the role of sex steroids in tumor pathogenesis. However, more recent investigations revealed an astonishing effect of gonadal hormones on tumorigenesis. Female somatic cells including tumor cells express receptors for sex steroid hormones such as estrogen and progesterone affecting growth of hormone-dependent breast cancer cells (Henderson and Canellos, 1980a,b). Women are well known as being less susceptible to tumors at organ sites not representing classical targets for gonadal hormones including the liver. Thus, hepatocellular carcinoma (HCC), the most common liver cancer, occurs mainly in men. The same gender disparity is seen in mice

Table 2 | Effects of cell–cell interactions in the tumor microenvironment.

Effector	Molecular/cellular outcome	Physiology/pathophysiology
IL-6, IL-10, TNF	Enhancement of tumor cell growth	Chronic inflammation
IL-10	Anti-inflammatory, blockage of IL-6, IL-12, TNF synthesis via NF-κB inhibition	Tumor suppression
IL-12	Activation of $CD8^+$ CTL and NK cells, expression of cytotoxic mediators (IFN, TRAIL, TGF-β)	Anti-tumor effect
IL-17	Induction of pro-inflammatory mediators (IL-1, IL-6, TNF)	Chronic inflammation, tumor progression
IL-23	Induction of IL-12/IFN-γ release from activated T cells, TNF/IL-12 from APC, IL-17 from Th17 cells, MMP-9 up-regulation, decrease of $CD8^+$ CTL recruitment, increase in angiogenesis	Chronic inflammation, tumor progression
TGF-β	Enhancement of tumor cell invasiveness and angiogenesis, inhibition of NK cells, CTL, macrophages	Tumor progression
	Anti-inflammatory effects on T cells, tumor suppressor/cytotoxic activity	Anti-tumor effect
TRAIL	Induction of apoptosis	Tumor suppression
Treg cells	IL-10 release from Treg, suppression of $CD8^+$ CTL	Anti-tumor effect
	Induction of IL-17 release from Th17	Chronic inflammation
TNF	Promotion of angiogenesis and metastasis, impairment of immune surveillance via T cell and macrophage blockage	Tumor progression, chronic inflammation
	Destruction of tumor vasculature and induction of necrosis	Anti-tumor effect
IL-23, TGF-β, IL-6, TNF	Th17 cell development	Chronic inflammation
IL-6, TNF, TGF-β	Impact on stromal cells and metastasis	Tumor progression
IL-17, TNF	Impact on endothelial cells, increase in angiogenesis	Tumor growth

given a chemical carcinogen, diethylnitrosamine (DEN) resulting in liver parenchymal damage followed by activation of Kupffer cells (KC; Naugler et al., 2007). As demonstrated in this study, DEN induces NF-κB-dependent production of pro-inflammatory and growth-promoting IL-6 in KC via IL-1 and TLR signaling cascades, respectively, finally leading to tumor development. Estrogen inhibits activation of NF-κB and blocks secretion of IL-6 from KC thus protecting against liver cancer in females.

Prostate cancer is an androgen-dependent cancer whose susceptibility to gonadal hormones is regulated by selective androgen-receptor modulators (SARM) attenuating the proliferative properties of androgens on tumor cells. Pro-inflammatory IL-1 derived from tumor cells or cells of the microenvironment reverses the properties of SARM from being inhibitory to activatory (Zhu et al., 2006). This de-repression effect requires TGF-β-activated kinase 1 (TAK-1)-binding protein (TAB) 2 (=TAB-2; Takaesu et al., 2000). At the molecular level, IL-1 signaling induces phosphorylation of TAB-2. TAB-2 acts as a sensor for inflammatory signals by serving as a molecular beacon for recruitment of MEKK1, which in turn mediates dismissal of the nuclear receptor co-repressor (N-CoR) holoco-repressor complex from the androgen-receptor and permits de-repression of androgen and estrogen receptor target genes. According to Zhu et al. (2006), this strategy might have come into notice in order to trigger reversal of gonadal hormone-dependent repression of a limited cohort of target genes in response to inflammatory signals linking inflammatory, and nuclear receptor ligand responses to essential reproductive functions. Treatment of prostate cancer by androgen deprivation either by suppression of testicular androgen production or by the use of pharmacological SARM such as flutamide or bicalutamide remains the standard systemic therapy. It has been shown previously that bicalutamide does not function as an androgen-receptor (AR) antagonist by preventing AR binding to DNA but instead stimulates the assembly of a transcriptionally inactive receptor on DNA (Masiello et al., 2002). Recent reports demonstrate that AR can also bind to co-repressor proteins, including N-CoR, and that this binding is enhanced in the presence of bicalutamide indicating that co-repressor binding could further contribute to the *in vivo* antagonist activity of bicalutamide (Cheng et al., 2002; Shang et al., 2002; Yoon and Wong, 2006). Interestingly, the group of Hollenberg clearly demonstrated that the AR/N-CoR interaction is not enhanced by AR antagonists used currently for the treatment of prostate cancer, but can be markedly enhanced by mifepristone (RU486) *in vitro* (Hodgson et al., 2005). RU486 can thus be considered as a novel AR antagonist that will likely have novel activities *in vivo*. However, clinical trials of RU486 or related drugs are needed to determine whether these may be more efficacious than currently available AR antagonists in the treatment of prostate cancer, particularly advanced androgen-independent prostate cancer. A recent phase II study was conducted to assess the efficacy of mifepristone as an AR antagonist in patients with castration-resistant prostate cancer (CRPC). In this study, RU486 showed only limited activity in patients with CRPC, but stimulated a marked increase in adrenal androgens (Taplin et al., 2008). From these findings the authors hypothesized that inhibition of glucocorticoid receptors by mifepristone might lead to an increase in adrenocorticotropic hormone followed by an increase in adrenal androgens, and that their conversion by tumor cells to testosterone and DHT might have limited the efficacy of mifepristone. As stated by Taplin et al. (2008), a therapeutic approach that combines mifepristone with a second drug harboring a complementary mechanism might be effective in blocking the compensatory rise in adrenal androgens seen in patients with CRPC. These data clearly demonstrate the impact of inflammation and gonadal hormones in tumor progression thus consolidating the fundamental work of Rudolf Virchow and George Thomas Beatson in the field of tumor-associated inflammation.

INFLAMMATION AND RADIATION

Several lines of evidence indicate that inflammation plays a pivotal role in modulating radiation responsiveness of tumors. Radiation treatment is obviously a two-edged sword. On the one hand, sublethal doses of ionizing radiation (IR) induces a nuclear DNA damage response. On the other hand, they trigger a cellular damage response in tumors by inducing pro-inflammatory pathways predominantly mediated via activation of NF-κB, the central linker between inflammation, carcinogenesis, and radioresistance. Apart from NF-κB activation, radiation activates/up-regulates the expression of immediate early genes encoding for, e.g., c-Fos, c-Myc, c-Jun (Hong et al., 1997) as well as TNF (Zhou et al., 2001), GM-CSF (Akashi et al., 1992), PGHS-2 (Steinauer et al., 2000), and ICAM-1 (Son et al., 2006). Radiation also induces activation of receptor tyrosine kinase pathways (Fedrigo et al., 2011) and mitochondria-associated responses (Aykin-Burns et al., 2011). As demonstrated by Valerie et al. (2007), radiation-induced activation of plasma membrane receptors occurs via generation of ionizing events in the liquid phase of the cytosol that are amplified, possibly via mitochondria, generating large amounts of ROS and RNS that inhibit protein tyrosine phosphatase (PTPase) activities. Moreover, radiation activates acidic sphingomyelinase and increases the production of ceramide. Inhibition of PTPases leads to activation of non-receptor and receptor tyrosine kinases (RTK) including epidermal growth factor receptor (EGFR) and the activation of down-stream signal transduction pathways (Goldkorn et al., 1997; Szumiel, 2008). Radiation-induced ceramide was found to promote membrane-associated receptor activation by facilitating the clustering of receptors within lipid rafts (Maziere et al., 2001; Galabova-Kovacs et al., 2006). As a consequence, activated RTK induce down-stream pro-survival pathways (e.g., Akt) that might act as promising targets in enhancing radiosensitivity of tumors.

Furthermore, various inflammatory mediators are reported as being up-regulated during radiation responses. In human glioblastoma cells, exposure to gamma-irradiation stimulated release of IL-6 and IL-8 into culture supernatants (Pasi et al., 2010). Radiation and chemotherapy led to a remarkable increase in the production of these cytokines in human oral carcinoma cells (Tamatani et al., 2004). IR induced a tremendous increase in IL-1, IL-6, and GM-CSF production by human lung cancer cells (Zhang et al., 1994). A similar effect was observed in patients with head and neck cancer where increased IL-6 and IL-8 levels can be detected after chemoradiotherapy (Meirovitz et al., 2010). Elevated IL-6 serum levels are also reported in patients with locally advanced non-small cell lung cancer undergoing concurrent chemoradiation therapy (Wang et al., 2010).

Some concern had been raised about the effect of IR on the expression of the pleiotropic cytokine TNF. The majority of the studies points to an activatory action of IR. For instance, Chendil et al. (2004) reported on a radiation-induced up-regulated TNF protein in prostate cancer cells PC-3 leading to an increase in NF-κB activity followed by an induction of Bcl-2 protein. Furthermore, IR persistently induced NF-κB DNA-binding activity and NF-κB-dependent TNF transactivation and secretion (Veeraraghavan et al., 2011a). In contrast, three-dimensional conformal blocking radiation therapy did not alter serum levels of TNF in patients with prostate cancer (Lopes and Callera, 2011). It is interesting to note that in an immunocompetent animal model of pancreatic cancer the combination of the radio inducible TNF-expressing adenovector Ad.Egr-TNF with IR resulted in significant anti-tumor effects mediated by the immune system (Meng et al., 2010). As demonstrated in this study, Ad.Egr-TNF/IR therapy contributed to local tumor control through TNF production in the tumor microenvironment. TNF induced expression of the known potent immune regulator IFN-β that, in turn, stimulated the production of chemokines leading to the recruitment of $CD8^+$ T cells to the tumor. Several clinical and preclinical studies with Ad.Egr-TNF/IR have suggested that this local approach suppresses the growth of distant metastases (Moral and Tomillero, 2008). However, a Phase III trial comparing Ad.Egr-TNF (TNFerade™) along with standard of care therapy (defined as infusion 5-FU and radiation therapy, followed by gemcitabine or gemcitabine/erlotinib maintenance therapy) versus standard of care therapy in the treatment of locally advanced, unresectable pancreatic cancer failed. Since the interim analysis did not provide sufficient evidence of the clinical effectiveness of TNFerade, the supplying company, GenVec, announced the discontinuation of the trial in 2010. From these observations one can hypothesize that up-regulated TNF probably enhances the impact of tumorigenic stimuli within the tumor or the tumor microenvironment thereby forcing a critical amplification mechanism in tumor-associated inflammation triggered by pro-inflammatory mediators.

It should be kept in mind that radiation therapy represents an efficient local anti-cancer approach leading to elimination of both, tumor cells and cells of the tumor microenvironment such as endothelial cells and tumor-induced suppressor T cells (North, 1984). IR also affects function of immune cells culminating in homing of APC and effector T cells (Ganss et al., 2002). According to the study of Ganss et al. (2002), the combination of irradiation and adoptive tumor-specific T cell therapy ensures antigen-driven tumor cell eradication with anti-angiogenic effects on tumor endothelium. It has been shown previously that sublethal doses of IR stimulates anti-tumor T cell responses and up-regulates MHC class I/II expression in melanoma cells rendering the cells more sensitive to T cell recognition (Abdel-Wahab et al., 1996), obviously through tumor-specific antigen presentation by DC (Ciernik et al., 1999). Clinical phase I/II trials are ongoing and will shed light on the efficiency of low-dose single phase fraction radiotherapy on tumor-infiltrating T cells responses in patients with liver metastasis derived from colorectal caner (Reissfelder et al., 2011) and primarily operable pancreatic cancer (Timke et al., 2011).

PGHS-2 INHIBITION

PGHS-2, the rate-limiting enzyme involved in converting arachidonic acid to prostanoids, has emerged as another crucial NF-κB-dependent pro-inflammatory mediator in tumorigenesis. Aberrant up-regulation of PGHS-2 is frequently observed in various pre-cancerous and malignant tissues. Because most of the PGHS-2-induced effects are mediated by its product PGE_2, down-regulation of prostaglandins in tumor tissues by PGHS-2 inhibition blocks several neoplastic pathways restricting tumor growth. Radiation is known to induce inflammation and NF-κB consequently up-regulating/activating PGHS-2. In this context, PGHS-2 inhibitors have been tested for their anti-tumor efficiency in combination with radiation or chemotherapy. It was found that these inhibitors exert promising anti-cancer effects in a variety of human tumor cells and increase the sensitivity of tumor cells toward chemotherapy and/or radiation therapy. For instance, the PGHS-2 inhibitor NS398 enhanced the radiosensitivity of radioresistant esophageal cancer cells CSC-like Eca109R50Gy most likely by down-regulating the expression of β-catenin as well as inhibiting activation of Akt and inducing apoptosis (Che et al., 2010, 2011). NS398 was also found to radiosensitize human melanoma cells through G2/M arrest of the cell cycle, predominantly via necrotic mechanisms (Johnson et al., 2008). A further PGHS-2 inhibitor, celecoxib, increased radiation-induced cell death, and clonogenic kill of prostate cancer cells *in vitro* providing a rationale for clinical evaluation of celecoxib in combination with irradiation in prostate cancer patients (Handrick et al., 2009). Celecoxib also enhanced radiosensitivity of bronchial and colon carcinoma cells by inhibiting EGFR-mediated mechanisms of radioresistance independent of PGHS-2 activity implying that PGHS-2 inhibition might ameliorate the therapeutic outcome of radiation therapy even in patients with PGHS-2-independent tumor radioresistance (Dittmann et al., 2008). In line with this observation, targeting PGHS-2 by different pharmacological inhibitors led to radio enhancement of human glioma cells in the absence of the PGHS-2 protein (Kuipers et al., 2007). Nimesulide is another PGHS-2-selective inhibitor that has been found to increase the efficacy of radiation therapy in non-small cell lung cancer cells possibly via suppression of NF-κB-mediated, radiation-induced cytoprotective genes (Grimes et al., 2006).

However, selective PGHS-2 inhibitors have come under scrutiny because of reports suggesting an increased cardiovascular risk associated with their use (Solomon et al., 2008). The thitherto used high therapeutic concentrations of these drugs may contribute to a pro-thrombotic state in patients with higher risk for serious cardiovascular events. A novel approach to overcome the limitations associated with the toxicity of PGHS-2 inhibitors might be the combination of pharmacological PGHS-2 inhibitors at low doses with naturally occurring compounds such as the catechin EGCG which is a promising chemopreventive agent derived from green tea (Cerella et al., 2010; Härdtner et al., 2012). Of note, nutraceuticals such as plant-derived polyphenols have been studied intensively for their potential chemopreventive properties and have been found as being pharmacologically safe. These compounds comprise genistein, curcumin, resveratrol, silymarin, caffeic acid phenethyl ester, flavopiridol, emodin, green tea polyphenols (e.g., EGCG), piperine, oleandrin, ursolic

acid, and betulinic acid. These phytochemicals sensitize tumor cells to chemotherapeutic agents and radiation therapy by inhibiting pathways responsible for treatment resistance (Garg et al., 2005; Nambiar et al., 2011). Among them, curcumin derived from the rhizomes of *Curcuma longa* has been identified to improve the anti-tumor effects of IR by blocking NF-κB pathways, down-regulating anti-apoptotic Bcl-X_L and survivin as well as increasing G2/M phase arrest in the cell cycle distribution in Burkitt's lymphoma cells (Qiao et al., 2012). Curcumin also potentiates radiation therapy-induced cell death by targeting radiation therapy-induced NF-κB activation in pancreatic cancer cells (Veeraraghavan et al., 2011b).

STAT-3 AND HIF-1 INHIBITION

Apart from NF-κB and PGHS-2, STAT-3 is a further inflammatory molecule crucially involved in radioresistance of tumors. Since enhanced radioresistance of cancer cells is additionally related to radiation-induced activation of the JAK/STAT pathway, inhibition of STAT by, e.g., phytochemicals might sensitize tumors to radiation therapy. It has been shown previously that STAT-3-mediated radiosensitization obviously occurs via down-regulation of anti-apoptotic survivin (Kim et al., 2006a). In this context, resveratrol, a polyphenolic phytoalexin, selectively targets numerous cell signaling pathways and decreases clonogenic survival primarily via an apoptotic mechanism. In melanoma cells, resveratrol inhibits STAT-3 and NF-κB-dependent transcription, culminating in suppression of c-FLIP and Bcl-X_L expression, while activating the MAPK and the ATM-Chk2-p53 pathways (Johnson et al., 2008). Resveratrol also up-regulates TRAIL promoter activity and induces TRAIL surface expression in some melanoma cell lines, resulting in a rapid apoptosis development (Johnson et al., 2008). As also demonstrated in this study, sequential treatment of melanoma cells, first with gamma-irradiation to up-regulate TRAIL receptor surface expression, and then with resveratrol to suppress anti-apoptotic proteins c-FLIP and Bcl-X_L and induce TRAIL surface expression, dramatically up-regulated apoptosis in some melanoma cell lines. Nitidine chloride, a natural phytochemical alkaloid derived from *Zanthoxylum nitidum*, was identified as a potent STAT-3 signaling inhibitor suppressing angiogenesis and growth of human gastric cancer (Chen et al., 2012a). From these data one can hypothesize that phytochemicals in combination with IR may play a significant role in enhancing the therapeutic efficacy of cancer treatment.

As already mentioned, HIF-1, the key mediator in hypoxia signaling pathways, is crucially involved in hypoxia-induced tumor radioresistance. This obviously includes radiation-induced activation of HIF-1 (Moeller et al., 2004; Harada et al., 2009a,b), HIF-1-dependent induction of VEGF and protection of endothelial cells from radiation-induced cytotoxicity by VEGF (Gorski et al., 1999) as well as delivery of oxygen and nutrients to tumor cells by radioprotected tumor blood vessels (Zeng et al., 2008). N-Myc down-stream-regulated gene 2 (*NDRG2*) was recently identified in cervical cancer cells as a new HIF-1 target gene acting down-stream of HIF-1 to promote radioresistance via suppression of radiation-induced Bax expression (Liu et al., 2010). Therefore, it would be reasonable to study the efficacy of HIF-1 and NDRG2 blockage as radiosensitizer for tumor therapy. Strategies to over-come radioresistance of hypoxic tumor cells comprise, among others, hyperbaric oxygenation, gene therapy approaches, fractionated radiotherapy, radiosensitization by mimicking the effect of molecular oxygen using nitroimidazole derivates as well as suppression of hypoxic tumor cell radioresistance by HIF-1 inhibitors (for a review, see Harada, 2011). As described previously, administration of the HIF-1 inhibitor YC-1 to hypoxic cobalt-treated cells derived from squamous-cell carcinoma of the larynx effectively inhibited HIF-1α expression, and enhanced the sensitivity of cells to radiation, decreasing the surviving fraction to that of normoxic cells (Moon et al., 2009). YC-1 was found to reduce the number of tumor lesions after tumor cell inoculation in nude mice (Shin et al., 2007). Compared to radiation therapy alone, inhibition of radiation-induced HIF-1 activation by YC-1 led to a significant reduction in tumor cell growth (Harada et al., 2009b).

Another HIF-1 inhibitor, acriflavine, was found to inhibit tumor growth and angiogenesis in a xenograft tumor model for human prostate cancer through blockage of HIF-1 dimerization (Lee et al., 2009). Since the same agent blocked lung metastasis in an orthotopic breast cancer model (Wong et al., 2012), it would be of interest to test the efficacy of this HIF-1 inhibitor in increasing radioresistance of certain tumors. Interestingly, inhibition of Hsp90 function by 17-allylamino-17-demethoxygeldanamycin or deguelin, a novel natural inhibitor of Hsp90, suppressed increases in HIF-1α/Hsp90 interaction and HIF-1α expression in radioresistant lung cancer cells (Kim et al., 2009). Hsp90 interacts with HIF-1α in competition with receptor of activated protein C kinase 1 (RACK-1) and inhibits oxygen-independent degradation of HIF-1α (Semenza, 2007). The study by Kim et al. (2009) also demonstrated that the combined treatment of radiation with deguelin significantly decreased the survival and angiogenic potential of radioresistant lung cancer cells *in vitro* and inhibited tumor growth and angiogenesis *in vivo*.

Even phytochemicals have the capacity to inhibit radiation-induced HIF-1 activation. Pre-treatment of prostate cancer cells with soy isoflavones inhibited Src/STAT-3/HIF-1α activation by radiation and nuclear translocation of HIF-1α. These findings correlated with decreased expression of APE1/Ref-1 and DNA-binding activity of HIF-1α and NF-κB (Singh-Gupta et al., 2009). Apurinic/apyrimidinic (AP) endonuclease 1/redox factor-1 (APE1/Ref-1) is a multifunctional protein involved in DNA repair that also functions as a redox activator of cellular transcription factors. Emodin, a natural anthraquinone enriched in the traditional Chinese herbal medicines and novel small HIF-1 inhibitor, was found to improve efficacy of chemotherapeutic drugs by inhibiting transactivation of HIF-1 without impairing mRNA expression and stability of HIF-1α protein in prostate cancer cells (Huang et al., 2008). Another approach to HIF-1 blockage might be the use of cell-permeable HIF-1 antagonists (Shi et al., 2007). As mentioned before, HIF-1 contributes to tumor radioresistance by up-regulating survivin expression under hypoxic conditions. Moreover, in hypoxic tumor cells the HIF-1 signaling pathway is activated and could be further enhanced by radiation, thereby providing survival signals to adjacent vascular endothelial cells by up-regulation of VEGF and basic fibroblast growth factor (bFGF) and resulting in tumor radioresistance through vascular

radioprotection. Thus, HIF-1 antagonists might decrease tumor angiogenesis and sensitize tumor cells to radiotherapy.

Targeting HIF-1 for radiosensitization can also be achieved by inhibiting its up-stream mediators. In this respect, the PI3K/Akt/mTOR pathway can be considered as the most relevant target in sensitizing tumors to radiation therapy. Mutations in this pathway can be found in various human cancers and have been found to up-regulate HIF-1α expression (Zhong et al., 2000). The PI3K/Akt/mTOR pathway also affects the NF-κB-mediated expression of PGHS-2 (Yang et al., 2009) and radiation-induced MMP-9 expression (Cheng et al., 2006). Several studies demonstrated that specific blockage of this pathway by certain inhibitors such as erufosine (Rudner et al., 2010), LY294002 (Nakamura et al., 2005; Kao et al., 2007), RAD001 (Albert et al., 2006), and rapamycin (Majumder et al., 2004) ensures efficient radiosensitization of distinct tumors cells.

NF-κB INHIBITION

In recent years NF-κB inhibition by synthetic compounds as well as nutraceuticals of different sources has been approved for tumor radiosensitization. In particular the use of nutraceuticals became a popular approach due to the broad anti-tumor and anti-inflammatory properties in conjunction with low toxicity risks of these compounds (Deorukhkar et al., 2007; Deorukhkar and Krishnan, 2010). How these drugs block NF-κB activation is becoming increasingly apparent. Among them, curcumin has emerged as one of the best studied plant-derived polyphenols. In a phase II clinical trial curcumin showed beneficial effects in patients with advanced pancreatic cancer (Dhillon et al., 2008). Curcumin down-regulated expression of NF-κB, PGHS-2, and phosphorylated STAT-3 in peripheral blood mononuclear cells from these patients. Furthermore, curcumin has been shown to suppress TNF-mediated NF-κB activation by inhibiting inhibitor of kappaB alpha (IκBα) in human myeloid ML-1a cells (Singh and Aggarwal, 1995). Curcumin also confers radiosensitizing effects in prostate cancer cells by inhibiting TNF-mediated NF-κB activation resulting in Bcl-2 protein down-regulation and concomitant activation of cytochrome c and caspase-9 and -3 (Chendil et al., 2004). More recently, curcumin was found to sensitize colorectal cancer cells to radiotherapy by suppressing radiation-induced NF-κB activation via inhibition of radiation-induced phosphorylation and degradation of IκBα, inhibition of inhibitor of kappaB kinase (IKK) activity, and inhibition of Akt phosphorylation (Sandur et al., 2009) consequently leading to down-regulation of several tumorigenic factors in colorectal cancer xenografts in nude mice (Kunnumakkara et al., 2008). Apart from its IKK-inhibitory capacity, curcumin also blocks p65 phosphorylation and acetylation and represses the p300/CREB-binding protein (CBP) HAT activity-dependent transcriptional activation from chromatin (Balasubramanyam et al., 2004). Similarly, anacardic acid (6-pentadecylsalicylic acid) derived from traditional medicinal plants, such as cashew nuts, has been identified to inhibit NF-κB activation, to suppress the activation of IκBα kinase that led to abrogation of phosphorylation and degradation of IκBα and to inhibit acetylation and nuclear translocation of p65 (Sung et al., 2008). The same study demonstrated that down-regulation of the p300 HAT gene by RNA interference abrogated the effect of anacardic acid on NF-κB suppression. Further nutraceuticals including the soy isoflavone genistein (Raffoul et al., 2006), the isoquinoline alkaloid berberine from medicinal plants such as *Berberis aristata*, *Coptis chinensis*, *Coptis japonica*, *Coscinium fenestratum*, and *Hydrastis canadensis* (Pandey et al., 2008), piceatannol (3,3′,4,5′-trans-trihydroxystilbene), a naturally occurring hydroxylated analog of resveratrol found in various plants (Son et al., 2010), the principal prenylated flavonoid xanthohumol from *Humulus lupulus* (Harikumar et al., 2009), and the polyphenol butein (3,4,2′,4′-tetrahydroxychalcone) from *Rhus verniciflua* Stokes (Pandey et al., 2007) have been identified as blocking NF-κB by direct interaction with IKKβ on cysteine 179 residue. NF-κB inhibition by direct interaction with one of its subunits occurs in the presence of numerous phytochemicals including sesquiterpene lactones (Garcia-Pineres et al., 2001). In radiation-resistant human CGL1 cells, parthenolide, a major active component of the herbal medicine feverfew (*Tanacetum parthenium*), enhanced radiosensitivity through NF-κB inhibition and apoptosis induction via p53 stabilization, induction of pro-apoptotic Bax, and phosphorylation of pro-apoptotic Bid (Mendonca et al., 2007). The radiosensitization effect of parthenolide is enhanced in the presence of tumor suppressor protein PTEN (phosphatase and tensin homolog deleted on chromosome 10), in part, by suppressing the absolute amount of activated p-Akt in human prostate cancer cells (Sun et al., 2007). The group of Aggarwal recently found out that crotepoxide (a substituted cyclohexane diepoxide), isolated from *Kaempferia pulchra* (peacock ginger), inhibited activation of TGF-β-activated kinase (TAK)-1, which led to suppression of IκBα kinase, abrogation of IκBα phosphorylation and degradation, nuclear translocation of p65, and suppression of NF-κB-dependent reporter genes encoding for anti-apoptotic (Bcl-2, Bcl-X_L, IAP1/2, Mcl-1, survivin, TRAF-1), pro-apoptotic (Bax, Bid), pro-inflammatory (PGHS-2), proliferation- (cyclin D1, c-Myc), invasion- (ICAM-1, MMP-9), and angiogenesis-promoting (VEGF) factors (Prasad et al., 2010). Moreover, Tamatani et al. (2007) analyzed the effects of radiation therapy in combination with cepharanthine on NF-κB activation and expression of its down-stream effector molecules in human oral squamous-cell carcinoma cells. Cepharanthine is a biscoclaurine alkaloid extracted from the roots of *Stephania cepharantha* Hayata, and is widely used in Japan for the treatment of patients with leucopenia, nasal allergy, and venomous snakebites. The authors could show that treatment of cancer cells with cepharanthine combined with exposure to IR enhanced radiosensitivity via NF-κB inhibition and concomitant down-regulation of IL-6, IL-8, and anti-apoptotic proteins such as cellular inhibitor of apoptosis protein (cIAP)-1 and -2. Moreover, the pleiotropic effects of cepharanthine also includes inhibition of STAT-3 in the human osteosarcoma cell line SaOS2 (Chen et al., 2012b). These examples highlight the crucial role of naturally occurring compounds in targeting inflammatory signaling pathways for sensitizing tumors to radiation therapy. Beside them, a variety of further compounds have been identified to enhance radiosensitivity via inhibition of NF-κB including celecoxib (Raju et al., 2005), pitavastatin (Tsuboi et al., 2009), docosahexaenoic acid (Zand et al., 2008), and the novel organoselenium thioredoxin reductase inhibitor ethaselen (Wang et al., 2011).

TUMOR CELL RE-POPULATION

It is commonly accepted that aggressive radio-/chemotherapy often results in a negative selection toward highly aggressive tumor clones. In recent days, the induction of radiation-induced apoptosis, the principal purpose of radiation therapy, has come under scrutiny due to reports suggesting that dying tumor cells use the apoptotic pathway to stimulate the re-population of tumors subjected to radiation therapy. The process of re-population was originally discovered in the second half of the twentieth century and is commonly accepted as playing a crucial role in radio-/chemotherapy (Hermens and Barendsen, 1969; Stephens et al., 1978). Interestingly, the group of Li from Aurora (USA) demonstrated that radiation-induced apoptosis comprises caspase-3 cleavage and concomitant activation of Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) followed by PGE_2 release promoting tumor cell re-population *in vitro* and *in vivo* (Huang et al., 2011). The relevance of this mechanism for the caspase-mediated tumor cell re-population was confirmed by caspase-3 determination in different cancer patients. Herein, elevated expression levels of activated caspase-3 in tumor tissues correlated with poor clinical outcome in patients with head and neck cancer as well as advanced stage breast cancer. However, the contribution of macrophages and the subsequently generated clearance-related anti-inflammatory milieu to radiation-induced tumor cell re-population and poor therapeutic outcome should be taken into consideration. As discussed by Tauber and co-workers, apoptotic manifestations such as externalization of phosphatidylserine, bleb formation, and DNA fragmentation are crucially involved in macrophage activation and depend on caspase-3 activation (Jänicke et al., 1998; Coleman et al., 2001; Sebbagh et al., 2001). The authors conclude that caspase-3-positive apoptotic cells can recruit more macrophages and are more efficiently internalized by the phagocytes ("silent clearance") culminating in a strong anti-inflammatory and growth-promoting phagocyte response via release of clearance-associated cytokines, e.g., PGE_2 than their caspase-3-negative counterparts. However, further preclinical and clinical studies using certain caspase inhibitors are required to strengthen this hypothesis. In this context, the novel caspase inhibitor GS-9450 was found to down-regulate caspase-3 expression on peripheral T cells from chronically HCV-infected patients in a phase II clinical trial (Arends et al., 2011). It would therefore be of interest to study a putative beneficial effect of a combinatorial treatment with caspase inhibitors and radiation therapy on the clinical outcome of patients with various advanced cancer types.

PATTERN RECOGNITION RECEPTORS IN RADIOIMMUNITY

It is well known that chronic inflammation induced by non-infectious agents can also contribute to carcinogenesis and act as a driving force in tumorigenesis. Several factors such as growth factors, oncoproteins, and toxins can affect the host via an activation of pattern recognition receptors (PRR) interacting with exogenous pathogen-associated molecular patterns (PAMP; Kawai and Akira, 2011). Apart from PAMP, the same receptor superfamily also recognizes endogenous "alarmins" both of them comprising the group of danger-associated molecular patterns (DAMP; Bianchi, 2007). Receptor ligation leads to activation of inflammatory cells and initiation of host responses that tend to eradicate invading microorganisms (Akira et al., 2006; Karin et al., 2006). Not surprisingly, inadequate pathogen elimination, recurring tissue injury, prolonged inflammatory signaling, and failure of anti-inflammatory mechanisms can all culminate in chronic inflammation promoting cancer development.

Signaling via PRR also makes an impact on radiation responses. It has been shown previously that both, low (0.075 Gy) and high (2 Gy) doses of IR causes sustained stimulation of IL-12 and IL-18 secretion by mouse macrophages (Shan et al., 2007) with concomitant activation of NF-κB accompanied by elevated cytoplasmic MyD88 levels and an up-regulated surface expression of CD14 and TLR-4/MD-2 (Shan et al., 2007), the latter acting as LPS sensor. From these findings the authors hypothesized that IR can stimulate the secretion of IL-12 and IL-18 presumably via activation of the Toll signaling pathway in macrophages. A detailed description of the TLR-4 signaling pathway is visualized schematically in **Figure 2**.

Interestingly, RP105 (radioprotective 105 kDa), a TLR-related molecule, was recently identified on human B cells and DC (Fugier-Vivier et al., 1997) and characterized as being similar to TLR-4 in that the extracellular leucine-rich repeats associate with MD-1, an MD-2-like molecule (Miyake et al., 1995; Fugier-Vivier et al., 1997). MD-2 directly binds to lipid A, the active center of lipopolysaccharide (LPS), leading to dimerization of TLR-4/MD-2 (Ohto et al., 2011). An antibody raised against surface-bound RP105 was found to drive B cell proliferation and protection from subsequent radiation- or dexamethasone-induced apoptosis (Miyake et al., 1994). Studies by Divanovic et al. (2005) demonstrated that: (a) RP105 is a specific inhibitor of TLR-4 signaling in human embryonal kidney cells HEK293; (b) RP105/MD-1 interacts directly with TLR-4/MD-2, thus abolishing the LPS-binding capacity of the complex; (c) RP105 regulates TLR-4 signaling in DC and macrophages; and (d) RP105 regulates *in vivo* responses to LPS supporting the assumption that RP105 acts as a physiological negative regulator of TLR-4 responses. This brief overview makes it clear that, on the one hand, pro-inflammatory responses to radiation and TLR signaling enhance the impact of tumorigenic factors in the tumor and the tumor microenvironment and, on the other hand, might represent pivotal target structures in radiation therapy. As discussed by Schaue and McBride (2010), radiation-induced DAMP signaling via TLR-2/-4 has emerged as a critical component in affecting the outcome of anti-cancer therapies. In this context, radiation was found to induce secretion of the prototypical DAMP, the high-mobility-group box 1 (HMGB-1) "alarmin" protein from dying tumor cells as a prerequisite for the development of a tumor antigen-specific T cell immunity mediated by an interaction of HMGB-1 with TLR-4 on DC (Apetoh et al., 2007). The same study revealed that patients with breast cancer who carry a TLR-4 loss-of-function allele relapsed more quickly after radiotherapy and chemotherapy than those carrying the normal TLR-4 allele implying a clinically relevant immunoadjuvant pathway triggered by tumor cell death. An intriguing novel finding comprises the reduction of metastatic ability and MMP-9 expression in MGC-803 gastric cancer cells by silencing of the HMGB-1 expression using an HMGB-1-specific RNAi lentiviral vector (Song et al., 2012). As also shown in this study, HMGB-1 silencing decreased cell proliferation and sensitized cells to

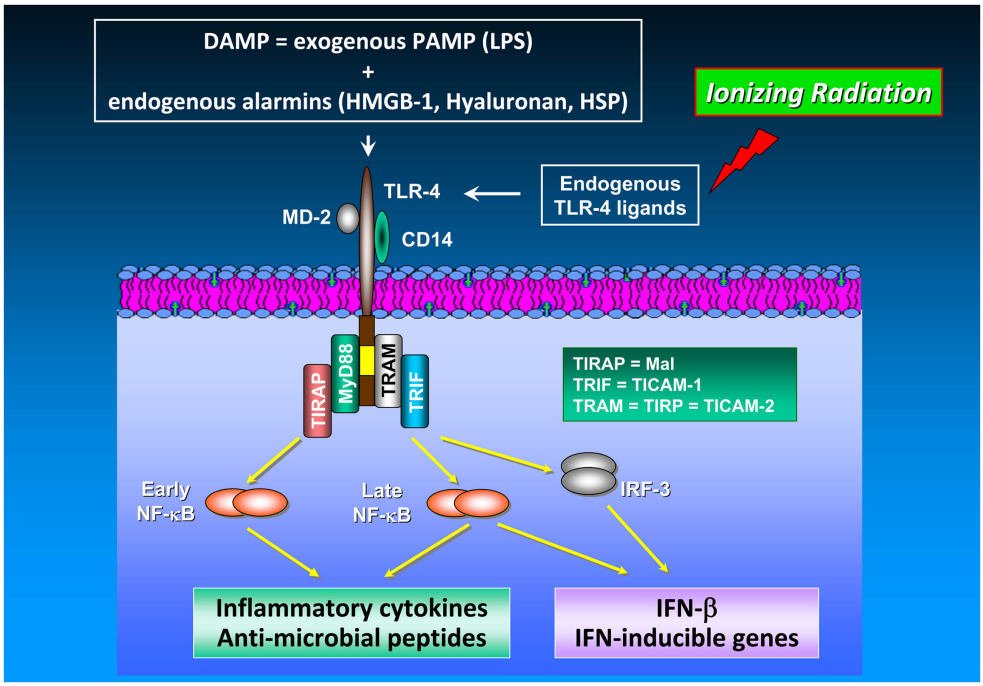

FIGURE 2 | TLR-4 signaling in cancer. The TLR-4/MD-2 receptor complex recognizes and binds exogenous PAMP (e.g., endotoxins such as LPS) as well as endogenous alarmins (HMGB-1, hyaluronan, heat shock proteins). Release of DAMP into the extracellular space is achieved by a number of different mechanisms including (i) leakage from necrotic cells, (ii) increased synthesis and post-translational modification in response to inflammation, and (iii) degradation of inactive precursors into TLR-mimetic degradation products in inflammatory environments (Mencin et al., 2009). TLR-4 induces two distinct signaling pathways controlled by the TIRAP/MyD88 and TRAM/TRIF pairs of adaptor proteins, which elicit the production of pro-inflammatory cytokines and type I interferons, respectively. The cytosolic adapter molecules mentioned above comprise myeloid differentiation protein 88 (MyD88), Toll/IL-1R resistance domain-containing adapter inducing IFN-β (TRIF), TIR domain-containing adapter protein (TIRAP), and TRIF-related adaptor molecule (TRAM). TIRAP is also termed Mal (MyD88 adaptor-like), TRIF is also known as Toll/IL-1R homology domain-containing adaptor molecule 1 (TICAM-1), whereas TRAM is alternatively entitled TIR-containing protein (TIRP) and TICAM-2, respectively. TLR-4-mediated signal transduction occurs via MyD88-dependent and MyD88-independent (i.e., TRAM/TRIF-dependent) pathways. Both, MyD88-dependent and MyD88-independent pathways induce expression of genes involved in pro-inflammatory and anti-microbial responses (Akira and Takeda, 2004). In TLR-4 signaling, MyD88 up-regulates inflammatory cytokines via NF-κB activation. Moreover, the MyD88-independent pathway does not only induce inflammatory gene expression in an NF-κB-dependent manner but also up-regulates type I interferon expression via the transcription factor IRF-3. NF-κB activation and subsequent inflammatory cytokine production are mediated by different mechanisms and kinetics in the MyD88-dependent and the MyD88-independent pathway: NF-κB activation in the MyD88-dependent pathway is an early event occurring with fast kinetics whereas NF-κB activation via the MyD88-independent pathway represents a late event occurring with slower kinetics. Unlike TLR-4 signaling in immune cells which has been found to enhance anti-tumor immunity by, e.g., IL-12/IFN-γ up-regulation and promotion of DC maturation and function, TLR-4 signaling in cancer cells increases their tumorigenic capacity under certain circumstances (Oblak and Jerala, 2011). Noteworthy, HMGB-1 which is released from irradiated tumor cells functions as an endogenous TLR-4 ligand leading to the development of a tumor antigen-specific T cell immunity mediated by an interaction of HMGB-1 with TLR-4 on DC.

apoptosis implying HMGB-1 as being a potential target for the therapeutic intervention of certain cancers such as gastric cancer.

TARGETING OF HEAT SHOCK PROTEINS IN RADIOTHERAPY

A promising approach in cancer therapy also might be targeting heat shock proteins (HSP), a class of proteins which are induced under physiologic stress to promote cell survival in the face of endogenous or exogenous injury. Compared to normal cells, tumors frequently have elevated basal Hsp70 levels which are further enhanced in response to a number of pathological and environmental stresses such as nutrient deficiency, hypoxia, heavy metals, irradiation, and/or chemotherapeutic agents. Also normal cells show an increase in the synthesis of Hsp70 following stress in order to mediate protection against lethal damage and to maintain protein homeostasis. Screening of nearly 1,000 primary human tumor biopsies and the corresponding normal tissues revealed that human carcinomas, but none of the tested normal tissues, frequently present Hsp70 on their cell surface (Multhoff et al., 1995; Multhoff, 2007). A membrane Hsp70-positive tumor phenotype has been found to be associated with a significantly decreased overall survival in tumor patients. Therefore, the expression of this molecule could serve as a negative prognostic marker (Pfister et al., 2007).

Apart from their intracellular localization, Hightower and Guidon Jr. (1989) reported on an ER/Golgi-independent release of Hsp70 from viable cells with intact cell membranes already in the late 1980s. Extracellular HSP are considered as molecules with immunomodulatory functions (Pockley and Multhoff, 2008; Pockley et al., 2008) either as cross-presenters of immunogenic peptides (Srivastava, 1997; Asea et al., 2000) or in a peptide-free

version as chaperokines (Asea et al., 2002) or stimulators of innate immune responses (Multhoff et al., 1995). Despite these well-documented immunological functions, the mechanisms of HSP export are still controversially discussed since cytosolic HSP lack a consensus signal for secretion. However, apart from Hsp70, other molecules lacking a secretory signal such as IL-1α, IL-1β, and HMBG-1 are also found outside of cells (Nickel and Seedorf, 2008; Eder, 2009). Hsp70 also has been found to be located on the cell surface although lacking a transmembrane domain (Multhoff and Hightower, 1996). Membrane Hsp70 might help to maintain stability of tumor cells and thus might protect tumors from lethal damage induced by environmental stress (Horvath et al., 2008; Horvath and Vigh, 2010). The fundamental work of the group of Antonio De Maio has demonstrated an interaction of members of the HSP70 family with artificial membranes containing phosphatidylserine (PS; Arispe and De Maio, 2000; Arispe et al., 2002; Vega et al., 2008; De Maio, 2011). Our group reported on an interaction of Hsp70 with the sphingolipid globotriaosylceramide (Gb3) in the plasma membrane of non-stressed human gastrointestinal stromal tumors (Gehrmann et al., 2008). Gb3 is found in cholesterol-rich microdomains, also termed as lipid rafts, which serve as signal transduction platforms. Following irradiation or hypoxia-induced stress Hsp70 was found to be associated predominantly with PS outside of lipid rafts in the plasma membrane of tumor cells (Schilling et al., 2009). These data indicate that environmental stress might result in a re-organization of the lipid bilayer and might modulate the interaction of Hsp70 with lipid components. Surprisingly, only tumors but not the corresponding normal tissues were found as being membrane Hsp70-positive using the IgG1 mouse monoclonal antibody cmHsp70.1. In contrast, other Hsp70-specific antibodies failed to bind to membrane Hsp70 on viable tumor cells (Stangl et al., 2011). The discovery that neither high/low salt concentrations nor changes in the pH were able to release Hsp70 from the plasma membrane of tumor cells (Gehrmann et al., 2008; Vega et al., 2008) confirmed our hypothesis that in tumor cells Hsp70 is an integral membrane protein which can associate with raft (Gb3) and non-raft (PS) lipid components.

We recently observed an increased surface expression of Hsp70 in colorectal tumor cells after IR alone or in combination with hyperthermia (HT) while the amount of extracellular Hsp70 was only increased when HT was given additionally (Schildkopf et al., 2011). Moreover, a high up-regulation of the co-stimulation molecule CD80 and the chemokine receptor CCR7 on DC was measured after contact with supernatants of X-ray plus HT-treated cells. This was dependent on extracellular Hsp70. Combined treatments further led to significantly increased phagocytosis rates of macrophages and DC and increased pro-inflammatory cytokine (IL-8, IL-12) secretion. From these findings we conclude that X-ray combined with HT induces Hsp70-dependent activation of immune cells and might generate a tumor microenvironment beneficial for cure.

HSP over-expression in tumor cells plays a pivotal role in tumorigenesis by inhibiting apoptosis and senescence. Recent studies indicate an involvement of HSP such as Hsp70/Hsp72 and Hsp90 in the recognition of PAMP by binding to TLR-4 within lipid rafts (Triantafilou and Triantafilou, 2004; Wheeler et al., 2009). Since extracellular residing Hsp70 acts as a danger signal for the immune system (Matzinger, 1998), this stress protein has been added to the list of "alarmins" comprising the group of DAMP together with PAMP, hyaluronan, and other HSP members. Consequently, developing means of abrogating HSP expression may provide a way to render cancer cells more susceptible to radiation or chemotherapy. Various attempts are underway to target these proteins, particularly small HSP, in developing potent radiation and chemotherapy sensitizers (Guttmann and Koumenis, 2011). For instance, Hsp27 has been found as being implicated in the resistance to chemotherapy in several types of cancers. The group of Moriwaki analyzed the effects of a gemcitabine treatment in pancreatic cancer cells (Nakashima et al., 2011). Gemcitabine is an anti-tumor drug and currently considered to be the standard of care for the treatment of advanced pancreatic cancer, but the clinical outcome is still not satisfactory. It was shown that gemcitabine suppressed growth of pancreatic cancer cells by inducing apoptosis. Gemcitabine also caused activation of p38 mitogen-activated protein kinase (MAPK), MAPK-activated protein kinase 2 (MAPKAPK-2) with concomitant serine phosphorylation of Hsp27 at position 15, 78, and 82 without affecting total Hsp27 levels. From these results the authors conclude that the phosphorylation status of Hsp27 obviously plays a pivotal role in gemcitabine-induced growth suppression of pancreatic cancer. Of Note, the HSP inhibitor KNK437, a benzylidene lactam compound, was observed to dramatically reduce expression of Hsp27 in gemcitabine-resistant pancreatic cancer cells KLM1-R and to enhance the *in vitro* anti-tumor cytotoxic effect of gemcitabine on KLM1-R compared to single-agent gemcitabine (Taba et al., 2011). KNK437 also sensitizes prostate cancer cells to the apoptotic effect of hyperthermia by down-regulating heat-induced Hsp70 mRNA expression (Sahin et al., 2011). Moreover, in patients with locally advanced squamous-cell esophageal cancer neoadjuvant radiochemotherapy (NRCT) led to a decreased expression of Hsp16.2, Hsp90, and heme-binding protein 2 (SOUL), and an increased Bax/Bcl-2 ratio was found in the responding tumors (Farkas et al., 2011).

Also Hsp90 may represent a potentially attractive target for specific molecular anti-cancer agents, because Hsp90 expression is up-regulated in tumors as compared with normal tissues, which implies that tumor cells might be preferentially affected by Hsp90-targeted therapies (Ferrarini et al., 1992). In this context, geldanamycin (GA), a naturally occurring ansamycin antibiotic, along with its clinically used analogs such as 17-allylamino-17-demethoxygeldanamycin (17-AAG), has been evaluated in preclinical and clinical trials for its significant anti-tumor properties. These agents disrupt Hsp90 association with client proteins by occupying the nucleotide-binding site of Hsp90 (Grenert et al., 1997; Prodromou et al., 1997; Stebbins et al., 1997), thereby preventing binding of Hsp90 with ATP and profoundly affecting the composition of Hsp90-containing multimolecular chaperone complexes (Obermann et al., 1998; Maloney and Workman, 2002). As demonstrated by the group of Gius, treatment of two human cervical carcinoma cell lines (HeLa, SiHa) with geldanamycin and 17-AAG resulted in cytotoxicity and, when combined with IR, enhanced the radiation response. In addition, mouse *in vivo* models using 17-AAG at clinically achievable concentrations yielded results that paralleled the *in vitro* radiosensitization studies of both

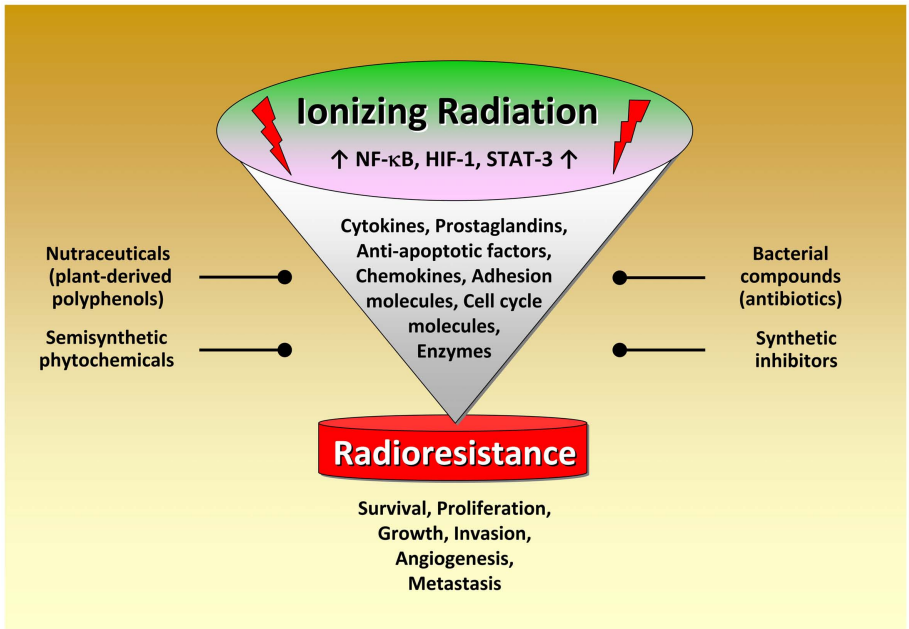

FIGURE 3 | Radiation-induced activation of inflammatory pathways in tumor cells. Schematical simplified representation of the complex intracellular mechanisms leading to radioresistance. Exposure to ionizing radiation leads to activation of several transcription factors modulating the expression of numerous factors promoting cancer development. Novel therapeutic approaches thus aim to interfere with the activity or expression of these factors, either in single-agent or combinatorial treatment or as supplements of the existing therapeutic concepts. Noteworthy, targeting the pro-inflammatory signaling pathways for tumor radiosensitization represents a promising novel therapeutical approach in cancer. A great variety of classical or novel drugs including nutraceuticals have the capacity to interfere with the inflammatory network in cancer and are considered as putative radiosensitizers.

single and fractioned courses of irradiation (Bisht et al., 2003). We recently analyzed the effects of the novel Hsp90 inhibitor NVP-AUY922 compared to 17-AAG on the HIF-1α/HIF-2α expression in combination with radiosensitivity in lung cancer cell lines under normoxic and hypoxic conditions (Schilling et al., 2012b). NVP-AUY922 is a synthetic, isoxazole/resorcinol-based second generation Hsp90 inhibitor exhibiting an enhanced metabolic stability and a tighter binding to Hsp90 compared to 17-AAG (Brough et al., 2008). As given in our study, both inhibitors reduced basal and hypoxia-induced HIF-1α levels in EPLC-272H lung carcinoma cells. However, despite a down-regulation of HIF-1α upon Hsp90 inhibition, sensitivity toward irradiation remained unaltered in EPLC-272H cells under normoxic and hypoxic conditions. In contrast, treatment of H1339 lung carcinoma cells with NVP-AUY922 and 17-AAG resulted in a significant up-regulation of their initially high HIF-1α levels and a concomitant increase in radiosensitivity indicating the ability of an HIF-1α-independent radiosensitization of normoxic and hypoxic H1339 lung cancer cells via Hsp90 inhibition. From these observations it can be concluded that treatment strategies combining HSP targeting and radiochemotherapy appear to be a high potential therapeutic benefits for cancer patients.

CONCLUDING REMARKS

Although radiation therapy, alone or in combination with chemotherapy, is the primary treatment for several tumors, radioresistance dramatically attenuates radiocurability. Several lines of evidence indicate that inflammation plays a pivotal role in modulating radiation responsiveness of tumors. As discussed in this review, exposure to IR leads to activation of several transcription factors modulating the expression of numerous factors promoting cancer development. Novel therapeutic approaches thus aim to interfere with the activity or expression of these factors, either in single-agent or combinatorial treatment or as supplements of the existing therapeutic concepts. Among them, NF-κB, STAT-3, and HIF-1 play a crucial role in radiation-induced inflammatory responses. A great variety of classical or novel drugs including nutraceuticals have the capacity to interfere with the inflammatory network in cancer and are considered to function as putative radiosensitizers (**Figure 3**). Thus, targeting the inflammatory signaling pathways induced by IR offers the opportunity to improve the clinical outcome of radiation therapy by enhancing radiosensitivity and decreasing putative metabolic effects. Since inflammation and sex steroids also impact tumorigenesis, a therapeutic approach targeting glucocorticoid receptors, and radiation-induced production of tumorigenic factors might be effective in sensitizing tumor cells to IR in certain cases.

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How does ionizing irradiation contribute to the induction of anti-tumor immunity?

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Radiotherapy (RT) with ionizing irradiation is commonly used to locally attack tumors. It induces a stop of cancer cell proliferation and finally leads to tumor cell death. During the last years it has become more and more evident that besides a timely and locally restricted radiation-induced immune suppression, a specific immune activation against the tumor and its metastases is achievable by rendering the tumor cells visible for immune attack. The immune system is involved in tumor control and we here outline how RT induces anti-inflammation when applied in low doses and contributes in higher doses to the induction of anti-tumor immunity. We especially focus on how local irradiation induces abscopal effects. The latter are partly mediated by a systemic activation of the immune system against the individual tumor cells. Dendritic cells are the key players in the initiation and regulation of adaptive anti-tumor immune responses. They have to take up tumor antigens and consecutively present tumor peptides in the presence of appropriate co-stimulation. We review how combinations of RT with further immune stimulators such as AnnexinA5 and hyperthermia foster the dendritic cell-mediated induction of anti-tumor immune responses and present reasonable combination schemes of standard tumor therapies with immune therapies. It can be concluded that RT leads to targeted killing of the tumor cells and additionally induces non-targeted systemic immune effects. Multimodal tumor treatments should therefore tend to induce immunogenic tumor cell death forms within a tumor microenvironment that stimulates immune cells.

Keywords: low and high dose ionizing irradiation, immune modulation, immunogenic cancer cell death, dendritic cells, abscopal effects, immune therapy, AnnexinA5, hyperthermia

INTRODUCTION

The old theory about immunological tumor control has been revived in the last decades. Especially preclinical experiments with immune deficient mice coined the immune editing hypothesis of cancer. Recombinase-activating gene 2 (RAG2) deficient mice lack functional T and B cell receptors and develop tumors more quickly and with greater frequency than immune competent wild-type mice (Shankaran et al., 2001). The cancer immune editing model provides a well reflected explanation regarding how cancer cells get eliminated by the immune system, stay calm (equilibrium phase), or escape immune surveillance. This knowledge is a valuable basis for the design of immunotherapies against cancer (Vesely et al., 2011).

Clinical studies evaluating the frequency of cancer in immune suppressed patients further support that the immune system is involved in tumor control (summarized in Mueller, 1999; Zitvogel et al., 2010). Changes in the surface expression of major histocompatibility complex (MHC) I molecules have a major impact on the prognosis of tumor patients. Since only the tumor peptide/MHC I complexes are recognized by cytotoxic T lymphocytes (CTLs), the contribution of the immune system to a successful cancer therapy has become evident. Importantly, the surface modifications of tumor cells result from the selection pressure (immune editing) exerted also by cells from the innate and adaptive immune system (Pages and Kroemer, 2011).

Nowadays the pivotal question is not whether the immune system contributes to tumor control but rather in which phase of tumor disease and after which treatment combinations. Since immune cells cannot cope with big tumor masses, additional therapies are needed to reduce the tumor volume or to render the cancer cells visible for immune attack. Certain chemotherapeutic agents (CT) and ionizing irradiation (X-ray) that is applied in radiotherapy (RT), mostly in combination with further immune stimulation, may render the tumor cells immunogenic. We assume that low and high doses of X-ray modulate the immune system and focus on abscopal anti-tumor immune responses that are induced by combinations of standard tumor with immune therapies.

IMMUNE MODULATION BY LOW AND INTERMEDIATE DOSE RADIATION

Exposure to radiation always has been a point of concern for many people, especially in times when nuclear power plant accidents occur. Radiation is often associated with being a threat to humans causing cancer and other diseases. Another rising source of radiation is medical applications. The latter increased the total effective collective dose of irradiation to humans by 70% over the last years

[United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) Report 2008]. Fortunately, the effects of radiation on the immune system being a first line defense system against malignancy have attracted the notice of researchers and clinicians.

LOW DOSE RADIOTHERAPY INDUCES ANTI-INFLAMMATION – THE ROLE OF MACROPHAGES

One should distinguish between high dose (single-dose >1.0 Gy), intermediate dose (single-dose >0.1 and ≤1.0 Gy), and low dose radiation (≤0.1 Gy; Salomaa et al., 2010). Low and intermediate dose radiation (low dose RT, LDR) is used to treat acute and chronic painful inflammatory diseases. LDR induces anti-inflammation by, e.g., hampering leukocyte adhesion to endothelial cells (ECs), induction of apoptosis, reducing the activity of the inducible nitric oxide synthase, and by lowering the oxidative burst in macrophages (summarized in Rodel et al., 2012).

Monocytes and macrophages are key players in initiation, maintenance, and resolution of inflammation (Fujihara et al., 2003; Hume, 2006; Valledor et al., 2010). They support the inflammatory host response by secreting pro-inflammatory cytokines such as tumor necrosis factor (TNF)α, interleukin (IL)-1β, and IL-6. The immune response is further amplified by release of reactive oxygen species (ROS) and nitric oxide (NO). On the other hand they may initiate the healing process and are involved in resolution of inflammation by phagocytosis of apoptotic cells and cell residues as well as by secreting anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF)β (Martin and Leibovich, 2005; Anders and Ryu, 2011). Inappropriate regulation of the resolution process can result in severe chronic inflammation and autoimmune diseases. Improvements of inflammatory diseases and pain after LDR have been observed in patients for over 100 years (summarized in Kern et al., 1999). This suggests that macrophage-mediated modulations of inflammation can be influenced by LDR.

Macrophages are considered to be radio-resistant while monocytes are more sensitive to radiation (Hildebrandt et al., 1998; Bauer et al., 2011). Monocytes are impaired in DNA double-strand break (DSB) repair; however even their apoptotic rate merely increased up to 10% following LDR (≤1.0 Gy). It therefore can be assumed that the anti-inflammatory effects of LDR are not caused by dying phagocytes themselves (Voll et al., 1997), but rather by regulatory mechanisms. Discontinuous dose dependence with local peaks within a dose range of 0.3–0.7 Gy has been observed in many assay systems where macrophages were exposed to LDR.

X-ray treatment with single-doses between 0.3 and 0.6 Gy reduces the production of ROS by activated macrophages. ROS enhances the destruction of pathogens, but could also lead to serious destructions of own tissue, if deregulated like in chronic inflammation and autoimmune diseases (Schaue et al., 2002). Further, a reduced activity of the inducible Nitric Oxide Synthase (iNOS) and a lowered concentration of its immune regulatory product NO take place in activated macrophages following LDR. The changes occurred on protein and not on mRNA level (Hildebrandt et al., 1998, 2003; Rodel et al., 2002). NO is a key mediator of cytotoxic and immune stimulating effects. It is produced and secreted by inflammatory macrophages. A significant inhibition of NO production in macrophages was observed after LDR, while X-rays with doses ≥5 Gy increased it (Hildebrandt et al., 1998, 2003). Since NO influences the expression of inflammatory cytokines (Abramson et al., 2001), it may serve as a link between LDR and inflammatory cytokine expression.

Decreased levels of the pro-inflammatory cytokine TNFα were measured when Lipopolysaccharide (LPS)-activated macrophages were irradiated with 0.5 or 0.7 Gy of X-rays (Tsukimoto et al., 2009; Rodel et al., 2012). An involvement of the ERK1/2 and p38-MAPK pathways in triggering such anti-inflammatory responses is likely. Both pathways are deactivated by dephosphorylation via the protein phosphatase MKP-1. Tsukimoto et al. (2009) reported that 0.5 Gy of γ-irradiation significantly increases the expression of MKP-1, inactivates p38-MAPK, and finally suppresses the TNFα production in mouse RAW264.7 macrophages. Actually many of such anti-inflammatory properties of LDR are regulated by nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) on a transcriptional level. A reduced translocation of NFκB into the nucleus has been observed in various inflammation models after exposure to LDR (summarized in Rodel et al., 2012).

The amount and nature of cytokines which are produced and released by macrophages following LDR also depend on the presence of dying cells in the microenvironment. Activated monocytes/macrophages secrete anti-inflammatory cytokines (IL-10, TGFβ), rather than pro-inflammatory ones (IL-1β, TNFα) in the presence of apoptotic cells (Voll et al., 1997). The apoptotic rate of peripheral blood mononuclear cells (PBMCs) increased following LDR with a maximum in the dose range of 0.3–0.7 Gy (Kern et al., 1999). The clearance of such apoptotic cells and/or cell residues is predominantly carried out by macrophages. More information on how LDR influences the phagocytosis of apoptotic and necrotic cells is urgently needed. First investigations with latex beads as prey revealed that low dose X-irradiation of LPS-activated macrophages reduces the phagocytosis. In contrast, higher single-doses (≥5 Gy) slightly increase the uptake of beads by activated macrophages (Conrad et al., 2009). The phagocytosis of colorectal tumor cells by macrophages and dendritic cells was shown to be reduced when the tumor cells (and not the macrophages) had been irradiated with higher doses of X-ray (2, 5, or 10 Gy). It should be stressed that the phagocytosis can be significantly enhanced when X-ray is combined with heat treatment (hyperthermia) of the tumor cells (Schildkopf et al., 2011).

Such impacts of LDR on macrophages, displaying repeatedly local peaks in a dose range of 0.3–0.7 Gy, could be a consequence of one central process affected by LDR. Also in other immune cells such as PBMCs, polymorphonuclear cells (PMNs), and ECs similar immune modulations induced by LDR have been observed, including a discontinuous dose-dependent translocation of NFκB into the nucleus (Prasad et al., 1994, 1995; Kern et al., 1999; Roedel et al., 2002;Rodel et al., 2004a,b, 2009; Gaipl et al., 2009). Since NFκB is a key transcription factor for a variety of immune factors such as cytokines, adhesion molecules, and growth factors and additionally is a potent post-transcriptional regulator of iNOS (Vodovotz and Bogdan, 1994), its modulation may therefore play a prominent role in the induction of an anti-inflammatory response

following LDR. We have recently reported that a reduced secretion of IL-1β by stimulated macrophages after exposure to LDR correlates with a reduced nuclear translocation of p65 (RelA) of the NFκB-complex (Lodermann et al., 2012). Nevertheless, LDR has been shown in experimental animal models to temporarily suppress immune functions by a variety of other mechanisms. Examples are the disturbance of cells of the cellular and humoral immunity or the reduction of the viability of mature blood cells by affecting the hematopoiesis (Yagunov et al., 1998; Serhatlioglu et al., 2004).

LDR STIMULATES IMMUNE FUNCTIONS

Other experiments link chronic and acute irradiation with low and intermediate doses with an enhanced immune function (Liu et al., 1987; James and Makinodan, 1988; Liu, 2007). Liu and colleagues showed that a variety of immune functions are stimulated by LDR such as natural killer (NK) cell and macrophage activity, or proliferation of T cells. Another feature of chronic exposure to LDR is the induction of an altered cytokine profile in the peripheral blood that can arise from activation of innate immune responses and not from changing the total number of white blood cells, red blood cells, and platelets (Shin et al., 2010).

The enhanced immune functions induced by LDR could explain why whole body LDR exposure of mice can reduce tumor outgrowth of B16 melanoma and Lewis lung cancer as well as metastasis formation after tumor cell inoculation (Hosoi and Sakamoto, 1993; Liu, 2003). Furthermore, the carcinogenic effect of high dose irradiation can be suppressed by a previous whole body irradiation with a dose of 0.075 Gy in C57BL/6 mice to a certain extent, mostly likely due to LDR-induced immune activation against tumor cells (Ina et al., 2005). These findings could shed some light on why people who are exposed to LDR by a higher background of earth radiation or through work situation display a decreased incidence for certain cancers or an elevated life span. This hypothesis is further supported by epidemiological studies, such as the British nuclear workers 51 year study (McGeoghegan and Binks, 2001; Atkinson et al., 2004) or the Hanford downwind inhabitants 50 years' survey (Boice et al., 2006).

The knowledge that LDR also activates immune functions is not only helpful for radiation safety questions and associated guidelines, but also for clinical applications where cancer patients are treated with high dose radiation therapy (RT). A hint that whole body irradiation with LDR could improve the effects of standard RT is provided by animal studies of Jin and colleagues. They compared a fractionated local RT of 6×5 Gy of Lewis lung cancer in C57BL/6 mice with a modified fractionated RT in which the second/fifth and third/sixth fraction of the locally applied irradiation with 5 Gy was substituted by a whole body irradiation with 0.075 Gy (Jin et al., 2007). Since the tumor outgrowth reflecting the therapeutic effect was comparable in both schemes, the total irradiation dose could be reduced by two-third when LDR was included. In another experiment, a radiation scheme of 6×2 Gy over 2 weeks was compared with a local 2 Gy irradiation and a double administrated whole body irradiation with 0.075 Gy, which was given twice in the same time frame $[2 \times (2\,\text{Gy} + 0.075\,\text{Gy} \times 2)]$. In this case, a significant slower tumor outgrowth in the whole body irradiated group of mice was observed, although the total dose was reduced to one-third (Jin et al., 2007).

IMMUNE ACTIVATION BY HIGH DOSE RADIATION

The immune stimulating potential of high dose radiation does initially not appear obvious since RT induces a time-restricted immune suppression by directly destroying immune cells (Anderson and Warner, 1976). However, in contrast to CT, the immune suppressive effects of RT are lower and more localized (Hodge et al., 2008). Nevertheless, CT and RT can both signal to the immune system tumor cells that had previously escaped immune surveillance (Ma et al., 2010). The phenotype of cancer cells has to be modified by therapeutic tools in a way that immune cells are attracted, induced to mature, and activated. For example, RT enhances the degradation of existing proteins inside the cells and also concomitantly the surface expression of MHC class I molecules (Reits et al., 2006). Complexes of MHC I molecules with peptides are recognized by CTLs that specifically kill tumor cells. RT further promotes the priming of antigen-specific DCs (Lee et al., 2009) and may increase the number of antigen presenting cells within tumor-draining lymph nodes (LN; Lugade et al., 2005) where antigen presentation by DCs and activation of CD8+ CTLs takes place.

IMMUNOGENIC TUMOR CELL DEATH

It was shown that higher radiation doses are associated with increased antigen expression (Santin et al., 1997) and induction of necrotic forms of tumor cell death (Mantel et al., 2010). Since necrotic cells have lost their membrane integrity, formerly hidden molecules such as DNA, chaperones, and proteins involved in stabilization of the DNA are released (Beyer et al., 2012). They operate as damage-associated molecular patterns (DAMPs) or alarmins and alert the immune system that "danger" has occurred (Matzinger, 1994). The immunogenicity of necrotic cells is strongly determined by the danger signals high-mobility group box 1 protein (HMGB1) and heat shock protein 70 (Hsp70). However, also CT- and/or RT-induced apoptotic tumor cells can be rendered immunogenic besides exerting their phosphatidylserine (PS)-dependent anti-inflammatory effects (Frey and Gaipl, 2011). The expression of the endoplasmic reticulum (ER)-derived protein calreticulin (CRT) on the tumor cell surface acts as recognition and uptake signal for DCs (Obeid et al., 2007). Further, Hsp70 is released within membranous structures after stressing the cells (Vega et al., 2008). The release of such microvesicles shows strong similarities to those of danger signals. Both events occur during cell death and may lead to stimulation of distinct Toll-like receptors (TLRs) on DCs (Pisetsky et al., 2011). RT-induced necrotic and apoptotic tumor cells may finally stimulate systemic innate (NK cell-mediated) and adaptive (DC and CTL-mediated) immunity against the tumor (**Figure 1**). Immunogenic tumor cell death induced by RT alone or in combination with further immune stimulation is one elicitor of abscopal anti-tumor responses (summarized in Frey et al., 2012).

ABSCOPAL ANTI-TUMOR EFFECTS

This conclusion is supported by clinical observations showing that RT achieves not only local tumor control by stopping the

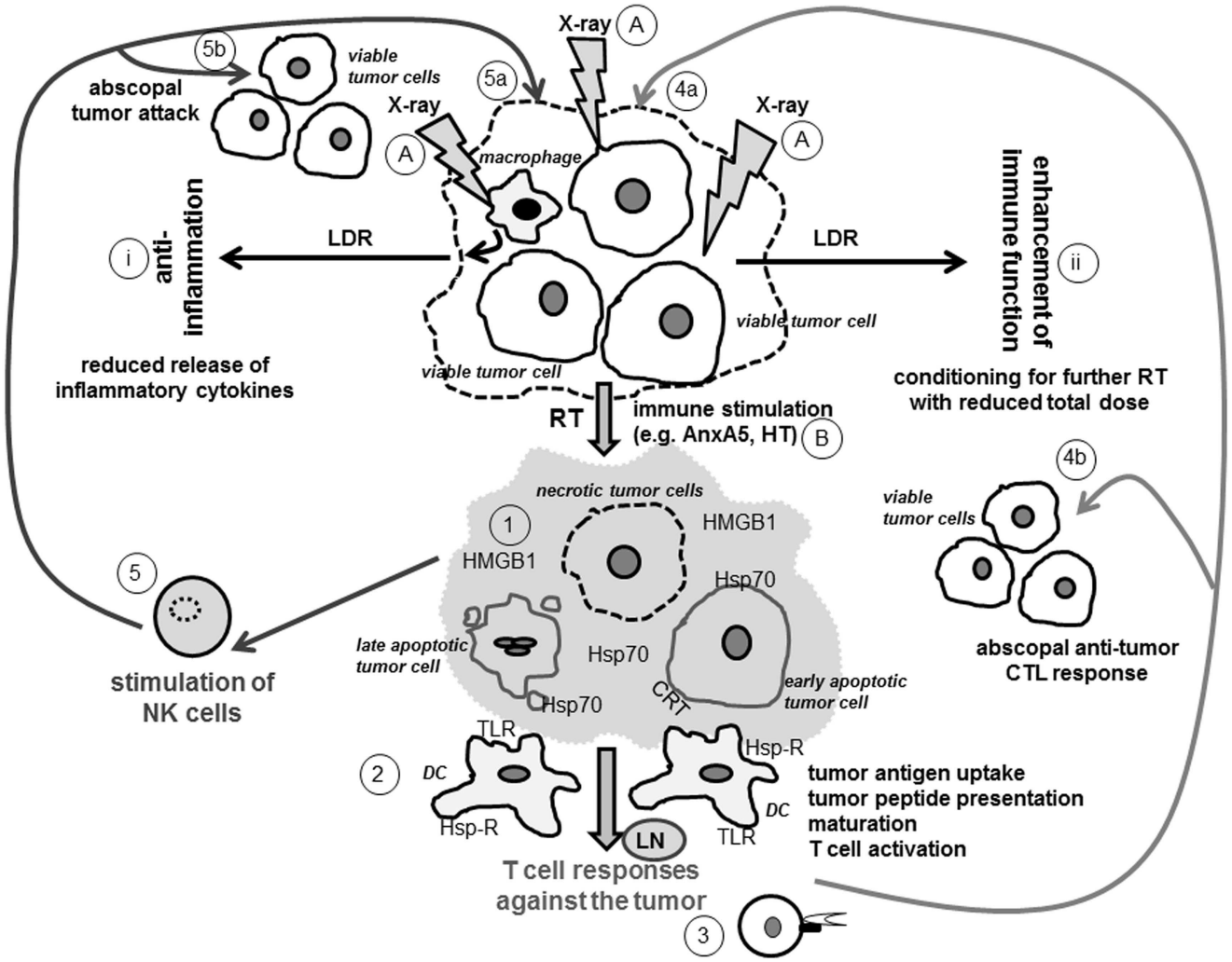

FIGURE 1 | Ionizing radiation modifies the tumor cell phenotype and induces a tumor microenvironment that fosters innate and adaptive immune responses against the tumor. Viable tumor cells or cells of the tumor microenvironment, as exemplarily displayed here for macrophages, modify their phenotype when exposed to X-rays (A). LDR leads to reduced secretion of inflammatory cytokines by macrophages and induces an anti-inflammatory environment (i). It additionally conditions the tumor for further treatment with higher doses of X-ray (ii). The latter are applied in RT as part of cancer treatment and induce distinct forms of tumor cell death (1). Early apoptotic tumor cells expose CRT, a recognition molecule for their phagocytosis by DCs. Late apoptotic cells release blebs that carry danger signals such as Hsp70 and thereby activate DCs. Necrotic tumor cells have lost their membrane integrity and therefore release immune activating danger signals (e.g., HMGB1 and Hsp70) that interact with receptors such as TLR and Hsp-R on DCs. Necrotic cells might also directly be induced by combination of stress stimuli such as RT and HT (B) or be secondary necrotic ones when the uptake of apoptotic tumor cells by macrophages is blocked with AnxA5 (B). The distinct forms of dying and dead tumor cells create an immune stimulatory tumor microenvironment (displayed in light gray) that fosters tumor antigen uptake and maturation of DCs (2). The latter process tumor antigens and (cross-) present tumor peptides via MHC class I and class II molecules to CD8+ and CD4+ T cells, respectively (3). CD8+ CTLs recognize tumor peptides presented in MHC class I molecules on tumor cells and specifically attack therapy-modified tumor cells (4a) as wells as non-treated ones (4b). The tumor microenvironment and the tumor cells resulting from RT and further immune stimulation may further directly activate NK cells (5) against the tumor (5a and b). Abbreviations: AnxA5, AnnexinA5; CRT, calreticulin; DC, dendritic cell; Hsp70, heat shock protein 70; Hsp-R, Hsp receptor; HMGB1, high-mobility group box 1 protein; HT, hyperthermia; LDR, low dose radiotherapy; LN, lymph node; NK cell, natural killer cell; RT, radiotherapy with high single-doses applied in tumor therapy; TLR, Toll-like receptor; X-ray, ionizing radiation.

proliferation of and destroying the tumor cells directly at the irradiated site, but additionally results in indirect anticancer effects in non-irradiated areas of the patients. Abscopal effects in the clinics have been observed for various cancer types including hepatocellular carcinoma (Ohba et al., 1998; Okuma et al., 2011), chronic lymphocytic leukemia (Sham, 1995), renal cell carcinoma (Wersall et al., 2006), malignant lymphomas (Nobler, 1969; Antoniades et al., 1977), and melanomas (Kingsley, 1975; Postow et al., 2012).

The phenomenon "abscopal effect" or "distant bystander effect" was originally described by Mole (1953) and the term comes from the latin "ab-" (position away from) and "scopus" (mark or target).

Mole defined it "at a distance from the irradiated volume, but within the same organism." In contrast to the radiation-induced bystander effect, which is mediated via cell-to-cell gap junctions (Azzam et al., 2001) or by secreted soluble factors (TGFβ, NO; Iyer et al., 2000) of irradiated cells that thereby communicate with non-irradiated neighboring (bystander) cells, the abscopal effect is an indirect and systemic effect in non-irradiated areas distant of the irradiated field. Taken together, ionizing radiation induces both local (targeted and bystander effects) and systemic effects (abscopal effects) in cancer patients. However, the cellular and molecular mechanisms of abscopal effects still remain to be clarified.

Various preclinical and clinical studies sustain the assumption that a spontaneous regression of tumors, metastases, or enlarged LN outside of the irradiated field is mediated by the immune system. Konoeda observed an abscopal effect in metastatic LN of breast carcinoma in 15 out of 42 patients. The effect was most frequently noticed when infiltrating CD4+ and CD8+ T cells were present around degenerated tumor cells of the irradiated primary tumor (Konoeda, 1990). Spontaneous regression of a relapsed nodular lesion in a patient with NK cell lymphoma without any treatment was documented by Isobe et al. (2009) after massive infiltration of CD8+ CTLs in the relapsed lesion. Interestingly, the patient was initially treated with radio- and chemotherapy against an eyelid tumor. The susceptibility of tumor cells to CTL-mediated lysis may result from the RT-induced altered tumor cell phenotype associated with increased expression of MHC class I molecules on the surface and an increased intracellular peptide pool (Reits et al., 2006). Demaria et al. (2004) have actually demonstrated in a mouse model of mammary carcinoma that the systemic anti-tumor effect is mediated by the immune system. T cells are required for distant tumor inhibition after combined therapy of the primary tumor with RT and the DC growth factor Fms-like tyrosine kinase receptor 3 ligand (Flt3-L). They concluded that RT alone is a poor inducer of abscopal effects, but that combinations with further immune stimulants are more effective. In their preclinical examinations, the Flt3-L increased the number of DCs at the tumor site. There, DCs assimilate tumor antigens for tumor peptide (cross)-presentation. Maturation signals for DCs are delivered in form of cytokines or other inflammatory stimuli released by the damaged cancer cells resulting after RT (Demaria et al., 2004). In summary, local RT damages tumor cells and generates large amounts of tumor antigens in apoptotic and necrotic tumor cells as well as cellular debris, which, either alone or together, provide immune stimulatory signals for DCs (**Figure 1**). The latter mature and migrate to draining LN, where they present tumor peptide antigens to naïve T cells. RT further increases the number of interferon (INF)-γ producing T cells in draining LN (Lugade et al., 2005). Despite this, Kim and co-workers demonstrated, in accord with Demaria et al. that conventional RT alone is not sufficient to eliminate tumor masses distant from the irradiated site. The reason could be an inadequate antigen presentation in LN which they overcome by injection of DCs into the irradiated tumor tissue (Kim et al., 2004). Anti-tumor immunity can further be potentiated with an additional administration of the DC danger/maturation signals LPS or TNFα.

The studies outlined above indicate that a proper DC maturation and activation is essential for the induction of an effective anti-tumor T cell response. Apetoh and colleagues identified the TLR4 as one crucial receptor on DCs stimulating the cross-priming of CD8+ CTLs. In addition to LPS, TLR4 also recognizes HMGB1, a nuclear protein passively released as danger signal by late apoptotic or necrotic cells (Apetoh et al., 2007). Using a similar experimental design as Kim et al. (2004), Akutsu et al. (2007) identified the heat shock protein gp96 as a target molecule involved in the abscopal effect. Radiation-induced gp96 is capable to activate DCs via TLR2 and TLR4 (Vabulas et al., 2002). Based on the studies of Lee et al. (2009), Shiraishi et al. (2008), and Dewan et al. (2009), Takeshima et al. showed that CD8+ T cells play a major role in growth inhibition of non-irradiated tumors in combining Th1 cell therapy with local RT. This combination of RT with immune therapy did not only induce the generation of tumor-specific CTLs at the primary tumor site and complete eradication of the tumor, but also prevented the outgrowth of distal tumors (Takeshima et al., 2010).

Likewise combinations of RT with cytokine therapy have the potential to control metastases. The local and systemic effect of ECI301, a human macrophage inflammatory protein-1 alpha variant, in combination with RT was investigated by Shiraishi and colleagues. Their results indicate that the combined therapy reduces the primary tumor growth at the irradiated site and that of distal, non-irradiated tumors. This abscopal effect was dependent of CD8+ lymphocytes, CD4+ lymphocytes, and NK1.1 cells, but independent of the tumor-type and genetic background (Shiraishi et al., 2008). An additional administration of Interleukin-2 (IL-2) to RT also results in a better local tumor control and regression of the not irradiated tumor within the same mouse (Everse et al., 1997; Jurgenliemk-Schulz et al., 1997). Others demonstrated an abscopal effect after manipulation of tumor cells with transgenes expressing several cytokines such as IL-2 (Kwong et al., 1997) or Flt3-L (Dong et al., 2003).

Combinations of RT with antibodies blocking inhibitory negative regulatory molecules on T cells, such as the monoclonal antibody ipilimumab against CTLA-4, are promising to induce systemic anti-tumor immune responses (Dewan et al., 2009). Ipilimumab was approved by the FDA (U.S. Food and Drug Administration) in 2011. In two randomized phase 3 trials, an overall survival benefit in patients with metastatic melanoma was observed (Hodi et al., 2010; Robert et al., 2011). However, autoimmune reactions have to be kept into mind as possible severe side effect of such combined therapies (Mellman et al., 2011). **Table 1** summarizes the literature about abscopal effects observed in preclinical and clinical studies after RT and/or immune therapy. It has to be stressed that until today only one major hint for a molecular mechanism for RT-induced abscopal effects has been suggested. Camphausen and co-workers showed that p53 and downstream signals are key mediators of this process. In contrast to the studies mentioned above, a dose-dependent abscopal effect induced by RT alone was observed in wild-type but not in p53 knockout mice (Camphausen et al., 2003).

In conclusion, cancer treatments which activate enough DCs and eventually the adaptive immune system and further directly cells of the innate immune system (see below) are promising approaches to improve the eradication of primary tumors and metastases (**Figure 1**). An optimized radiation regimen combined

Table 1 | Abscopal anti-tumor effects observed in preclinical and clinical studies after RT and/or immune therapy.

Tumor-type	Treatment	Abscopal effect	Mediator of abscopal effect	Reference
CLINICAL REPORTS				
Hepatocellular carcinoma	RT of thoracic vertebral bone metastases, *dose:* 36 Gy	Regression of primary tumor	TNF-alpha	Ohba et al. (1998)
Hepatocellular carcinoma	RT of mediastinum, *dose:* 27 × 2.25 Gy	Regression of lung metastases		Okuma et al. (2011)
Renal cell carcinoma	RT of primary tumor, *dose:* 12 × 8Gy	Regression of enlarged lymph nodes and lung lesions		Wersall et al. (2006)
Mammary carcinoma	RT of primary tumor	Regression of metastatic lymph nodes	CD8+ and CD4+ T cells	Konoeda (1990)
NK-ENKL	RT of eyelid tumor	Regression of NK cell lymphoma	CD8+ T cells	Isobe et al. (2009)
MOUSE MODELS				
Mammary carcinoma (67NR)	RT of primary tumor, *dose:* 2, 6 Gy, i.p. administration of Flt3-L (10×)	Growth delay of non-irradiated 67NR tumors, tumor-type dependent	DCs, T cells, RT + Flt3-L	Demaria et al. (2004)
Squamous cell carcinoma (SCCVII)	RT of primary tumor, *dose:* 3 × 4 Gy, i.t. administration of DCs	Growth inhibition of non-treated tumor	DC, gp96, RT+ i.t. DCs	Akutsu et al. (2007)
Mammary carcinoma (4T1)	RT of primary tumor, *dose:* 1 × 20 Gy	Elimination of lung metastases	CD8+ T cells	Lee et al. (2009)
Adeno-carcinoma (Colon26)	RT of primary tumor, *dose:* 6Gy, i.v. administration of ECI301	Growth inhibition of non-irradiated tumor, tumor-type independent	CD8+ and CD4+ T cells, NK1.1 cells, IFN-γ, RT + ECI301	Shiraishi et al. (2008)
Mammary carcinoma (TSA), mouse colon carcinoma (MCA38)	RT of primary tumor, *dose:* 20 Gy, 3 × 8 Gy, 5 × 6 Gy, administration of anti-CTLA-4 mAb	Growth inhibition of non-irradiated tumor	CD8+ and CD4+ T cells, IFN-γ, Fractionated RT + anti-CTLA-4 mAb	Dewan et al. (2009)
Lymphoma (EG7)	RT and Th1 cell therapy	Growth inhibition of non-irradiated tumor	CD8+ T cells	Takeshima et al. (2010)
Lymphoma (SL2), mammary carcinoma (M8013)	RT of primary tumor, *dose:* 20 Gy, 10 × 2.5 Gy, peritumoral administration of rIL-2	Regression of non-irradiated tumor	Radio-immunotherapy	Jurgenliemk-Schulz et al. (1997)
Lymphoma (SL2)	RT of primary tumor, *dose:* 20 Gy, 10 × 2.5 Gy, peritumoral administration of rIL-2	Regression of non-irradiated tumor	Radio-immunotherapy	Everse et al. (1997)
Lewis lung carcinoma (LL2)	LL2 transfected with viral vector expressing IL-2	Regression of hepatic lung cancer metastases		Kwong et al. (1997)
Squamous cell carcinoma (B4B8)	B4B8 transfected with plasmid encoding Flt3-L	Growth inhibition of non-treated tumor		Dong et al. (2003)
Lewis lung carcinoma (LLC), fibrosarcoma (T241)	RT of normal tissue, *dose:* 5 × 10 Gy, 12 × 2 Gy	Growth inhibition of non-irradiated tumor, tumor-type independent	p53	Camphausen et al. (2003)

with immune therapy makes indeed anticancer therapies more efficient.

ANTI-TUMOR IMMUNITY INDUCED BY COMBINATION OF RT WITH FURTHER IMMUNE STIMULATION BY ANNEXINA5 OR HEAT

Additional approaches to induce a CTL-mediated tumor cell killing are based on the *in vivo* activation of DCs, which should take up tumor antigens and consecutively present tumor peptides to T cells to achieve co-stimulation. However, macrophages recognize and phagocytose dying tumor cells swiftly and silently and thereby remove tumor antigens (Gaipl et al., 2007). They are recruited by find-me signals such as lysophosphatidylcholine (Lauber et al., 2003) secreted by RT-induced apoptotic cells. The latter may even cause caspase 3-dependent tumor cell repopulation by generating potent growth-stimulating signals (Huang et al., 2011). Moreover, an anti-inflammatory milieu results from the clearance of those apoptotic cells by macrophages (Lauber et al., 2011). Since DCs and macrophages partly utilize different clearance mechanisms (Hoves et al., 2011), one possibility to enable enhanced access of DCs to RT-induced apoptotic and necrotic tumor cells is to block their clearance by macrophages with the PS-binding protein AnnexinA5 (AnxA5; Bondanza et al., 2004; Frey et al., 2009). The growth of syngeneic tumors is significantly retarded by a single injection of AnxA5 around the tumor. Combination of RT with AnxA5 resulted in the most effective inhibition of tumor growth (Frey et al., 2009). *In vivo* experiments with immune competent mice bearing syngeneic tumors have proven that AnxA5 increases the immunogenicity of tumor cells. The injection of irradiated tumor cells pre-incubated with AnxA5 cured established tumors in about 50% of the animals, while the injection of irradiated tumor cells only resulted in less than 10% of tumor free mice (Bondanza et al., 2004). Since RT induces tumor cell death and thereby the exposure of PS on dying tumor cells and on tumor blood vessels, it represents an adequate combination partner with PS-targeting agents such as AnxA5, and monoclonal antibodies such as the murine 2aG4 antibody (He et al., 2007). Phase I and II clinical trials with bavituximab, the human analog to 2aG4, in combination with standard therapies for the treatment of solid tumors are currently performed (Derose et al., 2011). In preclinical rat models, combination of PS-targeting with RT resulted in long-term anti-tumor immunity even against glioblastoma in over 10% of the animals (He et al., 2009). Recently Riedl et al. (2011) showed that PS is also exposed by non-dying tumor cells, preferentially in metastases. Targeting of PS on therapy-induced dying and on viable metastatic cells could therefore both lead to efficient anti-tumor immune responses by promoting uptake of the tumor cells by DCs, to mention here one of multiple possible modes of action resulting from the shielding of PS (summarized in Frey et al., 2012).

Cross-presentation of tumor peptides by DCs requires antigen uptake and additionally a maturation signal for DCs to avoid tolerance induction. The maturation of immune stimulatory DCs is stimulated by necrotic tumor cells (Sauter et al., 2000). Extracellular heat shock proteins act as immune activating danger signals and fulfill both functions: they are means of transport for tumor antigens and elicitors of DC maturation (Basu et al., 2000; Somersan et al., 2001). Appropriately, DCs pulsed with tumor cells that have been heat-shocked mediated a significant enhanced cellular T cell cytotoxicity response against the tumor cells compared to pulsed DCs with lysates of non-heat-shocked cells (Schueller et al., 2003). In addition, combination of RT with HT increased the amount of released danger signals such as HMGB1 and Hsp70 and further fosters the maturation of DCs (Schildkopf et al., 2009a,b, 2011; **Figure 1**). Future research should focus on preclinical *in vivo* models to examine which immune cell subsets get recruited into the tumor after local treatment with RT plus HT and under which treatment combinations the maximum DC-mediated and MHC-dependent CTL activation takes place. Chen et al. (2009) have already demonstrated in mouse models that heat-stressed tumor cells are capable of initiating anti-tumor immune responses by inducing activation of DCs. Immunological back-up or parallel mechanisms for tumor cell killing should be considered, since tumor cells often shed MHC I molecules. The exposure of Hsp70 on the tumor cell surfaces serves as a recognition signal for activated NK cells (Stangl et al., 2008). NK cells are activated against the tumor when tumor cells have shed MHC class I molecules to escape killing by CTLs, since the inhibitory receptors of NK cells are no longer triggered by MHC I molecules. Following HT treatment, NK cells have been found to be enriched at the tumor site (Burd et al., 1998), showing that innate immune responses also contribute to the fight against the tumor.

OUTLOOK

We have outlined that immunogenic tumor cell death forms are induced by RT with additional immune stimulation. **Figure 1** schematically depicts how ionizing radiation (X-ray) could stimulate innate and adaptive immune responses against the irradiated tumor as well as against non-irradiated ones (abscopal effects). The current knowledge suggests that induction of tumor cell necrosis including necroptosis (Vanlangenakker et al., 2012) and apoptosis by RT and further immune stimulators is most beneficial for the induction of a specific and long-lasting anti-tumor immunity (Kepp et al., 2009). Since the phenotype of the individual tumor of a distinct patient is modified by RT, the best possible personalized treatment approach is realized. Although tumor regression is often the main indicator for a successful therapy, this may not always translate into improved survival rates. Since the immune system needs time to act, the success of immune therapies is often observed at later time points after the treatment and connected to long-term survival rates, as shown in clinical trials with the CTLA-4-blocking antibody ipilimumab (Mellman et al., 2011). Since *in vivo* assays revealed that DCs require approximately 48 h for migration into the tumor, tumor antigen uptake, maturation, and consecutive migration to the sentinel lymph node (Wheeler et al., 2004), innovative irradiation schemes could be that hypofractionated ones expand the days where no irradiation takes place. This could avoid that activated DCs in the tumor microenvironment are killed by RT. Further studies are needed to document which combinations of RT and immune therapies and which time windows of combination are most effective to induce specific and long-lasting anti-tumor immune responses.

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Influence of tumors on protective anti-tumor immunity and the effects of irradiation

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Innate and adaptive immunity plays important roles in the development and progression of cancer and it is becoming apparent that tumors can influence the induction of potentially protective responses in a number of ways. The prevalence of immunoregulatory T cell populations in the circulation and tumors of patients with cancer is increased and the presence of these cells appears to present a major barrier to the induction of tumor immunity. One aspect of tumor-mediated immunoregulation which has received comparatively little attention is that which is directed toward natural killer (NK) cells, although evidence that the phenotype and function of NK cell populations are modified in patients with cancer is accumulating. Although the precise mechanisms underlying these localized and systemic immunoregulatory effects remain unclear, tumor-derived factors appear, in part at least, to be involved. The effects could be manifested by an altered function and/or via an influence on the migratory properties of individual cell subsets. A better insight into endogenous immunoregulatory mechanisms and the capacity of tumors to modify the phenotype and function of innate and adaptive immune cells might assist the development of new immunotherapeutic approaches and improve the management of patients with cancer. This article reviews current knowledge relating to the influence of tumors on protective anti-tumor immunity and considers the potential influence that radiation-induced effects might have on the prevalence, phenotype, and function of innate and adaptive immune cells in patients with cancer.

Keywords: tumor immunity, T cells, NK cells, tumor microenvironment, immunoregulation

INTRODUCTION

For many years, the paradigm on which the majority of immunotherapeutic approaches for the treatment of cancer has been based, is that adaptive immune responses to tumors are similar to those that are induced in the generation of immunity to infectious pathogens. Although this is, in part at least, the case, a fundamental difference is that responses induced by infectious pathogens are driven by exogenous ("foreign") proteins/molecules, whereas those to tumors must be induced by endogenous ("self") proteins. Although not all tumor antigens are "self" antigens (e.g., EBV antigens, mutated p53), the majority are. For effective immunity, it is therefore necessary to overcome the well-developed capacity of the immune system to regulate responses to self-antigens and tissues.

Tumors can produce immunosuppressive factors such as IL-10, TGF-β, and vascular endothelial growth factor (VEGF). Tumor-derived cytokines such as IL-10 and TGF-β might protect against the development of anti-tumor immunity by influencing the functional capacity of antigen presenting cells (APCs) such as dendritic cells (DCs), and by promoting the generation/differentiation/expansion of immunoregulatory T cell populations which have the capacity to control and prevent immune responses (Ghiringhelli et al., 2005b; Liu et al., 2007; Mahnke et al., 2007a; Biollaz et al., 2009; Conroy et al., 2012; Multhoff and Radons, 2012). The biology of different immunoregulatory T cell populations and the functional significance of tumor-associated immunoregulatory T cells have recently been reviewed (Sakaguchi, 2011; Shevach, 2011; Facciabene et al., 2012; Savage et al., 2013). The net result is a complex relationship between tumors and elements of the protective immune system which has profound influences on the progression and treatment of cancer. It is on the influence of tumors on innate and adaptive immunity by tumors that this article focusses.

IMMUNOREGULATORY T CELLS

It is now known that thymic deletion of potentially self-reactive T cells cannot explain the lack of immune responsiveness to self-tissues and antigens, as the normal T cell repertoire includes low affinity T cells that are reactive against a number of self-peptides. Mechanisms that are capable of controlling the development of immune responses to self-antigens in the periphery must therefore be present. Over the last few years an anti-inflammatory activity has been shown to segregate, in part at least, into a naturally occurring $CD4^+$ T cell subset which constitutively expresses

the α chain of the IL-2 receptor (CD25) (Shevach, 2002; Gavin and Rudensky, 2003; Wood and Sakaguchi, 2003; Lee et al., 2004; Waldmann et al., 2004).

In addition to CD25, these cells also express other antigens including the intracellular transcription factor forkhead box p3 (Foxp3) (Sakaguchi, 2004), glucocorticoid-induced TNF receptor family-related gene (GITR), the immunoregulatory antigen CTLA-4, neuropilin-1 (Bruder et al., 2004) and, in the case of humans, low cell surface levels of CD127 (Liu et al., 2006b; Seddiki et al., 2006). The depletion or absence of these cells triggers autoimmune destruction of a variety of tissues (Asano et al., 1996; Suri-Payer et al., 1998; McHugh and Shevach, 2002). Although a multitude of immunoregulatory T and B cell populations have, and are, being discovered and reported upon, $CD4^+CD25^{high}$ Treg cells are regarded as being intimately involved in the governance of peripheral self-tolerance (Shevach, 2002, 2011; Nelson, 2004; Sakaguchi, 2004, 2011; Facciabene et al., 2012; Savage et al., 2013).

$CD4^+CD25^{high}$ Treg cells develop in the thymus and represent 5–10% of the peripheral $CD4^+$ T cell compartment. Although the expression of Foxp3 is currently accepted as being the most effective marker of Treg cells in mice and humans (Graca, 2005), there is evidence that some Foxp3 negative cells can be suppressive, and Foxp3 is not therefore a definitive marker for Treg cells (Gavin et al., 2007; Curiel, 2007a; Wan and Flavell, 2007). The suppressive effects of naturally-occurring Treg cells are mediated via relatively far-reaching soluble factors and more intimate cell–cell contact, as well as direct cytotoxic effects on effector cell populations (Schmetterer et al., 2012). These cells are also key regulators of anti-tumor immunity (Facciabene et al., 2012; Lindau et al., 2013; Savage et al., 2013).

According to Savage et al. (2013), the biology of tumor-associated Treg cells involves two developmental pathways: (1) the recognition of self-antigen by developing thymocytes within the thymus leads to the development of naturally-occurring $Foxp3^+$ Treg (nTreg) cells and (2), naïve $CD4^+$ T cells recognize a tumor-associated or tumor-specific antigen at extrathymic sites and, after being activated, develop into an inducible $Foxp3^+$ Treg cell (iTreg or Tr1) under a variety of conditions that facilitate/enable tumor immune evasion. These conditions include not only antigen presentation under sub-immunogenic or non-inflammatory conditions, but also chronic inflammation and infections. The observed selective accumulation of Treg cells in the tumor microenvironment suggests that this process can also be driven by tumors.

As hypothesized by Adema and colleagues (Jacobs et al., 2012), four non-mutually exclusive mechanisms can account for the accumulation of Treg cells in the tumor microenvironment: (1) Chemokine secretion induces the selective migration and retention of Treg cells that constitutively express high levels of CCR4 (CCL22, CCL2). (2) Secretion of anti-inflammatory mediators such as TGF-β and indoleamine 2,3-dioxygenase (IDO) converts conventional (naïve) T cells to Treg cells, either directly or via the actions of antigen-presenting cells; (3) A selective survival advantage of Treg cells over other tumor-infiltrating lymphocytes occurs when negative costimulatory signals selectively influence effector T cells (PD-L1, FasL). Treg cells also induce receptor-mediated or cytotoxin-mediated Teff cell depletion; (4) tumor-derived immunosuppressive factors such as IL-10 and TGF-β promote the expansion of nTreg cells and the *de novo* generation of iTreg cells.

The different origins of iTreg cells (non-inflammatory, inflammatory) results in distinct properties of these cells which include differential stabilities (Bilate and Lafaille, 2012). iTreg cells are also generated during homeostasis of the gut and in cancer, although some cancers favor the expansion of nTreg cells. Both pathways converge in the tumor environment and this leads to context-dependent Treg cell functions such as the promotion of metastasis and angiogenesis, as well as the limitation of inflammation and blockage of anti-tumor immunity in response to inflammatory conditions (tissue/organ-specific) and the tumor microenvironment, respectively. The suppressive effect of nTreg cells is mediated via cell contact-dependent mechanisms such as granzyme B/perforin and Fas/FasL (Jonuleit et al., 2001). In contrast, iTreg cells mediate suppression in a cell contact-independent manner (Roncarolo et al., 2006; Bergmann et al., 2008; Mandapathil et al., 2010).

IMMUNOREGULATORY T CELLS AND ANTI-TUMOR IMMUNITY

As stated above, a wealth of historical and more recent evidence now suggests that $CD4^+CD25^{high}$ Treg cell populations influence the presence, induction, and maintenance of protective anti-tumor immunity (Raimondi et al., 2007; Facciabene et al., 2012; Lindau et al., 2013; Savage et al., 2013), and their association with the progression of malignant disease has been highlighted by a number of observations (**Table 1**).

These cells present a significant barrier to the induction of tumor immunity (Raimondi et al., 2007; Facciabene et al., 2012; Lindau et al., 2013; Savage et al., 2013), and reducing their numbers and/or function is therefore likely to be of therapeutic potential. The evidence that the depletion of $CD4^+CD25^{high}$ Treg cells enhances the capacity to induce cellular and humoral immunity to Her-2 which is expressed on primary and metastatic breast cancer cells (Fulton et al., 2006) confirms the importance of these cells and highlights the importance of improving our understanding of the influence of the breast tumor microenvironment on protective innate and adaptive anti-tumor immunity.

It is also important to appreciate that other immunoregulatory T cells such as the adaptive or inducible populations (iTreg) are phenotypically and functionally different to the population discussed above and resistant to apoptosis or oncological therapies (Whiteside, 2012). The potent capacity of these cells to suppress effector T cell function involves immunosuppressive cytokines such as TGF-β, IL-10, prostaglandin E_2, and adenosine that can be produced by solid tumors and/or Treg cells themselves (Erdman et al., 2003; Roncarolo et al., 2006; Bergmann et al., 2008; Mandapathil et al., 2010; Conroy et al., 2012). As mentioned before, differentiation of naïve $CD4^+$ T cells into iTreg cells in the periphery is encouraged by tumor antigen in the presence of certain cytokines such as IL-2, IL-10, and TGF-β (Levings et al., 2001b; Bergmann et al., 2007, 2008).

Table 1 | Influence of $CD4^+CD25^{high}T_{reg}$ cells on anti-tumor immunity.

Observation	References
$CD4^+CD25^{high}$ Treg cells are potent inhibitors of anti-tumor immune responses and the depletion of Treg cells promotes the rejection of several transplantable murine tumor cell lines including melanoma, fibrosarcoma, leukaemia, and myeloma	Sakaguchi et al., 1995; Onizuka et al., 1999; Shimizu et al., 1999; Steitz et al., 2001; Jones et al., 2002
$CD4^+CD25^{high}$ Treg cells impair responses to tumor-associated antigens that are expressed as self-antigens	Sutmuller et al., 2001; Golgher et al., 2002
Increased numbers of functionally suppressive $CD4^+CD25^{high}$ Treg cells are present in the peripheral blood of patients with breast cancer, and also in the tumor microenvironment	Liyanage et al., 2002; Wolf et al., 2003
$CD4^+CD25^{high}$ T cells from patients with epithelial malignancies are anergic to T cell receptor stimulation and suppress the proliferation of $CD4^+CD25^-$ T cells	Wolf et al., 2003
Using an experimental murine system and CT26 tumor cells, depletion of $CD25^{high}$ Treg cells has been shown to allow the host to induce both $CD4^+$ and $CD8^+$ anti-tumor responses following tumor challenge. The capacity of the host to mount this anti-tumor response is lost once the number of $CD25^{high}$ Treg cells is restored over time	Casares et al., 2003
The depletion of $CD25^{high}$ Treg cells before immunization with AH1 (a cytotoxic T cell determinant from CT26 tumor cells) permits the induction of a long-lasting anti-tumor immune response which is not observed if immunization is conducted in the presence of $CD25^{high}$ Treg cells	Casares et al., 2003
$CD4^+CD25^{high}$ Treg cells alone can prevent effective adoptive immunotherapy	Antony et al., 2005
$CD4^+CD25^{high}$ Treg cells can impair $CD8^+$ T cell immunity against tumor/self-antigens	Antony et al., 2005
Depletion of $CD4^+CD25^{high}$ Treg cells promotes a tumor-specific immune response in mice bearing pancreatic cancers	Viehl et al., 2006
The depletion of $CD4^+CD25^+Foxp3^{high}$ Treg cells increases the efficacy of vaccination approaches that are aimed at increasing cellular and humoral immunity to Her-2 which is expressed on primary and metastasized breast cancer cells	Fulton et al., 2006
The proportion of $CD4^+CD25^{high}T_{reg}$ cells is elevated in the peripheral blood of patients with hepatocellular carcinoma (HCC), and their levels positively correlate with tumor burden	Cao et al., 2007
Depletion of $CD25^+$ cells results in an accumulation of $CD4^+$ and $CD8^+$ T cells and NK cells producing IFN-γ in mesothelioma tumor tissue	Rudge et al., 2007
In a syngeneic murine glioma model, combining Treg cell depletion with administration of blocking CTLA-4 mAbs further boosted glioma-specific $CD4^+$ and $CD8^+$ effector T cells resulting in complete tumor eradication without any signs of autoimmunity. These data illustrate that intratumoral accumulation and activation of $CD4^+FoxP3^+$ Treg cells act as a dominant immune escape mechanism for gliomas	Grauer et al., 2007
The frequency of $CD4^+CD25^{high}$ Treg increases during disease progression and also following cancer therapy in HNSCC patients with no evident disease compared to untreated patients with active disease	Strauss et al., 2007
$CD4^+CD25^{high}T_{reg}$ secrete IL-10 and TGF-β and mediate immunosuppression in the tumor environment in a cell contact-independent manner	Strauss et al., 2007
Low doses of IL-2 in combination with DC vaccination are able to expand $CD4^+CD25^+Foxp3^+$ Treg cells in metastatic renal cell carcinoma patients suggesting that a combination of DCS-mediated immunotherapy and Treg depletion may be a promising approach in enhancing the ability of vaccination therapy to elicit effective anti-tumor responses in cancer patients	Berntsen et al., 2010
$FOXP3^+$ Treg cells predict poor survival in patients with cyclooxygenase-2–positive uveal melanoma	Mougiakakos et al., 2010
AML and high-risk MDS patients have significantly larger $CD4^+CD25^{high}$/CD4 and $CD4^+CD25^{high}FoxP3^+$/CD4 populations in the periphery compared to patients with autoimmune hematologic diseases and controls, respectively	Moon et al., 2011

(Continued)

Table 1 | Continued

Observation	References
Chemotherapy significantly decreased $CD4^+CD25^{high}$ Treg cell numbers and FOXP3 mRNA expression in advanced esophageal cancer patients	Xu et al., 2011
The frequency of $CD4^+CD25^{high}$ Treg cells is elevated in HNSCC patients and may be modulated by radiochemotherapy	Schuler et al., 2011
Neoadjuvant sorafenib treatment significantly reduced the percentage of tumor-infiltrating Treg cells in renal cell carcinoma patients	Desar et al., 2011

INFLUENCE OF TUMORS AND TUMOR-RELATED FACTORS ON IMMUNOREGULATORY T CELL POPULATIONS

There is now a wealth of evidence indicating that factors present in the tumor microenvironment can foster immune tolerance by generating and inducing the functional capacity of $CD4^+CD25^{high}$ Treg cell populations (Zou, 2005) and the induction of antigen-specific regulatory T cells from naïve cells (Zhou and Levitsky, 2007). However, the mechanism(s) underlying the recruitment, expansion and/or activation of these cells remain unclear.

The preferential accumulation of functional regulatory T cell populations in tumors could result from an increased recruitment, decreased emigration, and/or the local conversion of naïve T cells to regulatory populations by tumor-derived factors. The conversion of naïve T cells into inducible immunoregulatory T cells involves TGF-β and other factors, and has been reviewed elsewhere (Dons et al., 2012). Should such factors be involved, then their presence might be patient/tumor and/or treatment-specific. Chemokines play a role in migratory events, as tumor cells and microenvironmental macrophages produce the chemokine CCL22, which mediates trafficking of Treg cells to the tumor (Curiel et al., 2004). More recently, it has been shown that tumor hypoxia promotes the recruitment of regulatory T cells to tumors via the induction of the chemokine CCL28 (Facciabene et al., 2011).

A number of tumor-related events could be influential for the induction of regulatory T cell populations. Regulatory T cells can be induced by antigenic stimulation both *in vitro* and *in vivo* [induced regulatory T cells (Bluestone and Abbas, 2003; Vigouroux et al., 2004)], and these can mediate tumor-specific T cell tolerance (Zhou and Levitsky, 2007). Tumors might therefore release antigens and/or other non-antigen-specific factors that activate Treg cells, thereby mediating tumor-related immunoregulation (Antony et al., 2005). It is also possible that factors expressed on, or released from tumors, might promote the development and expansion of $CD4^+CD25^{high}$ Treg cells.

In this regard, it is known that the prevalence of $CD4^+CD25^{high}$ T cells in tumor draining lymph nodes and the spleens of mice bearing the pancreatic adenocarcinoma Pan02, increases with tumor growth (Liyanage et al., 2006). Furthermore, tumor-related factors activate $CD4^+CD25^{high}$ Treg cells (Li et al., 2005), expand $CD4^+CD25^{high}$ Treg cells and enhance their suppressive capacity (Cao et al., 2007). Gastric cancer cells induce human $CD4^+Foxp3^+$ regulatory T cells via the production of TGF-β (Yuan et al., 2011). It has also been shown that tumor-related factors activate $CD4^+CD25^{high}$ Treg cells (as indicated by increasing their expression of CD69) (Li et al., 2005), expand $CD4^+CD25^{high}$ Treg cells and enhance their suppressive capacity (Cao et al., 2007).

It is also possible that the mode of tumor cell death, whether this is induced by normal cell turnover or by therapeutic intervention can influence the qualitative nature and effectiveness of the immune response induced. Cellular necrosis is an inflammatory stimulus, whereas apoptosis can have anti-inflammatory consequences, at least some of which appear to be mediated via the induction of immunoregulatory T cell populations (Groux et al., 1997; Steinbrink et al., 1997, 1999; Lee et al., 1998; Levings et al., 2001a; Yamagiwa et al., 2001).

EFFECT OF DECREASING REGULATORY T CELLS ON ANTI-TUMOR IMMUNITY

Modifying the numbers and function of immunoregulatory T cell populations could be of significant therapeutic benefit to patients with cancer (Ménétrier-Caux et al., 2012). One approach which has been considered is the use of DAB(389)IL-2 (also known as denileukin diftitox and ONTAK). This is a recombinant IL-2/diphtheria toxin fusion protein which delivers diphtheria toxin to $CD25^+$ cells and thereby abrogates the immunoregulatory influence of $CD4^+CD25^+$ Treg cells (Dannull et al., 2005). Following internalization, protein translation is inhibited and targeted cells undergo apoptosis (Foss, 2000). The administration of ONTAK has been shown to reduce the number of circulating Treg cells and to enhance the magnitude of vaccine-induced, tumor-specific immune responses in patients with renal cell carcinoma (Dannull et al., 2005). It also improves immunity in patients with melanoma (Chesney et al., 2006; Mahnke et al., 2007b). ONTAK has also been shown to decrease the number of circulating $CD4^+CD25^+$ Treg cells and the suppression mediated by these cells in patients with ovarian, lung, breast, and pancreatic cancer (Curiel, 2007b). It also improves immunity and induces tumor regression in a murine model of breast cancer (Knutson et al., 2006).

Daclizumab (Zenapex®) and basiliximab (Simulect®) are anti-human CD25 monoclonal antibodies (mAbs) which have been approved for use in autoimmune disease, transplantation, and cancer, including HTLV-1-induced adult T cell lymphoma/leukemia (reviewed in Ménétrier-Caux et al., 2012). Daclizumab treatment durably reduces circulating

$CD25^{high}FOXP3^{+}$ Treg cell numbers and promotes the emergence of cancer-specific cytotoxic T cells after vaccination with cancer antigen peptides (hTERT/survivin) in patients with metastatic breast carcinoma (Rech and Vonderheide, 2009). However, one issue which has to be considered using such approaches is the potential to influence the activated effector cells which also express CD25.

It is also possible to block the function of Treg cells via a number of cell surface receptors (reviewed in Ménétrier-Caux et al., 2012). These approaches include the use of the anti-CTLA-4 antagonist mAb, two humanized forms of which are available—BMS (MDX-100: ipilimumab®, Yervov®) and Pfizer (CP675206: tremelimumab®). These have been evaluated in patients with melanoma, renal cell carcinoma, and prostate cancer (amongst others), with response rates of 10–15% (reviewed in Ménétrier-Caux et al., 2012). The observation that ipilimumab® remarkably improved 1- and 2-year survival of patients with Stage IV melanoma in clinical Phase II studies resulted in approval for this indication by the US Food and Drug Administration. However, the use of CTLA-4 blocking agents has been associated with an increased risk of adverse effects including hypophysitis (Blansfield et al., 2005), diarrhea (Wolchok et al., 2010), colitis (Berman et al., 2010), thyroiditis and arthritis (Bronstein et al., 2011), and inflammatory skin rashes (Klein et al., 2009). Recent observations demonstrate the capability of anti-CTLA-4 mAb to enhance the number of Treg cells without affecting overall immune capacity (Maker et al., 2005; Ralph et al., 2010), implies a direct activation of effector T cells by anti-CTLA-4 mAb (Ménétrier-Caux et al., 2012).

Other options include the use of Vascular Endothelial Growth Factor Receptor (VEGFR) antagonists (sutent, sorafenib) (Ozao-Choy et al., 2009; Adotevi et al., 2010). Sorafenib dramatically reduces the number of peripheral and tumor-infiltrating Treg cells in patients with metastatic renal cell carcinoma (Busse et al., 2011; Desar et al., 2011) and sunitinib monotherapy decreases the number of peripheral Treg and has been shown to improve overall survival in >70% of the patients (Adotevi et al., 2010). One can hypothesize that combining CTLA-4 blockage with immunotherapy might improve the overall effectiveness of these approaches. Indeed, ONTAK-mediated elimination of Treg cells followed by vaccination with RNA-transfected DCs significantly improves the stimulation of tumor-specific T cell responses in patients will renal cell carcinoma when compared with vaccination alone (Dannull et al., 2005).

The use of agonistic mAbs against OX40 (CD134), a co-stimulatory molecule of the TNF receptor family (Piconese et al., 2008), represents a further approach to abrogate Treg cell-mediated suppression of anti-tumor immunity (Kitamura et al., 2009) and facilitate tumor rejection (Piconese et al., 2008).

Toll-like receptor (TLR2, TLR8, TLR9) agonists can also be considered as promising tools for blocking Treg cell-mediated immunosuppression. TLRs are involved in the recognition of pathogen-associated molecular patterns (PAMPs) and the activation of processes that lead to innate immune recognition. TLRs are expressed by a range of immune and non-immune cells, including Treg cells, and they play an important role in tumor immunotherapy (van Maren et al., 2008). Pre-treatment of human Treg cells with a mixture of TLR2 ligands (Pam_2CSK_4, Pam_3CSK_4, and FSL-1) abolishes Treg cell function by down-regulating the Cdk inhibitor $p27^{Kip1}$ and restoring Akt phosphorylation (Oberg et al., 2011). The synthetic bacterial lipoprotein $Pam_3Cys\text{-}SK_4$, a TLR1/2 agonist which is capable of modulating T cell immune responses, has been found to induce the expansion of $CD4^{+}$ $CD25^{+}$ Treg cells and $CD4^{+}$ $CD25^{-}$ effector T cells in the absence of APCs (Liu et al., 2006a). Expanded Treg cells showed a transient loss of suppressive activity. Furthermore, $Pam_3Cys\text{-}SK_4$ renders effector cells resistant to the suppression of Treg cells by increasing IL-2 secretion. The group of Chu convincingly demonstrated that $Pam_3Cys\text{-}SK_4$ treatment of mice with established lung carcinoma, leukemia, and melanoma, respectively, induced tumor regression and a long-lasting protective response against tumor re-challenge (Zhang et al., 2011).

$Pam_3Cys\text{-}SK_4$ treatment also reduces the suppressive function of Treg cells and enhances the cytotoxicity of tumor-specific cytotoxic T lymphocytes *in vitro* and *in vivo* (Zhang et al., 2011). Treg cell function can be reversed by synthetic and natural ligands for human TLR8 by a mechanism which is independent of DCs, and the adoptive transfer of TLR8 ligand-stimulated Treg cells into tumor-bearing mice enhances anti-tumor immunity (Peng et al., 2005). Furthermore, a preoperative local administration of the TLR9 agonist CpG B-type oligodeoxynucleotide (ODN) PF-3512676 (formerly known as CPG 7909) lowers the frequency of $CD4^{+}CD25^{high}$ Treg cells in the sentinel lymph node of 23 patients with Stage I to III melanoma (Molenkamp et al., 2007).

Another promising immunotherapy involves blockage of PD-1. Programmed death 1 (PD-1, CD279) is a key immune-checkpoint receptor expressed by several T cell subsets including Treg cells which plays an important role in the balance and regulation of adaptive immune responses. Programmed death ligand 1 (PD-L1) is constitutively expressed by B cells, DCs, macrophages, and T cells and can also be found on different tumor cells of human cancer (Jacobs et al., 2009). Activation-induced upregulation of PD-L1 occurs via TLR4 and STAT1 signaling (Loke and Allison, 2003; Freeman et al., 2006). Inhibition of PD-1 and PD-L1 interactions enhances T cell responses *in vitro* and mediates anti-tumor activity in pre-clinical models (Topalian et al., 2012a). Upregulation of PD-1 and its ligand might therefore be associated with immune evasion and inhibition in tumor-bearing hosts. Levels of T cells expressing PD-1 are upregulated in patients with high-risk renal cell carcinoma and patients with PD-1-positive T cells are at a significantly higher risk of cancer-specific death compared with patients harboring low PD-1-expressing T cells (Thompson et al., 2007). The blockage of the PD-1/PD-L1 pathway using anti-PD-L1 mAbs abrogates Treg cell-mediated immune regulation *in vitro* and tolerance induction *in vivo* in mice (Kitazawa et al., 2007). Treg cell and PD-1 pathway signals have been studied in tumor-bearing patients. The function and phenotype of Treg cells and the expression of PD-1 and PD-L1 on different cell populations from the peripheral blood of patients with high-risk-resected stage III and IV melanoma has been studied, and PD-1 blockage found to augment the generation of melanoma antigen-specific cytotoxic T cells by stimulating their proliferation and, indirectly, by masking their suppression by Treg cells (Wang et al., 2009). PD-1

blockage of Treg cells also diminished their inhibitory function (Wang et al., 2009).

In a dose-escalation study, the anti-PD-1 mAb BMS-936558 (also termed MDX-1106 and ONO-4538) has been used as single-agent in patients with advanced solid refractory tumors (Brahmer et al., 2010). This Phase I study demonstrated a favorable safety profile and preliminary evidence of clinical activity, thereby establishing the basis for a multiple-dose Phase I trial (Topalian et al., 2012b). PD-1 blockage extended the spectrum of clinical activity by immunotherapy beyond immunogenic tumor types, such as melanoma and renal-cell cancer, to treatment-refractory, metastatic non-small-cell lung cancer that is commonly not considered as being responsive to immunotherapy. This study and a companion study with anti-PD-L1 antibody (Brahmer et al., 2012) describe clinical activity with these agents validating the impact of the PD-1/PD-L1 pathway for the treatment of certain cancers. Phase II trials are under way (ClinicalTrials.gov numbers, NCT01354431 and NCT01358721), and Phase III studies with anti-PD-1 antibody for the treatment of non-small-cell lung cancer, melanoma, and renal cell cancer are being designed. Such treatment regimens offer a promising therapeutic approach for other tumor entities.

Given the difficulties that are associated with specifically targeting regulatory T cells, interest in the use of cyclophosphamide for inhibiting regulatory T cells and enhancing the induction of anti-tumor immune responses has developed (Le and Jaffee, 2012). A current perspective on the potential use of cyclophosphamide for reducing/eliminating the negative impact of regulatory T cells on protective anti-tumor immunity and thereby enhancing the efficacy of immunotherapeutic strategies has been provided elsewhere (Le and Jaffee, 2012). The key elements of the approach are that low-dose cyclophosphamide can enhance tumor-specific immune responses, despite the fact that it transiently decreases the frequency of regulatory T cell populations (Machiels et al., 2001; Motoyoshi et al., 2006; Emens et al., 2009) and that a metronomic (iterative, low-dose) approach results in a more prolonged suppression of regulatory T cells which returns to baseline within 4–6 weeks, even in the presence of continued administration (Cerullo et al., 2011; Le and Jaffee, 2012). It is also interesting to note that the downstream effects of cyclophosphamide treatment include the appearance of high avidity T effector cells (Ercolini et al., 2005; Laheru et al., 2008; Le and Jaffee, 2012).

It is important to appreciate that the influence of regulatory T cell populations on the induction of protective immunity might extend beyond their effects on adaptive T cell immunity, as $CD4^+CD25^{high}$ Treg cells also inhibit the cytotoxic activity of freshly isolated natural killer (NK) cells via their production of TGF-β (Ghiringhelli et al., 2005a). $CD4^+CD25^{high}$ Treg cells from cancer patients effectively inhibit NK cell-mediated cytotoxicity (Wolf et al., 2003) and the depletion of $CD4^+CD25^+$ Treg cells enhances NKT cell-mediated anti-tumor immunity in a murine mammary breast cancer model (Hong et al., 2010). It is also apparent that the relationship between Treg cells and NK cells is reciprocal, as NK-dependent increases in CCL22 secretion selectively recruits Treg cells to the tumor microenvironment (Mailloux and Young, 2009).

Given the apparent ability of the tumor microenvironment to foster immune tolerance by generating and inducing the functional capacity of regulatory T cell populations (Zou, 2005; Whiteside, 2012), it is essential that we better understand the influence that the tumor microenvironment and treatment modalities have on the induction and progression of the protective anti-tumor immunity which is mediated by T cells and NK cells.

MHC CLASS I EXPRESSION, NK CELLS, AND TUMOR IMMUNITY

Approximately 40–90% of human tumors derived from various MHC class I positive tissues are reported to be MHC class I deficient, and MHC class I downregulation is an important mechanism of tumor escape from T cell-mediated immune responses (Garrido et al., 1993; Restifo, 1996; Algarra et al., 1997; Zheng et al., 1999; Johnsen et al., 2001; Groth et al., 2011). Decreased or absent MHC class I expression is frequently associated with the invasive and metastatic tumor phenotype (Garrido and Algarra, 2001; Bubenik, 2003).

Although the modulation of MHC class I expression has a significant potential impact on the application of T cell-based immunotherapies (Marincola et al., 2000), it does render tumor cells to be more susceptible to NK cells which target MHC class I negative (missing self) cells (Kärre et al., 1986; Ljunggren and Kärre, 1990). Proof for the receptor inhibition model of the "missing self" hypothesis comes from the work of Karlhofer and Moretta who identified MHC I-specific inhibitory receptors on the surface of NK cells such as human p58 (later termed KIR2DL; Moretta et al., 1993), mouse-specific inhibitory Ly-49 receptors (Karlhofer et al., 1992) and the multi-species heterodimeric receptor complex CD94/NKG2D (Aramburu et al., 1990; Carretero et al., 1997).

NK cells provide an essential defence against this response and their importance is illustrated by the observation that downregulation of HLA class I is associated with an improved survival of patients with non-small cell lung carcinoma (Ramnath et al., 2006), uveal melanoma (Jager et al., 2002), breast carcinoma, (Madjd et al., 2005) and colon cancer (Menon et al., 2002).

NK cells are large granular innate immune cells which account for 5–20% of human lymphocytes and can spontaneously recognize virally-infected and cancer cells (Kiessling et al., 1975; Langers et al., 2012). NK cells are characterized by the absence of the characteristic T cell antigens CD3 and CD4 (Stobo et al., 1973). Based on their expression of CD56 (Neuronal Cell Adhesion Molecule) and CD16 (Fcγ receptor III; involved in antibody-dependent cellular cytotoxicity, ADCC) peripheral blood NK cells are grouped into two populations by the literature. The predominant population (90%) of NK cells comprises $CD56^{low}CD16^{high}$ cells which express perforin and granzymes, and exert cytotoxic functions including ADCC. $CD56^{high}CD16^-$ NK cells are mainly found in lymph nodes and comprise only 5% of the NK cells in peripheral blood. Stimulated by macrophage and DC-derived type I interferons (IFNs), IL-2 or IL-15, $CD56^{high}CD16^-$ NK cells are the primary producers of IFN-γ and promote the activation of immune effector cells. An additional population of $CD56^{high}CD16^+$ cells which accounts

for 5% of peripheral blood NK cells exhibit unknown functions (Nagler et al., 1989; Beziat et al., 2011). However, they have been suggested to be an intermediate in the maturation from $CD56^{high}CD16^{-}$ to $CD56^{low}CD16^{+}$ (Beziat et al., 2011).

NK cells express a large profile of inhibitory and activating receptors, the latter also comprising receptors for cytokines, chemokines, and adhesion molecules (**Table 2**), and NK cell functional activity depends on the balance of the signals that are delivered (Vivier et al., 2011). The intracellular domains of activating receptors consist of immunoreceptor tyrosine-based activation motives (ITAMs) or DAP10 with its transmembranic aspartic acid residues, and these receptors include NKG2D and CD16 (Vivier et al., 2004).

NKG2D belongs to the C-type lectin like family and recognizes stress-inducible ligands such as MICA/B or ULBPs (Martinović et al., 2011) and CD16 is the main inducer of ADCC. Receptors that are characteristic of NK cells include natural cytotoxicity receptors (NCRs) such as NKp46 which exert activating functions. Inhibitory receptors are monomeric, associated with immunoreceptor tyrosine-based inhibiting motifs (ITIMs) and exert their function via the recognition of MHC class I and related molecules (Vivier et al., 2004). One inhibitory receptor which recognizes HLA-E either alone or in complex with CD94 is NKG2A (Langers et al., 2012).

Receptors of the killer-immunoglobulin (Ig) like receptor family (KIRs) possess two or three extracellular Ig-like domains and integrate incoming activating or inhibitory signals, but recognize MHC class I molecules with higher affinity than ligands of activating KIRs (Martinović et al., 2011). Autoimmunity is prevented by the low expression of stress-induced ligands and the high expression of MHC class I molecules (Vivier et al., 2008; Langers et al., 2012).

Table 2 | NK cell receptors.

Activating receptors	Inhibitory receptors	Chemotatic receptors	Cytokine receptors	Adhesion receptors
mAct.Ly49	CEACAM-1	CCR2	IL-1R	CD2
2B4	CD94/NKG2A	CCR5	IL-2R	DNAM-1
CD16	mInh.Ly49	CCR7	IL-12R	β1 integrins
CD84	hKIR-L	CXCR1	IL-15R	β2 integrins
CD94/NKG2C	KLRG-1	CXCR3	IL-18R	
CRACC	LAIR-1	CXCR4	IL-21R	
hKIR-S	hLILRB1	CXCR6	IFNAR	
Ly9	mNKR-P1B	CX3CR1		
NKG2D	mNKR-P1D	hChem23R		
mNKG2D-S	TIGIT	S1P5		
NKp46				
hNKp30				
hNKp44				
hNKp80				
mNKR-P1C				
NTBA				

NK cells use a wide array of activating and inhibitory receptors which recognize specific ligands expressed by target cells. MHC class I molecules expressed on self cells are engaged by NK cell inhibitory receptors such as Ly49 in mice (mInh.Ly49) and killer immunoglobulin-like receptors (KIR) in humans (h). In contrast, expression of stress or pathogen-induced ligands, downregulation of MHC class I on target cells as well as transformation-mediated ligand expression are recognized by NK cell activating receptors (Table adapted from Vivier et al., 2011, Science 331, 44–49).

NK CELLS AND THE TUMOR MICROENVIRONMENT

One way via which tumors might avoid oncolysis is by altering NK cell surface receptors. The expression of inhibitory receptors NKG2A and CD85 on NK cells is upregulated in patients with breast cancer and melanoma (Mamessier et al., 2011a,b). Furthermore, the expression of activating receptors including NKG2D, DNAM-1, Nkp30, and CD16 is downregulated in patients with invasive breast cancer and metastasis to distant sites, with the poorest prognosis being linked to the downregulation of almost all activating receptors (Mamessier et al., 2011a,b; Martinović et al., 2011).

NKG2D expression is reduced in patients with gastric cancer and this is likely to have clinical relevance given that NK cells in patients with lymph node metastases exhibit lower NKG2D expression than those patients with no metastases (Saito et al., 2012). The same study demonstrated a lower expression of NKG2D on NK cells within the primary tumor than their circulating counterparts, and also that NKG2D expression increased after surgery (Saito et al., 2012). Expression of the activation receptors NKp30, NKp46, and NKG2D, but not NKp80 and 2B4 have been reported to be reduced in patients with cervical cancer and with precursor lesions when compared to healthy controls (Garcia-Iglesias et al., 2009). *In vitro* studies have demonstrated that factors released by cervical cancer cell lines significantly reduce NKG2D expression on NK cells and their cytotoxic activity (Jimenez-Perez et al., 2012) and that ovarian and cervical cancer cell lines expressing CD155 can downregulate the expression of the DNAX accessary molecule 1 DNAM-1 on NK cells (Carlsten et al., 2009).

It has recently been shown that the expression of tumor necrosis factor superfamily ligands (TNFSFLs) by NK cells and apoptotic tumor activity are suppressed in patients with head and neck cancer. This suppression is tumor-dependent and possibly mediated by soluble TNF superfamily receptors (solTNFSFRs) (Baskic et al., 2012).

NK cell activity can also be modified by increasing the expression of inhibitory receptor ligands. Ovarian and cervical cancers have been reported to exhibit an upregulated expression of HLA-E, a ligand for the inhibitory CD94/NKG2A complex and the upregulation of HLA-E and the infiltration of cytolytic T cell populations could be neutralized by strong overexpression of the NKG2A ligand (Gooden et al., 2011). Expression of the inhibitory receptor CD158b and the proportion of NK cells expressing it are increased in patients with non-small cellular lung cancer (Al Omar et al., 2011).

With regards to NK cell function, the tumor microenvironment and/or factors derived therefrom have been found to impair NK cell-mediated anti-tumor protection. NK cell numbers can be reduced, as has been found in patients with non-small cellular lung cancer (NSCLC) and melanoma (Al Omar et al., 2011; Martinović et al., 2011), and this might impact on the

overall potential of patients to elicit NK cell mediated immunity. Degranulation and NK cell-mediated killing, as well as IFN-γ and TNF-α secretion and induction of ADCC is impaired in patients with metastatic breast cancer via a mechanism which appears to involve TGFβ-1 and prostaglandin E_2 (PGE_2) (Holt et al., 2011; Mamessier et al., 2011a,b). These findings have been confirmed by studies that have related an NK cell functional profile with clinical stages in melanoma and demonstrated that CD107a (a marker for degranulation), IFN-γ and TNF-α levels as well as NKG2D were downregulated, whereas CD158b, an inhibitory receptor was upregulated (Martinović et al., 2011). An interesting finding has been that NK activity in the peripheral blood of patients with breast cancer is lower than that in controls and also that the activity of NK cells in patients with HER2- cancers is significantly lower than that in patients with HER2+ tumors (Dewan et al., 2009). NK cell activity has also been shown to be reduced in patients with cervical cancer (Garcia-Iglesias et al., 2009).

Tumors and macrophages produce H_2O_2 which can be detected by flow cytometry using intracellular formation of 2′,7′-dichlorodihydrofluorescein as a measure of reactive oxygen species. There is a negative correlation between $CD56^{dim}$ cell numbers and H_2O_2 concentration in gastric and oesophageal cancer (Izawa et al., 2011). This population is more susceptible to apoptosis than $CD56^{bright}$, and an observed impairment of ADCC could be reversed by catalase (Izawa et al., 2011). PGE_2 secretion depends on the rate limiting enzyme cyclooxygenase-2 (COX-2) which is overexpressed in some cancers. PGE_2 also has a negative influence on NK cell function via the receptors CD16, NCRs, and NKG2D (Holt et al., 2011).

However, it remains unclear whether the impaired functionality of patient-derived NK cells is permissive of the development of cancer or whether the observed altered phenotype and function result from its presence.

EFFECT OF RADIOTHERAPY ON A TUMOR'S CAPACITY TO INFLUENCE PROTECTIVE INNATE AND ADAPTIVE IMMUNITY

The direct effect of irradiation on immune cells and its influence of protective anti-tumor immunity has been considered elsewhere in this Research Topic (Manda et al., 2012; Multhoff and Radons, 2012; Rödel et al., 2012; Rubner et al., 2012; Schmid and Multhoff, 2012). However, there is much less information relating to the influence of radiotherapy on the tumor's capacity to modify anti-tumor immunity, particularly with regards to direct effects of tumor-derived factors on infiltrating NK and immunoregulatory T cell populations. In contrast to the immunosuppressive effects of whole body irradiation, focal radiation such as that used for treatment of many types of solid tumors has the capacity to influence the tumor microenvironment in a way which can enhance the infiltration and the activation of immune cell types which might foster and/or suppress tumor development (de Visser et al., 2006; Shiao and Coussens, 2010).

Radiotherapy induces the expression of nuclear factor (NF)-κB and this has a number of downstream effects with regards to the expression of molecules that promote a pro-inflammatory environment. Radiotherapy stimulates the migration and function of leukocytes via the release of cytokines such as TNF-α (Shakhov et al., 1990), IL-1 (Mori and Prager, 1996), chemokines (Wickremasinghe et al., 2004), the expression of adhesion molecules such as ICAM-1 and E-selectin on vascular endothelial cells within the tumor microenvironment (Iademarco et al., 1992; Caldenhoven et al., 1994; Schindler and Baichwal, 1994; Hallahan et al., 1996; Handschel et al., 1999). *In vivo* experiments using a murine model of mammary carcinoma have demonstrated that radiotherapy-induced expression of the chemokine CXCL16 is an important mechanism which mediates the infiltration of $CD8^+$ T effector cells following treatment, as the recruitment of $CD8^+$ T cells and responsiveness to treatment are reduced in mice that are deficient for its ligand (CXCR6) (Matsumura et al., 2008).

In vitro experiments using a range of different tumor cell types also suggest that the induction of CXCL16 is a common response to radiotherapy (Matsumura and Demaria, 2010). This could have far-reaching effects with regards to the efficacy of radiotherapy and the immunological mechanisms that are involved. Stromal cell-derived factor (SDF)-1α also appears to an important factor for to the immunological consequences of radiotherapy, as inhibiting its pathway prevents macrophage infiltration and delays tumor regrowth (Kozin et al., 2010).

Ionizing radiation also impacts signaling via pattern recognition receptors (PRRs). These receptors interact with exogenous PAMPs such as endotoxin and endogenous "alarmins" such as high-mobility-group-box 1 (HMGB1), hyaluronan, and heat shock (stress) proteins which together comprise the group of danger-associated molecular patterns (DAMPs) (Bianchi, 2007; Kawai and Akira, 2011). The export of DAMPs is achieved by several mechanisms such as (1) leakage from necrotic cells, (2) increased synthesis and post-translational modification in response to inflammation, and (3) degradation of inactive precursors into TLR-mimetic cleavage products in inflammatory environments (Mencin et al., 2009).

The impact of PRR signaling on radiation responses has been documented by Shan and colleagues who demonstrated that radiation-induced release of IL-12 and IL-18 from macrophages is accompanied by NF-κB activation and an upregulation of CD14 and TLR4/MD2 expression, thereby implying the involvement of the Toll signaling pathway (Shan et al., 2007). On B cells and DCs, TLR-related radioprotective 105 kDa (RP105) was identified as being similar to TLR4 because of its ability to interact with the MD2-like adaptor MD1 (Miyake et al., 1995; Fugier-Vivier et al., 1997). RP105/MD1 directly interacts with TLR4/MD2, thus abolishing the LPS binding capacity of the complex (Divanovic et al., 2005). Together with its ability to regulate TLR4 signaling *in vitro* and LPS responses *in vivo*, RP105 can be considered as being a negative regulator of TLR4 responses.

From these observations it can be assumed that pro-inflammatory responses to radiation and TLR signaling not only increase the impact of tumor-promoting factors in the tumor and the microenvironment, but also might function as crucial targets in radiotherapy. It is interesting to note that the radiation-induced release of HMGB1 by dying tumor cells enables the manifestation of tumor antigen-specific T cell immunity (Apetoh et al., 2007). This pathway depends on the interaction of HMGB1 with TLR4 expressed on DCs. During chemotherapy or radiotherapy, DCs require signaling through TLR4 for efficient processing and

cross-presentation of antigen from dying tumor cells. Moreover, patients with breast cancer who carry a TLR4 "loss-of-function" allele relapse more quickly after radiotherapy and chemotherapy than those carrying the normal TLR4 allele.

These results delineate a clinically-relevant immunoadjuvant pathway which is triggered by tumor cell death. Silencing of HMGB1 expression by an HMGB1-specific RNAi lentiviral vector has been shown to reduce matrix metalloproteinase 9 (MMP9) expression and metastatic capacity in MGC-803 gastric carcinoma cells (Song et al., 2011). HMGB1-specific silencing also significantly decreased cell proliferation and sensitized cells to oxaliplatin-induced apoptosis mediated via the caspase-3 pathway (Song et al., 2011), rendering HMGB1 a promising target structure in cancer therapy.

An intriguing novel observation has reported by Kono and colleagues who demonstrated that 38% of patients with oesophageal squamous cell carcinoma harbored elevated HMGB1 serum levels after chemoradiotherapy and showed concomitant tumor antigen-specific T cell responses (Suzuki et al., 2012). The same study revealed an upregulated HMGB1 expression within the tumor microenvironment in patients with ESCC after preoperative chemoradiotherapy, but not in those without chemoradiotherapy, and the degree of HMGB1 positively correlated with patient survival (Suzuki et al., 2012). Both, irradiation and chemotherapy induces upregulation of HMGB1 and the chaperone protein calreticulin. Furthermore, HMGB1 is able to induce maturation of DCs, implying that chemoradiation induces tumor antigen-specific T cell responses, and that chemoradiation-mediated HMGB1 production is related to clinical outcome (Suzuki et al., 2012).

Using an *in vitro* approach, in which the transmigration of T cell populations toward supernatants derived from primary cultures of tumor cells derived from patients with head and neck carcinomas, Schmidtner et al. (2009) have demonstrated that supernatants from irradiated cells significantly decrease the transmigration of $CD4^+CD25^{high}Foxp3^+$ Treg cells, yet had no effect on the transmigration of $CD4^+CD25^-$ T cells. The observed effects on cell migratory properties have been attributed to treatment-associated increases chemokine (C-C motif) ligand 22 (CCL22) levels in the tumor cell supernatants (Schmidtner et al., 2009). These findings contrast with the effects that are seen following hyperthermia treatment which appears to promote the migration of $CD4^+CD25^{high}Foxp3^+$ Treg cells (Schmidtner et al., 2009). These potentially important findings indicate that radiotherapy may play some role in reducing the capacity of tumors to promote the prevalence of $CD4^+CD25^{high}Foxp3^+$ Treg cells in the tumor microenvironment and might, as a consequence, facilitate the induction or protective innate and adaptive tumor immunity.

In an attempt to increase the therapeutic benefit of irradiation, several studies have combined molecular oncology therapeutics with radiation. Radiation sensitization, in which cytotoxic enhancers co-operate with radiation within the radiation field, aims to produce a greater (synergistic) anti-tumor effect than would be expected from simple additive cell killing (Zaidi et al., 2009). Oncolytic viruses represent prime candidates for enhancing the immunogenicity of the tumor microenvironment. Oncolytic virotherapy may be immunomodulatory via tumor cell death, production of endogenous danger signals, the release of tumor-derived cytokines and direct effects upon cells of the innate immune system (Prestwich et al., 2008).

Pre-clinical models suggest that tumor viral therapy mediates an early influx of immune cells, such as macrophages and NK cells (Benencia et al., 2005; Diaz et al., 2007). These changes within the tumor hold the potential to alter the pre-existing immunosuppressive microenvironment, in favor of the generation of therapeutic immune responses. DCs are critical for the subsequent generation of antigen-specific or adaptive immune responses. According to Prestwich et al. (2008), the outcome of the innate response is finely balanced between promotion of tumor clearance and viral clearance that limits the efficacy. Strategies that involve combining oncolytic virotherapy with external beam radiotherapy may help to exploit synergies between the two treatment modalities (Hingorani et al., 2007; Harrington et al., 2008).

Melcher and colleagues have demonstrated a synergy between oncolytic reovirus RT3D, a naturally occurring nonpathogenic, double-stranded RNA virus isolated from the respiratory and gastrointestinal tracts of humans and external beam radiotherapy in tumor cell lines *in vitro* and in three different *in vivo* tumor models (Twigger et al., 2008). The same group has now completed a Phase I dose-escalation study of this combination strategy in patients receiving two different dose schedules of palliative radiotherapy and confirmed the safety and tolerability of this approach (Harrington et al., 2010). The study further showed that virus is not shed after administration, thereby opening the way for outpatient treatment regimens. Most importantly, the ease of virus administration and the fact that there was no exacerbation of radiation-induced toxicity strongly support development of this combinatory treatment in patients with newly diagnosed, radiocurable cancers.

Several potentially positive theoretical interactions exist between RT3D and radiotherapy. Tumor radiation resistance is, at least partly, mediated by the Ras signaling pathway (McKenna et al., 2003). Moreover, activating Ras mutations, EGFR overexpression, and phosphorylation of Akt and phosphoinositide-3-kinase have been found as being associated with radioresistance *in vitro* and, with respect to EGFR and Akt, to the failure of radiotherapy in cancer patients (Gupta et al., 2002, 2003; McKenna et al., 2003). Blockage of the Ras signaling pathway sensitizes cells to radiation-induced cytotoxicity (Bernhard et al., 1996; Russell et al., 1999).

Radiotherapy in combination with oncolytic reovirus also represents a promising immunotherapeutic approach, as radiotherapy enhances T cell trafficking (Lugade et al., 2005), antigen presentation, and T cell recognition of tumor cells (Reits et al., 2006). Radiotherapy is also locally immunosuppressive by killing lymphocytes, and the optimal combination to enhance anti-tumor immune responses will require careful consideration of dose fractionation and treatment scheduling.

Another potentially important element of the tumor microenvironment which might be altered following the induction of cell death following radiotherapy relates to the release of heat

shock proteins. Although typically regarded as being intracellular proteins, stress proteins, including members of the 60 and 70 kDa families (Hsp60, Hsp70) can be released from a variety of cell types and have been identified in the peripheral circulation in a number of healthy and diseased states (Pockley et al., 1998, 1999, 2000; Rea et al., 2001; Pockley, 2003; Henderson and Pockley, 2010; Henderson et al., 2010). In addition to acting as potent inducers of inflammatory immunity, these proteins can also ameliorate inflammatory events/conditions by inducing/activating regulatory T cell populations (Borges et al., 2012) or by reducing T cell responses and the stimulatory capacity of monocyte-derived DCs (Stocki et al., 2012).

Hsp70 can be released from human glioma, prostate cancer cell lines, the human erythroleukemic cell line K562, and the 4T1 breast carcinoma cell line (Guzhova et al., 2001; Wang et al., 2004; Bausero et al., 2005; Evdonin et al., 2006; Mambula and Calderwood, 2006a,b). Although the relationship between intracellular Hsp70 expression and release has yet to be clarified, increasing the expression of Hsp70 by transfecting prostate cancer cells with cDNA encoding for human Hsp70 enhances their release of Hsp70 (Wang et al., 2004), as does heat treatment (Mambula and Calderwood, 2006a). Non-lethal heat, IFN-γ, and IL-10 also increase Hsp70 release from K562 and 4T1 cells (Bausero et al., 2005). Although in a small cohort of patients, it has been reported that radiotherapy increases circulating Hsp70 levels in patients with prostate cancer and it might be that this reflects increases in Hsp70 levels within the tumor microenvironment (Hurwitz et al., 2010).

Although one study reports that Hsp70 release from prostate cancer cells protects against tumor growth (Wang et al., 2004), presumably via its capacity to induce tumor-specific immunity (Calderwood et al., 2005, 2006), Hsp70 might also adversely influence anti-tumor immunity by activating $CD4^{+}CD25^{high}$ Treg cells via its capacity to interact with TLRs expressed thereon. Released Hsp70 might also have direct effects on tumor cell survival following radiotherapy, as radiation-induced tumor cell killing has been shown to be significantly enhanced by the addition of Hsp70 protein via a mechanism which appears to involve necrosis rather than apoptosis (Schilling et al., 2009).

In addition to the secretion of Hsp70 from viable tumor cells and its inevitable release from necrotic cells within the tumor mass, a form of Hsp70 is frequently expressed on the membranes of a number of cancers, and metastases derived therefrom, but not on their non-malignant counterparts (Multhoff et al., 1995, 1997; Botzler et al., 1996, 1998; Multhoff and Hightower, 1996; Multhoff et al., 2001; Gehrmann et al., 2002; Shin et al., 2003; Multhoff, 2007, 2009). It is also the case that the expression of this membrane form of Hsp70 is enhanced by clinically applied interventions such as radio- and chemotherapy (Gehrmann et al., 2008a,b, 2010) (**Figure 1**).

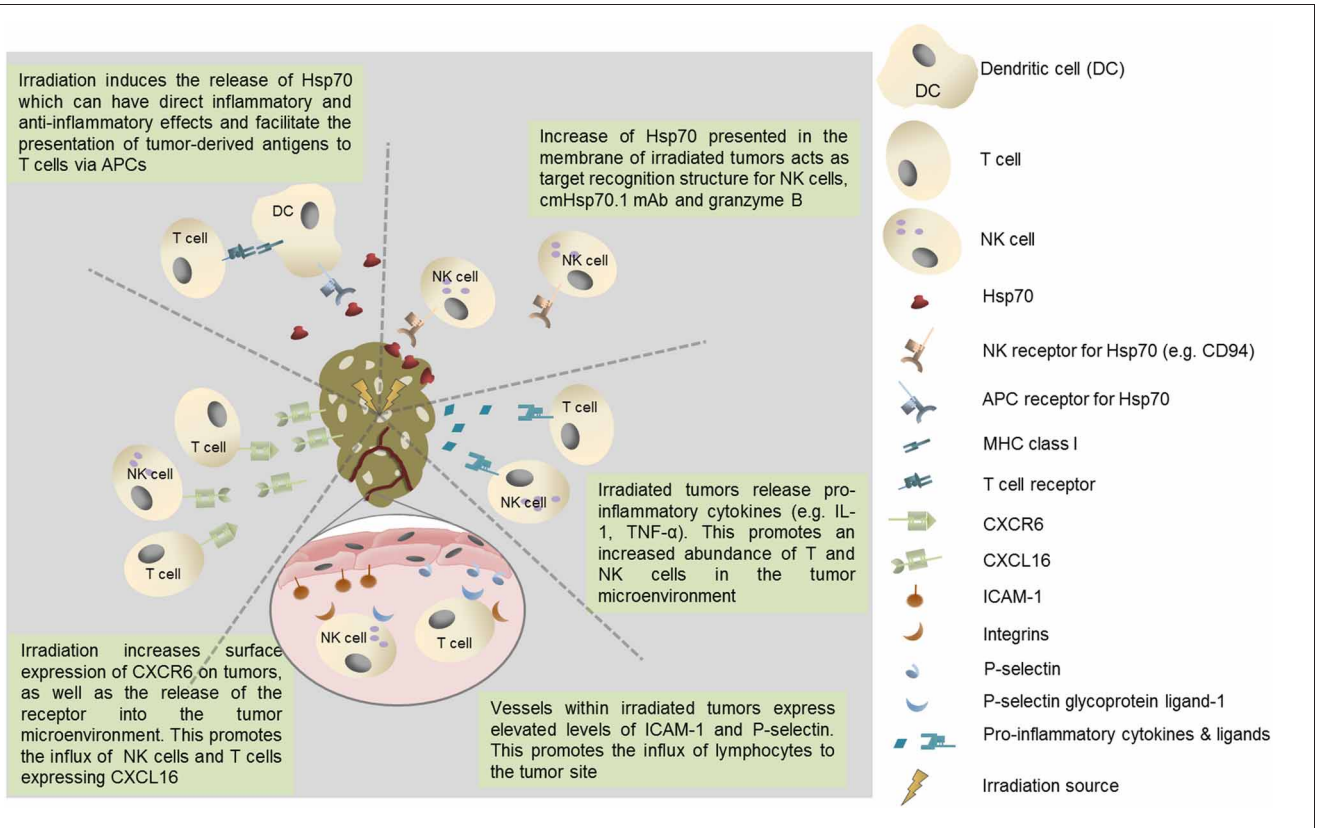

FIGURE 1 | Potential immunological benefits of tumor irradiation. Increased recruitment of immunoregulatory T cell populations could negatively impact on protective anti-tumor immunity.

Membrane Hsp70 acts as a tumor-specific recognition structure for CD94$^+$ NK cells and, in the presence of Hsp70 and cytokine (IL-2/IL-15) co-activation, it enhances the capacity of NK cells these cells to kill membrane Hsp70 positive tumor cells (Botzler et al., 1996, 1998; Gross et al., 2003a,b). Membrane Hsp70 expressing tumors can also be imaged and targeted using a specific monoclonal antibody (cmHsp70.1) or a glycosylated recombinant human granzyme B (Stangl et al., 2010, 2011; Gehrmann et al., 2011, 2012). It might therefore be that the release of heat shock proteins such as Hsp70 into the tumor microenvironment by radiation-induced cell death has a number of immunoregulatory effects that influence many aspects of protective anti-tumor immunity (**Figure 1**).

CONCLUDING STATEMENT

Although it is known that tumors can have a range of immunoregulatory effects that can have tumor-suppressive and tumor-stimulatory properties, an improved insight into the mechanisms and factors involved is required in order to design strategies for promoting the efficacy of therapeutic interventions such as radiotherapy. Focal radiotherapy can elicit anti-tumor immunity by the following mechanisms: (1) boosting trafficking of APCs to the tumor site, (2) augmenting antigen uptake of irradiated tumor cells; (3) increasing the maturation of APCs, (4) inducing maturation of immune effector cells in order to generate a robust immune response, and (5) limiting the immunomodulatory capacity of Treg cell populations.

Accumulation of Treg cells in cancer patients is a major factor in tumor immune escape. Targeting of these cells might thus provide a mechanism by which anti-tumor immune responses could be restored. Immunotherapeutic strategies which target Treg cells and deplete functional Treg cells from the tumor microenvironment cells appear to shift the immune balance in support of effective anti-cancer immune responses. It is likely that effective anti-cancer strategies will depend on multimodal approaches which combine immunotherapy with targeted therapies that stimulate protective immune effector activity and downregulate the suppressive effects of Treg cells.

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Role of T lymphocytes in tumor response to radiotherapy

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Over thirty years ago, Helen Stone and colleagues compared the effects of local tumor irradiation in immunocompetent and T cell deficient mice, providing the first evidence that tumor response to radiotherapy is impaired in the absence of a normal T cell repertoire. In the following three decades there has been an exponential growth in understanding T cells and the complex molecular mechanisms that regulate their activation, migration to tumors and effector functions. We now also know that tumor progression is intrinsically linked to the development of multiple immunosuppressive mechanisms that allow cancer cells to escape immune control. Recent evidence about the role of T cells in determining the prognosis and outcome of patients at any clinical stages of cancer has been instrumental in re-directing the concept of immunosurveillance and immunoediting from the realm of preclinical models to the reality of clinical observations. Importantly, cell death induced by standard anti-cancer therapies like chemotherapy and radiation has been demonstrated to involve the immune system and, in certain specific settings, enable a specific immune response. It is, therefore, not surprising that the last few years have seen an increase in investigations exploring how to harness the ability of radiation to induce anti-tumor immune responses. We will review here the experimental evidence that anti-tumor T cells are key players in tumor control achieved by radiotherapy. The effects of radiation on the tumor that have been shown to enhance the priming and effector phases of anti-tumor immunity will be discussed. Finally, we will highlight promising combinations of immune response modifiers that enhance T cell function with radiotherapy which are being tested in the clinic.

Keywords: abscopal, adjuvant, CD8 T cells, dendritic cells, immunoediting, immunotherapy, ionizing radiation, *in situ* vaccine

INTRODUCTION

Ionizing radiation has been employed as a cancer treatment based on its cytocidal effects, and the response to radiotherapy linked mostly to the delivery of irreparable DNA damage to tumor cells. Therefore, research to improve the efficacy of radiotherapy has been dominated by studies of the mechanisms of DNA repair, their regulation in normal and neoplastic cells, and the tumor cell factors that affect radiosensitivity, such as the phase of the cell cycle. While *in vitro* these parameters are determinants of the inhibition of tumor cell growth by radiation, *in vivo* they are essential but not sufficient to explain the response of a tumor to local radiotherapy. In fact, a report published in 1979 by Helen Stone and colleagues demonstrated that *in vivo*, factors extrinsic to the cancer cell are key determinants of tumor radiosensitivity (Stone et al., 1979). Instead of studying the response of human tumor xenografts that grow only in immunocompromised mice, a mouse tumor was injected in syngeneic animals. Radiosensitivity was then compared in immunocompetent and T cell-deficient animals. The difference was striking: tumors growing in mice that lacked T cells required over 60 Gy to achieve the same tumor control obtained with 30 Gy in immunocompetent mice. More than thirty years later, the key role of T cells as anti-tumor effectors is unquestionable in experimental mouse models as well as in humans. There is evidence from clinical trials that adoptive transfer of tumor-specific T cells can eliminate tumors even at advanced stages (Porter et al., 2011; Restifo et al., 2012). Significant progress has also been made in understanding how a treatment considered immunosuppressive such as radiation can induce anti-tumor T cells, as reviewed in this article. While the clinical evidence of systemic anti-tumor responses from local radiotherapy is rare, the uncommon observation of tumor regression outside of the radiation field was recognized by R. H. Mole and named, in 1953 as abscopal effect from the latin "ab scopus," i.e., away from the target (Mole, 1953). Based on the hypothesis that the abscopal effect is due to radiation-mediated induction of anti-tumor T cells (Demaria et al., 2004), interventions that improve T cell activation have shown abscopal effects when combined with radiotherapy in mice and humans (Demaria et al., 2005; Formenti and Demaria, 2009; Postow et al., 2012).

To understand the role of T lymphocytes in the tumor response to radiotherapy it is useful to review the evidence on the reciprocal influence that tumor and immune cells have on each other during tumor progression.

TUMOR-HOST IMMUNE SYSTEM: A DYNAMIC EQUILIBRIUM

The fundamental task of the immune system is to maintain tissue homeostasis. This is an active process that requires a delicate balance between tolerance and active surveillance to detect any tissue change that is potentially dangerous. Since tissue turnover and physiological remodeling, for example in the breast post-weaning, are often associated with significant cell death, the immune system has developed sensors to distinguish it from pathogenic cell death. A key class of receptors devoted to triaging cell death are pattern recognition receptors (PRR). Expressed by innate immune cells they bind to pathogen-associated molecular pattern (PAMP) molecules derived from infectious agents and damage-associated molecular pattern (DAMP) molecules derived from cells dying a stressful death (Janeway and Medzhitov, 2002; Zeh and Lotze, 2005; Mills, 2011). The ability to resist cell death has been identified as one of the hallmarks of cancer (Hanahan and Weinberg, 2000), suggesting that, in addition to resulting in tumor growth, this property may also account for a failure of recognition of the pathogenic features of transformed cells by the immune system. However, there is plenty of evidence to the contrary and, in fact, immune recognition of cancer cells is so common that the ability to evade immune destruction has been increasingly recognized as an essential biological capability required by tumors in order to become clinically apparent (Hanahan and Weinberg, 2011). The cancer immunoediting theory provides a rationale for this apparent paradox (Dunn et al., 2002).

Neoplastic transformation is invariably associated with genomic instability and cell stress. Genomic instability leads to the generation of neoantigens-containing epitopes that can be recognized by T cells (Segal et al., 2008) and cell stress leads to the expression of molecules such as members of the family of NKG2D ligands that are recognized by natural killer (NK), γδ T cells and effector CD8 T cells (Diefenbach et al., 2000; Hayakawa et al., 2002). Local disruption of the stroma and of normal tissue architecture generates danger signals in the form of DAMPs, including degraded extracellular matrix components (e.g., heparin sulfate, hyaluronan) (Lotze et al., 2007) that attract innate immune cells. Recognition of the stressed neoplastic cells by NK or other innate immune cells results in production of interferon (IFN)-γ, a cytokine shown to play a key role in immunosurveillance against tumors (Street et al., 2001; Dunn et al., 2006). Killing of the neoplastic cells by NK cells or macrophages activated by IFN-γ to produce cytocidal reactive oxygen and nitrogen species, eventually leads to cross-presentation by dendritic cells (DC) of antigens from the dying tumor cells to T cells and activation of the adaptive immune system. Tumor-specific T cells may be able to completely destroy the incipient tumor, thus functioning as an extrinsic tumor suppressor mechanism that reduces the incidence of spontaneous and carcinogen-induced tumors. This is supported by unequivocal evidence in experimental models and indirect evidence in humans with various immunodeficiencies [reviewed in Dunn et al. (2004) and Vesely et al. (2011)]. However, if complete elimination of genomically unstable cells is not achieved, the immunological pressure results in selection of clones of neoplastic cells that have acquired, via mutations or epigenetic changes, resistance to immune rejection, i.e., are "edited" by the immune system to become poorly immunogenic. This transition from elimination to escape can occur directly, or sometimes can occur after a long period of equilibrium, during which the immune response is able to prevent or limit the progression of cancer. The concept of equilibrium, initially formulated to explain clinical observations of occult tumors and tumor dormancy (Myron Kauffman et al., 2002; MacKie et al., 2003), has been confirmed in experimental models: depletion of T cells leads to growth of occult tumors that are more immunogenic, indicating that much of the immunoediting occurs during the equilibrium phase (Koebel et al., 2007). Importantly, recent evidence demonstrates that CD8 T cells play a key role in "editing out" strongly immunogenic tumor antigens (DuPage et al., 2012; Matsushita et al., 2012).

The same property that allows tumors to escape immune control may become their Achille's Heel. Tumors with high levels of genomic instability due to microsatellite instability (MSI) are prone to generate novel tumor antigens. They are often highly infiltrated by T cells and their carriers often enjoy better clinical outcomes, an association suggestive of better immune control (Buckowitz et al., 2005; Chiaravalli et al., 2006). Importantly, the association between infiltration by CD8 T cells and improved prognosis is not exclusive to tumors with MSI (Zhang et al., 2003; Galon et al., 2006; Pagès et al., 2010). This observation suggests that the degree and type of immune response matters at every stage of tumor progression, including metastatic disease. For example, the ability of immunotherapeutic strategies to improve survival of patients with metastatic melanoma (Hodi et al., 2010) indicates that even in advanced stages, when tumors have escaped immune control, it is possible to enhance anti-tumor T cell reactivity to revert to a phase of equilibrium, even in the presence of more extensive tumor burden.

Tumor's escape from immune control is a complex process, which does not only occur via antigenic loss. To avoid immune rejection tumors exploit multiple pathways that physiologically maintain immune tolerance to "self" and protect healthy tissues from immune destruction during acute inflammatory reactions. The recruitment of suppressive, tolerogenic and regulatory innate and adaptive immune cells, the secretion of immune suppressive cytokines and the induction of dysfunctional differentiation of T cells can be seen in most, if not all tumors [reviewed in Demaria (2012)]. In addition, cancer cells downregulate major histocompatibility complex (MHC) class I molecules that are required for recognition by CD8 T cells (Chang and Ferrone, 2007), and upregulate immunosuppressive receptors that preclude their destruction by T cells (Dong et al., 2002). The tumor vasculature also presents multiple barriers to T cell infiltration, through an abnormal architecture and a relative paucity of endothelial adhesion molecules (Chen et al., 2003). Overall, the tumor microenvironment evolves into a protective hub for the neoplastic cells, that actively prevents tumor rejection. In this context, ionizing radiation acts as a modifier of the microenvironment with the potential to switch the immunosuppressive hub into an immunogenic one (Demaria and Formenti, 2007).

ROLE OF THE IMMUNE SYSTEM IN RESPONSE TO LOCAL RADIOTHERAPY

Although radiation has been known to have pro-inflammatory and immunomodulatory effects for a long time (McBride et al., 2004), it is only recently that some of these changes have been elucidated at a molecular level. These studies have provided evidence for the counterintuitive concept that local radiotherapy, rather than suppressing anti-tumor immunity, can promote it. A series of important findings in relation to the main barriers to immune rejection that are affected by radiation have emerged.

As mentioned above, the correct assessment of cell death by innate immune cells as "dangerous" or "non-dangerous" dictates which downstream pathways are triggered to either activate adaptive immunity or maintain tolerance. The traditional dichotomy of cell death as apoptotic and non-inflammatory versus necrotic and inflammatory has been challenged by the demonstration that apoptotic death can be associated with release of pro-inflammatory and danger signals (Galluzzi et al., 2007). The stressful death of cancer cells induced by some types of chemotherapy and by ionizing radiation can be quite immunogenic and promote the cross-presentation of tumor-derived antigens by DC to T cells, leading to development of anti-tumor responses (Ma et al., 2010; Zitvogel et al., 2010). Among the three molecular signals identified as critical for the successful induction of immunogenic cell death, both, translocation of calreticulin (CRT) to the surface of the dying cell and release of high-mobility group protein B1 (HMGB1), which binds to the PRR Toll-Like Receptor (TLR) 4, are induced by ionizing radiation (Apetoh et al., 2007; Obeid et al., 2007). The third signal, active release of ATP by cells committed to apoptotic death, which is required to activate the NLRP3 inflammasome (Ghiringhelli et al., 2009) is still awaiting confirmation in irradiated cells. Given recent evidence that autophagy is required for ATP release (Michaud et al., 2011), and that ionizing radiation promotes autophagy (Rieber and Rieber, 2008; Rodriguez-Rocha et al., 2011), this third signal is likely to be generated by radiotherapy when autophagy precedes cell death. Overall, experimental evidence supports the contention that radiation can induce a tumor cell death that is perceived by the immune system as dangerous and, therefore, generates an *in situ* cancer vaccine.

Once activated, T cells have to be able to home to and infiltrate the tumor. Radiation has been shown to promote this process in multiple ways. For instance, radiation-induced remodeling of the abnormal tumor vessels, resulted in efficient tumor infiltration by adoptively transferred anti-tumor T cells in a spontaneous mouse tumor model (Ganss et al., 2002). In a murine experimental model of melanoma, up-regulation of vascular cell adhesion molecule (VCAM)-1 induced by radiation increased infiltration by T cells, in a process requiring IFN-γ production (Lugade et al., 2005, 2008). Our group demonstrated in a poorly immunogenic mouse carcinoma that radiation-induced up-regulation of the chemokine CXCL16 was required for the efficient recruitment to the tumor of $CXCR6^+$ effector CD8 T cells, resulting in optimal tumor inhibition (Matsumura et al., 2008). Other important effects of radiation include the up-regulation of MHC class I molecules, adhesion molecules, NKG2D ligands, and Fas/CD95, enhancing the ability of effector T cells to bind to and kill the cancer cells (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). Thus, radiation is a significant modifier of tumor microenvironment with specific effects that facilitate tumor rejection (**Figure 1**).

Despite the multiple pro-immunogenic effects, radiation by itself is usually insufficient to generate strong and lasting T cell responses that in addition to contributing to eradicate the irradiated tumor can control the growth of established metastases. Multiple immunosuppressive pathways make it very difficult to overcome these barriers by radiotherapy alone, in the absence of additional interventions. However, addition of antibodies to block a negative regulator of T cell activation, the checkpoint receptor cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), induced therapeutically significant anti-tumor immunity to a poorly immunogenic carcinoma treated with local radiotherapy, while each treatment by itself was not effective (Demaria et al., 2005). In addition, radiation induces effects that can dampen the immune response, like the activation of transforming growth factor (TGF)β (Jobling et al., 2006), and a relative increase in regulatory T cells (Kachikwu et al., 2011). Altogether, the pre-existing balance between tolerogenic and effector anti-tumor mediators, and the degree to which radiation can induce activation without stimulating suppression, converge to determine the outcome in terms of local and systemic tumor control. Intriguingly, there is at least some evidence that the type of the radiation regimen employed may have a role in determining whether a favorable pro-immunogenic response is elicited (Dewan et al., 2009).

HARNESSING THE PRO-IMMUNOGENIC EFFECTS OF RADIATION IN CANCER TREATMENT: A NEW PARADIGM

Progress in understanding the function and dysfunction of the immune system in cancer has identified specific targets for intervention, based on the dominant immunosuppressive mechanism in a given tumor type and/or patient (Zitvogel et al., 2011). The growing evidence that local radiotherapy can generate an *in situ* vaccine supports its use in concert with personalized immunotherapy, since the killed tumor cells provide the entire antigenic diversity of a patient's own tumor.

Since DC function is often suboptimal in tumors, studies have tested strategies to increase DC numbers and function by administering DC growth factors in combination with radiotherapy. Experimental work in two syngeneic mouse models, a lung and a mammary carcinoma, employed Flt-3 ligand as growth factor to expand DC, and demonstrated the induction of a T cell-mediated response that reduced tumor growth outside the field of radiation (Chakravarty et al., 1999; Demaria et al., 2004). Based on this data, we conducted a clinical trial that used s.c. GM-CSF to increase the percentage of DC and their maturation and facilitate cross-presentation of newly released antigens, after cell death at the site of radiotherapy. We selected patients with at least 3 metastatic sites from solid tumors. With a standard radiation fractionation of 3.5 Gy X10 fractions delivered to one tumor site we were able to measure an out-of field (abscopal) response in 30% of the patients with metastatic solid tumors accrued to the trial

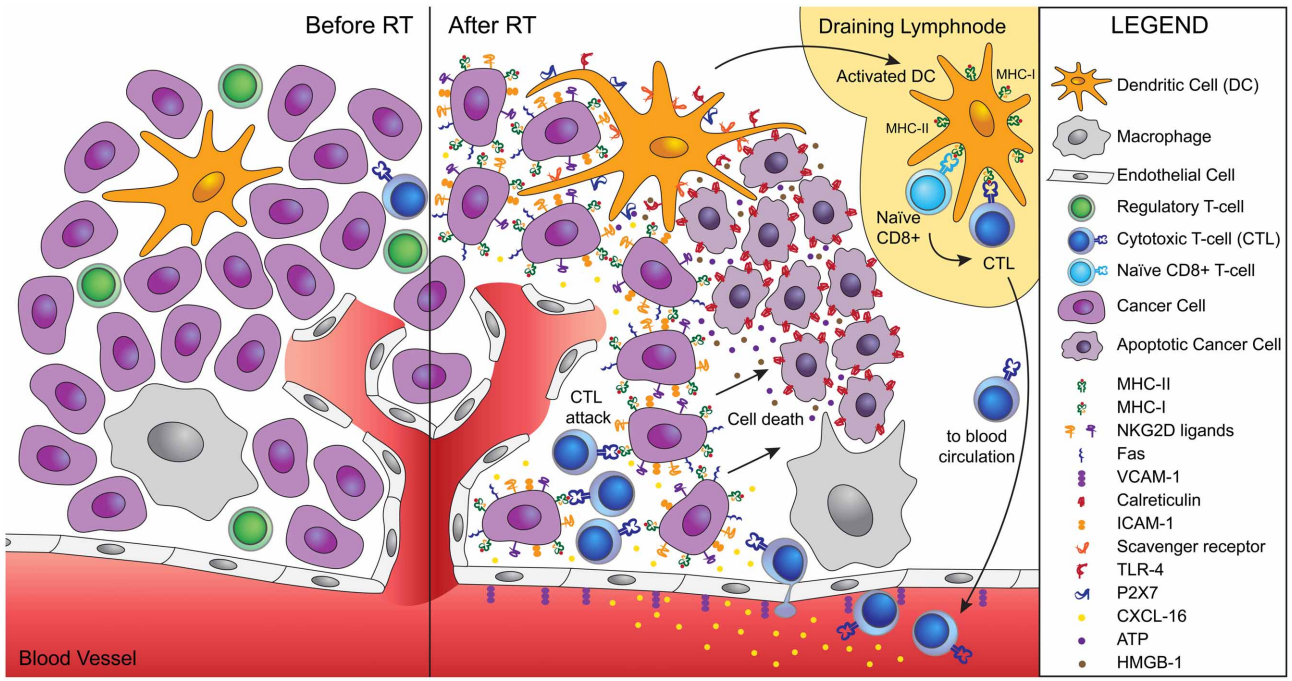

FIGURE 1 | Ionizing radiation acts as a modifier of the tumor microenvironment converting the tumor into an *in situ* vaccine. Radiation induces an immunogenic cell death of tumor cells characterized by calreticulin translocation to the surface of dying cells, and release of HMGB-1 and ATP. Calreticulin allows uptake of dying cells by dendritic cells via scavenger receptor(s). HMGB-1 binds to TLR4 and promotes the cross-presentation of tumor antigens, while ATP binds to P2X7 and triggers the activation of the inflammasome. Activated dendritic cells migrate to the draining lymph node, where they activate naïve T cells specific for tumor antigens. Activated CD8 T cells acquire effector functions and traffic to the tumor guided by radiation-induced chemokines. Tumor infiltration by CTLs is facilitated by radiation-induced upregulation of VCAM-1 on the vascular endothelium. Once in the tumor, CTLs interact efficiently with tumor cells expressing increased levels of MHC-I, ICAM-1, NKG2D ligands, and Fas that promote the formation of stable immunological synapses between targets and effectors and facilitate the killing of tumor cells by CTLs. Tumor cells killed by CTLs become a source of antigens for cross-presentation, thus fueling the process.

(Formenti and Demaria, 2009). In murine models, exogenously prepared DC injected in the tumor following radiation induced anti-tumor immune responses (Nikitina and Gabrilovich, 2001; Teitz-Tennenbaum et al., 2003; Kim et al., 2004). These effects were translated in the majority of patients with hepatoma and high risk sarcoma treated in two early clinical trials (Chi et al., 2005; Finkelstein et al., 2012). In preclinical models molecular mimics of the danger signals associated with pathogens, like olygodeoxynucleotides containing CpG motifs that bind to TLR9, when injected intratumorally enhanced DC activation and ability to cross-present tumor antigens released by radiation (Milas et al., 2004; Mason et al., 2005). A similar combination of local radiotherapy and CpG administration was tested in 15 patients with low-grade B-cell lymphoma, showing abscopal responses, associated with development of tumor-specific T cells (Brody et al., 2010). Taken together, the data support the ability of radiation to generate an *in situ* vaccine: the efficacy of this approach is dependent on DC fitness and can be enhanced by interventions directed at improving DC.

A complementary strategy is based on targeting checkpoint co-inhibitory receptors or co-stimulatory receptors expressed by T cells with blocking or agonistic antibodies, respectively, to achieve stronger and more sustained responses of anti-tumor T cells. Our group tested the hypothesis that inhibiting a key checkpoint receptor, CTLA-4, in combination with radiotherapy would induce therapeutically effective anti-tumor responses. While CTLA-4 is a dominant inhibitory receptor for T cells, as demonstrated by the development of uncontrolled T cell proliferation in mice deficient in CTLA-4 (Chambers et al., 1997), CTLA-4 blockade as monotherapy failed to induce regression of poorly immunogenic tumors, requiring its use in combination with vaccination (Peggs et al., 2008). Therefore, we hypothesized that radiotherapy would synergize with anti-CTLA-4, due to its ability to generate an *in situ* vaccine. This hypothesis was confirmed in mice models of poorly immunogenic carcinomas (Demaria et al., 2005; Dewan et al., 2009). The therapeutic efficacy of the anti-tumor T cells activated by treatment was enhanced by other effects of radiation such as an improved tumor infiltration by effector T cells, confirming it's beneficial effects at both the priming and effector phase of anti-tumor responses (Matsumura et al., 2008). A recent case report suggests that the success of the combination of local radiotherapy and anti-CTLA-4 can be translated in melanoma patients (Postow et al., 2012), with multiple clinical trials being conducted to confirm these results.

Targeting of other co-stimulatory or co-inhibitory receptors expressed by T cells, CD137 and programmed death (PD)-1, respectively, has also shown some success in combination with radiation in mice models (Newcomb et al., 2010; Verbrugge et al., 2012), supporting more studies to develop these strategies for clinical use.

A number of other studies exploited the pro-immunogenic effects of local radiotherapy that promote the effector phase of tumor rejection, by combining radiation with either vaccination or adoptive immune therapy (AIT). Increased expression of MHC class I antigens by irradiated glioma cells was implicated in the synergy of peripheral vaccination with whole brain radiation (Newcomb et al., 2006). In a mouse carcinoma, radiation-induced Fas expression was shown to synergize with T cell AIT and with vaccination, by facilitating tumor cell killing by T cells (Chakraborty et al., 2003, 2004). Interestingly, following the combination of vaccine and local radiation there was an induction of T cells specific for tumor antigens not present in the vaccine, a phenomenon known as antigen cascade or antigenic spread. Similarly, antigen cascade was also observed in prostate cancer patients treated with standard definitive radiotherapy and vaccination (Gulley et al., 2005).

CONCLUSIONS

Immune response modifiers (IRM) have been defined by the National Cancer Institute Translational Research Working Group as "immunotherapy agents that mimic, augment, or require participation of the host immune system for optimal effectiveness" (Cheever et al., 2008). Although host T cells contribution to the optimal tumor response to radiation was demonstrated over three decades ago (Stone et al., 1979), it is only in the last decade that the underlying mechanisms begun to be understood. Increasing number of publications testing new combinations of radiation and immunotherapy testify to the growing interest toward a new role of radiation as an "immunological adjuvant". Most exciting is the emerging evidence that radiation may indeed function as an IRM in patients, suggesting that it may be time to consider a paradigm shift in the use of radiotherapy.

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Effects of ionizing radiation on the immune system with special emphasis on the interaction of dendritic and T cells

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Dendritic cells (DCs), as professional antigen-presenting cells, are members of the innate immune system and function as key players during the induction phase of adaptive immune responses. Uptake, processing, and presentation of antigens direct the outcome toward either tolerance or immunity. The cells of the immune system are among the most highly radiosensitive cells in the body. For high doses of ionizing radiation (HD-IR) both immune-suppressive effects after whole body irradiation and possible immune activation during tumor therapy were observed. On the other hand, the effects of low doses of ionizing radiation (LD-IR) on the immune system are controversial and seem to show high variability among different individuals and species. There are reports revealing that protracted LD-IR can result in radioresistance. But immune-suppressive effects of chronic LD-IR are also reported, including the killing or sensitizing of certain cell types. This article shall review the current knowledge of radiation-induced effects on the immune system, paying special attention to the interaction of DCs and T cells.

Keywords: dendritic cells, T cells, ionizing radiation, low dose, immune system

INTRODUCTION

The interactions of dendritic cells (DCs) and T lymphocytes are a link between the innate and adaptive cell-mediated immunity. Therefore, radiation-induced disturbances may have serious consequences on the whole immune system. This article provides an overview of DC and T cell function and particularly reviews the effects of low-dose ionizing radiation (LD-IR; <1 Gy) and high-dose ionizing radiation (HD-IR; ≥ 1 Gy) exposure on the interrelationship of both cell types. Controversial data on the immune-modulatory effects of LD-IR and current knowledge about the immune-suppressive and pro-inflammatory effects of HD-IR are discussed in detail, together with the putative mechanisms behind them. Clinically relevant immunological aspects of ionizing radiation (IR) are presented and the possibility of their exploitation in combined immunotherapy are elucidated.

Abbreviations: Ag, antigens; APC, antigen-presenting cell; CCL, chemokine ligand; CCR, chemokine receptor; CD, cluster of differentiation; CTL, cytotoxic T cell; CTLA, cytotoxic T lymphocyte antigen; DC, dendritic cell; dLN, draining lymph node; HD-IR, high-dose ionizing radiation; ICAM, intercellular adhesion molecule; IFN, interferon; IG, immunoglobulin; IL, interleukin; IR, ionizing radiation; LD-IR, low-dose ionizing radiation; LD-RT, low-dose radiotherapy; LNT, linear, no-threshold; MCP, monocyte chemoattractant protein; MHC, major histocompatibility complex; MIP, macrophage inflammatory protein; NK cell, natural killer cell; T_{CM}, central memory T cells; TCR, T cell antigen receptor; T_{EM}, effector memory T cells; TGF, transforming growth factor; T_h cell, helper T cell; TNF, tumor necrosis factor; T_{reg}, regulatory T cell; STAT, signal transducer and activator of transcription; VEGF, vascular endothelial growth factor; WBI, whole-body irradiation.

DENDRITIC CELLS

Dendritic cells are antigen-presenting cells (APCs) which play a crucial role not only in inducing adaptive immune response to foreign antigens (Ags), but also in maintaining T cell tolerance to self-Ags, thus minimizing autoimmune reactions (Banchereau and Steinman, 1998). All DCs are derived from hematopoietic stem and progenitor cells in the bone marrow and give rise to distinct progenitors, which can be found in the blood, lymph, thymus, and most visceral organs. Their further development comprises differentiation into DC subsets, activation and maturation finally resulting in Ag presentation (Alvarez et al., 2008). Newly differentiated DCs are responsible for efficient Ag capture via a variety of mechanisms including macropinocytosis, endocytosis (Lim and Gleeson, 2011), or phagocytosis (Matsuno et al., 1996); these DCs are considered to be immature. To initiate immunity, immature DCs migrate throughout the body in order to take up several Ags, but expression of major histocompatibility complex (MHC) gene products and co-stimulatory molecules such as cluster of differentiation (CD) 80 and CD86, and thus presentation to T cells, is initially weak (Mellman and Steinman, 2001). Upon arrival at secondary lymphoid organs, such as draining lymph nodes (dLN) and the spleen, they have to undergo a maturation process initiated by several environmental stimuli or danger signals including bacterial DNA (Sparwasser et al., 1998) or viral products and proinflammatory cytokines (Mellman and Steinman, 2001). Maturation is characterized by an increase in surface marker expression responsible for co-stimulation, including CD40, CD54, CD58, CD80, CD83, and CD86 (Banchereau

and Steinman, 1998; Faries et al., 2001; Würtzen et al., 2001) along with the ability to present Ag more effectively to T cells. As a consequence of maturation, Ag uptake of DCs is reduced through a loss of Ag receptors and down-regulation of phagocytosis (Albert et al., 1998).

T CELLS

T lymphocytes are main player in the cell-mediated adaptive immune response. After migration of progenitor T cells from the bone marrow to the thymus T cells differentiate, resulting in the expression of the typical co-receptors CD4, CD8 and the assembly of functional T cell Ag receptors (TCRs). T cells then undergo a positive and negative selection process based on MHC receptor restriction and on the affinity threshold of their TCR to self-peptides presented by MHC molecules on the thymic epithelial cortical cells (Starr et al., 2003; Arens and Schoenberger, 2010). Naive, but mature T cells migrate to the secondary lymphoid organs where they survey the Ags presented by APCs. The TCRs recognize Ag fragments bound to MHC molecules on the surface of an APC. As a consequence of Ag binding and interaction with cytokines and co-stimulatory molecules, naive $CD4^+$ or $CD8^+$ T cells become activated, proliferate, and differentiate into effector T cells (Lee et al., 2012).

Whereas the majority of $CD4^+$ T cells are helper T (T_h) cells selectively binding to MHC class II proteins, the majority of $CD8^+$ T cells are cytotoxic T cells (CTLs) restricted to binding to MHC class I proteins (Banchereau and Steinman, 1998). T_h cells assist other cells of the immune system such as B cells and macrophages and can be further categorized into T_h1, T_h2, and T_h17 subsets (Reiner, 2007). T_h1 cells are primarily involved in cell-mediated inflammatory reactions including activation of macrophages and CTLs. T_h2 cells aid the humoral and allergic arms of the immune response and are associated with eosinophilia (Kimber and Selgrade, 1998). T_h17 cells are important for attacking extracellular microorganisms by activating neutrophils with interleukin (IL)-17. In addition to T_h cells, $CD4^+$ T cell also comprise subsets which have the ability to regulate inflammatory immune responses and are therefore termed regulatory T cells (T_{regs}; Lee et al., 2012). These cells express CD25, the IL-2 receptor and play a major role in maintaining immunological self-tolerance (Sakaguchi, 2004). Subsets of T_{reg} have also been demonstrated to inhibit both T_h1 and T_h2 functions, crucial to the outcome of infections and inflammatory diseases (Xu et al., 2003).

The main function of Ag-specific $CD8^+$ T cells (CTLs) is to eradicate infected or tumor cells through the release of cytolytic molecules and CD95 ligation, eventually leading to the programed cell death (apoptosis) of the target cell. Besides antigenic stimulation (signal 1) and co-stimulation by APCs (signal 2), inflammatory cytokines such as IL-12 and type I interferons (IFNs) are important for driving effector T cell expansion and function (Arens and Schoenberger, 2010). $CD4^+$ T cells effectively support CTL response, especially during the secondary expansion phase, and by generating long-term CTL immunity (Behrens et al., 2004).

As part of the adaptive immune response, both $CD4^+$ and $CD8^+$ T cells comprise also memory T cell subsets which have already encountered Ag during a prior infection and escaped apoptosis. After a second encounter with Ag or pathogen, memory T cells are able to work quickly without even requiring proliferation (Bevan, 2011). They are further categorized into effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) based on their capacity to migrate to secondary lymphoid tissue (T_{CM}) and infected or inflamed peripheral sites (T_{EM}). The main distinctive feature of the two memory T cell subsets is the expression of chemokine receptor 7 (CCR7), which exists on T_{CM} but is lacking on T_{EM} cells (Sallusto et al., 1999).

INTERACTION OF DCs AND T CELLS

The presentation of Ags by DCs plays a crucial role in effective T cell activation and initiation of an adaptive immune response. Naive $CD8^+$ cytotoxic and $CD4^+$ T_h cells circulate through secondary lymphoid tissues where they meet activated mature DCs presenting processed Ags to them via MHC class I and II molecules, respectively. Both cell types need to interact physically to induce T cell activation and proliferation. The subsequent outcome of T cell activation depends on the activation state of DCs. Activated, mature DCs induce T cell priming, whereas resting, non-activated but fully differentiated mature Ag-presenting DCs may induce tolerance (Tan and O'Neill, 2005; Hugues, 2010). The latter is a process which is required to eliminate self-reactive T cells in the thymus during a process known as central tolerance. However, some self-reactive T cells often bearing low affinity TCR for self-Ags escape clonal deletion in the thymus. A number of tolerance mechanisms have evolved in the periphery to prevent autoimmune disease. DCs capturing and presenting numerous self-Ags to T cells in secondary lymphoid tissues are an important part of this peripheral tolerance (Walker and Abbas, 2002).

The current model of T cell activation in general requires three signals. The first signal is the establishment of a cellular contact between a T cell and a DC occurring through TCR interactions with MHC complexes present on the DC surface. In this process, $CD4^+$ T_h cells can effectively support the Ag-specific $CD8^+$ CTL responses via activation of CD40 on DCs when both T_h cells and CTLs recognize Ag on the same DCs. The second signal comprises the engagement of different receptor–ligand bindings such as those of co-stimulatory and intercellular adhesion molecules (ICAMs). Important co-stimulatory molecules are CD80 and CD86, expressed on activated but not on resting APCs, which need to bind to the cell surface receptor CD28 on T cells for effective T cell activation and to cytotoxic T lymphocyte antigen 4 (CTLA-4) for suppression. These interactions finally lead to the third signal consisting of the secretion of mediators. The integration of all signals finally matches the outcome of T cell activation, resulting in the clonal expansion and differentiation of naive T cells into effector and memory T cells (Sharpe and Abbas, 2006; Arens and Schoenberger, 2010; Hugues, 2010).

Given the important role of DC and T cell interaction in the adaptive immune responses, it is not surprising that many pathogenic microorganisms exert immunomodulatory effects that may impair the ability of DCs to initiate T cell responses. Virus-induced interference with Ag presentation pathways, induction of cytopathogenesis, T_h1/T_h2 cytokine shifts, and CD4 depletion are examples of this (Clerici and Shearer, 1993; Arens and Schoenberger, 2010).

RADIATION-INDUCED EFFECTS ON THE IMMUNE SYSTEM AND THE INTERACTION OF DCs AND T CELLS

Since the spleen is a very highly radiosensitive organ (Gridley et al., 2009), cells of the immune system are considered to be among the most highly radiosensitive cells. The biological effects of IR are not completely understood, especially the effect of LD-IR. For a long-time IR was assumed to act mostly on target cells. DCs are one of the immune cells which have been studied the most. Nearly all processes mediated by DCs depend on their differentiation and maturation state. These processes involve migration to peripheral lymphoid organs as well as expression of MHC molecules, co-stimulatory molecules and cytokines resulting in T cell stimulation. Thus, IR-induced changes in the state of DC maturation and activation would affect the whole immune system. Additionally, the radiosensitivity of T cells generally depends on their state of activation. Resting (non-activated) lymphocytes are much more affected by IR than their activated counterparts (Anderson and Warner, 1976). Apart from these targeted effects, in recent decades the indirect (non-targeted) effects of IR such as bystander effects, adaptive response, abscopal effect, and genomic instability, have also been described. The reported non-targeted cellular responses to IR were modulating inflammatory and immune responses (Hildebrandt, 2010). The response of the immune system to IR depends, however, on the dose and the dose rate (Amundson, 2008) as well as on the irradiation quality and the immune cell types (Rödel et al., 2012).

EFFECTS OF HIGH-DOSE IRRADIATION

For this review HD-IR was defined as using single doses of 1 Gy or more. The immunosuppressive effects of HD-IR on the immune system are well known. Epidemiological and patient data show that acute radiation syndrome occurs after whole-body irradiation (WBI) of more than 1 Gy delivered at a high-dose rate (Goans and Waselenko, 2005). Higher radiation doses (>2 Gy) result in a massive killing of blood cells such as lymphocytes (Donnelly et al., 2010) and even in a halting of the proliferation of hematopoietic progenitors, thereby causing hematological crisis (Goans and Waselenko, 2005). Dainiak et al. (2003) stated that the shortage of leukocytes finally leads to suppression of immune function, increasing the risk of infections and impairing wound healing following irradiation with doses more than 2–3 Gy. Besides immunosuppression, one of the most common effects of HD-IR is the induction of pro-inflammatory processes. Long-term studies conducted on blood samples taken from survivors of the atomic bombings of Hiroshima and collected between 1995 and 1997 showed altered tumor necrosis factor alpha (TNF-α), and INF-γ levels which increased with rising doses (Hayashi et al., 2005). Nevertheless, also anti-inflammatory cytokine levels, such as that of IL-10, were increased with increasing dose.

Pecaut et al. (2001) published animal data which correlates with the situation in humans; they showed that HD-IR led to a loss of spleen and thymus mass. They observed decreasing leukocyte and lymphocyte ($CD4^+$ as well as $CD8^+$ subpopulations) numbers in the blood and spleen of mice treated with WBI, applying doses up to 3 Gy.

In vitro investigations showed radiation-induced (20 Gy, ^{137}Cs source) alterations of human DC function, including a less efficient Ag-presenting function (Anton et al., 1998) and a lower capacity of induction of T cell proliferation (Cao et al., 2004). There is evidence that very HD-IR (single dose of 30 Gy) reduces the co-stimulatory receptor expression in immature DCs (Reuben et al., 2004) and down-regulates the expression of CD86 and CD80 on human DCs compromising their ability to capture and present Ag (Cao et al., 2004). These results were supported by Liao et al. (2004), who found, in murine DCs treated with 10 Gy, a down-regulation of proteasome activity which is responsible for the processing of Ags for presentation. Also, alterations in the cytokine release of T cells were found in a co-culture with irradiated human DCs compared to naive (unirradiated) DCs (Cao et al., 2004). These alterations include increased IL-2 and IL-4 levels resulting in a lower capacity of HD-IR treated DCs to promote T cell proliferation efficiently. Liao et al. (2004) found marginally decreased MHC class II and CD86 expression on murine DCs 24 h after HD-IR with 2 or 10 Gy. There are also studies revealing a shift of T_h cells toward T_h2 instead of T_h1 differentiation after HD-IR, paralleled by changes in the cytokine expression profile (Han et al., 2002; Park et al., 2005). It has been suggested that gamma irradiation regulates the level of cytokine-mediators through transcriptional modulation, including signal transducer and activator of transcription (STAT) phosphorylation (Han et al., 2002, 2006). Members of the STAT proteins are involved in the activation of different cytokines and mice with altered STAT genes were shown to have enhanced T_h2 response and consequently, a lack of T_h1-type cytokines. This shift toward T_h2 differentiation after HD-IR may be important – Westermann et al. (1999) suggest that T_h2 cells might play a critical role in the pathogenesis of radiation-induced pneumonitis in rats. Furthermore, various organ-specific autoimmune diseases were reported after fractionated total lymphoid HD-IR (2.5 Gy, 17 times) on mice, probably caused by modification of T cell dependent control of self-reactive T cells (Sakaguchi et al., 1994).

Clinical aspects of high-dose radiation

High-dose ionizing radiation is applied in approximately 50% of all cancer patients and represents a major component of standard cancer therapy (Baskar et al., 2012). Recent investigations have demonstrated that the success in cancer treatment is contingent upon synergy of radiotherapy with the host's immune response. Whereas radioimmunotherapy uses antibodies directed against specific tumor Ags labeled with radioisotopes to deliver the radiation directly to the tumor, new combination approaches may use the effects of local HD-IR alone or especially in combination with further immune stimulation on the tumor cells or vasculature for a more efficient immune response.

High-dose ionizing radiation has been shown to up-regulate stress proteins which can function as neoantigens in target cells. These then might attract APCs or NK cells which have the capacity to recognize stress ligands and to selectively clear damaged or stressed cells by phagocytosis or cytolytic activity (Hallahan et al., 2001; Gastpar et al., 2005; Formenti, 2010). Also, radiation-induced distinct forms of cell death have been shown to be highly immunogenic and has already been suggested to improve the poor inherent capacity of glioma cells to stimulate APC response in

DC vaccination approaches (Ehtesham et al., 2004). It is thought that the exposure of pro-apoptotic proteins like calreticulin triggers the effective recognition and phagocytosis of tumor cells by DCs, leading to CTL response. In the brain, an immunologically privileged area, HD-IR treatment of brain tumors contributes toward the disruption of the blood–brain barrier (Nordal and Wong, 2005) and might synergize with vaccination therapy by facilitating the entry of immune cells. Radiation-induced "danger," death and inflammatory signals as increased MHC class I, Fas/CD95 expression and chemokine release can additionally attract activated T cells (Demaria et al., 2005a; Formenti, 2010).

Clinical results show that standard radiotherapy alone is inadequate in converting the existing immune suppression/tolerance of an established tumor. So far combination of radiotherapy with immunotherapy remains understudied in the clinic, but promising response rates have been achieved in preclinical settings including melanoma, mammary, and colon carcinoma. First clinical trials are underway (Formenti, 2010) and surely more will follow as soon as the clinical application of immunotherapy for cancer (Scott et al., 2004; Omay and Vogelbaum, 2009) moves forward.

In a murine model irradiation of cutaneous melanomas prior to resection led to a reduction in lung metastasis after systemic challenge with untreated melanoma cells (Ma et al., 2011). Similarly, immune-mediated inhibition of lung metastases after treatment with local radiation was described in a murine metastatic mammary carcinoma model using CTLA-4 blockade (Demaria et al., 2005b). Therefore we may assume that the host's immune response against the irradiated tumor might be the central player of the abscopal (outside the target) effects of radiotherapy if negative regulators of immune response are inhibited and the tumor-specific effector T cells target cancer cells at metastatic sites (Formenti, 2010).

EFFECTS OF LOW-DOSE IRRADIATION/CHRONIC LOW-DOSE IRRADIATION

The risk of cancer development or other effects of IR with low doses (<1 Gy; LD-IR) is often extrapolated from the results of epidemiological studies on more highly exposed individuals using the linear, no-threshold (LNT) hypothesis. The LNT model assumes that the radiation-induced risk of cancer is proportional to dose, with no threshold (Puskin, 2009). However, there are many studies indicating that dose–response curves for LD-IR are non-linear, displaying discontinuous dose dependencies, and that they reflect the hypersensitivity of cells to LD-IR not being predictable by extrapolation of the HD-IR response (Kern et al., 1999; Zaichkina et al., 2004; Rödel et al., 2007).

The underlying mechanisms of this discontinuous dose response remain unclear and may result from various overlapping individual processes (Rödel et al., 2010). One possible explanation may be that DNA structures might not be affected as harmfully by LD-IR, thus facilitating a better repair capacity (Rödel et al., 2002). But also epigenetic mechanisms like DNA methylation (Ma et al., 2010) or a differential protein expression (Rödel et al., 2012) may be possible explanations.

Since there is no general definition of LD-IR, we categorized the following paragraph into chronic IR with low-dose single fractions resulting in high total doses (>1 Gy; see Chronic Low-dose Irradiation with Total Doses of More Than 1 Gy) and chronic IR as well as single fraction IR with low total doses (≤1 Gy; see Single Low-dose Irradiation and Chronic Low-dose Irradiation with Total Doses of 1 Gy or Less).

Chronic low-dose irradiation with total doses of more than 1 Gy

In contrast to HD-IR, reports on the effects of LD-IR on the immune system are controversial. There are various animal studies showing that chronic low-dose irradiation with total doses of more than 1 Gy may lead to immunosuppression. Underlying mechanisms were revealed by Yagunov et al. (1998) and comprise a deficiency of hematopoietic stem cells, accelerated cell cycling of bone marrow precursors, or a decreased cell viability of mature blood cells in rats leading to ineffective hemopoiesis. These data were confirmed by studies of Seed et al. (2002) who found a suppression of blood leukocyte levels in dogs. Investigations of the blood samples of 50 radiology workers (age 21–57 years) exposed to long-term LD-IR showed decreased immunological parameters including lower levels of $CD4^+$ T lymphocytes as well as decreased total immunoglobulins (IgA, IgG, IgM) compared with non-exposed volunteers (Godekmerdan et al., 2004).

Other reports reveal immune stimulatory effects of chronic LD-IR in animals, including stimulation of growth rates in mice or rats (summarized in Luckey, 1982) and prolongation of the life span in MRL-*lpr/lpr* mice (Ina and Sakai, 2004). Ina and Sakai (2005) found increased numbers of $CD4^+$ cells as well as CD8 molecules on the surfaces of $CD8^+$ T cells after beginning with continuous WBI of C57BL/6 mice with low doses (1.2 mGy/h). The authors suggest that chronic LD-IR may be able to induce a moderate, but not excessive activation of the immune system.

Single low-dose irradiation and chronic low-dose irradiation with total doses of 1 Gy or less

Reports on single-fraction LD-IR or chronic LD-IR with total doses of 1 Gy or less are also contradictory. Recently Jahns et al. (2011) showed that LD-IR (0.5 and 1 Gy) of human DCs and T cells in co-culture lead to a decrease of T cell proliferation, which may suggest a suppressing effect on the immune system. In contrast, no changes in T cell proliferation were induced by IR of DCs alone. They also found no significant changes in DC cytokine release and reported similar to Shigematsu et al. (2007) no modulation of activation marker or co-stimulatory molecule expression, such as CD1a, CD40, CD80, CD86, ICAM, or MHC class II in murine DCs alone, treated with several irradiation doses (0.02–1 Gy). Hence, the authors suggested that LD-IR has no effect on the maturation of DCs.

In vivo studies demonstrated an increased tumor latency of lymphomas in radiation-sensitive, cancer-prone heterozygous *TRP53* mice (Mitchel et al., 2003) and a reduction of leukocyte adhesion (which was maximal at a dose of 0.3 Gy) in C75BL/6 mice (Arenas et al., 2006) were reported. Furthermore, suppression of metastasis could be confirmed in tumor-bearing rats after 0.2 Gy WBI; this was attended by an increased expression of genes coding for TNF-α and IFN-γ and a decreased expression of transforming growth factor beta (TGF-β; Hashimoto et al., 1999). The authors suggested immune augmentation as a reason

for the antitumor effect of LD-IR. Bogdándi et al. (2010) could demonstrate *in vivo* that low-dose radiotherapy (LD-RT) has an impact on the functional as well as quantitative parameters of murine splenocytes. They found a moderate decrease in the apoptosis of murine DCs after WBI with low doses of 0.01–0.1 Gy. These observations were likewise associated with alterations of the cytokine milieu, including partial down-regulation of IL-4 and IFN-γ. Molecular changes induced by LD-IR show a distinctly different pattern from those caused by HD-IR (Liu, 2003). Liu et al. (2001) showed stimulated expression of CD80 and CD86 on murine APCs after WBI with 0.075 Gy, and increased IL-12 secretion 4 h after IR. Additionally, they were able to demonstrate that the expression of CD28 on T cells was up-regulated and that of CTLA-4 was down-regulated in early time points after LD-IR. Considering the work of these authors together, in reference to suppressed production of IL-10 these findings indicate immunoenhancement by LD-IR. Since an increase of surface molecules on macrophages and an increased secretion of IL-12 results at both LD-IR and HD-IR, Liu (2003) suggests that the different immune reactions resulting from LD-IR compared to HD-IR might primarily depend on changes of T lymphocytes. This hypothesis is supported by studies of Jahns et al. (2011) who found a decrease of CD25, a typical marker for activated T cells, after IR of human DCs and T cells in co-culture after 0.5 and 1 Gy, whereas they reported no impact of LD-IR on DCs alone (see also above). The authors assume that this is an effect of LD-IR on T cells rather than on DCs.

The expression of leukocyte adhesion molecules such as L-selectin (Kern et al., 2000) as well as that of chemokines such as CCL20 (Rödel et al., 2008), all playing a fundamental role in leukocyte trafficking and thus are involved in the induction of inflammatory processes, is also reduced by LD-IR *in vitro*. Shin et al. (2010) reported about elevated levels of IL-3, IL-4, leptin, monocyte chemoattractant protein (MCP)-1, MCP-5, macrophage inflammatory protein 1 alpha (MIP-1α), thrombopoietin, and vascular endothelial growth factor (VEGF) along with slight reduction of IL-12p70, IL-13, IL-17, and IFN-γ in murine peripheral blood sera after chronic LD-IR with a total dose of 0.2 Gy (0.7 mGy/h). According to the authors, this pattern of cytokine release maybe facilitates the differentiation of naive T cells into T_h2, but not into T_h1 cell type.

Further LD-IR studies reported an increased *in vitro* proliferation response to mitogens such as Concanavalin A in lymphocytes, isolated after WBI of mice with 0.02 or 0.75 Gy (Ibuki and Goto, 1994; Liu et al., 1994a). Liu et al. (1994b) also reported a temporary stimulation of the protein kinase C activity of mouse splenic tissue and lymphocyte subpopulations after WBI with 0.75 Gy X-rays. In general, data indicate that immunoenhancement is restricted to a very narrow range of doses and is dependent on investigated endpoints (Safwat, 2000).

There also is evidence that exposure to LD-IR can result in radio-adaptation (reviewed in Jolly and Meyer, 2009). As a consequence of this process, known as "radiation hormesis," cells are more resistant to subsequent radiation events (Bhattacharjee and Ito, 2001; Mitchel, 2006).

With the current knowledge no threshold dose can presently be defined for the immune-enhancing effects of irradiation (Safwat, 2000). Variations due to the tested endpoints, animal species or the radiation dose rates applied may additionally complicate those investigations.

Clinical aspects of low-dose radiation

The clinical acceptance of LD-RT varies worldwide (Seegenschmiedt et al., 2004). Because of reports from the 1960s and epidemiological data about a possible carcinogenic late risk, especially of leukemia, the application of LD-RT is still a subject of controversial debate and less accepted in many countries (Leer et al., 1998). But in several European countries, LD-RT is practiced for the treatment of a variety of inflammatory and painful joint diseases (Hildebrandt et al., 2003; Seegenschmiedt et al., 2004), such as heel spurs (Heyd et al., 2007), osteoarthritis (Hildebrandt et al., 2000) or tendonitis (Adamietz et al., 2010). Total doses of LD-RT comprise 5–10% of those given to tumor patients, assuming different radiobiological mechanisms triggered by LD-RT compared with high-dose radiotherapy (HD-RT; see remarks above). In animal models it was demonstrated that repeated LD-RT can attenuate the pathology of autoimmune diseases. In collagen-induced arthritis mice, used as a model of rheumatoid arthritis, a suppression of IL6 and IL17 production and up-regulation of T_{regs} was demonstrated after repeated irradiation with 0.5 Gy (Nakatsukasa et al., 2010). LD-RT may also have a potential therapeutic effect for the attenuation of the pathology of other autoimmune inflammatory diseases, such as multiple sclerosis (MS). In experimental autoimmune encephalomyelitis mice, an established animal model of human MS, suppression of pro-inflammatory cytokines, reduction of $CD8^+$ CTLs, and induction of T_{regs} could be observed after repeated irradiation with 0.5 Gy (IR once per week for 4 weeks; Tsukimoto et al., 2008).

However, as long as there is insufficient knowledge about the precise biological effect of LD-IR, the old fears of tumor induction will remain. Currently, it is intended to investigate the mechanisms and biological impact of LD-IR on modulation of inflammatory response in the context of a sub project of the European project DoReMi (FP7-249689). Furthermore, several patterns of care studies as well as clinical investigations of anti-inflammatory and analgesic LD-RT in Germany are being conducted (summarized in Rödel et al., 2012). The results of all these investigations may help to gain reconsideration of LD-RT as an alternative option for the treatment of benign diseases, also in the countries where LD-RT is still less accepted.

CONCLUSION AND OUTLOOK

The effects of whole-body HD-IR on the immune system are well characterized, leading in the end to substantial immunosuppression. Underlying molecular mechanisms are inhibition of Ags-presenting function (Anton et al., 1998) by down-regulation of co-stimulatory receptors such as CD80 and CD86 in immature DCs (Reuben et al., 2004), alterations in cytokine release (Han et al., 2006) and radiation-induced depletion or proliferation stop of progenitor cells (Goans and Waselenko, 2005). A consolidated overview of the interactions of DCs with T cells and the effect of whole-body HD-IR on this is given in **Figures 1A,B**. A novel application of IR has emerged in the partnership of localized HD-RT with immunotherapy (Formenti, 2010). Further investigations

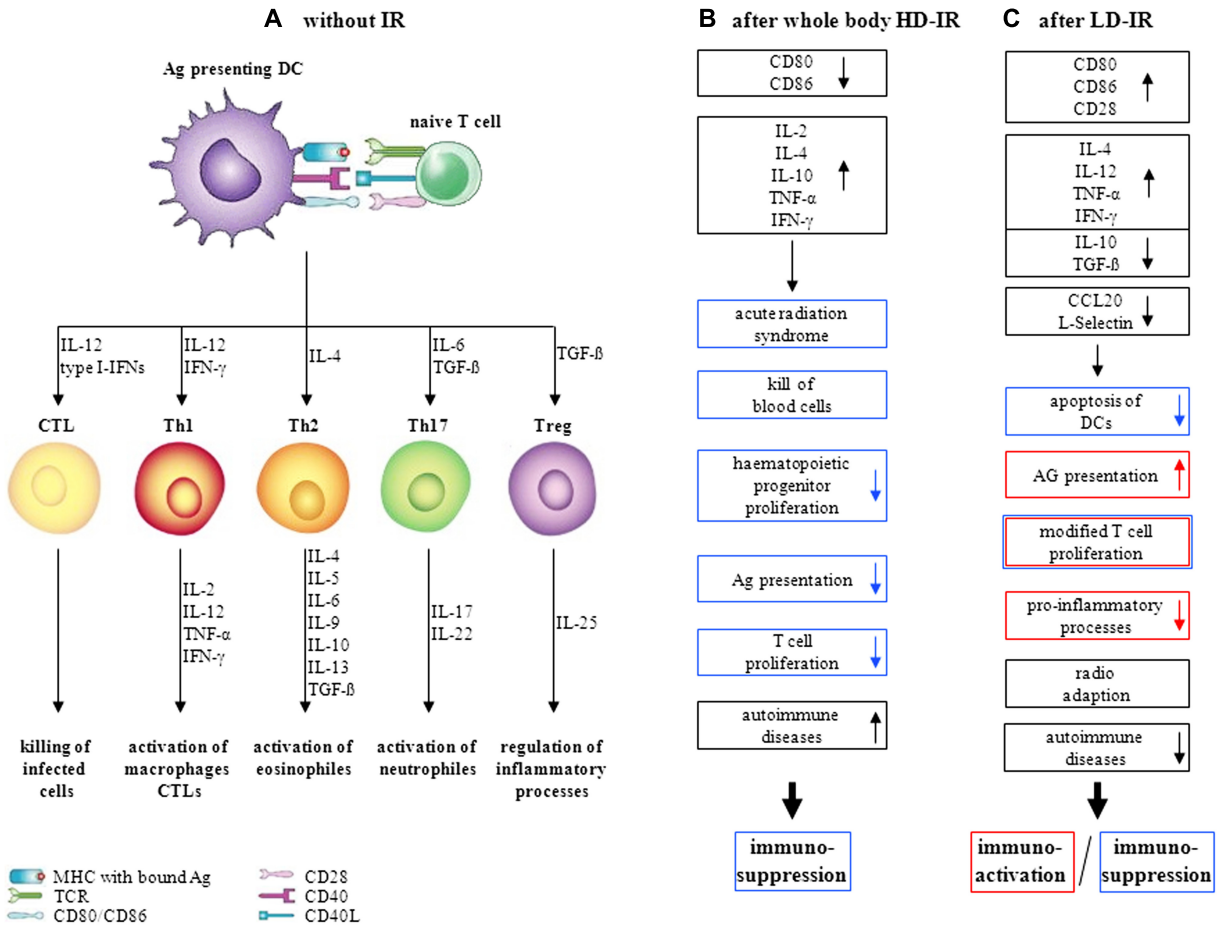

FIGURE 1 | Dendritic cell–T cell interactions and its variations upon irradiation, **(A)** without irradiation (modified after Moser and Leo, 2010), **(B)** influence of whole-body HD-IR, **(C)** influence of LD-IR.

regarding schedules, fractionation regimens, combination with chemotherapy, and the contribution of the innate immune system are urgently needed to achieve an optimal radiation-induced immunogenicity.

Until now, no consistent position exists with reference to the effects of LD-IR on the immune system. The observed effects are strongly dependent on the range of dose and dose rate as well as on the animal species and even the strain studied. The precise molecular mechanisms underlying single or chronic LD-IR are still a matter of contradictory discussion. As already mentioned above in more detail, on the one hand there are studies indicating immunosuppression, on the other hand studies suggesting stimulation of the immune system. The effect of LD-IR on the interactions of DCs and T cells is summarized in **Figure 1C**. Since LD-RT seems to have little or no effect on immune cells themselves, but rather on the interactions of DCs and T cells, further investigations will have to be made focusing on these findings. Due to several effects interfering with each other, *in vivo* experimental data often show very donor-specific results, necessitating the establishment of reliable *in vitro* models. These should consist of e.g. different immune cell types, ideally in three-dimensional configuration, to reveal the underlying mechanisms more precisely.

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Non-targeted effects of photon and particle irradiation and the interaction with the immune system

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Ionizing irradiation is an important clinical approach to treat solid tumors. Modern radiation technologies aim to selectively kill tumor cells and protect the surrounding normal tissue. The standard paradigm for radiation effects in cellular systems involves damage of the DNA including DNA double-strand breaks, which are considered as most effective in destroying tumor cells. Due to their enhanced physical and radiobiological properties, high-linear energy transfer radiation qualities are of special interest in tumor therapy. Future radiation therapy strategies aim to utilize carbon ions to effectively treat highly aggressive tumors. More recently, evidence is emerging for non-DNA targeted effects of radiation, including mutations, chromosomal aberrations, and changes in gene expression, which can occur in cells that were not directly exposed to radiation. Radiation oncologists are only gradually beginning to appreciate the clinical relevance of radiation-induced bystander effects, genomic instability, and abscopal effects. Since these effects are sensed by the immune system, a combination of immunotherapy and irradiation presents a new therapeutic opportunity in the future.

Keywords: immune system, LET, bystander effect, abscopal effect, genomic instability

INTRODUCTION

The long-standing conventional paradigm for radiobiology for radiation effects in cellular systems has involved DNA double-strand breaks (DSBs) as the triggering lesions leading to mutation, cell death, and transformation. Depending on the linear energy transfer (LET) and dose, ionizing radiation causes a variety of different DNA lesions, including single- and double-strand breaks, DNA–protein cross-links, and DNA base damages (Bouquet et al., 2006). Ionizing radiation causes DNA damage either by a direct attack or indirectly via the production of free radicals and reactive oxygen species (Rothkamm and Lobrich, 2003). DNA DSBs are most fatal for cells because they can induce a complete loss or rearrangement of genetic material which results in cell death (Lobrich et al., 2005).

In recent years, high LET irradiation is gaining greater interest in tumor therapy, due to their improved physical and radiobiological properties. It is well-known that a spatial focused deposition of high energy by heavy ions can cause complex damage types (Jeggo et al., 2011). The oxygenation status has been identified as a pivotal factor for achieving locoregional tumor control by radiotherapy (Vaupel and Harrison, 2004; Vaupel et al., 2011).

The oxygen enhancement ratio (OER) decreases with increasing LET (Ferguson et al., 2000). This suggests a potential clinical advantage of high-LET radiotherapy with heavy ions compared to low-LET photon irradiation. Also mutations in the tumor suppressor gene p53, which are frequently found in different tumor entities, exert negative effects on the clinical outcome of radiation therapy. Irrespectively of the p53 and oxygenation status of carbon ions have shown efficacy in gliomas, human tongue, and lung cancer cell lines (Jakob et al., 2011). Therefore, future radiation therapy strategies aim to utilize carbon ions to treat highly aggressive tumors.

Recently, non-targeted irradiation effects that are not a direct consequence of the initial lesions produced by damages of the cellular DNA have been reported (Shiraishi et al., 2008). Since these effects are dependent on a functional immune system, it is important to protect the immune system against irradiation-induced damage. At present no clinically applied therapeutic options exist to protect the patient's immune system. Most chemotherapeutic agents that are used in combination with radiotherapy to treat cancer exert immunosuppressive activities that might also suppress radiation-induced immunostimulatory effects (Shiraishi et al., 2008). Up to date, the effects of high-LET radiation on immune function have not been studied in detail. It is noteworthy that, unlike photon irradiation, particle irradiation may suppress the metastatic potential of cancer, suggesting that it may modify anti-tumor immunity via this treatment modality. Since high-LET cancer treatment using charged particles is performed only at very few sites worldwide, only little experimental information's are available yet (Cai et al., 2009).

RADIATION INDUCED NON-DNA TARGETED EFFECTS

Bystander effects, abscopal effects, and genomic instability are three phenomena which will lead to a paradigm shift in radiation biology (**Figure 1**). While the mechanisms underlying these effects are still not completely understood, it is very apparent that their implications are much wider than the field of classical radiobiology. The major adverse consequences caused by

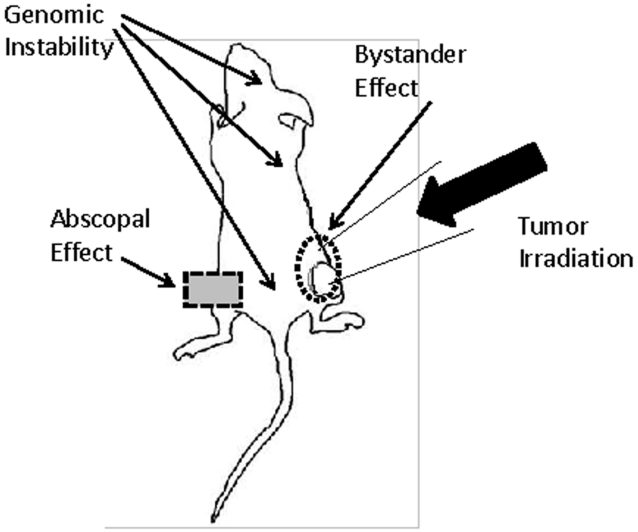

FIGURE 1 | The graph shows the different potential routes by which bystander, abscopal effects, and genomic instability may affect the outcome of radiation therapy in a tumor mouse model. Radiation-induced DNA damage in the tumor can be amplified by bystander signals in cells residing in close proximity to the irradiation field. In contrast, abscopal effects and genomic instability exert distant and systemic effects.

irradiation, such as initiation of secondary malignancies, are attributed to an inadequate repair in DNA damage in normal and tumor tissues. However, new studies have shown damage in cells that were not exposed to irradiation. These findings are explained by a potential interplay of irradiated and non-irradiated cells.

BYSTANDER EFFECT

Since the discovery of X-rays in 1895, it was assumed that the deleterious effects of ionizing radiation such as mutations and carcinogenesis are mainly due to a direct damage of the DNA. Radiation-induced bystander effects are defined as biological effects in cells that are in close proximity to cells that have been irradiated (Hei et al., 2011). In 1992, Nagasawa and Little reported about an experimental system in which after exposure of 1% of the cells to densely ionizing particles, sister chromatid exchanges were observed in approximately 30% of the cell population (Nagasawa et al., 2003). The damage that occurred in non-irradiated cells has been described as the "bystander effect." Unique microbeam facilities with the capacity to target subcellular areas within a cell such as the nucleus or the cytosol with a defined number of protons, photons or α-particles with high precision, play a pivotal role in a better understanding of the molecular mechanism of bystander effects (Hei et al., 2011). Using a microbeam in Columbia University, Wu et al. (1999) reported that a selective irradiation of the cytoplasm with four alpha particles results in killing of 10% of the cells and in increased gene mutations in the nucleus. It is speculated that either components of the cytoplasm or extracellular located components might be responsible for the observed increase in gene mutations in the nucleus.

Previous studies implicate that pro-inflammatory cytokine signaling is associated with *in vivo* chromosomal instability (Lorimore et al., 2008) and the involvement of COX-2 in the bystander response *in vitro* (Hei et al., 2008). The study of Lorimore et al. (2011) showed a connection of the bystander effect and the chromosomal instability that are mediated by signals involving COX-2 the initial enzymatic step in the metabolism of arachidonic acid to prostaglandins (Lorimore et al., 2011). Since NFκB is an important transcription factor for many signaling pathways including COX-2, it is likely that NFκB also participates in the bystander effect. There is clear evidence that alpha particle irradiation up-regulates the binding activity of NFκB via direct and bystander mediated effects (Zhou et al., 2008). Immune cells accumulate within and around tumors and cooperate with each other by utilizing specific cytokines. These results provide evidence that the COX-2 signaling pathway, which is essential in mediating a cellular inflammatory response, may be a critical signaling event for producing a bystander effect.

Importantly, *in vivo* experiments have demonstrated that cells of the innate immune system can be activated by ionizing radiation to produce pro-inflammatory mediators of genomic instability (Lorimore et al., 2008). Mutou-Yoshihara et al. (2012) showed that suppression of cytokine production was induced in the surrounding non-irradiated cells via the bystander effect (Mutou-Yoshihara et al., 2012). Bystander responses have been measured after exposures as low as a single proton or helium ion delivered to an individual cell. An important aspect is that the non-DNA targeted responses saturate with increasing dose to a single target cell (Prise et al., 2003).

The following conclusions can be drawn from experiments analyzing bystander effects: irradiation of the cytoplasm can induce genetic effects in the nucleus that was not directly exposed to radiation. It appears that the traversal of high-LET particles through the cytosol is more efficient than through the nucleus (Morgan and Sowa, 2009). Presumably, NF-κB, COX-2, and reactive oxygen species are involved in cytoplasmic irradiation-induced bystander effects.

ABSCOPAL EFFECTS

The term "abscopal" is derived from the Latin prefix "ab," meaning "away from," and the Greek word "scopos," meaning "target." An abscopal effect has been defined as a reaction of cells within an organism that had not been directly exposed to irradiation, but cause tumor regression of the non-irradiated tumors (Postow et al., 2012). These responses indicate that the target size of the responding tissue is much larger than the irradiated field.

It is assumed that the abscopal effect is mainly mediated by an activation of the immune system via cytokines. The abscopal effect refers to distant effects observed after local radiation therapy (Shiraishi et al., 2008). Therefore, some investigators argue that abscopal effects should be termed as "distant bystander effects." Although the immune system appears to be involved, the exact mechanisms of action of abscopal effects remain to be elucidated (Shiraishi et al., 2008).

Immune-mediated abscopal effects have been observed in mice with 67NR tumors after radiotherapy by studying the maturation status of dendritic cells (Formenti and Demaria, 2009). Radiation therapy seems to augment the ability of dendritic cells to capture and present tumor antigens and thereby mediating an anti-tumor-specific cytotoxic T cell response. Partial lung radiation experiments in rats demonstrated increased expression of tumor necrosis factor alpha (TNF-α), interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and transforming growth factor beta (TGF-β) in the shielded part of the lung that is adjacent but outside of the irradiation field (Langan et al., 2006). The generation of a sustained anti-tumor immune response at the irradiated tumor site, will not only determine the overall response of the irradiated tumor but also mediates an "abscopal effect" on the tumor sites outside of the treatment field (Formenti and Demaria, 2009). Apart from the activation of the immune system abscopal effects induce apoptotic signaling pathways. The irradiation of one tumor site resulted in the release of circulating tumor antigens and/or inflammatory factors that may then mediate an augmented immune response against non-irradiated, malignant lesions that express the same tumor antigens. It has been shown that local radiotherapy increases the activity of natural killer cells (Morgan and Sowa, 2007) that, as a result, can induce regression of non-irradiated tumors (Shiraishi et al., 2008). Importantly, irradiation of normal tissues of the mice did not induce abscopal anti-tumor immune responses. These findings suggest that radiation-induced stress and cell death responses of tumor but not normal cells are a prerequisite for the induction of specific anti-tumor immunity.

Clinical reports of abscopal effects after radiotherapy have been shown in different tumor types, including lymphoma, melanoma, and a variety of carcinoma (Hiniker et al., 2012; Postow et al., 2012). Abscopal effects are not restricted to ionizing irradiation but also have also been observed after surgery, hyperthermia, and laser therapy (Martin et al., 2011). It is assumed that abscopal effects require secreted factors to mediate systemic immune effects (Morgan and Sowa, 2009).

A better understanding of abscopal effects might improve the clinical outcome of radiotherapy.

GENOMIC INSTABILITY

Although ionizing radiation is known to induce secondary malignancies in many tissues, the underlying mechanisms at the cellular and molecular level are not completely understood. An attractive hypothesis is that radiation induces "genomic instability" in a subpopulation of cells harboring multiple mutational events that are required for the transformation of a normal tissue into an invasive tumor (Huang et al., 2003). DNA damage, as the biological consequence of irradiation, is observed within minutes post exposure. However, indirect effects of irradiation including genomic instability and carcinogenesis occur after months and years following irradiation (Morgan, 2003). Genomic instability is defined as an increased rate of acquisition of alterations in the genome, manifesting as chromosomal aberrations, micronucleus formation, gene mutations, and aneuploidy (Morgan, 2003). The current hypothesis to explain radiation-induced genomic instability is that radiation initiates sublethal damage in one cell that is communicated to other cells and as a result causes a destabilization in the genome (Huang et al., 2003).

Lorimore et al. (1998) demonstrated genetic instability in the surviving fraction of shielded, non-irradiated tumor cells residing in close proximity to cells exposed to alpha particles. These data clearly demonstrated that genomic instability could be induced by an interaction of irradiated and non-irradiated cells. Radiation-induced chromosomal instability appears to involve a significant epigenetic component and a link between non-targeted bystander effects resulting in chromosomal instability in non-irradiated cells (Huang et al., 2003; Lorimore et al., 2008). Intercellular signaling, production of cytokines, and free radicals are features of inflammatory responses that have the potential for both bystander-mediated effects and genomic instability (Lorimore et al., 2008).

The severity of genomic instability is influenced by the LET (Limoli et al., 2000, Smith et al., 2003). While there is a clear dose response for direct radiation effects immediately following exposure, there is no typical dose response for the delayed indirect effects of exposure to irradiation (Limoli et al., 2000). However, it is known that after high-LET radiation chromatid aberrations are more prevalent than chromosome aberrations.

As a clinical implication, genomic instability can serve as a marker for an increased risk to develop secondary malignancies after radiation therapy. Of particular interest is the observation that transmissible instability can be induced in somatic cells from normal individuals by exposure to ionizing radiation, leading to a persistent enhancement in the rate at which chromosomal aberrations arise in non-irradiated cells after many generations of replication (Little et al., 2002).

CONCLUSION

Current anti-cancer modalities such as surgery, chemo-, and radiation therapies have only limited success in the cure of solid tumors in advanced stages. During the past decade progress has been made in the understanding of the fundamental mechanisms and biological significance of the immune system in the control of cancer. The major challenge in the field is to understand the various molecular mechanisms involved in non-DNA-targeted irradiation effects that counteract tumor-related signaling pathways. Irradiation-induced abscopal and bystander effects have been shown to stimulate the immune system of cancer patients and thus might exert beneficial effects. A better understanding of the immune-modulatory effects of heavy-ion beam treatment will help to develop innovative and more effective strategies for charged-particle therapy in clinical settings.

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Dying cell clearance and its impact on the outcome of tumor radiotherapy

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The induction of tumor cell death is one of the major goals of radiotherapy and has been considered to be the central determinant of its therapeutic outcome for a long time. However, accumulating evidence suggests that the success of radiotherapy does not only derive from direct cytotoxic effects on the tumor cells alone, but instead might also depend – at least in part – on innate as well as adaptive immune responses, which can particularly target tumor cells that survive local irradiation. The clearance of dying tumor cells by phagocytic cells of the innate immune system represents a crucial step in this scenario. Dendritic cells and macrophages, which engulf, process and present dying tumor cell material to adaptive immune cells, can trigger, skew, or inhibit adaptive immune responses, respectively. In this review we summarize the current knowledge of different forms of cell death induced by ionizing radiation, the multi-step process of dying cell clearance, and its immunological consequences with special regard toward the potential exploitation of these mechanisms for the improvement of tumor radiotherapy.

Keywords: Radiotherapy, apoptosis, necrosis, necroptosis, senescence, mitotic catastrophe, dying cell clearance

Radiotherapy is an essential treatment option for various types of cancer due to its profound potential to kill malignant cells and to abrogate clonogenic survival. Ionizing radiation, including X-rays, gamma rays, and heavy ions, induces damages in the cellular DNA, which lead to the activation of a highly sophisticated and finely tuned signaling cascade designated the DNA damage response (DDR) and – depending on the extent of damage – to transient or permanent cell cycle arrest, and/or cell death, respectively.

THE DNA DAMAGE RESPONSE

The DDR is orchestrated by two conserved protein kinases, ATM (ataxia telangiectasia, mutated) and ATR (ATM and Rad3 related), which in concert control the cellular response to DNA double strand breaks (DSBs) and single-stranded (ss) DNA (Smith et al., 2010). ATM is recruited to DSBs by the Mre11–Rad50–Nbs1 (MRN) complex and phosphorylates the histone H2 variant H2AX, thus generating an interaction platform for other DDR constituents required for DSB repair (Shiloh, 2006). Concomitantly, ATM activates the DNA damage checkpoint by initiating the resection of the broken strand(s) leading to the generation of ssDNA, a DNA damage repair intermediate which, in turn, activates ATR kinase (Hurley and Bunz, 2007). ATR and ATM phosphorylate and activate two respective effector kinases termed Chk1 and Chk2. Collectively, these four protein kinases instigate multiple cellular pathways culminating in transient or permanent cell cycle arrest, DNA damage repair, and/or cell death (Jackson and Bartek, 2009). A crucial target of the ATM/ATR cascade is the tumor suppressor protein p53, a transcription factor, whose function is lost or compromised in more than 50% of all cancers (Levine, 1997). Under steady-state conditions, p53 protein is sustained at very low levels, since it is continuously being ubiquitinylated by the Mdm2 ubiquitin ligase and thus targeted for subsequent proteolytic degradation via the 26S proteasome. Yet, phosphorylation of p53 by kinases of the ATM/ATR pathway induces the dissociation of p53 from MDM2, and the subsequent reduction in p53 ubiquitinylation leads to a deceleration of proteasome-mediated degradation and hence a stabilization of p53 (Meek, 2009). Depending on the cell cycle phase and the type as well as the extent of DNA damage, p53 can function as a modulator of the DNA damage repair process, or as a transcriptional activator of genes, which are involved in transient or permanent cell cycle arrest, and/or cell death, respectively (Sengupta and Harris, 2005).

APOPTOSIS

Apoptosis is one type of programed cell death. It is commonly considered to be the prevalent form of cell death underlying daily tissue regeneration and renewal. Morphologically, it is characterized by cellular shrinkage, chromatin condensation, nuclear fragmentation, and membrane blebbing (**Figure 1**). In response to radiotherapy, apoptosis is predominantly observed in cells of the hematopoietic system, and it is critically regulated by the mitochondrial, intrinsic death pathway (Rudner et al., 2001; Eriksson and Stigbrand, 2010). The pivotal events in this context involve the permeabilization of the mitochondrial outer membrane (MOMP) and the release of various proteins, including cytochrome *c*, from the mitochondrial intermembrane space into the cytosol, thus stimulating the formation of the apoptosome and the activation of procaspase-9. Activated caspase-9, in turn, triggers the activation of downstream effector caspases, which execute the final stages of apoptosis and the disintegration of the cell (Taylor et al., 2008). Crucial regulators of

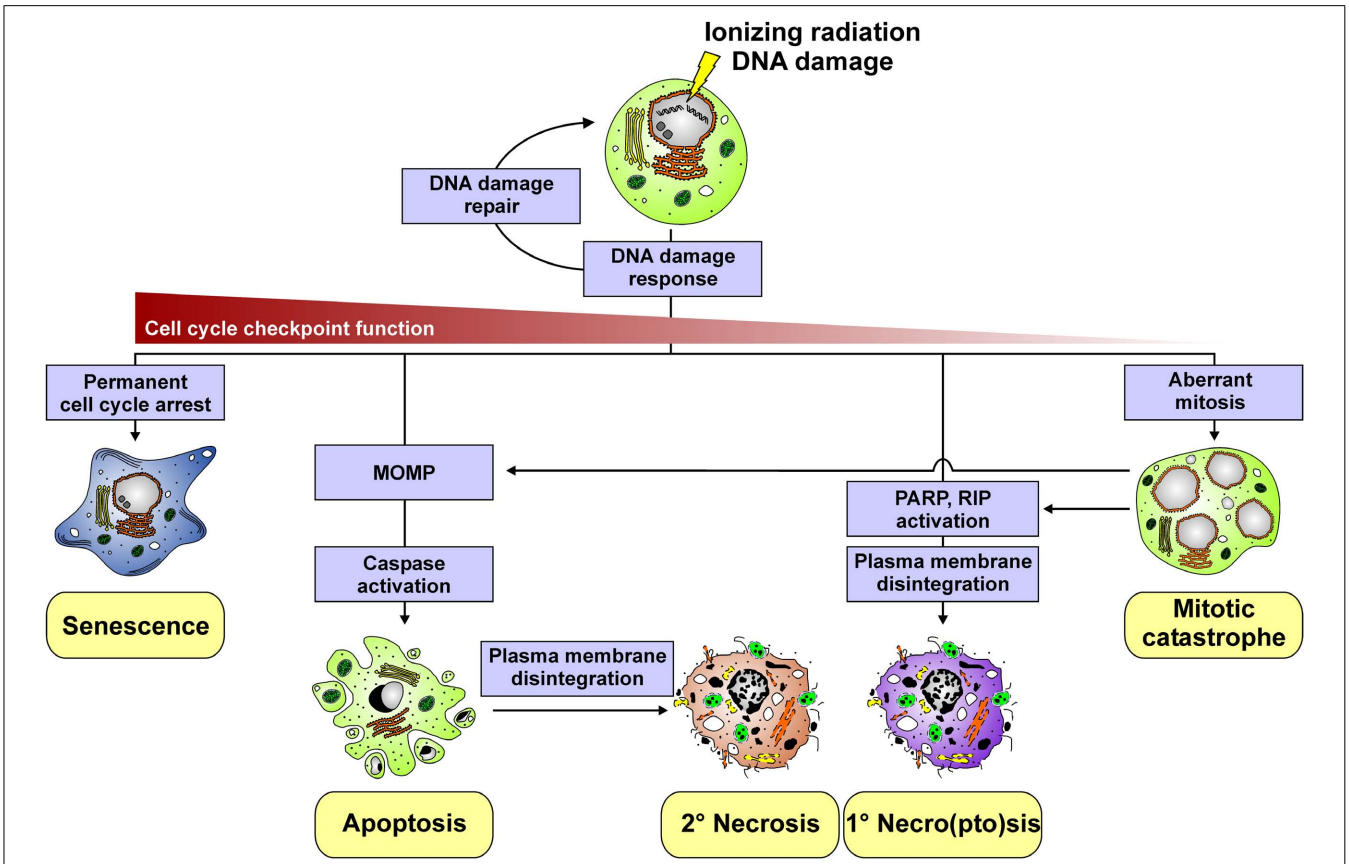

FIGURE 1 | Different cell death modalities induced by ionizing radiation.

mitochondrial permeabilization and cytochrome *c* release are proteins of the B cell lymphoma-2 (Bcl-2) family, including the pro-apoptotic BH3-only (e.g., Puma) and the anti-apoptotic (e.g., Bcl-2) family members, which control MOMP via their impact on the oligomerization of the effector members Bax and Bak (Youle and Strasser, 2008). p53 links this signaling pathway to radiation-induced DNA damage by transactivating the expression of pro-apoptotic Bcl-2 family members, such as Puma and Noxa (Sengupta and Harris, 2005). Apart from the intrinsic pathway, apoptosis can be induced extrinsically via the ligation of death receptors, such as CD95 or the TRAIL receptors 1 and 2, by their corresponding ligands (Debatin and Krammer, 2004). Receptor clustering leads to recruitment and activation of the pro-caspases-8 and -10, triggering of the caspase cascade, and thus to apoptosis. Various proteins of the death receptor pathway are known to be upregulated in response to ionizing radiation (p53-dependently as well as -independently) and thus might contribute to apoptosis induction (Belka et al., 1998; Haupt et al., 2003). However, the intrinsic death pathway appears to be the major signaling mechanism of irradiation-induced apoptosis (Rudner et al., 2001). Notably, although p53 essentially controls the expression of various key regulators of apoptosis, irradiation-induced apoptosis can be observed in cancer cells with defective p53 function. Here, mechanisms, such as p63-/p73-dependent induction of pro-apoptotic Bcl-2 members and p53-independent stimulation of death receptor signaling have been described to be involved (Afshar et al., 2006; Wakatsuki et al., 2008).

NECROPTOSIS AND NECROSIS

In tumor cells of epithelial origin, which reveal limited apoptosis induction in response to radiotherapy, radiation-induced DNA damage – especially when combined with hyperthermia - has been reported to stimulate necroptosis (Mantel et al., 2010; Schildkopf et al., 2010; **Figure 1**). The crucial events in this context include the hyperactivation of the DNA repair enzyme poly-ADP-ribose-polymerase (PARP) and the subsequent and substantial depletion of intracellular ATP levels (Vandenabeele et al., 2010; Vanlangenakker et al., 2012). This – in a so far poorly understood way – couples to the activation of receptor interacting protein (RIP), the formation of the high-molecular weight necrosome, and finally the execution of necroptosis as characterized by the production of reactive oxygen species (ROS), lipid peroxidation, swelling of organelles, rupture of the plasma membrane, and release of intracellular contents (Vandenabeele et al., 2010). Apart from necroptosis, ionizing radiation – particularly when applied in high single doses during ablative radiotherapy – can trigger necrosis, an accidental, uncontrolled form of cell death as a consequence of excessive physico-chemical stress (Vandenabeele et al., 2010). Moreover, secondary necrosis can occur when apoptotically

dying cells are not properly and timely engulfed by neighboring cells or professional phagocytes, respectively (Munoz et al., 2010a; Silva, 2010). This is of specific relevance when the local phagocytic compartment is overwhelmed due to massive apoptosis induction in the context of tumor radiotherapy. In both cases the integrity of the plasma membrane is lost and cellular contents, often in an oxidatively modified and partially degraded form, leak into the surrounding tissue.

MITOTIC CATASTROPHE

Mitotic catastrophe is a form of cell stress, which occurs in the context or as a result of aberrant mitosis owing to uncoordinated or improper entry into mitosis. It has been assigned to be the major death mechanism in response to irradiation-induced DNA damage of cells with defects in cell cycle checkpoints and impaired DNA repair mechanisms (e.g., cells with defective p53). In the course of mitotic catastrophe the formation of giant cells can be observed with aberrant nuclear morphology, centrosome hyperamplification, and multiple nuclei, and/or several micronuclei (**Figure 1**). These cells may survive for days, transit into senescence, or die by delayed apoptosis or delayed necro(pto)sis, respectively (Eriksson and Stigbrand, 2010).

SENESCENCE

Radiation-induced senescence is a condition of permanent cell cycle arrest, which can be observed in cells, where DNA damage is excessive and cell cycle checkpoints are still intact (**Figure 1**). The hallmarks of cellular senescence include an enlarged and flattened cellular morphology, increased granularity, upregulation of cyclin-dependent kinase inhibitors, such as $p16^{INK4a}$, $p21^{Waf1}$, and $p27^{Kip1}$, and positive staining for the senescence-associated β-galactosidase (SA-β-Gal). The key players in this scenario are p53 and pRB, yet senescent phenotypes have also been reported in the absence of functional p53 (Nardella et al., 2011). Senescent cells exit the cell cycle and do not further undergo cell division, but may remain metabolically active. Interestingly, they have been shown to release factors, which can be tumor suppressing as well as tumor promoting, and which can alter the immune response (Kuilman and Peeper, 2009; Coppe et al., 2010).

DYING CELL CLEARANCE

Higher organisms have developed impressively efficient mechanisms of dying cell clearance as can be appreciated from the fact that dying cells are rarely to be observed in normal tissues, although – according to careful estimations – approximately one million cells per second undergo apoptosis in the course of everyday tissue turnover and regeneration (Reed, 2006). These cells are swiftly phagocytosed and degraded, and the immune system had to acquire the capability to distinguish them from cells, which are dying in the context of an infection. Whereas several types of amateur phagocytes, including fibroblasts, endothelial cells, and mesothelial cells, have been described, professional phagocytes, such as macrophages and dendritic cells (DCs), apparently play a crucial role in this scenario, particularly when the local amateur phagocytic compartment is overwhelmed (Lauber et al., 2004; Ravichandran, 2011; Wagner et al., 2011). Macrophages and DCs serve as professional dying cell scavengers with discrepant tasks. While tissue resident macrophages can proficiently engulf and degrade huge amounts of dying prey cells, DCs act as sentinels, which are highly potent in presenting and cross-presenting antigens, thus stimulating, skewing, or inhibiting adaptive immune responses, respectively (Steinman, 2007; Biswas and Mantovani, 2010).

The sophisticated process of dying cell removal involves distinct phases: phagocyte recruitment, prey cell engulfment, and the post-phagocytic response (Ravichandran, 2010; **Figure 2**).

PHAGOCYTE RECRUITMENT

Phagocyte recruitment is accomplished by the release of soluble "find-me" signals from the dying cell (Munoz et al., 2010b; Peter et al., 2010; **Figure 3**). In case of apoptotic cells, these "find-me" signals comprise different molecular entities. As such, nucleotides, like ATP and UTP, have been described to trigger monocyte/macrophage recruitment via the purinergic $P2Y_2$ receptor (Elliott et al., 2009). Released proteins, including a covalently linked dimer of ribosomal protein S19 (dRPS19), endothelial monocyte activating polypeptide II (EMAP-II), soluble fractalkine (sFKN), and the ectodomain of the IL-6 receptor (sIL-6R) also contribute to phagocyte attraction and involve their cognate receptors CD88, CXCR3, CX_3CR1, and CD130 (Nishiura et al., 1998; Hou et al., 2006; Chalaris et al., 2007; Truman et al., 2008). Phospholipids, such as lysophosphatidylcholine (LPC) and sphingosine-1-phosphate (S1P), complement the molecular spectrum of apoptotic cell-derived "find-me" signals and have been reported to stimulate monocyte/macrophage chemotaxis via the G-protein coupled receptor G2A or the family of the S1P receptors (S1PR1–5), respectively (Lauber et al., 2003; Gude et al., 2008; Peter et al., 2008). Finally, also microblebs carrying ICAM-3 have been assigned a role in phagocyte recruitment by apoptotic cells (Segundo et al., 1999; Torr et al., 2012). Amongst the mediators being liberated during apoptosis, lactoferrin (LTF) plays a special role. Its *de novo* expression and release by apoptotic cells have been described, but in contrast to the "find-me" signals described above, LTF exerts the function of a granulocytic anti-attraction or "keep-out" signal, since it exerts a deterring effect on neutrophils and eosinophils and prevents them from invading the area of apoptotic cell death (Bournazou et al., 2009).

Of note, all these factors are released and/or secreted while the plasma membrane is still intact – a crucial hallmark of bona fide apoptosis, which fundamentally discriminates it from necro(pto)sis. In the course of the latter, the disintegration of the plasma membrane liberates the intracellular contents, and the most abundant factors within this "releasate" act as endogenous danger signals or damage-associated molecular patterns (DAMPs) that "inform" the immune system about the tissue damage (Matzinger, 1998; Lotze et al., 2007). The majority of danger signals have been reported to exert pleiotropic effects on the immune system, with phagocyte recruitment being one of them. As such, high mobility group box 1 protein (HMGB-1) and its close relative hepatoma-derived growth factor (HDGF), peroxiredoxins (Prx), members of the S100 protein family, and heat shock proteins (HSPs) can stimulate monocyte/macrophage attraction by engaging the Toll-like receptors 2 and 4 (TLR2/4), the receptor

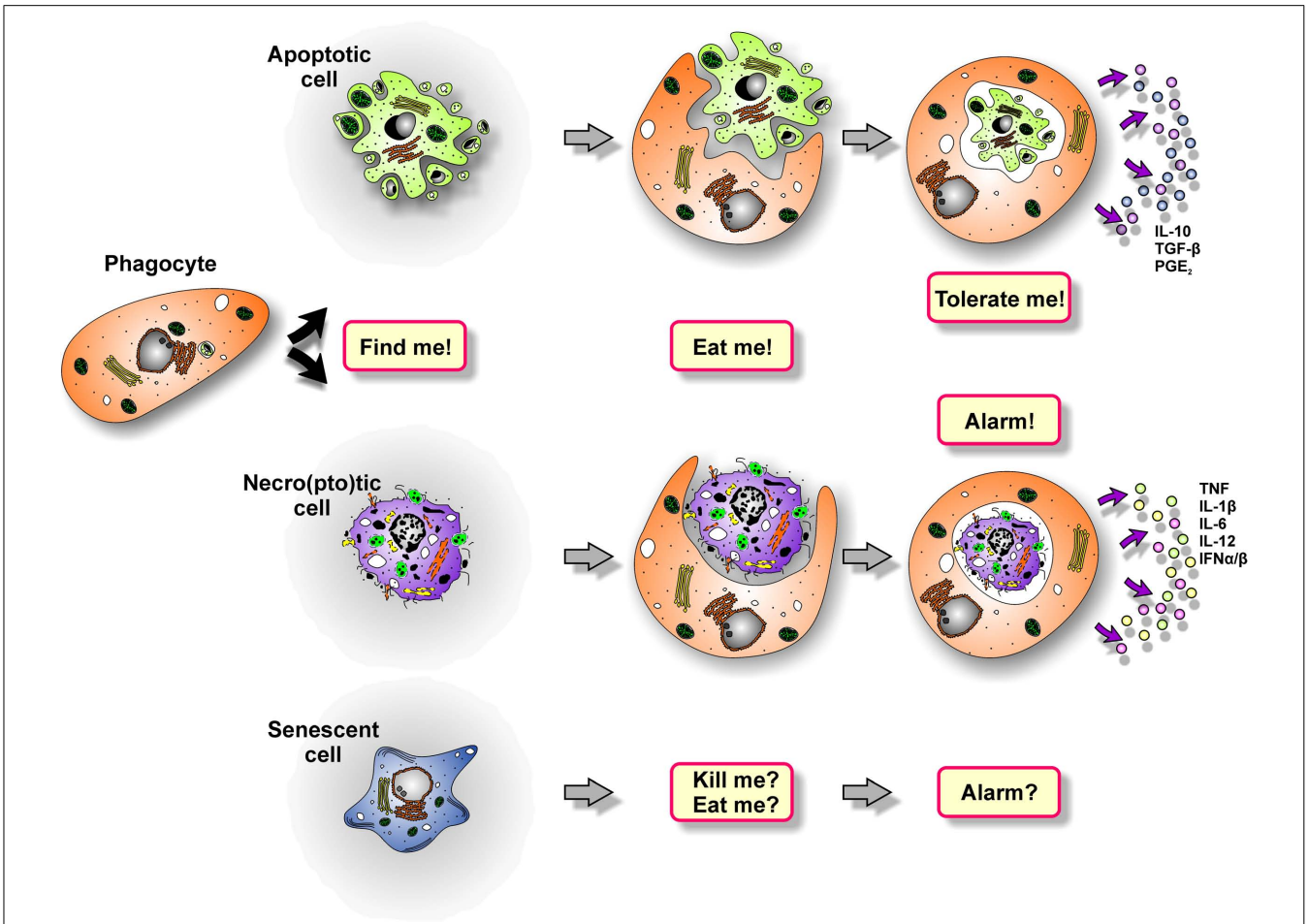

FIGURE 2 | The multi-step process of dying cell clearance.

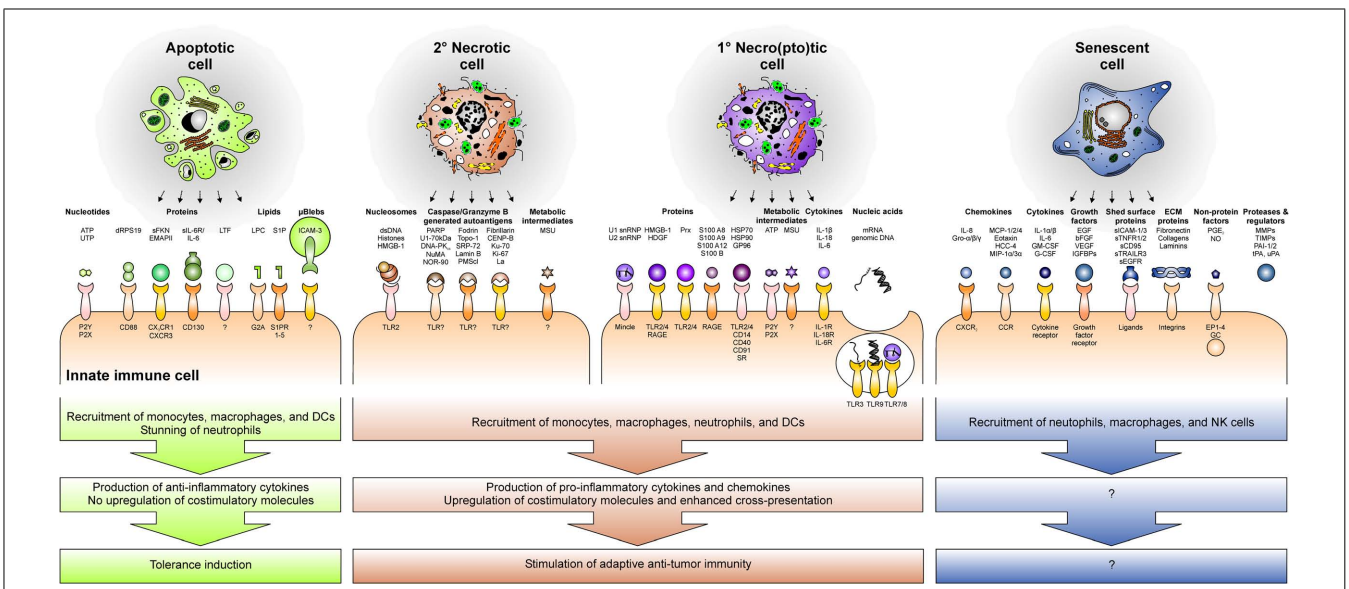

FIGURE 3 | The secretome and releasate of cells undergoing different forms of cell death and senescence recruit immune cells and skew the immune response toward tolerance induction or anti-tumor immunity.

for advanced glycation end products (RAGE), and members of the scavenger receptor (SR) family (Eue et al., 2000; Bianchi and Manfredi, 2007; Tsan and Gao, 2009; Shichita et al., 2012). Additionally, ATP and uric acid, which readily forms monosodium urate (MSU) crystals in the extracellular space, have been assigned a phagocyte attracting danger signal function. Yet, the receptor for MSU crystals, if there is any, remains to be identified (Shi et al., 2010).

In case of post-apoptotic, secondary necrotic cells, the process of phagocyte recruitment is only poorly understood. One could argue that the repertoire of soluble mediators released by secondary necrotic cells should be a sum of the factors released by apoptotic and primary necro(pto)tic cells. However, it has to be taken into account that dRP S19, EMAP-II, sFKN, sIL-6R, LPC, S1P, and micro-blebs are released early during apoptosis, and according to the ubiquitous presence of degrading enzymes in the extracellular space the stability of these compounds is presumably rather limited. Hence, it is questionable if these mediators are still present and active after the transition into secondary necrosis. Moreover, the factors liberated during primary and secondary necrosis should differ essentially, since the key danger signals described in the context of primary necrosis undergo modifications during apoptosis: ATP is consumed and intracellular proteins are proteolytically processed, which might enhance as well as abolish their danger signal function (Galluzzi et al., 2012). Yet, available data on this issue are limited. From the present point of view, the most likely candidates for secondary necrotic cell-derived "find-me" signals are MSU crystals, since the amount of releasable uric acid – owing to the degradation of chromatin – supposedly is even higher than in the case of primary necrosis, and HMGB-1 associated with secondary necrotic cell-derived nucleosomes (Urbonaviciute et al., 2008; Shi et al., 2010). In addition, annexin A1 has very recently been shown to translocate to the outer leaflet of the plasma membrane and to be proteolytically processed into a peptide, which subsequently is released and stimulates monocytes/macrophage chemotaxis during secondary necrosis (Blume et al., 2009, 2012).

DYING CELL ENGULFMENT

When phagocytes reach the dying cells, they identify and recognize their prey by "eat-me" signals, which are exposed on the cell surface. For apoptotic cells, the dominant "eat-me" signal appears to be phosphatidylserine (PS), an anionic phospholipid that during apoptosis translocates from the inner to the outer leaflet of the plasma membrane (Fadok et al., 1992; Krahling et al., 1999). PS is recognized directly by specific PS receptors, including brain angiogenesis inhibitor 1 (BAI-1), members of the T cell Ig and mucin family (TIM-1, -3, and -4), and the stabilins 1 and 2 (Kobayashi et al., 2007; Miyanishi et al., 2007; Park et al., 2007, 2008, 2009; Nakayama et al., 2009). Additionally, PS can be recognized indirectly via the mediation of soluble bridging proteins. They link PS to different engulfment receptors of the phagocyte, and originate from the phagocyte, the dying cell, or the interstitial body fluids, respectively. Milk fat globule EGF factor 8 (MFG-E8) and its close relative developmental endothelial locus 1 (Del-1) are examples for phagocyte-derived bridging proteins and ligate PS to $\alpha_v\beta_{3/5}$ integrins (Hanayama et al., 2002). In addition, annexin A1 can be released by the phagocyte for apoptotic cell opsonization, but it has also been reported to be the prototypical apoptotic cell-derived bridging protein, although its corresponding phagocyte receptor still has to be identified (Arur et al., 2003; Fan et al., 2004). The serum proteins β_2-glycoprotein 1 (β_2GP1), protein S, and growth arrest-specific gene 6 (Gas6) contribute to PS bridging as well and involve members of the LDL receptor-related protein family (LRP), or the protein tyrosine kinases Mer, Axl, and Tyro3, respectively (Ishimoto et al., 2000; Lu and Lemke, 2001; Scott et al., 2001; Anderson et al., 2003; Wu et al., 2005; Rothlin et al., 2007; Maiti et al., 2008; Xiong et al., 2008). Aside from externalized PS, several other "eat-me" signals on the surface of apoptosing cells have been described: sites resembling oxidized low density lipoprotein particles and sites binding thrombospondin-1, collectins or complement proteins have been reported to engage SRs, $\alpha_v\beta_{3/5}$ integrins, collectin and complement receptors in order to trigger dying cell engulfment. Finally, also the inactivation of "don't-eat-me" signals, such as CD31 or CD47 and its binding partner SIRPα, which prevent viable cells from mistakenly being ingested, contributes to proper dying cell recognition (Brown et al., 2002; Gardai et al., 2005).

In case of primary and secondary necrosis the mechanisms of dying cell recognition and engulfment are not as well understood as in the case of apoptosis. However, it appears that complement opsonization rather represents a hallmark of secondary necrosis than of early apoptosis, and the same seems to apply to annexin A1 externalization (Gaipl et al., 2001; Blume et al., 2009). Moreover, although mechanistically different from PS translocation during apoptosis, ruptures or holes in the plasma membrane lead to an exposure of PS by primary and secondary necrotic cells. Consequently, PS has also been attributed a role in necrotic cell engulfment (Brouckaert et al., 2004; Bottcher et al., 2006). Of note, very recently the first specific "eat-me" signal of primary necrotic cells has been identified: exposed actin filaments, which are recognized by the C-type lectin Clec9A (Sancho et al., 2009; Ahrens et al., 2012; Zhang et al., 2012).

Ligation of engulfment receptors triggers rearrangements in the actin cytoskeleton and thus the internalization of the dying cell. However, the underlying molecular mechanisms are only poorly understood and may differ fundamentally according to the structure of the respective receptor and its downstream signaling cascades. Probably the most detailed data are available for BAI-1, a seven transmembrane domain G-protein coupled receptor that signals via the evolutionarily conserved ELMO1–Dock180–Rac complex (Park et al., 2007). Undoubtedly, further studies are required in order to dissect the signaling cascades of each engulfment receptor. On the one hand this will help to understand why the morphology of apoptotic and necrotic cell ingestion is so different from each other, with apoptotic cells being phagocytosed by a "zipper"-like mechanism and necrotic cells – together with a substantial amount of extracellular material – being taken up by macropinocytosis (Krysko et al., 2006). On the other hand, it will contribute to elucidate, why the phagocytosis of apoptotic and necrotic cells is so fundamentally different in terms of the immunological outcome.

THE POST-PHAGOCYTIC IMMUNE RESPONSE

After engulfing dying cells, macrophages and DCs exert discrepant functions regarding the subsequent immune response. Whereas macrophages predominantly degrade the phagocytic cargo and shape the cytokine milieu for other immune cells, DCs traffic the ingested material to MHC-II- or MHC-I-dependent pathways of antigen-presentation or cross-presentation, respectively. Of note, the immunological outcome of dying cell phagocytosis fundamentally differs depending on the type of death the internalized prey has previously undergone (**Figure 2**). It can be said very simplistically that the uptake of apoptotic cells blunts pro-inflammatory and induces anti-inflammatory cytokine production in macrophages, including IL-10, TGF-β, and PGE_2 (Voll et al., 1997; Fadok et al., 1998). DCs take up apoptotic material and process, present, or cross-present apoptotic cell-derived antigens to T cells, thus resulting in the induction of immune tolerance (Albert et al., 1998; Stuart et al., 2002; McGaha et al., 2011). The "tolerate-me" signals involved in this complex process are currently being elucidated, and PS, which is exposed on the apoptotic cell surface, apparently is of pivotal importance in this regard (Huynh et al., 2002; Chen et al., 2004; Doffek et al., 2011). However, due to holes in the plasma membrane, PS is also exposed by primary and secondary necro(pto)tic cells, but in this case dying cell engulfment triggers a potent pro-inflammatory immune response. So, somehow the tolerogenic effect of PS must be overridden. Exposed F-actin, which ligates Clec9A, efficiently stimulates cross-priming of damaged cell-derived antigens, but alone it is not sufficient to induce an adaptive immune response to dying cells (Ahrens et al., 2012). Evidence for the nature of the required molecules comes from a study reporting on the lack of pro-inflammatory responses against necrotic cells, which have been separated from their secretome and/or releasate (Brouckaert et al., 2004). Hence, it is obviously the plethora of molecules released during primary or secondary necro(pto)sis that tip the scale. Different studies have shown that these liberated components, including HMGB-1-decorated nucleosomes, HSPs, ATP, ribonucleosome particles, and others, trigger TLR-, C-type lectin- and nod-like receptor-signaling, which is essential to promote pro-inflammatory cytokine production in macrophages and efficient cross-priming in DCs (Rovere-Querini et al., 2004; Blander, 2008; Ghiringhelli et al., 2009; Aymeric et al., 2010; Garaude et al., 2012; **Figure 3**). This is further substantiated by research in the field of autoimmunity as defects in the process of apoptotic cell clearance and the subsequent accumulation of secondary necrotic debris have been shown to represent hallmarks in the etiopathogenesis of chronic inflammatory and autoimmune diseases, such as systemic lupus erythematosus (SLE; Herrmann et al., 1998; Baumann et al., 2002; Gaipl et al., 2005, 2007a; Munoz et al., 2010a). Moreover, mice deficient in different engulfment genes or DNase II, which is required for the proper degradation of dying cell-derived DNA, reveal an accumulation of dying cell debris and develop a late-onset autoimmune phenotype, including the typical interferon (IFN) signature, closely resembling human SLE (Botto, 1998; Le et al., 2001; Cohen et al., 2002; Hanayama et al., 2006; Kawane et al., 2006).

In contrast to apoptotic and necro(pto)tic cells, the clearance of senescent cells is only poorly understood. Yet, a pioneering study has shown that senescence induction in established liver carcinomas results in the recruitment of macrophages, neutrophils, and natural killer (NK) cells, and this was sufficient for the clearance of the senescent tumor cells (Xue et al., 2007). However, which factors of the senescence-associated secretome are involved in this scenario (**Figure 3**), if senescent cells are "eaten up alive" or first are actively killed and then phagocytosed, and which "eat-me" signals play a role in this context, remains elusive (**Figure 4**; Kuilman and Peeper, 2009; Coppe et al., 2010). Moreover, senescence induction in pre-cancerous hepatocytes was observed to stimulate an adaptive immune response depending on the interaction of antigen specific $CD4^+$ T cells and macrophages (Kang et al., 2011). Whether this might be of use for the induction of anti-tumor immunity in established cancers has to be further evaluated.

It should be noted that the form of cell death or cell stress does not only shape the subsequent immune response, it has also profound impact on the surviving neighboring cells. In this regard, radiotherapy-induced apoptosis has been shown to potently promote tumor cell repopulation via mechanisms involving the caspase-3-dependent cleavage and activation of the calcium-independent phospholipase A_2 ($iPLA_2$) and the subsequent production of prostaglandin E_2 (PGE_2; Huang et al., 2011; Lauber et al., 2011). Moreover, DAMPs released from necro(pto)tic cells and particularly HMGB-1 have been reported to stimulate autophagy as a mechanism of programed cell survival and thus to confer resistance to radiotherapy and chemotherapy in different models of leukemia, pancreatic, and colon cancer (Tang et al., 2010a,b; Liu et al., 2011a,b). As mentioned above, also the senescence-associated secretome contains factors, which can suppress or promote the proliferation of surviving neighboring cells, respectively (Kuilman and Peeper, 2009; Coppe et al., 2010). Presumably, these proliferation-stimulating effects of dying cells and their releasates represent integral parts of a conserved wound-healing program, which controls tissue regeneration and repair under physiological conditions. However, in the context of cancer therapy they might be counterproductive as therapy-induced tumor cell repopulation and/or therapy resistance would strongly interfere with the therapeutic aim of tumor eradication.

DYING CELL CLEARANCE AND THE INDUCTION OF ANTI-TUMOR IMMUNE RESPONSES IN RESPONSE TO RADIOTHERAPY

It is well acknowledged that the induction of tumor cell death and the abrogation of clonogenic survival by ionizing irradiation are key determinants of its therapeutic success. However, there is accumulating experimental evidence that particularly in the context of ablative radiotherapy, during which radiation is applied in high single doses of 10 Gy or more, complex immune mechanisms contribute to tumor regression. One of the initial reports on this issue showed that local high dose radiation therapy of transplanted mouse B16 melanoma stimulates the generation of tumor antigen-specific, IFN-γ producing T cells (Lugade et al., 2005). In the same mouse model, ablative, but not fractionated radiotherapy was observed to drastically enhance T cell priming in tumor draining lymph nodes paralleled by a reduction/eradication of

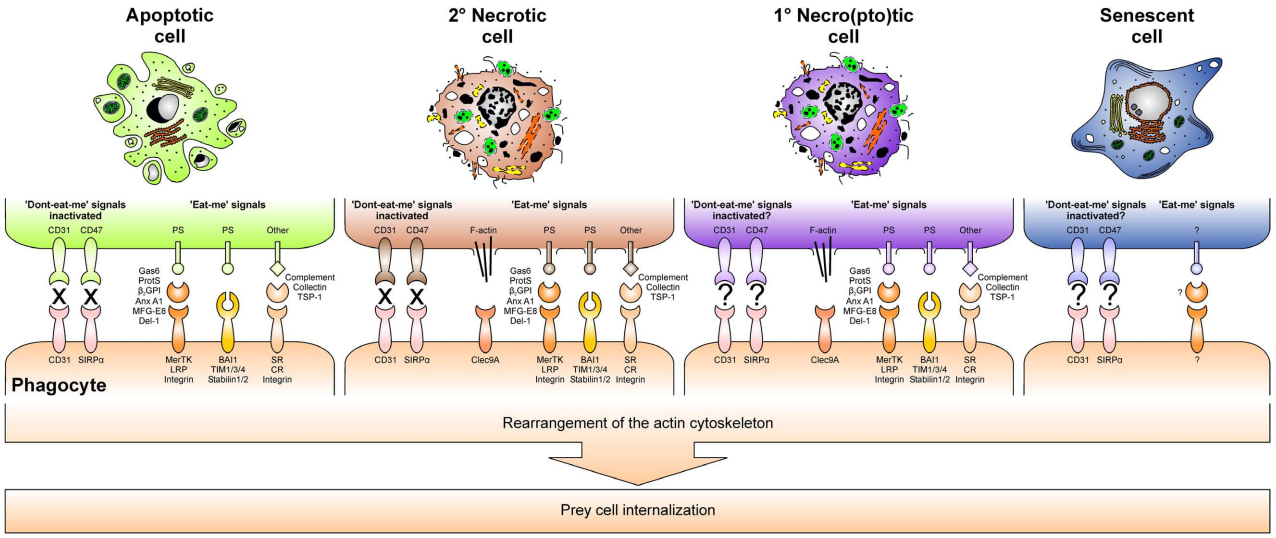

FIGURE 4 | "Eat-me" signals and inactivated "don't-eat-me" signals together with their respective phagocyte receptors orchestrate dying cell engulfment.

the primary tumor as well as distant metastases in a $CD8^+$ T cell-dependent manner (Lee et al., 2009). A mechanistic explanation of how these T cells are primed was provided by a recent study showing that the intra-tumoral production of IFN-α/β in response to high dose radiotherapy enhances the cross-presenting capacity of tumor infiltrating DCs (Burnette et al., 2011). Hence, it appears that ablative radiotherapy triggers a temporal cascade of IFNs, which is well-known from the field of tumor immunoediting, where IFN-α/β produced by $CD11c^+$ cells (presumably DCs and macrophages) enhances the cross-priming activity of $CD8\alpha^+$ DCs, thus stimulating the generation of IFN-γ producing $CD8^+$ T cells, and finally tumor rejection (**Figure 5**; Diamond et al., 2011; Fuertes et al., 2011). Notably, there is a clear difference in how IFN-α/β and IFN-γ contribute to a reduction in tumor burden. IFN-α/β predominantly affects macrophages, DCs, and NK cells leading to their activation and/or maturation, the upregulation of chemokine expression, the enhancement of antigen-presentation and cross-presentation by DCs and a robustly augmented induction of adaptive immune responses (Dunn et al., 2006). Consequently, in the context of cancer immunoediting, IFN-α/β-responsiveness is specifically required in DCs and macrophages, whereas it appears to be dispensable in tumor cells (Diamond et al., 2011; Fuertes et al., 2011). Nevertheless, tumor rejection in response to radiotherapy might also be improved – at least in part – by a direct effect of IFN-α/β on the tumor via its reported capacity of radiosensitization (Morak et al., 2011). In strong contrast to IFN-α/β, tumor cell responsiveness to IFN-γ obviously is an essential prerequisite for the development of anti-tumor immune responses. Various anti-tumor mechanisms have been reported to be exerted by IFN-γ, including inhibition of tumor cell proliferation, apoptosis induction, inhibition of angiogenesis, and an overall enhancement of tumor immunogenicity as characterized by an upregulation of the MHC-I pathway, modulation/extension of the MHC-I ligandome, and downregulation of NKG2D ligands (Dunn et al., 2006; Reits et al., 2006; Lugade et al., 2008). Notwithstanding these direct effects on the tumor, IFN-γ is key for the stimulation of an anti-tumor immune response. As such, IFN-γ is vitally involved in T_H1/T_C1 cell differentiation/activation, and it exerts similar effects as IFN-α/β in terms of innate immune cell activation and the promotion of DC-mediated antigen cross-presentation (Dunn et al., 2006).

Once more it should be emphasized that this IFN-controlled cascade of innate and adaptive immune responses was only observed in case of ablative but not conventional, fractionated radiotherapy (Lee et al., 2009). A feasible explanation for this might be that ablative and fractionated radiotherapy trigger different modalities of tumor cell death with only high dose irradiation stimulating primary and/or secondary necro(pto)sis. The corresponding liberation of danger signals, including HMGB-1 and ATP, in turn stimulates the TLR4-dependent production of IFN-α/β and other pro-inflammatory cytokines, initiating the above described IFN cascade and the DC-mediated instigation of anti-tumor T cell responses (Apetoh et al., 2007).

A key question that arises from an immuno-radiotherapeutic point of view at this point is: How can the process of dying cell clearance be instrumentalized or manipulated in order to enhance the efficacy of fractionated radiotherapy? Various putative approaches can be envisioned in this regard (**Figure 6**). The observations made with high dose ablative radiotherapy suggest that the temporary induction of primary/secondary tumor cell necro(pto)sis might be beneficial. Initial studies on this issue in fact provide evidence that the combination of radiotherapy with hyperthermia results in the induction of an immunogenic type of cell death as characterized by the release of danger signals, including HMGB-1 and HSP70, which foster the maturation of DCs *in vitro* (Schildkopf et al., 2009, 2010, 2011; Mantel et al., 2010). However, it remains to be elucidated, whether this translates into the productive stimulation of anti-tumor T cell responses

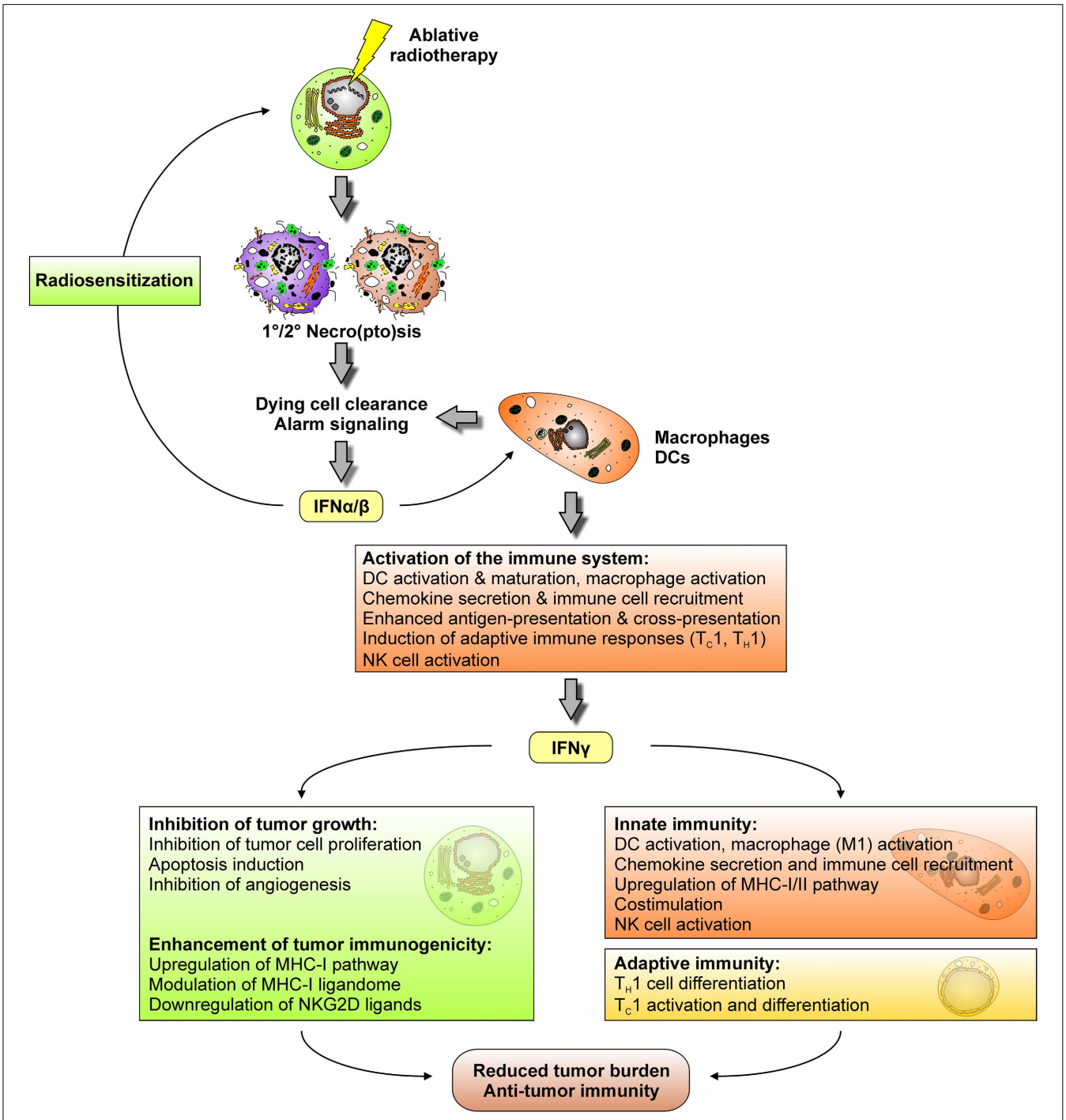

FIGURE 5 | Ablative radiotherapy stimulates a cascade of interferons, which contributes to a reduction in tumor burden and the induction of anti-tumor immunity.

in vivo. Alternatively, radiotherapy might be combined with photodynamic therapy (PDT), since PDT has been reported to induce the expression of HSPs and the release of danger signals (Castano et al., 2006). Alone or in combination with chemotherapy PDT leads to the stimulation of anti-tumor T cell responses *in vivo* (Castano et al., 2008; Kammerer et al., 2011). Yet, if this holds also true for its combination with radiotherapy awaits further clarification. For tumors, which predominantly undergo apoptosis in response to fractionated radiotherapy, caspase inhibition might represent an approach to overcome the tolerogenic nature of this form of cell death, since caspase inhibition in apoptosing cells has been reported to directly trigger necroptosis (He et al.,

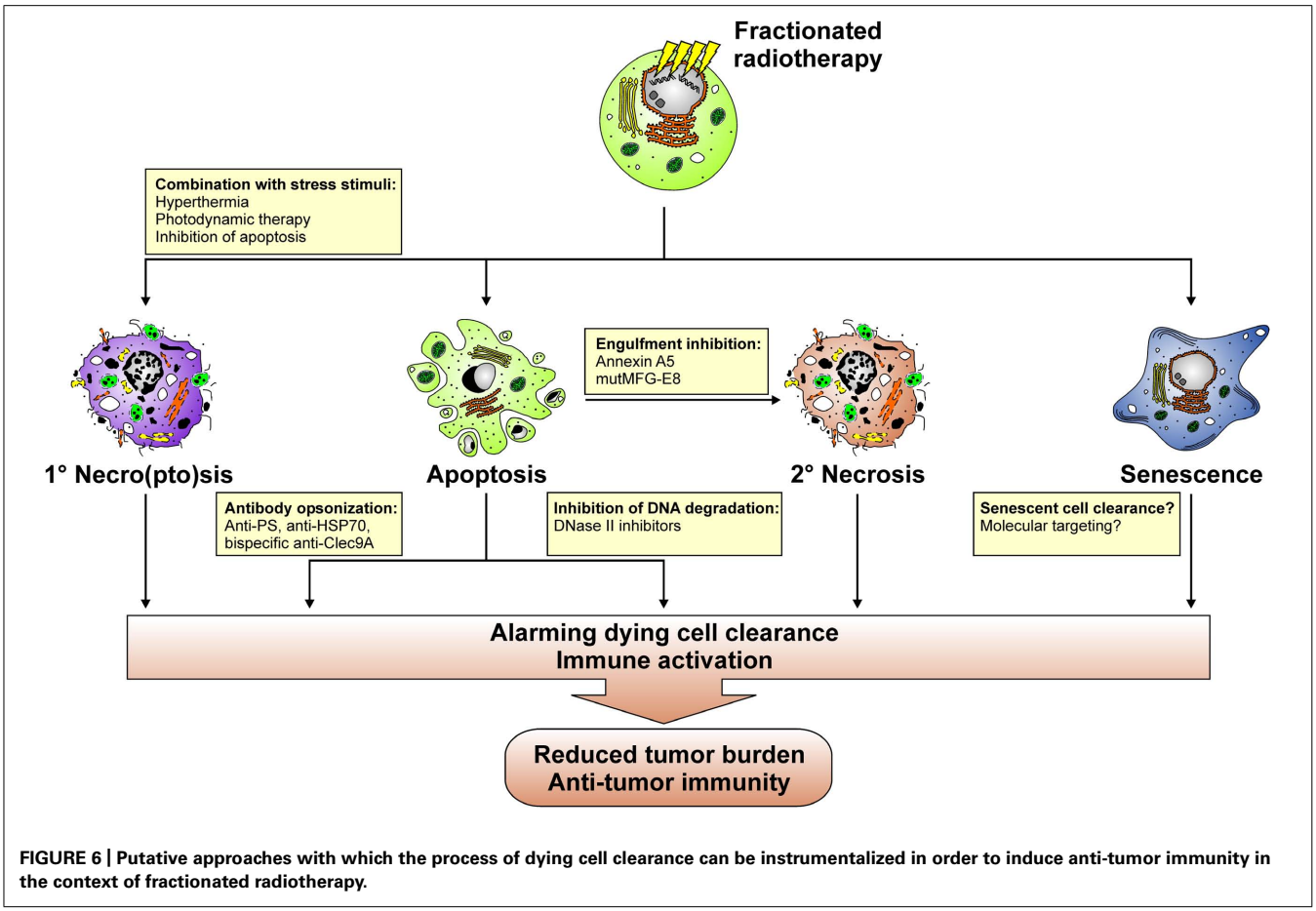

FIGURE 6 | Putative approaches with which the process of dying cell clearance can be instrumentalized in order to induce anti-tumor immunity in the context of fractionated radiotherapy.

2009b). However, caspase inhibition might also confer radioresistance due to an overall inhibition of cell death. Therefore, the applicability of this approach has to be very carefully evaluated.

Directly interfering with apoptotic cell clearance in order to promote the accumulation of secondary necrotic tumor cell material might be an alternative approach to instigate the IFN cascade described above and a concomitant anti-tumor immune response. In this regard, annexin A5 and a mutant form of MFG-E8, which no longer is able to bind to the vitronectin receptor, might be valuable tools (Hanayama et al., 2002; Bondanza et al., 2004; Gaipl et al., 2007b; Munoz et al., 2007a,b; Frey et al., 2009). In a mouse model of tumor vaccination, annexin A5 has been shown to impair the uptake of irradiated apoptotic lymphoma cells by macrophages and to specifically target them to $CD8\alpha^+$ DCs, thus inducing the release of proinflammatory cytokines and tumor-specific immune memory, which contributed to the regression of growing tumors and conferred resistance against tumor re-challenge (Bondanza et al., 2004). Redirecting dying cell clearance toward Fcγ receptor-mediated phagocytosis also appears a promising strategy for the induction of anti-tumor immune responses. For example, opsonization with autoantibodies has been reported to potently trigger FcγR-dependent phagocytosis of dying cells and subsequent pro-inflammatory cytokine production (Manfredi et al., 1998; Sarmiento et al., 2007; Grossmayer et al., 2008). Hence, "eat-me" signal specific antibodies, including anti-PS antibodies, or tumor antigen specific antibodies, like anti-HSP70 antibodies, might help to utilize pro-inflammatory dying cell phagocytosis for the induction of anti-tumor immunity (Ran et al., 2005; Gehrmann et al., 2008; He et al., 2009a; Stangl et al., 2011a,b). Engineering these antibodies into bi-specific antibodies coupling to Clec9A, is of special interest in this regard, since thereby dying tumor cell material could be specifically targeted toward the recycling endosomal compartment, thus favoring cross-presentation in $BDCA3^+$ DCs, the alleged human equivalent to mouse $CD8\alpha^+$ DCs (Schreibelt et al., 2012). Seminal data obtained with different mouse models of viral vaccination point to the direction that this represents a successful approach of inducing vaccine-specific immune responses (Idoyaga et al., 2011; Iborra et al., 2012; Zelenay et al., 2012). It should be noted that Clec9A ligation alone is not sufficient to induce DC maturation and a concomitant adaptive immune response against dying cells (Ahrens et al., 2012). Coligation of additional receptors for DAMPs instead appears to be required. Research in the field of autoimmunity has convincingly shown that dying cell-derived nucleosomal material is very potent in this regard (Frisoni et al., 2007; Urbonaviciute et al., 2008). Hence, interfering with DNase II activity, which in phagocytes mediates the proper degradation of engulfed prey cell DNA, could represent a promising strategy to stimulate the described IFN cascade required for the induction of productive immune responses and well-known for its crucial role in the etiopathogenesis of

autoimmune diseases, such as SLE (Kawane et al., 2006; Marshak-Rothstein, 2006; Deng and Tsao, 2010). To this end, cell-permeable, small molecule inhibitors of DNase II would be helpful, and it remains to be elucidated whether such compounds can technically be developed. Finally, further studies are required to improve and expand our knowledge on the process of senescent cell clearance with special focus on its applicability and potential utilization for the enhancement of tumor immunogenicity in the context of fractionated radiotherapy.

CONCLUSION

Undoubtedly, the induction of tumor cell death and the abrogation of clonogenic survival by ionizing irradiation are key to its therapeutic success. However, pioneering immuno-radiotherapeutic studies have convincingly shown that a contribution of complex immune mechanisms – particularly in the context of ablative radiotherapy – can no longer be neglected. The clearance of dying tumor cells by phagocytic cells of the innate immune system represents a crucial initiating step in this scenario. Valuable lessons can be learned from research in the field of autoimmunity on how the form of cell death skews the subsequent immune response, which cell types and molecules are involved in this context, and how these might be utilized for the induction of productive anti-tumor immune responses in combination with radiotherapy. Notably, these immune responses would not only target the local tumor but could also reach distant, out-of-field metastases. To this end, combined, multi-modal treatment regimes have to be developed with the capacity to induce immunogenic forms of tumor cell death and concomitantly activate the immune system. The correct timing will be an essential parameter affecting the therapeutic success of these approaches, since the priming of adaptive immune responses takes place in the tumor draining lymph nodes, which due to the risk of tumor cell spread and metastasis formation are commonly removed by surgery. Hence, it might be worth considering – carefully and on an individualized basis – to postpone lymph node surgery until the initial anti-tumor immune priming has successfully been accomplished. Not only in this regard, the close collaboration of clinical radiation oncologist, surgeons, radiobiologists, molecular oncologists, and immunologists is indispensable in order to develop and optimize the personalized therapeutic regime with the highest benefit for each individual patient.

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Selected anti-tumor vaccines merit a place in multimodal tumor therapies

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Multimodal approaches are nowadays successfully applied in cancer therapy. Primary locally acting therapies such as radiotherapy (RT) and surgery are combined with systemic administration of chemotherapeutics. Nevertheless, the therapy of cancer is still a big challenge in medicine. The treatments often fail to induce long-lasting anti-tumor responses. Tumor recurrences and metastases result. Immunotherapies are therefore ideal adjuncts to standard tumor therapies since they aim to activate the patient's immune system against malignant cells even outside the primary treatment areas (abscopal effects). Especially cancer vaccines may have the potential both to train the immune system against cancer cells and to generate an immunological memory, resulting in long-lasting anti-tumor effects. However, despite promising results in phase I and II studies, most of the concepts finally failed. There are some critical aspects in development and application of cancer vaccines that may decide on their efficiency. The time point and frequency of medication, usage of an adequate immune adjuvant, the vaccine's immunogenic potential, and the tumor burden of the patient are crucial. Whole tumor cell vaccines have advantages compared to peptide-based ones since a variety of tumor antigens (TAs) are present. The master requirements of cell-based, therapeutic tumor vaccines are the complete inactivation of the tumor cells and the increase of their immunogenicity. Since the latter is highly connected with the cell death modality, the inactivation procedure of the tumor cell material may significantly influence the vaccine's efficiency. We therefore also introduce high hydrostatic pressure (HHP) as an innovative inactivation technology for tumor cell-based vaccines and outline that HHP efficiently inactivates tumor cells by enhancing their immunogenicity. Finally studies are presented proving that anti-tumor immune responses can be triggered by combining RT with selected immune therapies.

Keywords: immunotherapy, vaccination, cancer therapy, multimodal, anti-tumor immunity, whole cell-based vaccines, high hydrostatic pressure, radiotherapy

IMMUNOTHERAPY IN CANCER TREATMENT

Today's cornerstones in cancer therapy are radiotherapy (RT), chemotherapy (CT), and surgery. Local tumor control and/or complete regression are achievable for many tumor entities, since these methods alone and especially combinations of them have been further improved. Nevertheless, development of tumor recurrence and metastases substantially deteriorates the patient's prognosis. To win the fight against cancer is therefore not only restricted to kill all tumor cells of the primary tumor, but also to act on the patient's whole body to achieve a long-lasting anti-tumor effect which keeps remaining and recurrent tumor cells in check. Therefore, systemic approaches are required that activate the patient's immune system against the tumor. The cells of the immune system have to be trained to control residual disease and hidden metastases (Sistigu et al., 2011).

Cancer immunotherapies (CI) aim to be, beneath their role in primary local tumor killing, the second line therapy against recurrent tumors and metastases by priming the patient's immune system to elicit an anti-tumor response (Sharma et al., 2011). First and foremost, the combination of standard therapies with immunotherapeutic strategies is auspicious to reach stable disease and to improve overall survival. Additionally, due to a lower toxicity compared with chemotherapeutic agents, immunotherapeutic approaches are more compliant for normal tissue and the whole organism. Especially combinations of RT and CI are promising since they are capable to elicit anti-tumor effects outside the radiation field, a phenomenon called abscopal effect (Demaria et al., 2004; Frey et al., 2012). RT with ionizing irradiation (X-ray) triggers the release of pro-inflammatory signals. It further indirectly contributes to the activation of dendritic cells (DCs) by

generating modified and new tumor specific antigens and by inducing the release of danger signals of irradiated tumor cells. Further, an enhanced loading of DCs with tumor antigens (TAs) of irradiated tumor cells has been observed (Teitz-Tennenbaum et al., 2008). Therefore, combination of RT with DC-based CI is considered to be ideal for the induction of tumor specific immune responses.

The field of immunotherapy offers a broad array of approaches including monoclonal tumor specific antibodies that have been already established in clinical treatment of several tumor entities (Scott et al., 2012), the application of immune activating cytokines (Dranoff, 2004), or even gene transfer of adoptive T-cell receptor (TCR) to obtain large numbers of tumor-reactive T cells. The *in vivo* stimulation of an anti-tumor response by vaccines is another important approach. Especially whole tumor cell-based vaccines offer a wide array of TAs. Contrary to peptide-based vaccines, defining and manufacturing of individual and immunogenic antigens is not required since whole cells comprise all immunologically relevant tumor peptides (**Figure 1**). Of special note is that this multiplicity decreases the risk of tumor escape.

Crucial in generating effective whole tumor cell vaccines is to induce, or even increase their immunogenicity (Frey et al., 2008). Since the way cells die is closely connected to their immunogenic potential, the inactivation process of tumor cells is often the determining factor for a vaccine's potency (Tesniere et al., 2008a,b). Currently, we investigate high hydrostatic pressure (HHP, meaning pressure stages >100 MPa) treatment as a novel inactivation technology of whole tumor cells. We already proved that various tumor cell lines can be efficiently inactivated by treating them with pressure ≥200 MPa and observed in preclinical mouse models that that HHP-killed tumor cells are immunogenic (Weiss et al., 2010b).

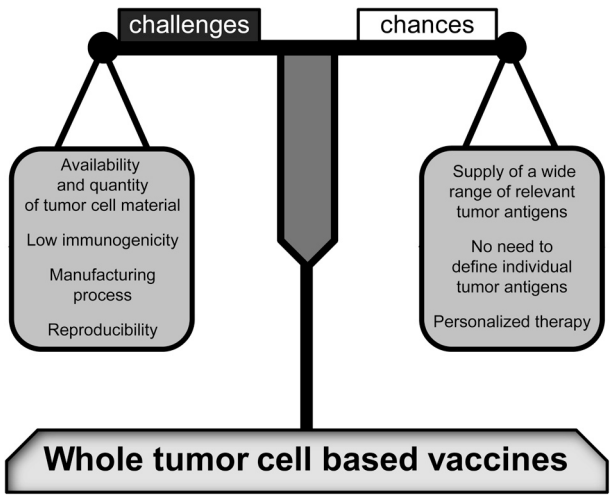

FIGURE 1 | Challenges and changes of whole tumor cell-based vaccines. As compared to peptide-based vaccines, a high variety of relevant tumor antigens is provided by whole tumor cell-based vaccines. Therefore, the tumor antigens have not to be individually defined. However, there are some crucial challenges that may decide about the vaccine's efficiency and productivity including the immunogenic potential of tumor cell material and a reliable manufacturing process associated with a high reproducibility. Furthermore, the limited availability of tumor cell material has to be taken into account. Nevertheless, when these resolvable problems have been resolved, whole tumor cell-based vaccines will be prime immune therapies for personalized tumor treatments.

IMMUNE THERAPIES WITH CYTOKINES AND MONOCLONAL ANTIBODIES

Before we go into detail how whole tumor cell vaccines induce anti-tumor immunity, we will shortly introduce further strategies of CI with "agents" that do not bear tumor peptides and antigens such as cytokines or monoclonal antibodies.

Cytokines in the tumor microenvironment have a strong influence on the host's immunity. They may foster or suppress tumor growth (Chometon and Jendrossek, 2009; Apte, 2010). Consequently, the administration of distinct cytokines in cancer therapy can modulate the microenvironment of a tumor in a way that leads to a better therapeutic outcome (Dranoff, 2004). However, their administration can also induce relevant side effects related with a moderate effectiveness (Kelley et al., 2003; Dantzer and Kelley, 2007). Hence, combination of cytokines with other strategies allows dose reduction. Clinically successful phase III trials have been carried out with systemic administration of interleukin (IL)-2, that enhances natural killer (NK)-cell and T-cell activity (Rosenberg et al., 1993; Fyfe et al., 1995), or stimulators for TA presentation like granulocyte-macrophage colony-stimulating factor (GM-CSF) (Dranoff et al., 1993), interferon (IFN)-α (Biron, 2001), or IFN-γ (Bach et al., 1997). Since immunity against cancer is a multi-step-process, the sole application of cytokines is insufficiently. The role of cytokines in cancer therapy and pathogenesis has been extensively discussed during the last years (Dranoff, 2004; Margolin, 2008; Mellman et al., 2011).

Beyond, immunity against malignant cells can be established with monoclonal antibodies that trigger tumor cell apoptosis by inducing antibody-dependent cellular or complement-mediated cytotoxicity. Further, those antibodies may block growth factor receptors or foster anti-tumor immune responses (reviewed in King et al., 2008; Scott et al., 2012; Weiner et al., 2012).

Rituximab, an antibody that targets CD20 on B-cells and causes B-cell apoptosis in B-cell lymphoma (Pescovitz, 2006), is one of the prominent examples for the application of monoclonal antibodies in CI. Others are antibodies such as Trastuzumab (Hudis, 2007; Valabrega et al., 2007), acting against human epidermal growth factor receptor 2 (HER-2) on cancer cells, or Cetuximab, that acts against the epidermal growth factor receptor (EGFR) (Cunningham et al., 2004; Bonner et al., 2006). Bevacizumab is directed against vascular endothelial growth factor (VEGF) and the anti-cytotoxic T-lymphocytes (CTL) A-4 antibody Ipilimumab abrogates the inhibitory effects of CTLA-4 on T-cell activation (Greenwald et al., 2001; Ferrara et al., 2004; Scott et al., 2012). Via binding to Fc gamma receptors (FcγR) on DCs, monoclonal antibodies contribute further to an induction of adaptive anti-tumor immune responses. An antibody-mediated enhanced cross-presentation of TAs was observed (Dhodapkar et al., 2002, 2005; Weiner et al., 2010). Similar to chemotherapeutics, monoclonal antibodies act directly in the whole tumor

mass, but are more compliant for the patient, due to their low toxicity. However, monoclonal antibodies have a narrow target antigen range. Nevertheless, a plenty of studies have been performed using such antibodies. Scott and colleagues recently summarized the advantages and disadvantages of this therapeutic option against cancer (Scott et al., 2012).

Besides the above mentioned mechanisms, the potential of antibodies and cytokines to activate the immune system accompanied with an establishment of immunological memory is still low. A combination of CI strategies might be beneficial to induce anti-tumor immunity and reduce the tumor cell growth and induce tumor cell death, respectively (Van Elsas et al., 1999; Takaku et al., 2010; Weiss et al., 2010a).

IMMUNOLOGICAL BASICS OF THE MODE OF ACTION OF TUMOR VACCINES

Contrary to monoclonal antibodies, tumor vaccine strategies aim to actively train the immune system going along with the development of a long-lasting immunological memory. Hence, particularly the appearance of metastases and tumor recurrences can potentially be counteracted or even avoided. Both, the innate and the adaptive arm of the cellular immune system can contribute to an effective attack toward tumor cells.

Lymphokine-activated killer (LAK) cells, NK cells, and macrophages are crucial players of innate immunity, acting with a low specificity but widespread against tumor cells with different histological background (Grimm et al., 1982; Rosenberg et al., 1985; Krause et al., 2002; Terme et al., 2008). Cells of the adaptive immune system, comprising CD4+ and CD8+ T-lymphocytes, are more suitable to elicit a long-lasting immune response, since they can specifically target TAs and differentiate to memory cells. The three most important processes leading to long-lasting tumor immunity were recently proposed by Mellman et al. (2011). In the first step, DCs must sample relevant TAs. Secondly, DCs have to mature and to initiate the T-cell response. Last but not least, the T-cells have to overcome the immunosuppression of the solid tumor and enter the tumor bed (Mellman et al., 2011).

Tumor vaccinations offer a variety of strategies to activate and train the immune system. Vaccination with distinct TAs, or whole tumor cells have been performed to activate DCs *in vivo*. Further, strategies with pulsing of DCs *ex vivo* with TAs are followed up. Nevertheless, the generation of an effective, specific, and long-lasting response against tumor cells is more challenging compared to that against pathogens, since the tumor had repeatedly escaped an immune surveillance (Novellino et al., 2005).

Transformed cells have several strategies to circumvent immune activation (reviewed in Dunn et al., 2002; Igney and Krammer, 2002). For example, presentation of TA on the tumor cells' surface is generally poor. This is even more present in metastases that are often characterized by frequent mutations; this helps transformed cells to escape from an initially induced specific response (Kim et al., 1975; Bailly et al., 1993). Moreover, presentation and protein loading of major histocompatibility complex (MHC)-class I molecules is often reduced or malfunctioned avoiding lysis by cytotoxic T cells (Hicklin et al., 1999). Further mechanisms in the microenvironment contribute to tumor escape, such as the secretion of immunosuppressive factors including TGF-β (Li et al., 2006), IL-10 (Wittke et al., 1999), or prostaglandin (Botti et al., 1998). These cytokines secreted by tumor cells favor an immune deviation, characterized by a shift toward a more Th2-polarized response that suppresses the establishment of an adaptive cellular response involving effector CD8+ T-lymphocytes (Maeda and Shiraishi, 1996; Shurin et al., 1999; Ribas et al., 2000). The cytokine profile, a low stimulation by TA and the missing stimulative conditions can finally lead to T-cell anergy, to the induction of regulatory T-cells, and also to T-cell-depletion (Staveley-O'Carroll et al., 1998; Zou, 2006). The major challenges of an effective cancer vaccine are therefore to overcome these immune suppressing mechanisms and to train the immune system to recognize and to attack tumor cells.

For the induction of an effective and long-lasting anti-tumor response, priming of CTL is crucial. CTLs are able to specifically target TAs and to destroy tumor cells directly. Moreover, they may differentiate into memory T-cells, which enable the development of a prolonged anti-tumor response (Podack, 1995; Shresta et al., 1998). For activation, T-cells have to meet their specific TA and additionally have to receive co-stimulatory signals. Recognition of TA is mediated by TCRs on naïve T-lymphocytes and antigen-MHC complexes on antigen presenting cells (APCs). The binding of the TA is restricted to MHC-class I or MHC-class II molecules on CD8+ and CD4+ T-cells, respectively. Furthermore, APCs provide co-stimulatory signals, including B7.1/B7.2 molecules that interact with CD28 on T-cells, or CD40 receptors on T-cells interacting with CD40 ligand (CD40 L). Without those additional signals T-cells are not able to proliferate and to produce cytokines; they even undergo apoptosis (Frauwirth and Thompson, 2002). Contrary to the activation of CD4+ T-cells, the priming of CTLs from naïve CD8+ T-cells is more complex and involves the interplay of DCs, T helper cells type 1 (Th1 cells), and cytokines (Mellman et al., 2011).

Immature DCs constantly migrate through tissues and blood, scanning their environment for potential pathogens or danger signals. DCs recognize invading pathogens with their pathogen-associated molecular patterns (PAMPs) recognition receptors (PRRs). But DCs are not only activated by pathogen-derived signals. According to the danger theory of Polly Matzinger (Matzinger, 1994), the immune system is able to distinguish between danger and non-danger. Dying mammalian cells release danger-associated molecular patterns (DAMPs) that act as potent stimuli for DCs. In the last years several DAMPs have been described, including high mobility group box 1 protein (HMGB-1), heat shock proteins (HSPs), and uric acid (Shi et al., 2003; Bianchi, 2007). After the recognition by DCs, the tumor cell is engulfed and antigen processing takes place. Maturation of DCs has been initiated and is accompanied by a decreasing potential of antigen assimilation combined with increased migration ability. Consecutively, DCs migrate to the lymph node (LN), where their peptide/MHC-class II complex is presented to the antigen specific TCR on naïve CD4+ T-cell. DCs have to reach a fully mature stage to power an effective immune response, because semi-mature DCs have rather tolerogenic features (Rutella et al., 2006; Mellman et al., 2011).

The fate of T helper cell subtypes and therefore the type of immune response are strongly determined by polarizing factors that are secreted by DCs (O'Garra and Arai, 2000). The cytokines IL-2, IL-12, and IFN-γ favor the differentiation of naïve CD4+ T-cells to T helper cell type 1 (Th1), while IL-10, IL-4, and IL-5 polarize a T helper cells type 2 (Th2) response (O'Garra and Arai, 2000). In regard to a CTL-mediated tumor response, the induction of a Th1 response is crucial since the interaction of Th1 cell with DCs renders the DCs themselves capable for the activation of naïve CD-8+ T-cells by CD40 and CD40-L interaction (Schoenberger et al., 1998). Consequently, the primary contact between tumor cells and DCs is pivotal for both the initialization and the polarization of adaptive immune responses.

The cytokine milieu may also favor several other T helper subclasses besides Th1 and Th2 cells. For example, the development of Th17 cells that secrete the cytokine IL-17 is promoted by TGF-β and IL-6. The role of Th17 cells in tumor progression and/or regression is under current investigation (Langowski et al., 2006; Hirota et al., 2012; Liu et al., 2012; Middleton et al., 2012). In conclusion, the course of adaptive immunity against cancer cells is already initialized by innate immune cells, in particular by DCs. The priming of DCs by malignant cells is mainly determined by microenvironmental factors of the "meeting point" (Kapsenberg, 2003; De Jong et al., 2005).

Since danger signals released by dead tumor cells dictate the DC's behavior, they are crucial for the induction of anti-tumor immune responses. Toll like receptors (TLR) on the DCs' surface can recognize both PAMPs and DAMPs and have a great impact on anti-tumor immunity (Tesniere et al., 2008a). It has been reported that ligation of TLR-4 and TLR-5 instructs DCs to stimulate naïve T helper cells to a Th1 polarization while binding of TLR-2 dictates a Th2 response (Agrawal et al., 2003). DAMPs that are released by dying and dead cells can bind to TLRs and are therefore capable to determine priming of DCs. For example, HMGB-1 favors binding to TLR-4, while HSP-70 can interact with TLR-2 and TLR-4 (Apetoh et al., 2007; Asea, 2008). Hence, the form of tumor cell death, induced either *in vivo* by direct cytotoxic therapy or *ex vivo* for vaccination purpose, strongly determines whether an anti-tumor immune response is elicited or not (Tesniere et al., 2008a). Inactivation technologies for the preparation of whole tumor cell vaccines should therefore aim to induce immunogenic tumor cell death forms.

CELL DEATH FORMS AN IMMUNOLOGICAL RELEVANCE

Today a variety of different cell death forms is described for mammalian cells. Some cell death forms are highly genetically determined; others display more accidental characteristics (Griffith and Ferguson, 2011). Most attention was given to the two main cell death forms, namely apoptosis and necrosis, since their immune modulatory potential helped to understand how chronic autoimmune diseases might develop and/or sustained (Gaipl et al., 2006; Gaipl, 2009). Usually, apoptotic cells are immunologically silent or even tolerogenic. They are part of a physiological process to maintain homeostasis of every multicellular organism. Apoptosis is characterized by several cell morphological and biochemical features like DNA fragmentation, cell blebbing, and condensation of the chromatin (Kerr et al., 1972; Griffith and Ferguson, 2011). The silent clearance of apoptotic cells is mediated by "find-me" signals that are released by apoptotic cells to promote the attraction of phagocytes (Lauber et al., 2004; Ravichandran and Lorenz, 2007). The latter recognize "eat me" signals on the apoptotic cells' surface, leading under healthy conditions to a swift clearance of the dying cells. Engulfment of apoptotic cells provokes in activated phagocytes even the secretion of anti-inflammatory signals such as IL-10 and TGF-β (Voll et al., 1997). However, it has recently turned out that under certain circumstances apoptosis may also exhibit immune-stimulatory features, in particular when treated with certain chemotherapeutics (anthracyclines) or γ-irradiation (Casares et al., 2005; Obeid et al., 2007; Tesniere et al., 2008a; Locher et al., 2010). The associated molecular mechanisms are not yet fully investigated, anyhow there is evidence that the early exposition of the ER resident chaperone calreticulin together with ERP57 on the cell surface is one key part of it (Obeid, 2008; Ma et al., 2011).

Contrary to the predominantly immunologically silent manner of apoptotic cells, necrosis is associated with (pro-) inflammation; hence it's conditioned by a pathological process (Golstein and Kroemer, 2007). The loss of membrane integrity results in the secretion of danger signals that may lead to the activation and maturation of immune cells and generally generates inflammatory conditions. Extracellular HSPs (Asea, 2008; Schmid and Multhoff, 2012) and HMGB-1 are prominent examples for such released immune activator proteins (Apetoh et al., 2007; Bianchi, 2009; Schildkopf et al., 2011). Interestingly, some danger signals can be released by necrotic as well as apoptotic cells. One has to consider that in the case of apoptosis the danger signals are often modified before their release resulting in a contrary immunological outcome (Griffith and Ferguson, 2011). For example, HMGB-1 is usually oxidized by reactive oxygen species during the apoptotic process and thereby loses its immunological potency (Urbonaviciute et al., 2009). This highlights that the dying cell itself and its microenvironment determines whether immune activation or immune suppression is triggered. The different forms of cell death are manifold and sometimes hard to differentiate. Garg and colleagues recently summarized what factors determine the immunogenicity of the dying cells (Garg et al., 2010). Even a programmed form of necrosis, the so called necroptosis, has been described and the immunological consequences of this cell death modality are currently under investigation (Galluzzi et al., 2012).

If whole tumor cell vaccines are prepared by inactivation of tumor cells, the immunogenicity of the dead cells should be enhanced or at least maintained by this procedure. A main focus of future cancer therapy concepts should be set on combination of classical anti-cancer therapies with immunotherapy in order to accomplish the anti-tumor effects of both concepts. Standard therapies such as RT or the treatment with particular chemotherapeutics should induce highly immunogenic dead cells which generate an immune activating microenvironment (Ma et al., 2011). Solely immunotherapeutic agents are not sufficient to efficiently shift a strong anti-inflammatory tumor microenvironment to an inflammatory one. Therefore, the tumor cell death form of the whole tumor cells used as vaccine as well as the cell death induced in the primary tumor by standard

therapies are of great importance to trigger an effective anti-tumor immunity.

AUTOLOGOUS AND ALLOGENEIC WHOLE TUMOR CELL-BASED VACCINES IN THE CLINIC

During the last years, several vaccination strategies were evaluated in pre-clinical and clinical phase I and II studies. However, most of the tested vaccination approaches finally failed to achieve clinical success in randomized phase III trials (summarized in Rosenberg et al., 2004; Itoh et al., 2009; Klebanoff et al., 2011). **Table 1** summarizes the phase III trials containing whole tumor cell-based vaccines. In 2010, an autologous DC-based vaccine (Sipuleucel-T), used in prostate cancer, achieved FDA approval in the USA (Cheever and Higano, 2011) and has led to a boost in the development of new vaccination strategies and agents. For an improvement of vaccine concepts, the big challenge for research is to figure out the reasons for previous clinical failings of the vaccines.

Most of the clinical studies reviewed by Rosenberg and Klebanoff applied strategies based on vaccination with a single peptide or protein. These vaccines have the disadvantage to be restricted to a single target or, in the case of proteins, to few epitopes. Identification of TAs and proof of their immunogenicity are challenging aspects. Difficulties may result from poor antigen presentation on tumor cells or the appearance of frequent mutations in metastases: Both may result in the loss of peptide-specific effector T-cells' ability to recognize the tumor cells. Additionally, the efficiency of peptide-based vaccines is mostly HLA-restricted, resulting in a constricted quantity of potentially responding individuals (Chiang et al., 2010). Whole tumor cell vaccines are promising to bypass complex procedures in defining individual antigens. The tumor cell surface comprises a huge amount of potentially relevant antigens. Besides, the provided antigen plurality impedes tumor escape. For an enhanced anti-tumor response the additional application of immune adjuvants such as Bacillus Calmette-Guérin (BCG) or cytokines is beneficial. *Ex vivo* genetic manipulations of inactivated cell material are also followed up, resulting in the secretion of GM-CSF (Simons and Sacks, 2006), other cytokines, chemokines, or in increased expression of MHC antigens (Pardoll, 1995).

Autologous tumor cell-derived material has already been demonstrated in clinical studies to be a promising cancer vaccine. An adjuvant renal tumor cell lysate-based vaccine (Reniale®) was applied in a phase III study for patients with renal-cell carcinoma after nephrectomy. The 5-year progression-free survival rate improved to 77.4% compared to 67.8% in the control arm (Jocham et al., 2004). In another study, a ten-year survival analysis was performed for renal carcinoma patients also treated with that autologous tumor lysate vaccine in an adjuvant setting. An overall survival benefit (OS rates of 68.9% in the study group versus 62.1% in the control group) was observed. Especially the subgroup of patients with pT3 stage tumors did profit from this adjuvant vaccination (OS rates of 53.6% in the study group versus 36.2% in the control group) (May et al., 2010). For those vaccinations, necrotic cell lysates were obtained by freeze/thaw cycles of whole tumor cells. These lysates consist of cell fragments

Table 1 | Overview of whole tumor cell-based vaccines that have been tested in Phase III trials.

Trade name	Type of vaccine	Cancer type	Phase	Included patients	Observation periode	Reference
Sipuleucel-T	Autologous dendritic cell-based vaccine incubated with PAP-GM-CSF fusion protein	Prostate cancer	III	512	3 years	Cheever and Higano, 2011
Reniale	Cell-lysate-based autologous vaccine by freeze/thaw cycles	Renal-cell carcinoma	III	379	5 years	Jocham et al., 2004
Reniale	Cell-lysate-based autologous vaccine by freeze/thaw cycles	Renal-cell carcinoma	III	692	10 years	May et al., 2010
Oncovax	Irradiated autologous tumor cell-based vaccine with BCG	Colorectal cancer	III	317	5 years	Simons and Sacks, 2006
Prostate-GVAX	Allogenic cell-based, GM-CSF gene transduced vaccine	Prostate cancer	III	408	Prematurely terminated	Higano et al., 2009
Prostate-GVAX	Allogenic cell-based, GM-CSF gene transduced vaccine	Prostate cancer	III	626	Prematurely terminated	Small et al., 2009
Canvaxin	Irradiated allogenic cell mix-based vaccine with BCG	Melanoma	III	Stage III: 1100	Prematurely terminated	Finke et al., 2007
Canvaxin	Irradiated allogenic cell mix-based vaccine with BCG	Melanoma	III	Stage IV: 670	Prematurely terminated	Finke et al., 2007

including parts of cellular membrane, RNA, and DNA as well as cell organelles. Since the cell membrane is disturbed, danger signals such as HSPs and HMGB-1 are released and may *in vivo* stimulate the maturation of DCs (Sauter et al., 2000; Somersan et al., 2001). Until today no generalized vaccination recommendations for patients with renal cancer are given since further controlled trials are recommended and necessary. Furthermore, it has become obvious, that subgroups of patients exist that might benefit most from such vaccinations (May et al., 2010).

For colorectal cancer, a vaccine consisting of irradiated whole autologous tumor cells (Oncovax®) has been evaluated in clinical studies (up to phase III) (Hanna Jr et al., 2001; Simons and Sacks, 2006). For immunotherapy of melanoma, a quite similar approach was tested. Patients diagnosed with metastatic melanoma stage III/IV ($n = 81$) were included. After resection, melanoma cell material was irradiated and re-injected together with BCG. A survival benefit of the patients who received the vaccine was observed but it was only restricted to patients without evidence of macroscopic disease during the vaccination period (Baars et al., 2000). The delayed type hypersensitivity response (DTH) correlated with the survival data of the patients.

These clinical trials illustrate that despite disappointing clinical results of cancer vaccines (Klebanoff et al., 2011) also positive ones exist. However, the clinical studies with whole tumor cell vaccines curtailed the high expectations that were put into them. Nevertheless, whole tumor cell-based vaccines have to be brought back to bench and then again back to bedside, because highly immunogenic autologous tumor cell-based vaccines may offer great changes to cure cancer, especially when used in an adjuvant setting to avoid tumor recurrences or metastases.

Besides autologous tumor cells, allogeneic ones might also be used as cancer vaccines. Autologous tumor cells have the advantage to provide the complete set of personalized TAs; including individual mutated ones (Fournier and Schirrmacher, 2009). Anyhow, relevant drawbacks exist with regard to the production of autologous cell vaccines. Resectable tumors are needed to obtain enough tumor material. The number of tumor cells obtained after resection is often limited, especially in the case of metastases or early stage tumors. To obtain an adequate tumor cell amount, *in vitro* cultivation and expansion is often required entailing other difficulties (e.g., maintenance of cell integrity, prolonged process duration) that may influence the immunogenicity of the vaccine (Copier et al., 2007).

GVAX technology is one example for vaccine development where genetically modified tumor cells were used for vaccination. Granulocyte macrophage colony-stimulating factor (GM-CSF) is transduced into the tumor cells since it has proven immune stimulatory properties. It promotes recruitment and maturation of DCs and further up-regulation of MHC-class II molecules, co-stimulatory molecules, and cytokine production in DCs. Both, preclinical trials in a melanoma model (Dranoff et al., 1993) and a clinical studies in melanoma patients have shown the potential of autologous GVAX cells to elicit a tumor specific long-lasting anti-tumor response (Hege et al., 2006). GVAX was tested in numerous tumor entities with autologous tumor cell lines including renal (Tani et al., 2004), melanoma (Kusumoto et al., 2001; Soiffer et al., 2003), and non-small lung carcinoma (Salgia et al., 2003). Finally, GVAX therapies were designed for allogeneic settings in pancreatic and prostate cancer (Copier et al., 2007). "Prostate-GVAX" is the most advanced approach and two randomized phase III studies have been running (Higano et al., 2009; Small et al., 2009). The vaccine consists of two prostate cancer cell lines that were genetically modified by adenoviral transfection to produce GM-CSF. In one trial, GVAX was compared with CT (docetaxel plus prednison), while the other one comprised GVAX plus CT as study arm, and CT alone as control arm. Disappointingly, both studies were terminated prematurely, because it has turned out that control arm was more beneficial compared to the study arm regarding OS (Joniau et al., 2012).

There are further approaches using allogenic, genetically modified whole tumor cells as tumor vaccine. The group of Nemunaitis et al. tested the administration of the TGF-beta2 antisense gene modified allogeneic tumor cell compound in the treatment of non-small lung carcinoma; promising results were obtained in a phase II trial (Nemunaitis et al., 2006). The administration of the vaccine resulted in partial response rates of 15%, but failed in initiating a significant improvement in the overall response rate in the vaccine group. It was therefore not pursued in clinical trials (Nemunaitis et al., 2006). Contrary, canvaxin, as an example for a whole cell tumor vaccine consisting of unmodified allogeneic tumor cells, made its way into clinical trials. Canvaxin is composed of irradiation inactivated three different cell lines that express various TAs for melanoma. Administrated with BCG as an adjuvant resulted in immunological responses detected by DTH response and IgM level in the sera of the patients. This response correlated with increased median survival (Morton et al., 1992). The canvaxin phase II studies were promising and had proven survival benefits for stage III melanoma patients and even complete remissions in patients with low volume disease (after surgical removal) (Hsueh et al., 1999; Morton et al., 2002). Nevertheless, this approach failed in a phase III study, which was terminated earlier because the efficiency of control arm (BCG + placebo) was stronger, compared to the study arm (Finke et al., 2007). To conclude, many of the allogeneic-based vaccine approaches failed in the end in clinical trials. Therefore, autologous tumor cell based vaccines will be more in the focus of future vaccine developments (Fournier and Schirrmacher, 2009).

HIGH HYDROSTATIC PRESSURE AS PROMISING VACCINE PREPARATION METHOD

Although there are some promising approaches in the field of whole cell-based vaccines, the clinical outcome still remains unsatisfactory. One major problem could be the low immunogenicity of the vaccines and/or the failure of establishing an immunological memory. The clinical success is mainly proven in phase I and phase II trials. Dalgleish suggests that differences in clinical centers may contribute to the disappointing outcome in the phase III randomized studies and assumes that parameters like diet, exercise, and supplements might determine anti-tumor effect more than supposed (Dalgleish, 2011).

The production of the vaccines is another major challenge. Long processing times and reproducibility are still major problems. Strategies are needed that allow both, simple processing of tumor cells by concomitantly rendering them immunogenic. Cell

death pathways and death stimuli are closely connected to the tumor cell's immunogenic potential. However, despite the numerous vaccine approaches, hardly any work has taken into account the immunogenicity of the tumor cells that is determined by the different inactivation methods (Tesniere et al., 2008a).

Therefore, our group has focused on how a distinct inactivation method renders the dead tumor cells immunogenic (Weiss et al., 2010a). We examine HHP as a novel and innovative inactivation method for the generation of whole tumor cell-based vaccines. Focus is set on inactivation efficiency and the potential to deliver tumor cells with high immunogenicity. The HHP-technology is a highly reproducible technology, since pressure force vectors act orthogonal, with equal absolute value on the cell surface. Further, pressure propagation is homogenous and quasi not delayed (sound-velocitiy in media, see **Figure 2A**). Treatment with HHP preserves the shape of the cells and induces a gel-like consistence of cytoplasm (Frey et al., 2008). Importantly, experiments with several tumor cell lines treated with pressure equal or above 200 MPa have already proven that tumor cells are totally inactivated (Weiss et al., 2010b). So the pressurization of cells with 200 MPa leads to a mixture of apoptotic and necrotic cells (Frey et al., 2004; Korn et al., 2004; Weiss et al., 2010a). This mixture of dead cells, the cell morphology, and the observed release of danger signals after HHP treatment suggest that the tumor cells have a sufficient high enough immunogenic potential after inactivation with 200 MPa of HHP. We have demonstrated that tumor cells that were inactivated with 200 MPa HHP release DAMPs such as HMGB-1 and Hsp70 (own unpublished data). We further already revealed that the administration of HHP-treated autologous tumor cells without any adjuvant leads to a reduction of tumor outgrowth as well as to an advantage in survival in CT26 colorectal tumor bearing Balb/c mice. This was observed in a prophylactic setting (Weiss et al., 2010b). Taken together, the main prerequisites (summarized in **Figure 2B**) for a whole cell-based tumor vaccine are fulfilled by the application of HHP.

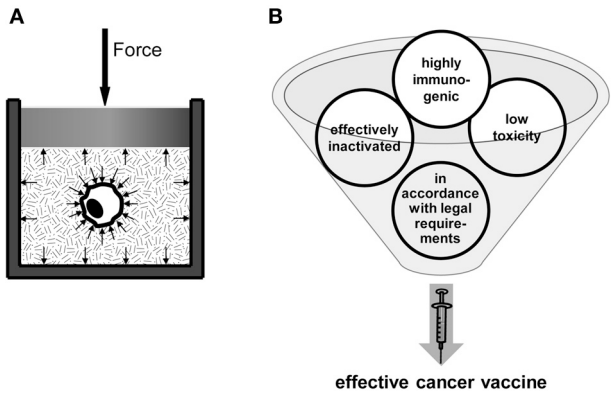

FIGURE 2 | High hydrostatic pressure (HHP) Technology as an innovative method for the preparation of whole tumor cell-based vaccines. The basic principle of the HHP technology is displayed in **(A)**. The tumor cell suspension, which has to be pressurized, has to be packed in a plastic wrapping. The pressure is applied to the treatment cavity and propagates through the pressure transmitting medium and also through the packaging (Yaldagard et al., 2008). Since the pressure is a vector product its acts on every material in the cavity with the same amplitude, independent of the shape of the probe. It was obvious in different experiments, that the pressure for inactivation should be at least equal or above 200 MPa. For the production of an effective whole cell-based vaccine, four main requirements have to be fulfilled **(B)**: the vaccine has to be safe, i.e., it has to exhibit low toxicity and, especially in the case of autologous tumor cell-based vaccines, the tumor cell material has to be effectively inactivated. The vaccine has to be further highly immunogenic, i.e., it has to trigger the immune system to elicit a strong anti-tumor response accompanied by the development of an immunological memory against the tumor. Finally, the vaccine and its processing have to be in accordance with statutory provisions.

RADIOTHERAPY COMBINED WITH IMMUNOTHERAPY FOR INDUCTION OF ANTI-TUMOR RESPONSES

Immunotherapy provided as mono-therapy won't be successful in destroying huger tumor masses. However in combination with standard therapies that reduce the tumor burden, it can be an effective tool to obtain a prolonged anti-tumor effect. Immune surveillance diminishes the risk for the development of metastases and tumor recurrence. The combination of RT with immunotherapy therefore offers great prospects to induce a strong and prolonged anti-tumor effect.

RT is, besides CT and surgery, one of the well-established methods in clinical cancer treatment. The primary effect of RT relies on the local killing of tumor cells by damaging the DNA. However, there is growing evidence that the impact of RT is not restricted to the local destruction of tumor cells, but also on the modification of the tumor's microenvironment. It may induce abscopal effects and positively influence the systemic therapeutic outcome (Frey et al., 2012).

Solid tumors are characterized by an atypical vascular network. Additionally, tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC) (Marigo et al., 2008), and regulatory T-cells may foster tumor progression. They secrete chemo- and cytokines such as VEGF, IL-10, and TGF-β to suppress the maturation of DCs. They therefore hamper APCs to establish an immune response against the tumor (Melief, 2008). Since irradiation modifies the tumor cell's phenotype and the tumor microenvironment, it may contribute via bystander effects to tumor regression (Lorimore et al., 2001). It has turned out that ionizing radiation can induce pro-inflammatory conditions in the tumor by causing immunogenic cell death, associated with the release of danger signals such HMGB-1. Further, an increased macrophage activation and neutrophil infiltration has been observed after RT (Lorimore et al., 2001). Antitumor effects outside of the radiation field were already observed in numerous studies (Ehlers and Fridman, 1973; Ohba et al., 1998; Okuma et al., 2011). Originally, this phenomenon (the so called abscopal effects of X-ray) was described by Mole in 1953. Even though the molecular mechanisms are not fully known, it has become evident that the immune system plays a significant role (Mole, 1953; Demaria et al., 2004; Formenti and Demaria, 2009; Frey et al., 2012).

Due to their immunogenic features induced by e.g., RT, the dead tumor cells are rendered visible for immune surveillance. This can be regarded as an "intrinsic vaccination." Preclinical experiments with tumor bearing mice that have been treated with RT and CTLA-4 blocking agents revealed that CTLA-4 therapy on its own is not able to elicit a systemic effect in regard to tumor

outgrowth of a second, non-irradiated tumor. However, combinatory treatment with CTLA-4 blocking agents and fractionated RT resulted in a significant growth retardation of the tumor outside the radiation field (Dewan et al., 2009). Furthermore, it was observed by Schaue et al. that irradiation resulted in a dose-dependent increase of IFNγ producing T-cells that correlated with tumor control (Schaue et al., 2012).

The pivotal role of DCs for radiation-induced abscopal immune effects was demonstrated by Demaria et al. (2004). Ionizing irradiation further directly influences the ability of DCs to load antigens being associated with an improved cross-priming of T-cells (Teitz-Tennenbaum et al., 2008). Knowing that irradiation may elicit immunological responses directed against tumor cells when combined with further immune activation, the application of an additional immunotherapy to RT might be a promising approach in tumor therapy (Demaria et al., 2005). Auspicious preclinical trials examining RT in combination with cytokines (especially IL-3 and IL-12) and DC applications, have already been performed (Maraskovsky et al., 1996; Chiang et al., 1997; Lynch et al., 1997; Seetharam et al., 1999; Teitz-Tennenbaum et al., 2003; Trinchieri, 2003; Oh et al., 2004). In a rat model of glioma, the combination of a vaccine composed of irradiated glioma cells and RT was tested. Despite vaccination alone results in a reduced survival compared to the control group, the combination of RT with the vaccine was superior in regard to survival than RT alone (Graf et al., 2002). Using a mouse model of glioma, the combination of a vaccine consisting of cytokine-producing autologous cancer cell with RT was tested. This combination resulted in cure of mice; unfortunately, a group just receiving RT alone was not included in this study (Lumniczky et al., 2002).

To summarize, some combination therapies of RT and immunotherapy have already reached the level of clinical studies. As one further example, in a phase II trial for prostate cancer, the combination of standard RT and vaccination with poxvirus encoding prostate-specific antigen was examined in a two arm setting (combination versus RT only). The combination therapy provoked a cellular immune response with T-lymphocytes that were not only restricted to the TAs provided by the vaccine. This implies a radiation-induced *in vivo* immunization effect (Gulley et al., 2005).

OUTLOOK

The outcomes of multimodal cancer therapies including immunotherapy are complex. While treatments with antibodies are already established in the daily clinical routine, the treatment of cancer with whole cell-based vaccines is still at an experimental stage. However, recent studies revealed that such immunotherapeutic agents may broaden the anti-tumor response in cancer patients (Mellman et al., 2011). CI can help to overcome the immune suppression emanating from the tumor. Nowadays, it has become clear that the standard tumor therapies elicit local and abscopal effects (summarized in Frey et al., 2012) that could be potentiated when combined with cancer vaccines (Sistigu et al., 2011). Future research is needed focusing on which combination of standard therapies (surgery, RT, CT, RCT) with CI are most beneficial and in which tumor stage and chronology they should be applied.

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Radiation-induced alterations in histone modification patterns and their potential impact on short-term radiation effects

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Detection and repair of radiation-induced DNA damage occur in the context of chromatin. An intricate network of mechanisms defines chromatin structure, including DNA methylation, incorporation of histone variants, histone modifications, and chromatin remodeling. In the last years it became clear that the cellular response to radiation-induced DNA damage involves all of these mechanisms. Here we focus on the current knowledge on radiation-induced alterations in post-translational histone modification patterns and their effect on the chromatin accessibility, transcriptional regulation and chromosomal stability.

Keywords: post-translational histone modifications, radiation, double-strand breaks, DNA damage response, chromatin

INTRODUCTION

The genetic information is stored in the DNA, which in eukaryotes is organized in chromosomes. In the first level of DNA packaging, DNA and histone proteins build the nucleosomes where about 147 bp of DNA is wrapped around an octamer of histone proteins. Each two copies of the four main histones, H2A, H2B, H3, and H4, form the nucleosomal core unit. Nucleosomes are linked to their adjacent nucleosome by the linker histone H1. Instead of the canonical histones, some nucleosomes contain histone variants (e.g., H2AX, H3.3, CENP-A) that confer specific functions (Talbert and Henikoff, 2010). The major functions of the canonical histones are DNA packaging and transcriptional regulation. Chromatin structure and function are associated with post-translational modifications (PTMs) of the histone proteins (both canonical and variant), such as acetylation, methylation, phosphorylation, as well as covalent addition of larger groups such as ubiquitin, SUMO (small ubiquitin-like modifier), or poly-(ADP-Ribose). The detection of new PTMs is ongoing (Turner, 2012). PTMs may directly affect the interaction of DNA and histones and thus influence the accessibility of chromatin. For example, it is widely accepted that extensive acetylation of histone tails neutralizes positive charge and thus reduces interaction with negatively charged DNA. There are, however, alternative explanations for the "opening" effect of acetylations (Turner, 2012). The presence of PTMs may increase or reduce binding of other proteins to histone tails and thus affect chromatin structure. It should be noted that direct causality between specific PTMs and a chromatin effect has been demonstrated only for few PTMs (Henikoff and Shilatifard, 2011).

Chromatin structure does not only affect transcription, but all processes requiring access to the DNA, including replication and repair. The paradigm "access-repair-restore," originally formulated for repair of UV-induced DNA damage (Gong et al., 2005), has also been applied to repair of other damage types, including DNA damage induced by ionizing radiation. Ionizing radiation induces a variety of damage types, among which DNA double-strand breaks (DSBs) are considered as being most relevant for the induction of chromosome rearrangements, cellular survival and long-term genomic stability. Interest in alterations of chromatin structure and PTMs following DSB induction has also been sparked by the fact that a PTM, namely phosphorylation of the histone variant H2AX, is widely used to visualize the chromatin regions surrounding DSBs and also for assessment and quantification of DSB (Dickey et al., 2009). The phosphorylation at serine 139 (S139) of H2AX in response to DSBs is mainly mediated by the kinase ATM (ataxia teleangiectasia mutated), which belongs to a family of phosphatidyl-inositol 3-kinase-related kinases. Two other family members, ATR (ATM- and Rad3 related) and DNA-PK (DNA-dependent protein kinase), can also phosphorylate H2AX. Since phosphorylation occurs in an Mbp-sized region surrounding the DSB, the localization of the DSB and its surrounding chromatin domain can be visualized as so-called foci by immunofluorescence with antibodies recognizing H2AXS139p, which is also termed γ-H2AX (Rogakou et al., 1999). The role of γ-H2AX as platform for the direct and indirect

recruitment of a large number of proteins involved in DSB signaling and processing has extensively been reviewed (e.g., Bekker-Jensen and Mailand, 2010; Lukas et al., 2011). For example, by binding to the phosphorylated serine 139 of γ-H2AX, retention of MDC1 in the chromatin domain surrounding the DSB is obtained (Stucki et al., 2005). MDC1 (mediator of DNA damage checkpoint protein 1) is a large mediator/adaptor protein playing a key role in the assembly of radiation-induced foci by its ability to bind various proteins (reviewed by Bekker-Jensen and Mailand, 2010; Jungmichel and Stucki, 2010). These include ATM, NBS1 (nibrin, which is encoded by a gene mutated in Nijmegen Breakage Syndrome) and RNF8 (RING finger protein 8).

So far, γ-H2AX is the best investigated modified histone associated with the cellular response to DSBs, but in recent years analysis of alterations in quantity and localization of other PTMs has gained large interest. Methods to investigate this include analysis of PTM patterns in the γ-H2AX decorated chromatin region by antibody-based immunofluorescence detection and microscopic visualization after DSB induction (e.g., Falk et al., 2007; Solovjeva et al., 2007; Ayoub et al., 2008). Since antibodies detecting histone modifications generally produce a pan-nuclear staining pattern modulated by local alterations in chromatin state, the study of co-localization or mutual exclusion of the PTM in question and γ-H2AX is not easy. While most studies are limited to qualitative assessment of co-localization or mutual exclusion of PTMs, Seiler et al. (2011) introduced methods for quantitative assessment. In addition to DSB induction by ionizing radiation or DSB-inducing chemical agents, such as neocarcinostatin, many IF-based studies used laser microirradiation to investigate PTM patterns at damage sites. A variety of laser microirradiation setups have been described to induce, in addition to other DNA damage types, DSBs (Grigaravicius et al., 2009). A disadvantage of the laser-based methods is that the amount and distribution of damage types induced are poorly characterized and that high laser energy densities may lead to unspecific chromatin damage. The high damage load induced by laser-irradiation may also result in microscopically detectable accumulations of proteins that are not found to visibly accumulate after DSB induction with ionizing radiation, not even after irradiation with heavy ions that produce clustered DSBs (for examples see Nagy and Soutoglou, 2009; Splinter et al., 2010; Seiler et al., 2011; Suzuki et al., 2011). Thus, the use of laser irradiation may lead to an over-estimation of response reactions in comparison to more physiological damage situations. An advantage of the targeted irradiation achievable with laser beams is, however, that the site of damage is determined in advance, which facilitates the detection of small alterations and differentiation of irradiated regions from spurious accumulations of damage markers. By using microirradiation with heavy ions, the advantage of localized irradiation can be combined with the production of physiologically relevant damage types (Durante and Friedl, 2011; Seiler et al., 2011).

Chromatin immunoprecipitation (ChIP) offers the possibility of high resolution analysis of chromatin-associated proteins and histone modifications. Its application for the analysis of alterations in chromatin patterns at DSB sites requires site-specific induction of DSBs (e.g., Murr et al., 2006; O'Hagan et al., 2008; Stante et al., 2009; Iacovoni et al., 2010). In general, the size of regions analyzed by ChIP is much smaller than that of regions analyzed by immunofluorescence. In some cases, DSB-associated alterations in PTMs can even be detected by Western blotting of nuclear lysates (e.g., Tjeertes et al., 2009; Seiler et al., 2011), in which case it is assumed that the PTM alterations detected affect also regions not directly adjacent to DSB sites (so-called global alterations). A concern with all antibody-based methods to PTM analysis is potential cross-reactivity. It is, therefore, expected that more specific methods based on mass spectrometry will increasingly be used in future.

Several excellent review articles have recently been published on the topic chromatin and DNA damage response (DDR) (van Attikum and Gasser, 2009; Ball and Yokomori, 2011; Bao, 2011; Xu and Price, 2011; Miller and Jackson, 2012). In the present article, we concentrate on the dynamics of PTMs in response to DSB induction. Over the last years it became clear that not only a large variety of "new" PTMs are formed in the vicinity of DSB sites during the DDR, but that other PTMs appear to be removed from these regions.

INVOLVEMENT OF PTMs IN IMMEDIATE EARLY DAMAGE DETECTION AND CHROMATIN OPENING

Early events of the DSB-induced DDR, starting with ATM-mediated phosphorylation of H2AX, are quite well understood. In contrast, the sequence of immediate early events upstream of ATM is more difficult to elucidate. A major player in recruitment and activation of ATM is the MRN complex, consisting of the proteins MRE11 (meiotic recombination 11), RAD50 (radiation sensitive 50) and NBS1 (for review, see Rupnik et al., 2010). In addition to a function depending on MDC1-mediated recruitment of MRN to the γ-H2AX domain, this complex acts as a DSB sensor, but how exactly it does sense the breaks is not yet clear. Kruhlak et al. (2006) demonstrate a rapid (within 20 s) local expansion of a chromatin region in which DNA damage including DSBs was induced by an UV laser. The expansion did not depend on ATM or H2AX, thus it cannot be explained by events downstream of ATM activation and H2AX phosphorylation. Since this expansion was dependent on ATP, it cannot be solely due to break-induced relaxation of torsional stress. Kruhlak et al. (2006) proposed that a damage sensor mediates decondensation of chromatin.

A candidate for such a sensor is PARP-1 (also known as ARTD1, ADP-ribosyltransferase diphtheria toxin-like 1), the major poly-(ADP-ribose)-polymerase in the cell. PARP-1 can bind to DSBs (and other DNA structures, including single-strand breaks) and is visibly recruited within 1 s to damage induced by laser-microirradiation (Haince et al., 2008). This is accompanied by an extensive poly-(ADP-ribosyl)ation of histones and other chromatin-bound proteins at DSB sites. All core histones, as well as H1, can be subject to poly-(ADP-ribosyl)ation. Recently, in an *in vitro* study Messner et al. (2010) identified the target sites H2AK13, H2BK30, H3K27, H3K37, and H4K16. Whether the same sites are targets of DSB-induced poly-(ADP-ribosyl)ation, remains to be tested. Anyway, *in vitro* and *in vivo*, poly-(ADP-ribosyl)ation of histones leads to increased accessibility of chromatin, which is explained by reduced DNA-histone interaction due to the high density of negative charge in

poly-(ADP-ribose) and by recruitment of nucleosome remodeling factors (Messner and Hottiger, 2011; Martinez-Zamudio and Ha, 2012). Unexpectedly, however, after laser-induced damage infliction, Timinszky et al. (2009) observed a higher chromatin density in the damaged region in spite of extensive poly-(ADP-ribosyl)ation, which was due to poly-(ADP-ribose)-dependent recruitment of macroH2A1.1. It is possible that this apparent compaction is preceded by a relative opening of chromatin following poly-(ADP-ribosyl)ation, but further elucidation would require systematic time course experiments.

Besides MRN complex and PARP-1, also the Ku heterodimer consisting of Ku70 and Ku86 has direct DSB end-binding ability. End-binding by the Ku heterodimer and subsequent activation of the catalytic subunit of DNA-PK are required for DSB repair via non-homologous end-joining. Up to now, there is no consistent picture of the interplay between PARP-1, MRN complex and the Ku heterodimer in the initial sensing of DSBs. Binding of Ku has been shown to inhibit binding of PARP-1 and MRE11 (Cheng et al., 2011), while others show an interaction between Ku complex and PARP-1 (Spagnolo et al., 2012). On the other hand, MRE11 binding depends on functional PARP-1 (Haince et al., 2008; Cheng et al., 2011).

Another pathway proposed to explain immediate early chromatin decondensation involves HMGN1 (high mobility group N1)-mediated activation of a histone acetyltransferase, resulting in a global increase of H3K14 acetylation (Lim et al., 2005; Kim et al., 2009). HMGN1, a factor binding to nucleosomes in a constitutive but highly dynamic fashion (Kim et al., 2009), appears to act upstream of ATM in the signaling cascade, since efficient ATM activation requires the presence of HMGN1. However, a role for HMGN1 in rapid local expansion of chromatin after DSB induction has not yet been addressed experimentally.

An impressively large number of ATP-dependent chromatin remodeling factors have been described to accumulate in the vicinity of DSB sites and/or to be involved in the DDR (Lans et al., 2012). Although some of these remodeling factors appear to act at very early steps of the DDR (Ahel et al., 2009; Gottschalk et al., 2009; Lan et al., 2010; Sánchez-Molina et al., 2011), so far no clear candidate has been defined which may be responsible for the rapid decondensation observed by Kruhlak et al. (2006).

In addition to the very early, ATM-independent, chromatin decompaction, also ATM-dependent relaxation mechanisms appear to act after DSB induction (Ziv et al., 2006). These mechanisms involve KAP-1 (Kruppel-associated box domain-associated protein-1), a transcriptional co-repressor involved in DNA condensation. After rapid ATM-dependent S824 phosphorylation of KAP-1 at damage sites, a quick pan-nuclear spreading of the phosphorylated KAP-1 leads to a global increase in nuclease accessibility of the chromatin (Ziv et al., 2006). Global increase in nuclease sensitivity after DNA damage induction has also been observed by others, but its significance is not clear. Since KAP-1 is a barrier to DSB repair in heterochromatin regions, its phosphorylation at S824 serves in addition a more localized role for the repair of DSB located in heterochromatin regions (Goodarzi et al., 2008; Noon et al., 2010) which involves dispersal of the long isoform of CHD3 (chromodomain helicase DNA binding protein 3), one of several possible catalytic subunits of the nucleosome remodeling and deacetylase (NuRD) complex (Goodarzi et al., 2011). Recently it was shown that a second phosphorylation of KAP-1 at S473, which depends on ATM and checkpoint kinase CHK2, promotes mobilization of the heterochromatin stabilizing protein HP1β (Bolderson et al., 2012). It is generally assumed that loss of CHD3 or HP1β facilitates repair in heterochromatin regions by facilitating access for repair factors. Local decondensation may, however, also serve to allow for DSB relocation to regions of lower density via physical forces (Jakob et al., 2011).

Interestingly, damage-associated local chromatin decondensation is not accompanied by a damage-induced localized or global loss of heterochromatin-specific PTMs, such as H3K9me3 or H3K9me2 (Ayoub et al., 2008; Luijsterburg et al., 2009; Sun et al., 2009; Noon et al., 2010; Seiler et al., 2011). It is, however, accompanied by a localized increase of histone H4 acetylation (Murr et al., 2006; Falk et al., 2007; Ikura et al., 2007; Ogiwara et al., 2011), which is mainly conferred by the histone acetyltransferases TIP60 (Tat-interactive protein; Murr et al., 2006) as well as p300 and CBP (CREB-binding protein; Ogiwara et al., 2011). Dispersal of HP1β allows binding of TIP60 which activates its acetyltransferase activity (Sun et al., 2009). DSB-induced hyperacetylation of H4 at lysines 5, 8, 12, and 16 may affect nucleosome stability either directly by reducing the interaction between H2A and H4, or indirectly by involving the NuA4 remodeling complex (reviewed by Xu and Price, 2011). Data on damage-induced hyperacetylation of H3 are conflicting: Ogiwara et al. (2011) observed hyperacetylation of H3K18, which depended on CBP/p300, but not of other N-terminal lysines, whereas H3K14 hyperacetylation was observed by others (Murr et al., 2006; Kim et al., 2009). CBP/p300-dependent hyperacetylation at damage sites has also been described for at H3K56 (Das et al., 2009; Vempati et al., 2010), whereas others observed H3K56 hypoacetylation at damage sites and on a global level (Tjeertes et al., 2009; Yang et al., 2009; Miller et al., 2010, our own unpublished observations). A local decrease of H3K56 acetylation in the vicinity of damage sites would agree well with the observation of local accumulation of histone deacetylases HDAC1 and HDAC2 at damage sites (Miller et al., 2010). However, all results on H3K56 acetylation obtained with antibody-based techniques recently were seriously challenged by Drogaris et al. (2012) due to potential cross-reactivity to other acetylation sites present on H3 N-terminal tails.

Ubiquitination of histones is involved in transcriptional regulation and DDR (Cao and Yan, 2012). The ubiquitin ligase RNF8 directly interacts with MDC1 and is thus recruited to the γ-H2AX domain. RNF8 has in recent years emerged as the starting point of a complex ubiquitin-dependent signaling response (reviewed by Bekker-Jensen and Mailand, 2011; Luijsterburg and van Attikum, 2012) which includes RNF168- and ubiquitin conjugating enzyme UBC13-dependent K63-linked poly-ubiquitination of H2A and H2AX (Huen et al., 2007; Mailand et al., 2007). K63-linked poly-ubiquitination is required for accumulation of downstream repair factors such as BRCA1 (breast cancer protein 1) and 53BP1 (p53 binding protein 1; Lok et al., 2012). Interestingly, after DSB induction RNF168 preferentially targets two novel N-terminal ubiquitination sites (H2AK13/K15)

rather than the canonical K119, whereas RNF8 appears to target all three sites (Gatti et al., 2012). RNF8-dependent histone poly-ubiquitination and recruitment of BRCA1 was shown to depend on prior nucleosome destabilization due to H4 hyperacetylation (Ikura et al., 2007; Xu et al., 2010). Others have, however, reported that RNF8- and CHFR (checkpoint with forkhead and ring finger domains)-dependent histone ubiquitination is required for MRG15 (MORF4-Related Gene on chromosome 15)-dependent recruitment of the histone acetyltransferases TIP60 and MOF (males absent on the first) to damage sites and subsequent hyperacetylation of H4K16 (Wu et al., 2011), which would place ubiquitination upstream of acetylation. In addition, RNF8 itself appears to have a role in unfolding higher-order chromatin structure which does not rely on its ubiquitin ligase function, but rather on recruitment of the chromatin remodeling factor CHD4 (Luijsterburg et al., 2012).

While recruitment of BRCA1 into foci involves direct interaction of its binding partner RAP80 (receptor-associated protein 80) with the poly-ubiquitin chain (reviewed by Kim and Chen, 2008), the molecular mechanisms of 53BP1 recruitment have long time been enigmatic (FitzGerald et al., 2009; Coster and Goldberg, 2010). 53BP1 binds via its tandem tudor domain to H3K79me1/me2 and/or H4K20me2 (Huyen et al., 2004; Botuyan et al., 2006; Spektor and Rice, 2009), but the damage-specific accumulation of 53BP1 and its dependence on RNF8 cannot solely be explained by damage-induced increase of these methylated sites in the vicinity of DSBs (Huyen et al., 2004; Pei et al., 2011). Interestingly, recent work showed that RNF8 and RNF168 mediate not only the formation of K63-linked poly-ubiquitination chains, but also the formation of K48-linked chains which label the target protein for proteosomal degradation (Meerang et al., 2011; Mallette et al., 2012). RNF8/RNF168-dependent degradation of the histone demethylases JMJD2A (lysine-specific demethylase KDM4A) and JMJD2B (KDM4B) unmasks H4K20me2, thus enabling binding of 53BP1 (Mallette et al., 2012). How these observations reconcile with 53BP1's dependence on K63-linked poly-ubiquitination as described by Lok et al. (2012) remains to be resolved. A similar unmasking mechanism involving the ubiquitin-selective segregase VCP/p95 and chromatin eviction of L3MBTL1 (lethal(3)malignant brain tumor-like protein 1), a chromatin compaction factor, was also described (Acs et al., 2011; Meerang et al., 2011). Interestingly, RNF8 does not only contribute to chromatin opening at damage sites, but may also contribute to the establishment of repressive patterns (see below).

Ubiquitination of H2B is a prime example of participation of mechanisms normally involved in other cellular reactions (in this case transcription) in the DDR. Shiloh et al. (2011) suggest that "borrowing" of factors and mechanisms from other cellular reactions in the case of emergency may help the cell to rapidly respond without having to wait for the synthesis and activation of damage-specific proteins. Mono-ubiquitinated H2BK120 (H2BK123 in *S. cerevisiae*) is associated with transcribed regions of highly expressed genes (Minsky et al., 2008) and mainly found downstream of the transcription start site, hinting at a function in transcriptional elongation rather than initiation. Using chemically defined nucleosome arrays, Fierz et al. (2011) showed that ubH2B interferes with chromatin compaction, leading to an open fiber conformation. The RING finger proteins RNF20 (hBRE1) and RNF40 form the E3 ligase complex responsible for this formation of ubH2B. Recruitment of RNF20/40 to active transcriptions sites appears to be mediated by linking to RNA Polymerase II (RNAPII), e.g., via WAC (WW domain-containing adaptor protein with coiled-coil) protein (Zhang and Yu, 2011). RNF20/40-dependent formation of ubH2B is also seen after induction of DNA damage. Moyal et al. (2011) demonstrate by Western analysis an increase in global levels of ubH2B after treatment with neocarcinostatin, a clastogenic agent, and also a local enrichment of ubH2B at damage sites induced by laser-microirradiation. Since ATM activation and recruitment of early signaling factors MDC1 and RNF8 are not affected by inactivation of RNF20, the authors proposed that RNF20/40 act downstream of signaling, but before initiation of repair. Indeed, recruitment of repair proteins to laser-induced damage sites appears to be reduced if RNF20 is inactivated. Nakamura et al. (2011) similarly demonstrate RNF20 localization to DSB sites, which does not depend on H2AX. They propose that RNF20 accumulation leads to chromatin relaxation, end resection and subsequent recruitment of recombination proteins, such as RAD51 and BRCA1. A role for RNF20/40 in conferring genomic stability was also found by others (Chernikova et al., 2012). So far, it has not been elucidated how RNF20 is recruited to damage sites.

Taken together, several redundant and, at least in part, communicating pathways have been identified which are associated with hyperacetylation, poly(ADP-ribosyl)ation and ubiquitination of histones and may lead to chromatin opening. This raises the question of whether transcriptional regulation is affected by damage-induced alterations in PTM patterns.

REPRESSIVE PATTERNS ESTABLISHED IN THE VICINITY OF DSB SITES

Early evidence that transcription may be inhibited in the vicinity of break sites in spite of the open chromatin configuration came from Solovjeva et al. (2007) who observed that BrUTP incorporation is strongly suppressed at γ-H2AX foci after ionizing irradiation. Similarly, Kruhlak et al. (2007) demonstrated reduced FUrd incorporation in nucleoli microirradiated with a laser beam, suggesting inhibition of RNA polymerase I (RNAPI)-dependent transcription. Transcription inhibition is not a global response, since FUrd incorporation was not affected in neighboring, un-irradiated nucleoli. Inhibition was found to depend on ATM, but not on Ku proteins, JNK (jun N-terminal kinase) pathway or proteasome activity. Shanbhag et al. (2010) developed an elegant system based on induction of site-specific DSBs upstream of a reporter gene to investigate whether the presence of a DSB affects the expression of a (RNAPII-transcribed) gene located *in cis*. By means of fluorescence-tagging, both the nuclease target site (i.e., the location of the DSB) and the nascent RNA of the reporter gene (which contains structures specifically bound by a viral protein) can be visualized. The authors demonstrate a drastically reduced production of nascent reporter transcript upon DSB induction *in cis*, but not a global reduction of transcription. Another similarity of this so-called "DSB-induced silencing *in cis*" to inhibition of RNAPI was that it depended on ATM,

but not on the DNA-dependent protein kinase (DNA-PK). In contrast, Pankotai et al. (2012) described dependence on DNA-PK, but not ATM, of DSB-induced transcriptional inactivation of RNAPII-transcribed genes containing target sites for site-specific nucleases. It should be noted that some authors did not observe repressed transcription in the vicinity of break sites (Iacovoni et al., 2010; Cramers et al., 2011). The significance of this discrepancy remains to be elucidated.

Different states of RNA polymerase II (RNAPII) are characterized by different patterns of PTMs in the large subunit, especially in its C-terminal domain (CTD) which consists of 53 copies of a heptapeptide (reviewed by Brookes and Pombo, 2009). Best characterized are the roles of CTD phosphorylation at serine 2 and serine 5. When recruited to the promoter, neither S5 nor S2 are phosphorylated. During initiation, S5 is phosphorylated. The presence of S5 does, however, not necessarily hint at activity of a gene, since paused genes also contain S5 phosphorylated RNAPII. Productive elongation is associated with S2 phosphorylation. To further characterize DSB-induced silencing, Shanbhag et al. (2010) investigated the elongating form of RNAPII. They observed a loss of actively elongating RNAPII with phosphorylated S2 in the vicinity of enzyme-induced DSB sites. At the same time, they did not observe a significant loss of the total amount of RNAPII, as assessed with antibody 8WG16, which recognizes unphosphorylated S2 in CTD repeats. Similarly, a loss of the elongating form, but no reduction in the total amount of RNAPII (in this case measured by an antibody detecting an epitope outside of the CTD), was observed by Seiler et al. (2011) at ion-induced γ-H2AX domains. Chou et al. (2010) and Chagraoui et al. (2011) observed a loss of the elongating form of RNAPII at laser-induced γ-H2AX domains and at γ-H2AX foci induced by UV irradiation after Hoechst 33258-sensitization, respectively. Similarly, underrepresentation of the elongating form of RNA polymerase II is seen in replication-stress-induced so-called OPT (Oct-1, PTF, transcription) domains (Harrigan et al., 2011). OPT domains contain, among other factors, γ-H2AX and 53BP1 and presumably contain damage sites awaiting repair in the next replication phase. At the same time an underrepresentation of the initiating form of RNAPII, which is phosphorylated at S5, was observed by Seiler et al. (2011) and Harrigan et al. (2011). A loss of S5-phosphorylated RNAPII at laser-induced γ-H2AX domains was first described by Miller et al. (2010). Taken together, a picture emerges that transcriptional repression at γ-H2AX domains is associated with a loss of S5- and also S2-phosphorylated RNAPII, but not with a loss of total RNAPII. This is in contrast to mechanisms described for transcriptional inhibition after induction of bulky DNA lesions, such as for example induced by UV irradiation, where RNA polymerase stalls at the damaged site and is then removed by proteosomal degradation after K48-linked ubiquitination (Heine et al., 2008; Hammond-Martel et al., 2012) in a process accompanied by hyperphosphorylation of the CTD, especially at S5. Very recent work (Pankotai et al., 2012) did, however, also imply proteasome-dependent displacement of RNAPII from broken genes after DSB induction.

Ubiquitination of H2A is a well-described PTM associated with transcriptional repression, e.g., in X inactivation, which correlates with ubiquitination of H2AK119 via polycomb repressive complex PRC1 (de Napoles, 2004; Fang et al., 2004). The E3 ubiquitin-protein ligase responsible within PRC1, RING1B (also called RING2 or RNF2), is stimulated by RING1A and BMI-1 (B lymphoma Mo-MLV insertion region 1; Cao et al., 2005), both of which also possess RING finger domains. BMI-1, RING1A, and RING1B are also involved in DSB-associated H2A ubiquitination. BMI-1 is recruited to γ-H2AX domains after site-specific DSB induction, ionizing or laser irradiation (Facchino et al., 2010; Ismail et al., 2010; Chagraoui et al., 2011; Ginjala et al., 2011). Whether it is also recruited to other types of damage, such as UV or hydroxyurea induced damage, is under debate (Ismail et al., 2010; Ginjala et al., 2011). Ismail et al. (2010) report that BMI1 and RING1B are recruited to DSB sites where they confer monoubiquitination of H2AX. BMI1 recruitment did not depend on γ-H2AX or RNF8, but on poly-(ADP-ribosyl)ation at the damage sites and the authors concluded (Ismail et al., 2010; Gieni et al., 2011) that the BMI1-mediated pathway to γ-H2AX ubiquitination acts in parallel to and independent of the RNF8-mediated pathway. In contrast, Ginjala et al. (2011) report that BMI1 recruitment depends on γ-H2AX and RNF8, but not on PARP-1. The work of Ismail et al. (2010) and Ginjala et al. (2011) differs also in the reported effects of inactivation of the BMI1-mediated pathway: while Ginjala et al. did not observe any effect on 53BP1 recruitment, Ismail et al. (2010) reported strong reduction of 53BP1 (as well as BRCA1 and RAP80) foci formation in the absence of BMI1. Chagraoui et al. (2011) reported BMI1 is required for the loss of elongating RNAPII at γ-H2AX domains, thus strengthening the link between recruitment of PRC1 factors and transcriptional repression.

Recent evidence suggests that in addition to PRC1, the polycomb repressive complex 2 (PRC2) is active in the vicinity of damage sites. O'Hagan et al. (2008) observed the appearance of silencing histone modifications, including H3K27me3, in the region surrounding an enzyme-mediated DSB. This was accompanied by the accumulation of several key proteins involved in establishing and maintaining transcriptional repression, including PRC2 core component EZH2 (enhancer of zeste homolog 2), which is the histone methyltransferase responsible for the majority of cellular H3K27me3 marks. Chou et al. (2010) observed recruitment of repressive polycomb complexes at damage sites induced by laser microirradiation, while Seiler et al. (2011) showed EZH2 accumulation at damage sites induced by ion irradiation. Whereas these observations support the involvement of polycomb-mediated silencing (Tang and Greenberg, 2010), no indications for the involvement of heterochromatin-based silencing have been observed. Thus, no DSB-induced increase of the repressive marks H3K9me3 or H3K9me2 could be observed (Ayoub et al., 2008; Luijsterburg et al., 2009; Seiler et al., 2011).

The activity of the polycomb repressive complex PRC2 is inhibited by active chromatin marks, including H3K4me3 (Schmitges et al., 2011). H3K4me3 is a well-characterized active mark primarily associated with the start site of transcription, which at least in part reflects tethering of the COMPASS histone methyltransferase complex to RNA polymerase during active transcription (Henikoff and Shilatifard, 2011; Shilatifard, 2012). H3K4 trimethylation depends also on mono-ubiquitination of H2B. Since ubH2B accumulates at DSB sites (see above), it was

suggested that a SET/COMPASS family histone H3K4 methyltransferase may also be involved in DSB repair (Shilatifard, 2012). Indeed, by ChIP analysis of chromatin regions directly flanking nuclease-mediated DSB sites, an increase of H3K4 methylation was seen (Faucher and Wellinger, 2010; Nakamura et al., 2011). In contrast, immunofluorescence analysis after DSB induction by ionizing radiation, coupled with elaborate image analysis methods including ultra-thin sectioning of cells, demonstrated a loss of H3K4me3 and H3K4me2 signals in the γ H2AX domains, which started within minutes after damage infliction and increased over time (Seiler et al., 2011). The loss of H3K4me2/3 signals was associated with a loss of another active mark, H3K9ac, and a loss of active RNAPII. These data are compatible with involvement of JARID1/KDM5 family histone demethylases capable of demethylating H3K4me3 and H3K4me2, presumably as part of complex that also contains histone deacetylases. It is also interesting to note that during ES cell differentiation the PRC2 complex recruits JARID1A (KDM5A/RBP2) to its target genes (Pasini et al., 2008), which couples generation of H3K27me3 with loss of H3K4me3. Recently, depletion of H3K4me3, H3K4me2, and H3K9ac was also observed upon binding of DNA methyltransferase DNMT1 in the context of transcriptional regulation (Clements et al., 2012). Interestingly, this function of DNMT1 was independent of its DNA methyltransferase activity. The authors suggested that depletion of the active histone marks results from DNMT1's interaction with histone deacetylases and demethylases. Since DNMT1 is known to accumulate at laser-induced damage sites (Mortusewicz et al., 2005), it may also serve to recruit histone modifying enzymes in the context of DSB response.

CONCLUSION

Growing evidence shows that histone modification patterns alter significantly in the course of the cellular response to DSB induction. Both, establishment of patterns suggesting open chromatin configurations and patterns suggesting more condensed configuration were described (see **Figures 1** and **2**). Many data in the literature are controversial and it is at present not yet possible to reconcile all observations. Observed differences may be explained by different damage types (e.g., clean enzyme-mediated DSBs vs. radiation-induced damage comprising (unclean) DSBs and other damage types), different subcompartments investigated (immediate vicinity of DNA ends vs. γ-H2AX domain) or different time scales. Clearly, more systematic analyses will be required to resolve the open questions.

Any radiation-induced alteration in histone modification pattern has to revert to the original state after successful completion of damage response and repair. The same holds for alterations on other levels of expression regulation such as DNA methylation status or miRNA expression. Otherwise, long-term epigenetic alterations may occur, which then may be causally linked to a carcinogenic process (Mothersill and Seymour, 2003; Loree et al., 2006; Kovalchuk and Baulch, 2008). It will be interesting to determine the relative impact of epigenetic alterations vs. DNA sequence alterations on radiation-induced carcinogenesis.

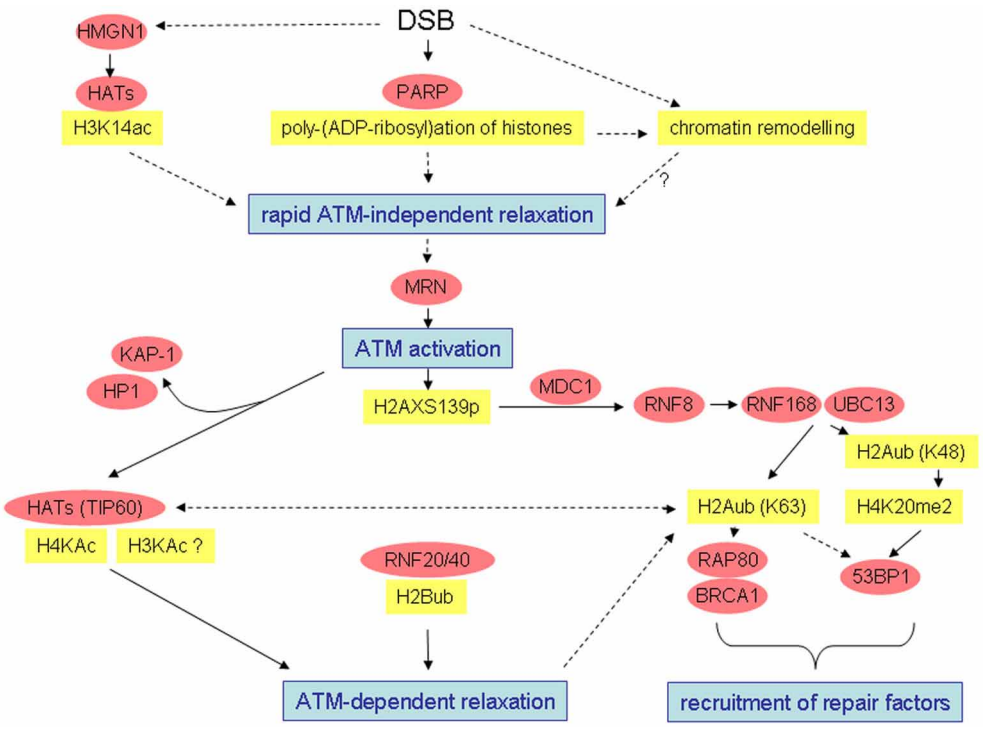

FIGURE 1 | Simplified overview of radiation-induced alterations possibly leading to open chromatin configuration or chromatin relaxation and recruitment of damage response factors. Well-characterized interactions and causal relationships are depicted by solid lines, less well-characterized interactions and relationships by broken lines.

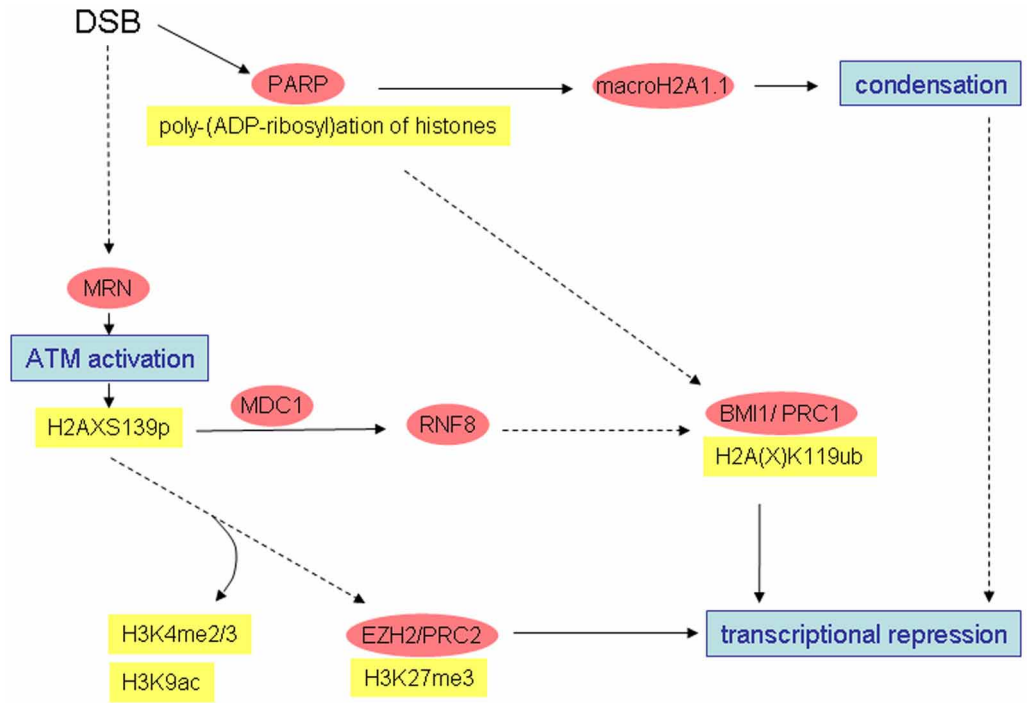

FIGURE 2 | Simplified overview of radiation-induced alterations possibly leading to chromatin condensation and transcriptional repression. Well-characterized interactions and causal relationships are depicted by solid lines, less well characterized interactions and relationships by broken lines.

Finally, since epigenetic alterations contribute to carcinogenesis, the idea of reverting these alterations by so-called epigenetic drugs has lead to the development of several drugs approved for the treatment of cancer (reviewed by Baylin and Jones, 2011). DNA demethylating agents and HDAC inhibitors are generally thought to combat tumour cells by reversing epigenetic silencing of tumor suppressor genes. In the context of the present article, it is interesting to note that these agents posses also radiomodulating activity (reviewed by De Schutter and Nuyts, 2009). Agents leading to DNA demethylation, such as cytidine analogs, possess radiosensitizing activity, at least *in vitro* (e.g., Dote et al., 2005; Brieger et al., 2012; Kim et al., 2012). Radiosensitizing activity of HDAC inhibitors has extensively been studied (reviewed by Camphausen and Tofilon, 2007), but in certain contexts also radioprotective effects were observed (e.g., Konsoula et al., 2011; Miller et al., 2011). Inhibition of poly-(ADP-ribose)-polymerases has emerged as paradigm of synthetic lethal treatment, which is thought to rely on inhibition of SSB repair. During replication, unrepaired SSB will convert into DSB, and in cells deficient in DSB repair via homologous recombination, e.g., breast cancer cells carrying mutations in BRCA1 and BRCA2, cell death will occur (reviewed by Chalmers et al., 2010). It remains to be tested to what extent radiosensitizing effects of PARP inhibition in recombination-proficient cells are caused by inhibition of histone poly-(ADP-ribosyl)ation. The ongoing identification of small molecule inhibitors of histone modifying enzymes opens the way of testing their potential radiomodulating effects in the future.

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Radiation-induced changes in microcirculation and interstitial fluid pressure affecting the delivery of macromolecules and nanotherapeutics to tumors

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The immature, chaotic microvasculature of most solid tumors can present a significant impediment to blood-borne delivery, uneven distribution, and compromised penetration of macromolecular anticancer drugs and diagnostic agents from tumor microvessels across the interstitial space to cancer cells. To reach viable tumor cells in relevant concentrations, macromolecular agents are confronted with several barriers to vascular, transvascular, and interstitial transport. Amongst those (1) heterogeneous and poor blood supply, (2) distinctly reduced or even abolished hydrostatic and oncotic pressure gradients across the microvessel wall abrogating the convective transport from the vessel lumen into the interstitial space (impairment of transvascular transport), and (3) impediment of convective transport within the interstitial compartment due to elevated interstitial fluid pressure (IFP) (resulting from hyperpermeable blood vessels coupled with non-functional lymphatics) and a dense structure of the interstitial matrix are the major mechanisms hindering drug delivery. Upon irradiation, changes in these barrier functions are inconclusive so far. Alterations in vascular transport properties following fractionated radiation up to 40 Gy are quite inconsistent in terms of direction, extent, and time course. Total doses above 45 Gy can damage tumor microvessels, additionally impeding vascular delivery. Vascular permeability for macromolecules might be enhanced up to a total dose of 45 Gy. However, this effect is counteracted/abolished by the elevated IFP in solid tumors. When assessing IFP during fractionated radiotherapy in patient tumors, inconsistent alterations have been observed, both in direction and extent. From these data it is concluded that modulations in vascular, transvascular, and interstitial transport by irradiation of solid tumors are rather unclear so far. Translation of experimental data into the clinical setting thus needs to be undertaken with especial care.

Keywords: irradiation, tumor microcirculation, transport barriers, tumor interstitial fluid pressure, macromolecular agents, intratumor pharmacokinetics

INTRODUCTION

The chaotic microvasculature of solid tumors leads to significant impediment of delivery, uneven distribution, and compromised penetration of macromolecules and nanotherapeutics from tumor microvessels across the interstitial compartment to cancer cells, especially to cells distant from microvessels. To reach viable tumor cells in relevant concentrations, diagnostic, and therapeutic agents are confronted with several obstacles: disturbed convective transport within the chaotic vascular compartment (*vascular transport*), spatio-temporally uneven distribution within the tissue, and significant shunt flow bypassing the exchange processes between the vascular bed and the extravascular space. Extravasation (*transvascular transport*) and extravascular convection (*interstitial transport*) of macromolecules and nanoparticles are mainly impaired by high interstitial fluid pressure (IFP). Furthermore, marked gradients in concentrations of macromolecules and nanoparticles exist within the extravascular space limiting anticancer therapies with increasing distance from tumor blood vessels (Jain, 1987, 1990; Vaupel, 2009b; Jain and Stylianopoulos, 2010; Vaupel and Multhoff, 2012).

Amongst the key pathophysiological abnormalities in solid tumors related to drug transport, chaotic vascular networks, abnormal blood flow, and elevated IFP (interstitial hypertension) seem to play the dominant roles (see **Figure 1**). Accumulated solid stress from the growing tumor (through unlimited proliferation of cancer cells and excessive production of collagen and hyaluran), a dense interstitial structure, and contractions of the interstitial matrix mediated by stromal fibroblasts add to the transport barrier to anticancer agents (Heldin et al., 2004; Chauhan et al., 2011; Wiig and Swartz, 2012).

While some data suggest that interstitial hypertension might not be a significant barrier to therapy as has generally been proposed (Wiig and Swartz, 2012), in the following sections the impact of irradiation on the key pathophysiological

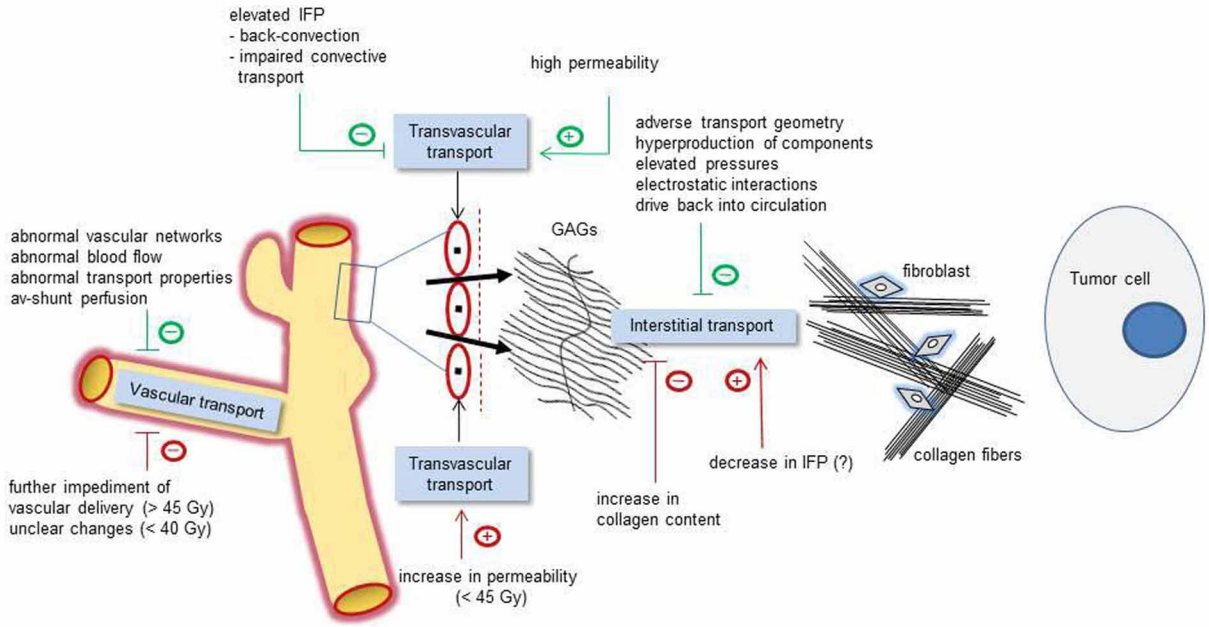

FIGURE 1 | Schematic representation of relevant pathophysiological mechanisms affecting the vascular (left), transvascular (center), and interstitial transport (right) of macromolecular compounds (e.g., anti-tumor and diagnostic large-size molecules). Green tags: basic pathophysiological obstacles (negative signs) or facilitating mechanisms (positive signs). Red tags: irradiation-induced modulations affecting the transport properties in a positive or negative direction. Interstitial transport of macromolecules is hindered by an adverse transport geometry (including enlarged interstitial volumes and transport distances), by a hyperproduction of interstitial components (e.g., stromal cells, collagen fibers, interstitial matrix), by elevated pressures (IFP, OP, and accumulated solid stress), electrostatic interactions, and drive back into the circulation. IFP, interstitial fluid pressure; OP, oncotic pressure; GAGs, negatively charged glycosaminoglycans.

characteristics mentioned above will be discussed with regard to their effect on the delivery of macromolecules and nanotherapeutics to primary and metastatic tumors.

VASCULAR TRANSPORT

Vascular transport, i.e., the delivery of anticancer and diagnostic agents via the blood stream, includes the convective transport to the tumor and the subsequent distribution within the tumor ("blood-borne delivery," Vaupel and Multhoff, 2012). The development of a disorganized microvasculature and significant arterio-venous shunt perfusion leads to an inefficient delivery of (macromolecular) agents and nutrients (e.g., oxygen, glucose) through the vascular system of the tumor (see **Table 1**). The situation is further aggravated by flow-dependent spatio-temporal heterogeneities in the distribution of plasma-borne agents (and their metabolites). These "4D-heterogeneities" are not static, but instead are quite dynamic, and therefore more complex than has been previously assumed (for reviews see Vaupel et al., 1989; Vaupel, 2006, 2009a,b, 2012).

The status of the tumor microvasculature and blood flow (direction, extent, and time course of changes) upon irradiation remains largely unclear, both for single large doses (12–50 Gy) and fractionated radiation (25 fractions, 5 weeks, up to a total dose of 75 Gy), but also appears to depend on the tumor type studied, the radiation dose, the time interval between exposures, and irradiation stage (during vs. post). The literature provides quite conflicting data on whether or not radiation-related biologically or clinically relevant changes in microvascular structures and functions occur.

Descriptive and morphometric studies performed between 1927 and 1977 using experimental tumors suggested that fractionated doses commonly led to an increase in vascular density, while single large doses often destroyed the vasculature and shut down blood flow (for details see Narayan and Cliff, 1982; Fajardo and Berthrong, 1988; Baker and Krochak, 1989; Dewhirst, 1991). However, experiments using single large dose irradiation are quite inconclusive since changes in tumor blood flow were both dose- and time-dependent (Vaupel et al., 1984). In a recent review, a very contradictory data set for single large dose local irradiation in the experimental setting has been presented (Kozin et al., 2012).

In conventional fractionation schedules, tumor microvessels are distinctly damaged above doses of 40–45 Gy (Zywietz et al., 1994). Above this "critical cumulated dose" tumor oxygenation and ATP levels progressively decreased (Thews et al., 1999), clearly showing that these parameters are critically determined by the efficacy of tumor blood flow. Continuous hyperfractionation (2 daily fractions of 2.5 Gy, up to 60 Gy), however, induced only relatively discreet alterations of the tumor microvasculature (Lorke et al., 1999).

Published data on changes in tumor blood flow and oxygenation upon radiation therapy in the clinical setting showed no clear direction in observed alterations (Feldmann et al., 2000; Molls et al., 2000). From this compilation of data, there is evidence that changes in tumor microcirculation (i.e., vascular transport

Table 1 | Obstacles in blood-borne delivery of macromolecular anticancer and diagnostic agents and modulations following irradiation (selection; Vaupel, 2006, 2009a).

A. ABNORMAL VASCULAR NETWORK ("MORPHOLOGICAL ABNORMALITIES")
Development of an immature, disorganized microvasculature
Spatial heterogeneities
Existence of avascular spaces
Enlarged intervessel distances
Blind vessel endings
Arterio-venous anastomoses
Convoluted, elongated, and dilated microvessels
Leaky microvessels
B. ABNORMAL BLOOD FLOW ("FUNCTIONAL ABNORMALITIES")
Excessive spatial and temporal heterogeneity in flow ("4D-heterogeneity")
Slowing of blood flow, flow stops
Poor, inadequate perfusion
Sluggish perfusion
Unstable flow velocities
Arterio-venous shunt perfusion
Flow reversals
Elevated geometric and viscous resistance to flow
C. IRRADIATION-INDUCED MODULATIONS OF BLOOD-BORNE DELIVERY
Changes in vascular transport properties following fractionated irradiation up to 40 Gy are rather unclear
Total doses above 45 Gy may damage tumor microvessels further impeding vascular delivery

properties) following γ-irradiation (fractionated doses, up to 40 Gy) are rather unclear so far due to obvious variabilities in the direction, extent, and time course of changes observed. There is at least some consensus that upon conventional fractionation with total doses above 45 Gy microvessels are damaged, further impeding vascular delivery of blood-borne anticancer (macro-) molecules.

TRANSVASCULAR TRANSPORT

Therapeutic (and diagnostic) molecules and nanomedicines cross the leaky vessel walls by two major mechanisms: diffusion and convection. Large pore sizes of tumor microvessels facilitate these transport processes. Diffusion is the prevailing molecular transport modality of small-size molecules driven by concentration gradients. Convection is driven by hydrostatic pressure gradients and is the dominant mode of transport for large molecules, liposomes, and other nanoparticles (Kuszyk et al., 2001). Due to the elevated interstitial fluid pressure (IFP, interstitial hypertension, see section below), transvascular pressure gradients are approaching zero. As a result of this "equilibration" of hydraulic pressures, significant hindering of the transport of macromolecules and nanoparticles into the extravascular space by convection has to be considered (see **Table 2**). For this reason, the main mechanism of mass transport across vessel walls is diffusion (for a review see Vaupel and Multhoff, 2012). This process is significantly slower than convection, especially for macromolecules and

Table 2 | Obstacles to transvascular transport (extravasation) of macromolecular therapeutic and diagnostic agents in solid tumors and modulations upon irradiation (selection, Vaupel and Multhoff, 2012).

A. MECHANISMS FACILITATING EXTRAVASATION
Presence of abundant fenestrae, wide channels, and large pores in the microvascular wall
High permeability (leakiness) of microvessels (vascular permeability is at least 10 times higher than interstitial permeability; Lunt et al., 2008)
B. MECHANISMS HINDERING EXTRAVASATION
Leakiness of microvessels is heterogeneous
Impaired transluminal convective transport of macromolecules (due to elevated IFP, see **Table 3**)
Decreased transfer of large-sized, anionic, and neutral particles
Intravasation back to vascular compartment (due to elevated IFP, see **Table 3**), "back-convection" from the interstitial space into the circulation
C. IRRADIATION-INDUCED MODULATION OF EXTRAVASATION
Radiation-induced increase in vascular permeability might enhance extravasation up to a total dose of 45 Gy
However, enhanced permeability is counteracted by elevated interstitial fluid pressure (IFP)
Due to elevated IFP transluminal transport can be reversed (intravasation instead of extravasation)

nanoparticles (Jain and Stylianopoulos, 2010). Vessel wall hyperpermeability (enhanced porosity) is thus counteracted by elevated IFP in tumors (and by the large size of nanoparticles).

Vascular permeability decreases with increasing size of the transported nanoparticles (according to the Organization for Standardization, nanomedical approaches use particles from 1 to 100 nm; e.g., gold nanoparticles 2.5 nm, monoclonal antibodies 10–15 nm, oncolytic viruses 30–40 nm, magnetic nanoparticles for drug targeting 15–100 nm, liposome-encapsulated doxorubicin 80–130 nm, gadolinium-based nanoparticles 115 nm, and albumin-paclitaxel nanoparticles 130 nm). Furthermore, permeability is higher for cationic compounds than for their anionic or neutral counterparts (Jain and Stylianopoulos, 2010).

Upon fractionated γ-irradiation, time- and dose-dependent changes in vascular permeability have been described in the experimental setting due to direct vessel wall damage and the action of indirect inflammatory stimuli (Lorke et al., 1999). A discrete increase in leakiness (associated with interstitial edema) has been observed already after a total dose of 15 Gy, with more pronounced leakiness at higher radiation doses. Upon radiation with a total dose of 30 Gy, hyperpermeability was further increased. Prolonged irradiation was eventually associated with progressive destruction of the vascular wall and disruption of the basal lamina.

In principle, radiation-triggered increases in vascular permeability may enhance extravasation of anti-cancer macromolecules up to a total dose of approximately 45 Gy. However, this facilitation is severely counteracted or totally abolished by mechanisms occurring in the interstitial compartment as outlined in the following section.

INTERSTITIAL TRANSPORT

The interstitial compartment of tumors differs significantly from that of normal tissue (Vaupel and Multhoff, 2012). As a result of (1) vessel leakiness, (2) lack of functional lymphatics, (3) interstitial fibrosis, (4) contraction of the interstitial matrix mediated by stromal fibroblasts, and (5) cell proliferation in a confined space, most solid tumors develop an elevated interstitial/hydrostatic fluid pressure (IFP), which is in contrast to normal tissues where IFP is close to atmospheric pressure (Jain, 1987, 1990; Heldin et al., 2004; Milosevic et al., 2004; Cairns et al., 2006; Wiig and Swartz, 2012).

As already mentioned above, increased IFP within solid tumors decreases extravasation. In addition, high IFP severely inhibits interstitial transport of larger molecules (e.g., antibodies, antibody drug conjugates, and liposomes) by convection (see **Table 3**). Macromolecules rely more heavily on convection as opposed to simple diffusional transport of low-molecular weight drugs. Compounds larger than 60 nm in diameter are not able to effectively diffuse through the extracellular matrix of highly fibrotic tumors. Interstitial transport of macromolecules is further impaired by a much denser network of interconnected collagen fibers in the extracellular matrix of tumors (as compared to normal tissues) leaving them in higher concentrations in perivascular areas only (Jain and Stylianopoulos, 2010). The transport of compounds with sizes of up to 1000 nm is further hindered by highly negatively charged heparan sulfate in the matrix.

Heterogeneous mobility and distribution of large-sized molecules is additionally caused by two phases in the matrix: a more aqueous phase is found in regions with low fiber content ("fast" compartment with relatively high diffusivity), and a more viscous phase is due to a high concentration of collagen fibers in a dense matrix ("slow" compartment with high retention of compounds). Collagen content in tumors is much higher and collagen fibers are much thicker than in normal tissue leading to an increased mechanical stiffness of the tissue (Netti et al., 2000; Heldin et al., 2004). The interstitium also contains abundant stromal cells and enzymes that can affect the activity and delivery of agents to the tumor cells (Kuszyk et al., 2001).

It is assumed that IFP is almost uniform throughout a tumor and that relevant gradients of IFP do not exist. However, IFP drops precipitously at the tumor/normal tissue interface. For this reason, the interstitial fluid oozes out of the tumor into the surrounding normal tissue, carrying away anticancer agents, growth factors or released heat shock proteins, and cancer cells with it (Fukumura and Jain, 2007). Shedded cancer cells may mediate metastasis. As another consequence of this peripheral drop in IFP, blood flow may be diverted away from the tumor center toward the periphery where anticancer agents may be lost from larger vessels.

Transmural coupling between IFP and microvascular pressure can critically reduce perfusion pressure between up- and downstream tumor blood vessels leading to flow stasis and thus, inadequate delivery of anticancer agents, in addition to the mechanisms impairing blood flow already mentioned above.

In the experimental setting, radiocurability of human tumor xenografts decreases with increasing IFP (Rofstad et al., 2009, 2010). In these experiments, IFP showed a strong positive correlation to the extent of acute hypoxia in the tumors investigated (Rofstad et al., 2009), an increased number of clonogenic cells (Rofstad et al., 2010), stimulation of proliferation, occurring presumably via modulation of signaling pathways

Table 3 | Obstacles in interstitial transport of macromolecular anti-cancer agents and nanomedicines and modulations following irradiation (selection, Vaupel and Multhoff, 2012).

A. PATHOMORPHOLOGICAL CHARACTERISTICS OF THE INTERSTITIAL COMPARTMENT
Enlarged interstitial volume
Enlarged interstitial transport distances
Hyperplasia of stromal cells
High stromal fraction
Dense network of collagen fibers
Hyperproduction of interstitial matrix
Non-functional lymphatics in the tumor center
B. PATHOPHYSIOLOGICAL FEATURES OF THE INTERSTITIAL COMPARTMENT
Elevated hydrostatic fluid pressure (IFP, 5–40 mmHg in solid tumors vs. −3 to +1 mmHg in most normal tissues)
Elevated oncotic (colloid osmotic) pressures (approximately 20.5 mmHg in tumors vs. 8 mmHg in subcutis; Stohrer et al., 2000)
Equilibrium between oncotic pressures of plasma and tumor interstitium
Transmural coupling between IFP and microvascular pressure leading to slowing/stoppage and even reversals of microvascular blood flow
Convective drive of anti-cancer agents back into the circulation
High visco-elasticity caused by glycosaminoglycans, e.g., hyaluronan
Severely hampered convective transport within the interstitial compartment
(Poor) diffusion largely responsible for interstitial transport in the bulk of tumors
Diffusivity (diffusion coefficient) decreases with increasing size of macromolecules
Diffusion rate for macromolecules correlates with orientation of collagen
Electrostatic interaction of charged particles with charged compounds of the interstitium
Electrostatic binding of macromolecules/nanoparticles by heparan sulfate
Escape of macromolecules at the tumor edge into the surrounding normal tissue
Diversion of blood flow from center to periphery of tumors due to elevated IFP
C. MODULATION OF INTERSTITIAL TRANSPORT UPON IRRADIATION
Inconclusive results when assessing IFP during fractionated radiotherapy in patients with cancers of the uterine cervix
(decrease in IFP in four out of seven patients, increase in IFP in three patients; Roh et al., 1991)
Decrease in IFP above a threshold of 10 Gy upon single dose or fractionated radiation of human colon cancer xenografts (Znati et al., 1996)
Reduced convective and diffusive transport of macromolecules following single dose or fractionated irradiation
(reduced interstitial fluid transport, increased collagen content; Znati et al., 2003)

(Nathan et al., 2005), and upregulation of VEGF-A expression (Nathan et al., 2008).

Studies in patients with cervix cancers have explored the relationship between IFP and outcome following radiotherapy (Milosevic et al., 2001; Yeo et al., 2009). In these studies, IFP was found to be a strong, negative, and independent prognostic factor for local control and distant metastasis. Several compounds have been shown to decrease tumor IFP in patients (for a review see Heldin et al., 2004). This reduction in IFP has been attributed to a substantial decrease in vascular permeability, lowered microvascular pressure and changes in the extracellular matrix.

Assessing interstitial hypertension during fractionated radiotherapy in patients with cervix cancers showed inconclusive results, since only 4 out of 7 patients experienced a drop in IFP during treatment, whereas in 3 patients IFP distinctly increased (Roh et al., 1991). Measurements after single dose or fractionated radiation in human colon cancer xenografts yielded a reduction in IFP above a threshold of 10 Gy. Below this threshold there was no significant change in IFP (Znati et al., 1996). A decrease in microvascular pressure has been discussed as a plausible explanation for the radiation-induced reduction in IFP by these authors. Furthermore, the authors argued that this radiation-related decrease in IFP may have been responsible for an improved uptake of monoclonal antibodies following single dose or fractionated irradiation as reported earlier by others. In contrast to these data, in a later publication by this group a reduced interstitial fluid transport and increased collagen content in tumors has been communicated (Znati et al., 2003), implicating a reduced transport of macromolecular agents in tumors upon radiation.

CONCLUDING REMARKS

Preceding cellular pharmacodynamics, three important pharmacokinetic steps govern the delivery of anti-cancer drugs and diagnostic agents to tumor cells: vascular, transvascular, and interstitial transport. Barriers to delivery of macromolecular drugs mainly arise from immature, chaotic vascular networks and abnormal tumor blood flow, hyperpermeability of leaky microvessels, and elevated fluid pressure within the interstitial compartment abrogating convective transport. Upon tumor irradiation, changes in these barriers and thus in transport properties are inconsistent so far, so that definite conclusions for the clinical (and experimental) setting cannot be drawn. Therefore, transport mechanisms for (macro-) molecules should increasingly receive attention. One of the goals of translational cancer research is to obtain a better understanding of the compromised delivery and distribution of anti-cancer compounds in solid tumors (i.e., intratumor pharmacokinetics) in order to improve patients' outcomes.

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Immunomodulatory properties and molecular effects in inflammatory diseases of low-dose X-irradiation

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Inflammatory diseases are the result of complex and pathologically unbalanced multicellular interactions. For decades, low-dose X-irradiation therapy (LD-RT) has been clinically documented to exert an anti-inflammatory effect on benign diseases and chronic degenerative disorders. By contrast, experimental studies to confirm the effectiveness and to reveal underlying cellular and molecular mechanisms are still at their early stages. During the last decade, however, the modulation of a multitude of immunological processes by LD-RT has been explored *in vitro* and *in vivo*. These include leukocyte/endothelial cell adhesion, adhesion molecule and cytokine/chemokine expression, apoptosis induction, and mononuclear/polymorphonuclear cell metabolism and activity. Interestingly, these mechanisms display comparable dose dependences and dose-effect relationships with a maximum effect in the range between 0.3 and 0.7 Gy, already empirically identified to be most effective in the clinical routine. This review summarizes data and models exploring the mechanisms underlying the immunomodulatory properties of LD-RT that may serve as a prerequisite for further systematic analyses to optimize low-dose irradiation procedures in future clinical practice.

Keywords: discontinuous dose dependency, inflammation, immune modulation, low-dose radiation therapy

INTRODUCTION

The relationship between ionizing radiation and an inflammatory response displays a dichotomous character and greatly depends on the radiation dose/quality and immune cell types investigated. When compared to a high dose exposure with pronounced inflammatory promoting effects (Williams et al., 2003), low-dose irradiation (single doses $\leq$1.0 Gy) reveals anti-inflammatory properties (Seegenschmiedt et al., 2008; Rödel et al., 2012). This implicates the involvement of complex mechanisms differentially operating at different dose levels (Marples et al., 2004). Although low-dose radiation therapy (LD-RT) for the treatment of inflammatory and degenerative diseases (Seegenschmiedt et al., 2008) is successful in clinical use since several decades, underlying immunological and molecular mechanisms are far from being fully explored, in part because of their unusual discontinuous dose dependency and non (DNA)-targeted properties. The present review focuses on immunomodulatory properties of LD-RT to document the anti-inflammatory efficacy with special emphasis on preclinical *in vivo* models.

CLINICAL APPLICATION OF LOW-DOSE RADIATION THERAPY

The first known description of the clinical implementation of LD-RT to treat patients with non-cancerous diseases was as early as 1898 when Sokoloff and Stenbek reported on pain relief in patients with juvenile arthritis (Schmid-Monnard, 1898; Stenbek, 1898). More than 100 years later, a pattern of care study performed in Germany was published with 37,410 patients treated for inflammatory diseases proving LD-RT to be an accepted conservative treatment option at least in this country (Seegenschmiedt et al., 2004). Concepts and doses in clinical practice have been established empirically in the early twentieth century (von Pannewitz, 1933) recommending local treatment with single doses of 0.3–1.0 Gy in 4–5 fractions for acute and 1–3 fractions for chronic inflammatory disorders adding to a total doses of 3–5 Gy (acute) and 12 Gy (chronic), respectively (Seegenschmiedt et al., 2008). Typical clinical indication comprise degenerative disorders like rotator cuff syndrome (impingement of the shoulder joint), tennis/golfer's elbow (Epicondylopathia humeri), painful heel spur (plantar fasciitis), exacerbated refractory, and painful osteoarthritis, or hyper-proliferative syndromes like Dupytren's disease or the prevention of heterotopic ossification (Seegenschmiedt et al., 2004, 2008). Concerning the most clinical relevant endpoints pain relief, response, and analgetic effects, LD-RT is reported to result in a 33–100%, a 47–100%, and a 12–89% efficacy, respectively (Kutzner et al., 2003; Micke and Seegenschmiedt, 2004; Niewald et al., 2007; Adamietz et al., 2010; Betz et al., 2010; Heyd et al., 2010). Moreover, due to the low-doses used in actual clinical practice, radiogenic acute or chronic side effects were not observed in the treatment of inflammatory diseases (Seegenschmiedt et al., 2008). By contrast, LD-RT is still considered unfashionable in

some (Anglo-American) countries due to elder reports on harmful side effects and increased mortality from leukemia and anemia (Cannon et al., 1959; Court-Braun Wm, 1965). Nevertheless, non-steroidal or steroidal drugs used as a pharmaceutical alternative also display numerous side effects and a considerable number of patients does not respond to the treatment (Rainsford, 2007). Consequently, after improving radiation protection, LD-RT is practised on an increasing number of patients as an effective and safe treatment routine. To underscore this, a more recent pattern of care study (Mücke et al., 2010) reported on 4.500 patients with osteoarthritis of the knee receiving LD-RT demonstrating an increased acceptance (95% referral) of this treatment alternative in Germany.

PRINCIPLES OF INFLAMMATION

Inflammation comprises a complex and basic immunological response to harmful stimuli, such as pathogens, damaged cells, or irritants to remove the stimuli and to initiate a healing process. Moreover, inflammation is a stereotyped response, and therefore is considered to be a mechanism of innate immunity, in contrast to a pathogen specific adaptive immunity (Murphy, 2011). A cascade of biochemical events propagates and matures the inflammatory response, involving the local vascular system. A pivotal molecular mechanism in the regulation of the inflammatory response is the secretion of regulatory cytokines. Whereas interleukin-1 (IL-1) or tumor necrosis factor-α (TNF-α) activate cellular components in a pro-inflammatory manner, anti-inflammatory peptides like the isoforms of transforming growth factor β (TGF-β_{1-3}) or IL-10 down regulate and thus limit the inflammatory cascade (Mosmann, 1994).

An early event in the inflammatory cascade is the recruitment of leukocytes from peripheral blood by activation of local endothelial cells (ECs) with pro-inflammatory mediators mainly produced by macrophages and dendritic cells (DCs) at the site of the damaged tissue (Speyer and Ward, 2011). The subsequent effector phase of inflammation is characterized by the accumulation of monocytes and their differentiation into DCs or inflammatory macrophages (Adams, 1989). These cells support the local inflammatory process by a plethora of functions like phagocytosis, cytotoxicity, antigen presentation, secretion of cytokines, and the production of nitric oxide (NO), or reactive oxygen intermediates (Ding et al., 1988). Inflammation can be further classified as either acute or chronic (prolonged), characterized by a progressive shift in the type of cells present at the site of inflammation and by simultaneous destruction of the tissue from the inflammatory process.

MODULATORY PROPERTIES ON ENDOTHELIAL CELLS OF LOW-DOSE IRRADIATION

As reported before, EC play a crucial role in the regulation of the local inflammatory process both by their ability to recruit leukocytes from peripheral blood and to express a variety of cytokines/chemokines and growth factors (Speyer and Ward, 2011). As a consequence, the effect of low-dose irradiation on the adhesion process was analyzed in *in vitro* assays using human (EA.Hy926) or murine (mlEND1) EC and peripheral blood mononuclear cells (PBMC). LD-RT prior to the stimulation by TNF-α resulted in a hampered adhesion of PBMC to 43–50% of the control level at 4 and 24 h but elevated values at 12 h after irradiation with a single dose of 0.3–0.6 Gy (Kern et al., 2000a; Hildebrandt et al., 2002; Rödel et al., 2002). This characteristic coincides with a biphasic kinetic and elevated expression of the anti-inflammatory cytokine TGF-β1 both on the levels of mRNA and protein in the same dose range. Moreover, abrogation of TGF-β1 by neutralizing antibodies restored adhesion of PBMC to irradiated EC (Rödel et al., 2004b), indicating the cytokine to be a key player in the modulation of adhesion following low-dose exposure. A hampered adhesion is further supported by a lowered expression of the adhesion molecule E-selectin on stimulated EC with a local minimum following a 0.3–0.5 Gy exposure. This indicates that the modulation of E-selectin may further contribute to the anti-inflammatory properties of LD-RT (Hildebrandt et al., 2002; Rödel et al., 2002).

MODULATORY PROPERTIES ON LEUKOCYTES OF LOW-DOSE IRRADIATION

The major cellular elements of the immune system comprise different lineages of lymphocytes (B and T cells) as members of an antigen-specific effector response, as well as polymorphonuclear (PMN) and mononuclear leukocytes (PBMC) as components of the innate immune system (Kobayashi et al., 2005). As outlined below, these immune cells are modulated by distinct low-doses of X-ray.

Apoptosis is a physiological endogenous cellular suicide program mediated by a variety of endogenous and exogenous stimuli including ionizing irradiation (Hengartner, 2000). Beside its central role in cellular homeostasis, apoptosis significantly impacts on immune regulation and radiation response. In line with that, cells undergoing apoptosis contribute to the modulation of activated mononuclear cell activity in a paradox manner by reducing the secretion of pro-inflammatory cytokines like TNF-α or IL-1. In addition, secretion of the anti-inflammatory peptide IL-10 is increased indicating an immune-suppressive potential of apoptotic cells (Voll et al., 1997). In 1999, Kern et al. (1999) were the first to report on a dose-dependent discontinuous increase of apoptosis in PBMC with a plateau or peak between a 0.3 and 0.7 Gy exposure. Additionally, the coincidence of a reduced PBMC/EC adhesion (as reported before) and induction of apoptotic cell death in PBMC prompted the group to investigate a putative link to the expression of adhesion molecules on the surface of the PBMC. They described a time-dependent proteolytic shedding of L-selectin that was associated with their early apoptotic phenotype (Kern et al., 2000b). More recently, a comparable performance of apoptosis induction was reported for PMN irradiated 2 h before stimulation with phorbol myristate acetate (PMA). Applying subG1 DNA content analyses, a discontinuous appearance of cell death was observed, showing a relative maximum at 0.3 Gy and a minimum at 0.5 Gy, respectively (Gaipl et al., 2009). Notably, the discontinuous course of apoptosis parallels a diminished protein level of mitogen activated protein (MAP) kinases and protein kinase B (or AKT), reported to be involved in the regulation of proliferation, transcription, and apoptosis (Yang et al., 2004).

Neutrophilic PMN accumulation has been implicated in the pathology of acute and chronic inflammatory diseases, such as rheumatoid arthritis (Witko-Sarsat et al., 2000) in part by the

secretion of chemotactic cytokines with the potential to amplify leukocyte infiltration (Scapini et al., 2000). Thus, the impact of LD-RT on chemokine secretion in PMN was analyzed. In comparison to CXCL8 and CCL18, CCL20 chemokine was shown to be exclusively induced in a TNF-α dependent manner by a cell–cell contact between PMN and EA.hy926 EC. Furthermore, irradiation with doses between 0.5 and 1.0 Gy resulted in a discontinuous reduction of CCL20 secretion that parallels a hampered PMN adhesion to EC with a pronounced effect at a 0.7 Gy exposure (Rödel et al., 2008).

Very recently, Bauer et al. (2011) reported that monocytes are severely impaired in base and DNA double-strand break repair that renders them highly vulnerable to ROS and irradiation induced cell death by apoptosis. Thus, it is tempting to assume that a selective killing of monocytes at doses below 1 Gy may cause a depletion of macrophages and DCs that may further contribute to the anti-inflammatory effects of LD-RT.

MODULATORY PROPERTIES ON MACROPHAGES AND DCS OF LOW-DOSE IRRADIATION

Monocytes, unlike PMN, differentiate into tissue resident DCs or macrophages (Adams, 1989). Due to their central role in the initiation and the resolution of inflammatory processes, these cells are considered as key players in the regulation of inflammation (Valledor et al., 2010). Macrophages for example support a local inflammatory process by a variety of functions including phagocytosis, antigen presentation, secretion of cytokines, and the expression of enzymes like inducible nitric oxide synthase (iNOS; Fujiwara and Kobayashi, 2005). The latter enzyme processes the synthesis of nitric oxide (NO) that in turn increases vascular permeability and is involved in inflammatory pain (Holthusen, 1997; Abramson et al., 2001). In that context, low-dose radiation ($\leq$1.0 Gy), if applied before stimulation with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) of murine RAW 264.7 cells (a mouse leukemic monocyte macrophage line) decreases iNOS protein and NO production without affecting iNOS mRNA expression (Hildebrandt et al., 1998). This may indicate a translational or post-translational regulation of the enzyme that is linked, at least in part, to the analgetic properties of LD-RT.

Tsukimoto et al. further examined signal transduction pathways in RAW264.7 macrophage cells following γ-irradiation (137Cs source) with doses of 0.5–1.0 Gy. Dephosphorylation of both extracellular-signal-regulated kinases 1/2 (ERK1/2) and p38 mitogen activated protein kinase (MAPK) was observed at 15 min after irradiation which was concomitant with a significant increase in the expression of the MAPK phosphatase-1 (MKP-1; Tsukimoto et al., 2009). Since activated p38 MAPK mediates pro-inflammatory cytokine expression, they further assayed the effect of low-dose radiation on TNF-α, showing that production of the cytokine induced by LPS was significantly suppressed in 0.5 Gy irradiated macrophages.

A further essential inflammatory cytokine is the IL-1 family member IL-1β, which shows numerous activities in the inflammatory process (Dinarello, 2011). Using RAW264.7 macrophages, a non-linearity in IL-1β production was observed after high-linear energy transfer (LET) carbon ion radiation (Conrad et al., 2009). Notably, as compared to other inflammatory cytokines, expression of IL-1β is tightly regulated by a three step process and requires two distinct stimuli (Tschopp et al., 2003). In more recent studies using human THP-1 derived macrophages which were stimulated by LPS and MSU (mono sodium urate crystals) a significantly decreased IL-1β secretion at doses of 0.5 and 0.7 Gy was confirmed by Lödermann et al. (2012). In their experiments, the IL-1β machinery (also called the NALP3 inflammasome) was not affected by the doses used, but a hampered secretion of the IL-1β correlates with a reduction in nuclear translocation of the transcription factor nuclear factor κB (NF-κB) subunit RelA (p65) in line with a decreased protein amount of upstream (p38 MAPK) and downstream molecules (AKT). Thus it is tempting to conclude, that the discontinuous regulation of IL-1β following LD-RT may occur in a NF-κB dependent manner.

Furthermore, activated macrophages are a major source of reactive oxygen species (ROS) when they mount an oxidative burst to destroy pathogens. Accordingly, the impact of low-dose irradiation on oxidative burst activity and superoxide production was investigated in RAW 264.7 macrophages after stimulation with TNF-α/IFNγ, PMA, or the yeast product zymosan. Low X-ray doses between 0.3 and 0.6 Gy significantly reduced the oxidative burst in these activated macrophages, whereas higher doses had little effect. This indicates that a diminished release of ROS may contribute to the local therapeutic effect of LD-RT (Schaue et al., 2002).

Finally, Jahns et al. recently analyzed the effect of LD-RT on the maturation, cytokine release, and T-Lymphocyte activation of human DCs. They indicated that irradiation of DC-precursors *in vitro* does not influence surface marker (CD80, CD83, CD86) expression or cytokine profile of immature DCs nor of mature DCs stimulated by LPS, neither did it influence the capacity of the DCs to stimulate T-cell proliferation (Jahns et al., 2011).

MODULATION OF TRANSCRIPTION FACTOR ACTIVITY BY LOW-DOSE IRRADIATION

As there is enormous evidence for the involvement of cellular transcription factors including TP53, activating protein 1 (AP-1), and NF-κB in both, cellular radiation response and inflammation (Habraken and Piette, 2006), they may also represent a crucial link between low-dose radiation and their immune modulatory properties. The family of NF-κB transcription factors comprises a heterogeneous group of homo- or heterodimeric members of the Rel family including p50, p52, p65/RelA, c-Rel, and RelB (Baeuerle and Baltimore, 1996; Oeckinghaus et al., 2011). NF-κB is located in the cytoplasm in an inactive form by binding to inhibitor molecules of the IκB family (IκBα, IκBβ, IκBγ/NEMO, IκBε, p100, and p105; Huxford and Ghosh, 2009). Upon dissociation of the inhibitors, NF-κB dimers translocate into the nucleus and bind specific sequence elements in the enhancer/promoter regions of a variety of effector genes. These include factors implicated in DNA damage repair, the execution, or inhibition of cell death by apoptosis (Oeckinghaus et al., 2011) and cytokines (e.g., IL-1, TNF-α), adhesion molecules (e.g., E-selectin), and enzymes (e.g., iNOS) essential in the regulation of the immune system (Kracht and Saklatvala, 2002). In the field of low-dose irradiation, Prasad et al. (1994) were the first to report on an activation of NF-κB in a discontinuous manner with peak activities at 8 and 36 h after irradiation analyzed in 244B lymphoblastoid and B16 melanoma

cells. In accordance to these findings, a comparable time dependence of DNA binding and transcriptional activity with a first peak at 4 h and a second peak at 24–30 h was confirmed in stimulated human EA.Hy926 EC (Rödel et al., 2004a). Based on these initial observations, factors engaged in the pathway(s) of NF-κB activation in stimulated EA.Hy926 EC were investigated. Among these regulatory proteins, X chromosome-linked inhibitor of apoptosis protein (XIAP) that enhances RelA/p65 nuclear translocation and promotes the degradation of IkB (Hofer-Warbinek et al., 2000; Jin et al., 2009) was investigated in more detail. Following irradiation, a discontinuous profile of XIAP-expression was observed with a relative maximum at 0.5 and 3.0 Gy which parallels a discontinuity in NF-κB induction. Furthermore, RNA-interference (siRNA) derived knockdown of XIAP resulted in a hampered NF-κB transcriptional activity, indicating a regulatory interrelationship between these factors (Rödel et al., 2010). These findings are in agreement with the observation that XIAP interacts with MAP Kinase Kinase 2 (MEKK2). The latter has previously been shown to be associated with a second wave NF-κB activation and the propagation of inflammatory processes (Winsauer et al., 2008). Moreover, a functional consequence of XIAP-expression and altered NF-κB activity on the adhesion process was obvious since XIAP attenuation showed an abrogation of the reduced PBMC binding normally observed following a 0.5 Gy exposure. This effect is, at least in part, driven by a reduced secretion of the cytokine TGF-β_1, a key player in the anti-inflammatory effects of low-dose irradiation (Rödel et al., 2007).

Members of the c-Fos and c-Jun protein family that collectively form the homo- or heterodimeric AP-1 complex (Criswell et al., 2003), are considered to be involved in the transcription of a variety of immune effector molecules, including TGF-β_1. By applying electrophoretic mobility shift assays (EMSA) and luciferase based transcriptional activity assays, a biphasic induction of AP-1 was detected in EA.Hy 926 EC (Rödel et al., 2009) that may further contribute to the immunomodulatory properties of LD-RT.

MECHANISMS UNDERLYING A DISCONTINUOUS CHARACTERISTIC OF LOW-DOSE IRRADIATION EFFECTS

The classical paradigm of radiation biology is based on the concept, that deposition of energy to the nucleus and the resulting DNA damage is responsible for the biological consequences of radiation exposure. By contrast, based on recent findings, there is growing evidence for non-(DNA) targeted effects that challenged this classical concept. Among these findings bystander or distant out of field (abscopal) mechanisms, as well as adaptive responses have been reported (Mothersill and Seymour, 2006; Hildebrandt, 2010). Notably, these novel concepts also take into consideration a complex intercellular communication and describe radiation responses on a tissue level (Barcellos-Hoff, 2005). The molecular mechanisms responsible for the discontinuous dose response characteristics, a common hallmark of these non-targeted effects, remain elusive at present and most likely originate from an overlap of several processes that may be initiated at various thresholds and operate in a staggered manner. There may also be parallels to the phenomenon of low-dose hyper-radiosensitivity and induced radioresistance, which have been reported for cellular survival at doses below 0.3 Gy and in the dose range of 0.3–0.6 Gy (Joiner et al., 2001; Marples and Collis, 2008). The current hypothesis on the regulation of this behavior is that the HRS region (<0.3 Gy) reflects an area of increased induction of apoptosis in cells that failed to undergo an ataxia telangiectasia-mutated (ATM)-dependent G2-phase cell cycle arrest. By contrast, a transition to induced radioresistance originates from a shift toward a G2-checkpoint induction, giving time for repair of DNA damage, and to increase cell survival. Corresponding to this, DNA double-strand breaks induced after very low-dose irradiation do not seem to be repaired, and cells containing residual damage were removed by a TP53-dependent apoptosis (Rothkamm and Löbrich, 2003). Interestingly, two seminal molecular studies (Xu et al., 2002; Bakkenist and Kastan, 2003) have shown discontinuous responses over the 0.1–1.0 Gy dose range, the most important being the activation and (auto)phosphorylation of the DNA damage sensor ATM. Once activated, ATM is implicated in several signaling cascades and is essential for the regulation of a cell cycle arrest, DNA damage repair, and acts as an inducer of the ATM–IκK–NF-κB signaling pathway (Hacker and Karin, 2006).

Based on these findings, it is reasonable to assume that beside DNA (repair)-mediated mechanisms, a non-linear dose-effect relationship may be associated with a differential protein expression. In line with that, Pluder and colleagues recently reported that exposure to Co^{60} γ-rays of EA.hy926 EC resulted in rapid change in the cytoplasmic proteome. The group identified 15 significantly differentially expressed proteins of which 10 were up- and 5 down-regulated. Pathways influenced by the factors include the RhoA pathway, fatty acid metabolism, and cellular stress response (Pluder et al., 2011).

ANTI-INFLAMMATORY PROPERTIES IN PRECLINICAL MODELS OF LD-RT

Beside an increasing knowledge concerning underlying mechanisms, *in vivo* models of experimentally induced arthritis (Gilroy et al., 1998) have been established to investigate a clinical anti-inflammatory efficacy of LD-RT. In 1933 von Pannewitz (1933) reported on a first series of animal studies, when rabbits with electro-coagulation of the knee joint cartilage or by mechanical bone destruction were treated with 1.0 Gy single dose irradiation. This treatment does not show a benefit on the degenerative changes, however, an improvement of the symptoms joint swelling (an indicator of reduced inflammation) and pain was observed. In subsequent years a variety of inducible models have been established to more closely mimic the situation in joints of patients suffering from rheumatoid arthritis (Smolen and Steiner, 2003). Experimental induction of arthritis in rodents treated either with zymosan (a yeast product) or inactivated mycobacterium tuberculosis (mtb) results in a fast (five days from injection) joint swelling associated with cartilage destruction and bone loss (Asquith et al., 2009). Using an intra-articular injection of papain, inactivated mtb or zymosan, Budras et al., Trott et al., and Fischer et al. induced an acute arthritis in rabbit knees. In their experiments five weekly fractions of 1.5 or 1.0 Gy significantly diminished the inflammatory proliferation of the synovial cover cells, the synthesis of synovial fluid, and thus swelling of the joint (Budras et al., 1986; Trott et al., 1995; Fischer et al., 1998). Although partially not reaching a level of significance, morphometric data further revealed a decreased number of the synovial cell layers and thus thickness of

the synovial membrane and a lowered distance between capillaries and synovial membranes following irradiation.

The effects of LD-RT on morphological progression of adjuvant induced arthritis in rats were further investigated by Hildebrandt et al. In their analysis, local irradiation of the arthritic joints reduced clinical symptoms, if given at days 15–19 after induction of arthritis by intradermal injection of mtb. Histopathological analysis performed at days 21 and 30 revealed a significant reduction of cartilage and bone destruction with minimal effect on the number of inflammatory cells in the periarticular tissue (Hildebrandt et al., 2000). In addition, the histologically observed prevention of disease progression appears to be related to a modulation of the iNOS activity with a reduction of the histochemical iNOS score and increase of heme oxygenase-1 (HO-1) expression (Hildebrandt et al., 2003).

Most of the analyses as reported before, however, used single fractions that exceed a dose of 1.0 Gy. In order to explore the lowest effective dose, the optimal time of treatment and the most favorable schedule, the effect of different fractionation schemes was analyzed by Liebmann et al. The most pronounced treatment effect was observed after two daily fractionated series of 5×0.5 Gy with an early treatment onset (days 10–14) and repetition after an interval of 8 days (days 22–26; Liebmann et al., 2004).

Relevantly, a variety of mechanisms, recognized in *in vitro* investigations to contribute to the immunomodulatory properties of LD-RT, were confirmed in the *in vivo* situation. Using an air pouch model in NMRI mice, the expression of TNF-α, IL-1β, and the proteins iNOS, HO-1, cyclooxygenase-2 (Cox2), and heat shock protein 70 (Hsp70) were investigated. Whereas the amount of exudates and number of inflammatory cells mainly remained unaffected, iNOS expression was decreased by irradiation concomitant with an increased expression of Hsp70 and HO-1 (Schaue et al., 2005). In addition, as binding of lymphocytes to blood vessel EC is crucial to drive an inflammatory process, the impact of LD-RT on adhesion and extravasation was analyzed by intra-vital microscopy in mice stimulated with LPS. In accordance to the *in vitro* findings, leukocyte adhesion in intestinal venules was diminished after irradiation with 0.1, 0.3, and 0.6 Gy, respectively (Arenas et al., 2006). This mechanistically correlated with increased levels of TGF-β_1 in the serum of the mice, and partially could be restored by neutralization of the cytokine.

In a model of collagen inducible arthritis (CIA), DBA/1J mice were irradiated once before induction and on consecutive 4 weeks with a single dose of 0.5 Gy. The authors described a significant improvement of the clinical symptoms associated with a reduced amount of antitype II collagen antibodies and reduced values of inflammatory cytokines TNF-α, IFN-γ, and IL-6 in the serum of treated mice (Nakatsukasa et al., 2008). In proceeding analyses, the group further reported on a significant increased proportion of CD4(+)CD25(+)FoxP3(+) regulatory (Treg) cells in the spleen of irradiated mice at 4, 6, and 8 weeks after immunization with collagen and a hampered secretion of inflammatory IL-17 and IL-6 (Nakatsukasa et al., 2010). Tregs in turn are reported to act as suppressors of osteoclast cell activity, which drive arthritis and bone loss and therefore may contribute to the reduction of the clinical symptoms in mice treated with low-dose irradiation (Zaiss et al., 2010). In accordance to these observations, Weng et al. (2010) reported that a therapeutic effect of low-dose irradiation was associated with an increment in the proportion of Treg cells despite the overall reduction in lymphocyte count. By contrast, depletion of CD25 or folate receptor (FR)4(+) cells with specific antibodies before the treatment abolished the beneficial effects of irradiation confirming a fundamental role of Treg.

The models mentioned beforehand, however, suffer from the fact of an artificial induction of pathologic conditions in former healthy animals, that does in detail not reflect the situation in arthritic patients which are suffering from the disease since years and display an autoimmune status (Imboden, 2009). To overcome these limitations, Frey et al. (2009) were the first to analyze effects of low-dose irradiation in human TNF-α transgenic (hTNFtg) mice (Frey et al., 2009). These animals overexpress the cytokine TNF-α during their whole lifetime and develop a genetically determined polyarthritis (PA), with an onset after 3–6 weeks of age (beginning PA). The disease pattern in animals shows similarity to a human rheumatoid arthritis such as joint swelling and deformation, synovial inflammation, cartilage damage, and bone erosion, and is fully blown at an age of 9–12 weeks (Keffer et al., 1991). Irradiation of these mice with five times 0.5 Gy at a beginning (4–6 weeks) PA demonstrates significantly temporal improved clinical symptoms like a reduced paw swelling and increased grip strength. Those effects are less pronounced at later stages of the disease (9–12 weeks, fully established PA; Frey et al., 2009).

More insight on the impact of low-dose irradiation further arises from experiments on genetically determined MRL-lpr/lpr mouse autoimmune diseases. These animals are characterized by a deletion in the TNF receptor superfamily member six (FAS) gene resulting in an impaired apoptosis of autoreactive lymphocytes and aberrant T cell proliferation, concomitant with massive autoantibody production, immune complex glomerulonephritis, and arthritis. If irradiated with a daily single dose of 0.5 Gy for 4 weeks to a total dose of 10 Gy the mass of the spleen of the MRL-lpr/lpr mice was significantly reduced in line with a drastically reduced amount of CD3(+)CD4(−)CD8(−)B220(+) T cells, which drive the splenomegaly (Tago et al., 2008). In an additional model of experimental autoimmune encephalomyelitis (EAE) animals were irradiated with a total dose of 5.5 Gy, subdivided in 0.5 Gy fractions over 5 weeks (one fraction before induction and four fractions once a week for 4 weeks) resulting in a reduced EAE incidence along with a significant improved clinical score and delayed onset of pathological changes (Tsukimoto et al., 2008). These effects may arise from a hampered ability of spleenocytes to produce the pro-inflammatory cytokines IL-6, and IL-17. Furthermore, irradiated mice spleenocytes exhibited a reduced IFN-γ secretion and shifted the Th1/Th2 balance to an anti-inflammatory phenotype.

In summary, *in vivo* data and models clearly confirmed anti-inflammatory effects and have proven true a variety of immune modulatory effects of low-dose irradiation as summarized in **Table 1**. They may display suitable platforms for an intensified research of the underlying mechanisms and on options to improve the clinical efficacy of LD-RT.

CONCLUSION

Considerable progress has recently been achieved in the understanding of cellular targets and molecular mechanisms involved

Table 1 | Preclinical models and clinical/experimental parameters for the analyses of the anti-inflammatory effects of low-dose irradiation.

Experimental model (M) Animal (A)	SD/TD	Time of irradiation	Clinical/biological effects	References
M: mechanically induced OA **A:** rabbit (knee joint)	SD 1.0 Gy	Different	↓ Inflammatory symptoms, ↓ pain ≈ degenerative changes	von Pannewitz (1933)
M: granugenol-induced OA **A:** rabbit (knee joint)	SD 1.5 Gy TD 7.5 Gy	1× Week immediately 6, 12 Weeks p.i.	↓ Joint swelling, ↓ cell proliferation within the SM, ↓ synovial fluid	Budras et al. (1986)
M: zymosan/Mtb-induced OA **A:** Wistar rat	SD 1.0 Gy/SD 5.0 Gy TD 4.0 Gy/TD 5.0 Gy	3 h p.i. or 4× daily	Zymosan: ↓ joint swelling ↓↓ cartilage/bone destruction Mtb: ↓↓ joint swelling (4× 1 Gy), ↑↑ bone destruction (1× 5 Gy)	Trott et al. (1995)
M: papain-induced OA **A:** rabbit (knee joint)	SD 1.0 Gy TD 5.0 Gy	24 h p.i. 5× Daily	↓↓ Joint diameter, ↓ SM-thickness and cell layers, ↓ distance between vessels and SM	Fischer et al. (1998)
M: Mtb-induced RA **A:** Lewis rat	SD 1.0 Gy/TD 5 Gy SD 0.5 Gy/TD 2.5 Gy	Day 15 p.i. 5× daily	↓↓ HPV, ↓↓ AS, ↓ ESR ↓↓ cartilage- and bone destruction, ↓ iNOS expression, ≈inflammatory infiltration	Hildebrandt et al. (2000, 2003)
M: Mtb-induced RA **A:** Lewis rat	SD 0.5 Gy/SD 1.0 Gy TD 2.5/TD 5.0 Gy	Days 10, 15, or 22 p.i 1× SD/ 5 days (FS1) 5× SD/9 days (FS2)	↓ HPV, ↓ AS, early IR (acute, day 10), and 0.5 Gy most effective. IR (chronic days 22–26) ≈ clinical signs	Liebmann et al. (2004)
M: carrageenan air pouch model **A:** NMRI mice	SD 0.5/1.0/2.0/5.0 Gy	6 h after challenge	≈Inflammatory exudates and cell number ↓ iNOS expression, ↓ IL-1β ↑ Hsp70, ↑ HO-1	Schaue et al. (2005)
M: LPS induced systemic response **A:** C57/Bl/6 mice	SD 0.1/0.3/0.6 Gy	1 h before LPS challenge	↓ Leucocyte adhesion in intestinal venules (max. 0.3 Gy), ↑ circulating levels of TGF-β1	Arenas et al. (2006)
M: systemic lupus erythematosus **A:** MRL-lpr/lpr mice	SD 0.5 Gy TD 2.5 Gy	24 h before induction and 4× week	↓ Weight of spleen, ↓ CD(+)CD4(+)CD8(−) B220(+) Tcells, ↑ FoxP3 T(reg) in spleen, ↓ anti-DNA antibodies	Tago et al. (2008)
M: collagen induced RA **A:** DBA/1 mice	SD 0.5 Gy TD 2.5 Gy	24 h before RA induction and 4× week	↓ Clinical symptoms, ↓ joint collapse, cytokines TNF-α, IFN-γ, IL-6 in serum	Nakatsukasa et al. (2008)
M: autoimmune encephalomyelitis (EAE) **A:** SJL/J mice	SD 0.5 Gy TD 2.5 Gy	24 h before EAE induction and 4× week	↓EAE incidence, ↓clinical score, ↓ TNF-a, IL-6, IL-17 in spleen, ↑ FoxP3 T(reg) in spleen, ↓ IFN-γ in serum	Tsukimoto et al. (2008)
M: genetically determined RA **A:** (hTNFtg) mice	SD 0.5 Gy TD 2.5 Gy	6–7 weeks and 10–12 weeks	↓↓ Ankle swelling, ↑grip strength at the beginning PA	Frey et al. (2009)
M: collagen induced RA **A:** DBA/1 mice	SD 0.5 Gy TD 2.5 Gy	24 h before RA induction and 4× week	↓ Arthritis score, ↓ antitype II collagen ab, ↑ FoxP3(+) T(reg) in spleen, ↓ IL-6, IL-17	Nakatsukasa et al. (2010)
M: collagen induced RA **A:** DBA/1 mice	SD 0.4 Gy	24 h before or at day 25	↓ AS, ↓ number of lymphocytes in circulation, ↑ FoxP3 T(reg) in circulation	Weng et al. (2010)

A, animal; AS, arthritis score; EAE, experimental autoimmune encephalomyelitis; ESR, erythrocyte sedimentation rate; HO-1, heme oxygenase; HPV, hind paw volume; hTNFtg, human TNF transgenic mice; Hsp70, heat shock protein70; IL-1, Interleukin-1; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; M, experimental model; Mtb, mycobacterium tuberculosis; IR, ionizing radiation; OA, osteoarthritis; RA, rheumatoid arthritis; SD, single dose; SM, synovial membrane; TD, total dose; TGF-β$_1$, transforming growth factor β1; ↑, increase; ↓, decline; ≈, not changed; ↑↑ highly significant increase; ↓↓ highly significant decline.

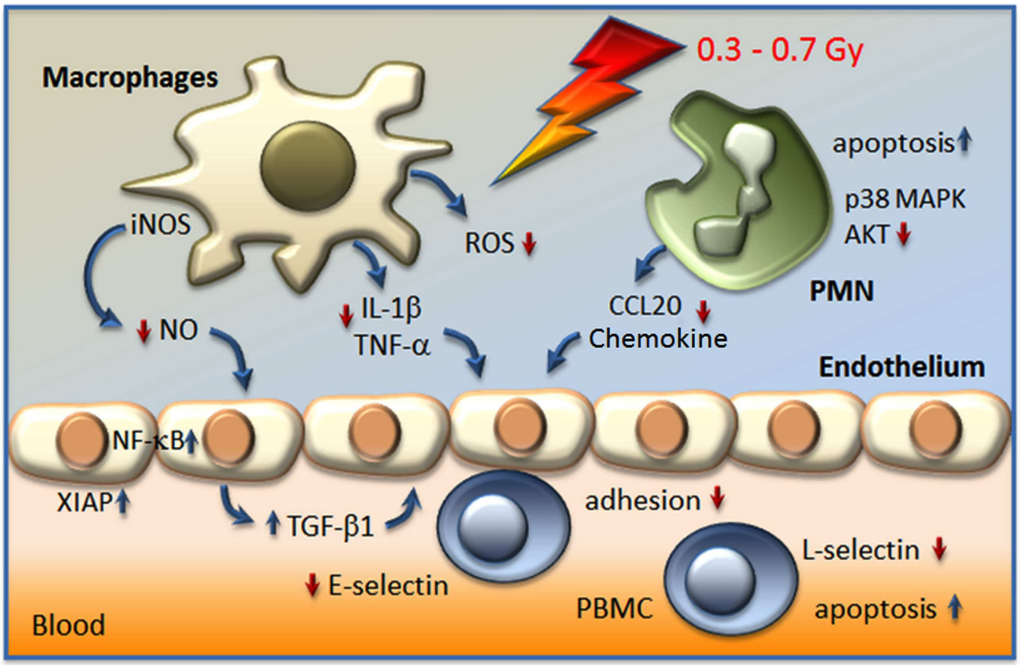

FIGURE 1 | Actual model on the modulation of inflammatory cell activity and factors involved in the anti-inflammatory effect of LD-RT (<1 Gy). Irradiation resulted in a hampered adhesion of peripheral blood mononuclear cells (PBMC) to the endothelium, due to the secretion of the anti-inflammatory cytokine transforming growth factor β1 (TGF-β1), a decreased expression of E-selectin on the surfaces of endothelial cells, a local increase of apoptosis, and the proteolytic shedding of L-selectin from PBMC. In stimulated macrophages a diminished activity of the inducible nitric oxide synthase (iNOS) in line with reduced levels of nitric oxide (NO), a lowered production of reactive oxygen species (ROS), and a diminished secretion of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) may contribute to local anti-inflammatory effects. Moreover, polymorphonuclear cells (PMN) respond to low-dose exposure with a locally increased rate of apoptosis, a hampered secretion of CCL20 chemokine and alterations in signal transduction pathways p38 mitogen activated protein kinase (MAPK) and protein kinase B (AKT).

in the immune modulatory properties of low-dose irradiation as depicted in **Figure 1**. However, as (chronic) inflammatory and degenerative diseases are based upon complex (patho)physiological networks, a variety of yet unresolved questions exists. Thus, intensive translational and clinical research efforts as well as the development of further basic models are seriously needed to recognize additional contributing factors and mechanisms. Moreover, on-going efforts should also focus on a putative relationship to tumor immune biology to optimize clinical use of radiation therapy of both benign and malignant diseases.

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Radiation-induced effects and the immune system in cancer

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Chemotherapy and radiation therapy (RT) are standard therapeutic modalities for patients with cancers, and could induce various tumor cell death modalities, releasing tumor-derived antigens as well as danger signals that could either be captured for triggering anti-tumor immune response. Historic studies examining tissue and cellular responses to RT have predominantly focused on damage caused to proliferating malignant cells leading to their death. However, there is increasing evidence that RT also leads to significant alterations in the tumor microenvironment, particularly with respect to effects on immune cells and infiltrating tumors. This review will focus on immunologic consequences of RT and discuss the therapeutic reprogramming of immune responses in tumors and how it regulates efficacy and durability to RT.

Keywords: cancer, cell death, immune response, low-dose radiotherapy, radiation therapy, tumor microenvironment

INTRODUCTION

The immune system maintains a complex regulatory balance, maintaining immunological composure despite powerful immunological stimuli. There can be intense immune activity at mucosal surfaces and relative inactivity in closely neighboring sites. Multiple inflammatory mechanisms control the location of immune responsiveness and serve to direct therapeutic responses to the site of immunological insult. The host remains in a state of controlled immune activity, regulating the initiation and termination of immune responses to prevent widespread pathology is exploited by tumors to overcome the immunogenicity caused by their antigenicity and aggressive growth. Despite the presence of immune suppression, even multiply treated patients with significant tumor burden are capable of generating *de novo* tumor-specific immune responses (Laheru et al., 2008). Over the course of radiation therapy (RT), patients have been shown to develop tumor antigen-specific immune responses that were not detectable before treatment demonstrating that immune suppression in cancer patients and any immune suppression caused by RT is relative rather than absolute (Nesslinger et al., 2007).

Ionizing radiation is a powerful cytotoxic force that can be manipulated to specifically kill cancer cells at target sites. In addition to the direct effect of radiation, focal radiation can have distant bystander effects that influence tumor growth outside of the irradiated region (Ohba et al., 1998). The abscopal bystander effect would be an important phenomenon, whether it is intended to target pre-existing distant metastases or to residual disease that was not removed by the primary therapy. RT is not always used alone and clinical translation of radiation therapies that incorporate immunotherapy must take into account their interaction with surgery or the multitude of chemotherapies. Both chemotherapy and RT impact growing cancers through their ability to induce cell death by disrupting various parameters of cell biology necessary for survival (Haynes et al., 2008; Tesniere et al., 2008; Zitvogel et al., 2008). Leukocytes detect cell death through immune-based receptors for molecules released by dying cells (often termed "danger signals"), such as Toll-like receptor 4 (TLR4) and its ligands including the high-mobility group box 1 protein (HMGB1; Apetoh et al., 2007).

Effective anti-tumor therapy should induce sufficient tumor cell death in order to release tumor-associated antigens (TAAs) as well as danger signals attracting professional antigen-presenting cells (APCs) phagocytes to uptake and present tumor antigen for specific adaptive immunity. Proper cell death modality should be triggered in both tumor cells, tumor stem cell, and stromal cells. RT clearly influences multiple immune-based programs in tissues, some of which lead to durable tumor regression, whereas others propel tumor development. It seems reasonable to conclude that identifying pathways mediating activation of myeloid-based protumor immunity induced by RT, will encourage development of novel therapeutics that suppress those activities to effectively bolster RT responses. Moreover, blockade of these protumor immune-based pathways may also present the opportunity to combine RT with anti-tumor immune-therapeutics to yield effective and durable suppression of tumors, resulting in improved outcomes for patients with cancer. In the palliative setting, for patients who have rituximab and chemotherapy-resistant disease

Abbreviations: APCs, antigen-presenting cells; ATM, ataxia telangiectasia mutated; CTL, cytotoxic lymphocytes; DAMP, damage-associated molecular patterns; DCs, dendritic cells; Flt3-L, Fms-like tyrosine kinase receptor 3 ligand; GM-CSF, granulocyte monocyte-colony stimulating factor; HMGB1, high-mobility group box 1 protein; HSP, heat shock proteins; ICAM, intracellular adhesion molecule; IFN-γ, interferon-gamma; IL, interleukin; LDRT, low-dose radiation therapy; LQ, linear-quadratic; LysoPC, lysophosphatidylcholine; MDSCs, myeloid-derived suppressor cells; MHC, major histocompatibility complex; NF, nuclear factor; NK, natural killer; OS, overall survival; PSA, prostate serum antigen; RT, radiation therapy; SABR, stereotactic ablative radiotherapy; SBRT, stereotactic body radiation therapy; TBI, total-body irradiation; TLR, Toll-like receptor; TNF, tumor necrosis factor; Tregs, T regulatory cells.

and bulky tumors, low-dose RT (LDRT; <1.0 Gy) is an active and non-toxic treatment modality that might alleviate symptoms for long periods. Conventional RT remains potentially toxic, particularly for patients whose disease is located in certain sites. As with LDRT, rituximab induces apoptosis which is suspected to contribute to the induction of a specific anti-lymphoma immune response in mice (Franki et al., 2008).

IMMUNOGENICITY OF CHEMOTHERAPY AND RADIOTHERAPY

Cancer research has primarily focused on the role of activating and/or inactivating mutations in genes regulating aspects of cell proliferation or cell death. Solid tumors contain neoplastic and non-neoplastic stromal cells embedded in a dynamic extracellular matrix (ECM) microenvironment. Cellular components of tumor stroma include hematogenous and lymphatic vascular cells, infiltrating and resident leukocytes, various populations of fibroblasts and mesenchymal support cells unique to each tissue microenvironment. Increased presence of extra follicular B cells, T regulatory cells (Tregs) and high ratios of CD4/CD8 and Th1/Th2 T lymphocytes in primary tumors or in draining lymph nodes correlates with tumor grade, stage, and overall survival (OS; Bates et al., 2006). Infiltration of macrophages into the tumor microenvironment particularly when $CD8^+$ cytotoxic lymphocytes (CTL) are also present correlates with increased OS (Kawai et al., 2008). Macrophages exposed to Th1 cytokines including interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and granulocyte monocyte-colony stimulating factor (GM-CSF) exhibit enhanced cytotoxic activity, production of pro-inflammatory cytokines, and antigen presentation (**Figure 1**; Mantovani et al., 2007). On the other hand, macrophages exposed to Th2 cytokines such as interleukin-4 (IL-4) and IL-13, immune complexes or immunosuppressive cytokines instead block CTL activity and promote angiogenesis and tissue remodeling (**Figure 1**; Ruffell et al., 2010). The immunological clinical success story in metastatic melanoma is high-dose IL-2, which causes durable regression of significant disease in a subpopulation of patients (Rosenberg et al., 1998). Phase I trials with chemotherapy-induced lymphodepletion and adoptive transfer have been performed with impressive results, showing a 50% response rate in patients with stage IV cancer (Dudley et al., 2005). Preclinical data suggested an enhanced benefit associated with lymphoablative doses of radiation requiring hematopoietic stem cell rescue and these data have been confirmed in clinical studies where the addition of myeloablative radiation with hematopoietic stem cell rescue increased response rates to 72% (Muranski et al., 2006).

Radiation can replicate the effect of vaccination by providing an alternate means to present tumor antigens, it is important to examine why vaccination and RT synergize. Chakraborty et al. (2004) demonstrated that RT influenced the tumor site to render cancer cells more susceptible to T cell-mediated cytotoxicity, potentially through upregulation of a range of adhesion molecules (Garnett et al., 2004). Radiation has been shown to increase the expression of major histocompatibility complex (MHC) class I, and accentuating this effect *via* gene therapy increases the therapeutic margin of radiation. While radiation may have a direct effect on MHC expression, tumor antigen-specific cells elicited by radiation can upregulate IFN-γ in the tumor, and responsiveness to IFN-γ has been shown to be required for radiation-induced MHC upregulation. Combination of radiation-induced local inflammation and tumor-specific effector T cells can together alter the tumor vasculature, providing an additional mechanism of tumor control (Ganss et al., 2002). In prostate cancer patients, Gulley et al. (2005) demonstrated that vaccination with an immunogenic virus combination expressing the prostate serum antigen (PSA), in combination with radiation and IL-2, resulted in prostate-specific immune responses. Direct injection of dendritic cells (DCs) into tumors undergoing radiation has been shown to increase tumor-specific T cell priming and extend survival in murine models (Teitz-Tennenbaum et al., 2008). CD8 T cell responses play an important role in the therapeutic outcome of RT in immune-competent animal models suggesting that therapies targeting T cells have the potential to enhance this component of therapy in patients (Lee et al., 2009). Chemotherapy and RT are commonly believed to kill cancer cells by apoptosis, which is generally considered as non-immunogenic. Irradiated tumor cells (cell lines or autologous dissociated tumor pieces) engineered to secrete GM-CSF are able to mobilize DCs, plasma cells, invariant natural killer (NK) T cells, and tumor-reactive $CD4^+$ and $CD8^+$ T cells, both in mice and in metastatic cancer patients (Hodi and Dranoff, 2006). Many chemotherapeutic agents used to treat malignant diseases damage lymphocytes and consequently suppress cell-mediated immunity. New cancer treatment agents such as tyrosine kinase inhibitors, thalidomide and its derivatives, proteasome inhibitors, and IFNs have been found to have diverse immunomodulatory activities blocking immune surveillance of the malignancy and permitting disease recurrence, or, favorably, by reprogramming immunity to increase autologous anti-tumor effects.

Traditional fractionated radiation is locally immunosuppressive, dampening local immune responses as they develop due in part to the fact that lymphocytes are sensitive to radiation doses and are cleared rapidly from the radiation field (Rosen et al., 1999). Fractionation makes it possible to achieve a therapeutic dose of radiation to cancer cells while relatively sparing normal tissues from late toxicities. For example, in standard fractionation for breast cancer, the radiation dose may be given in 1.8 Gy doses daily for 6–7 weeks. A single radiation dose of 1.8 Gy will result in minimal toxicity to normal tissues in the region of the tumor, with one of the notable exceptions being lymphocytes. Treatment plans go to significant lengths to minimize dose to radiosensitive populations outside of the tumor. Tumor antigen-specific T cells can be isolated from many tumors, amplified *in vitro* and restored to full cytolytic function and tumor-draining lymph nodes are a rich source of tumor antigen-specific T cells (Robbins et al., 1996). Therefore, continued tumor radiation for 7 weeks would severely diminish tumor antigen-specific T cell populations through constant site-specific cytotoxicity. T lymphocytes are exquisitely sensitive to ionizing radiation with their high turnover and radiation sensitivity, are effectively ablated by relatively low radiation doses in an effective immunosuppressive therapy. Depletion of Tregs can remove this suppressive mechanism and restore anti-tumor immunity (Onizuka et al., 1999). Any T cells introduced into a T cell-deficient environment find themselves replete

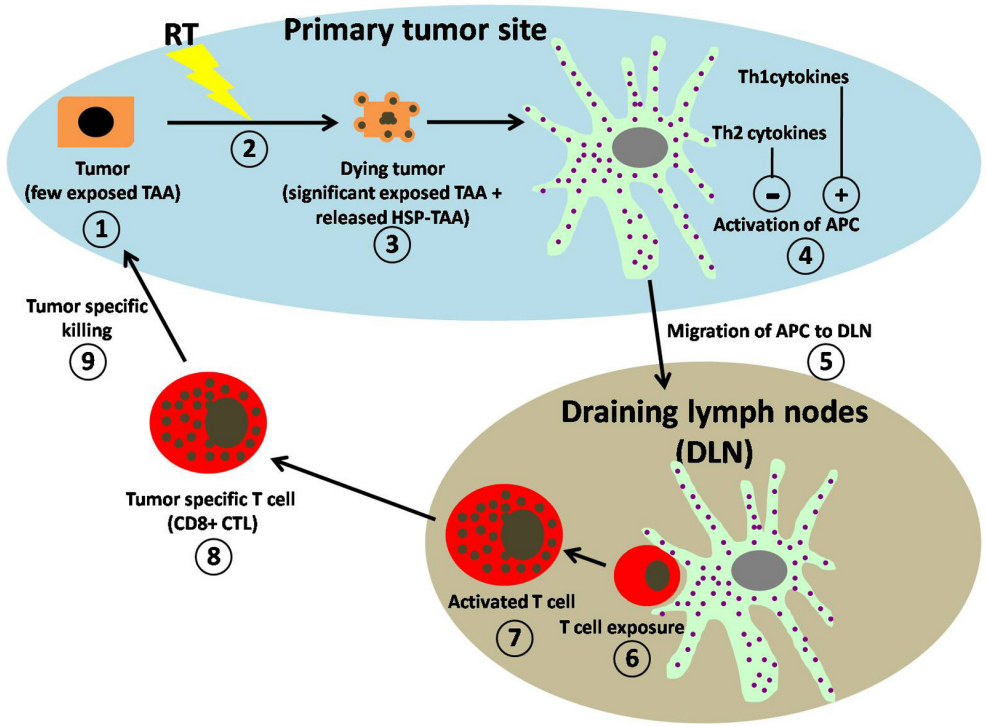

FIGURE 1 | Schematic representation of working hypothesis for RT-induced anti-tumor immune regulation. (1) Within the primary tumor microenvironment (blue area) untreated tumors express few exposed tumor-associated antigens (TAAs). (2) Exposure to RT induces the (3) dying tumors to express significantly more TAAs on their surface and to release DAMPS, (4) which are both taken up by professional APCs resulting in their activation. Activation of APC is greatly enhanced (+) by the presence of Th1-type cytokines and significantly suppressed (–) by the presence of Th2-type cytokine. (5) Activated APCs migrate to draining lymph nodes (DLN; gray area). (6) Within the DLN, T cell exposure to APC is achieved by direct contact with activated APCs. (7) Activated T cells increase in size and granularity. (8) The activated T cells migrate from the DLN as tumor-specific T cells ($CD8^+$ CTL) into the tumor microenvironment. (9) Within the tumor microenvironment $CD8^+$ CTL perform tumor-specific killing.

in both stimuli, resulting in homeostatic proliferative expansion to steady-state levels. In the process, T cells take on an activated phenotype, and have increased cytolytic activity to self and thus tumor antigens (Gattinoni et al., 2005). It was shown that in some mouse tumor models complete tumor regression was achieved following total-body irradiation (TBI). Gattinoni et al. (2005) likewise were able to show a significant increase in anti-tumor immunity associated with radiation-induced lymphodepletion and importantly, that radiation-induced lymphodepletion had a greater therapeutic benefit than was observed in a genetically deficient Rag1-lymphodepletion model. Immunostimulatory cytokines including IL-2, IL-12, and TNF-α have been used in combination with RT to stimulate anti-tumor T cell responses. Addition of these pro-inflammatory cytokines enhances RT efficacy by bolstering CTL cell responses (**Figure 1**). Interestingly, IL-3, a cytokine that activates monocytes and mast cells, delays tumor growth in response to RT (Oh et al., 2004). Intratumoral injection of CpG oligodeoxynucleotides that activate TLR9 on macrophages and DCs resulted in increased RT response and resistance to a second challenge with the same tumor, thus indicating development of a durable immune response. Antigen presentation on the surface of DCs to T cells requires both MHC and costimulatory molecules, B7 molecules and OX40. Inhibition of tumor growth and enhanced OS was also observed in a murine sarcoma model when RT was given in combination with an agonistic antibody for OX40, a costimulatory molecule found on activated T cells that stimulates T cell proliferation and differentiation. Inhibition of CTLA-4 co-stimulation also enhanced effectiveness of RT in 4T1 murine mammary carcinomas carcinoma resulting in diminished metastasis and increased survival, however, RT dose and timing were critical with regards to anti-CTLA-4 therapy (Dewan et al., 2009).

Cytokines are peptide-type regulatory proteins, such as the ILs and lymphokines, released by immune cells leading to generation of an immune response. Some cytokines act to inhibit immune responses, e.g., IL-10 and TGF-β, or instead stimulate immune responses, e.g., TNF-α or IL-1 (Germano et al., 2008). TNF-α mRNA and protein levels were increased in human sarcoma cells following RT, an effect that sensitized tumor cells to radiation-induced cell death. Macrophage-derived IL-1α and IL-1β have also been found increased in response to RT *in vivo* following sublethal TBI, as also have IL-6 and TGF-β. Consequences resulting from the release of these cytokines are recruitment and activation of leukocytes from peripheral blood and extravasation into tissue (tumor) parenchyma. Cell adhesion molecules such as ICAM-1, E-selectin and vascular cell adhesion molecule 1 (VCAM-1) are upregulated on endothelial cells during inflammation and are critical for leukocyte trafficking across endothelial barriers.

Vascular endothelial cells within tumor vessels respond to RT by upregulation of ICAM-1 and E-selectin and thereby facilitate leukocyte arrest and adhesion prior to transmigration. Blockade of CD11b, the ligand for ICAM-1, in a transplantable murine squamous carcinoma model significantly reduced tumor-infiltration by $CD11b^+$ myeloid cells following RT resulting in diminished tumor growth (Ahn et al., 2010). Similarly, examination of tumor tissue removed from head and neck cancer patients following RT revealed marked increase in endothelial ICAM-1 expression, in concert with increased β2 integrin-positive myeloid cell infiltration. The radiation-induced CXCL16 is an important mechanism by which RT promotes $CD8^+$ T cell infiltration leading to tumor suppression. Stromal cell-derived factor-1 alpha (SDF-1α) is also upregulated following RT in bone marrow-derived cells and cell lines derived from brain tumors. Inhibition of the SDF-1α pathway with a small molecule inhibitor blocking the interaction of SDF-1α and CXCR4 prevented infiltration of macrophages and significantly delayed tumor regrowth following RT (Kozin et al., 2010). Vaccination of prostate cancer patients with recombinant viral-based vaccines expressing PSA, in combination with the costimulatory molecule B7-1 and standard RT to the prostate (70 Gy of RT in 1.8–2 Gy fractions), resulted in a threefold increase in PSA specific T cells and evidence of generating T cells against other prostate-specific antigens in 76% of patients (Gulley et al., 2005).

Stereotactic body RT (SBRT)-dose radiation has been shown to generate tumor antigen-specific T cells in mice bearing B16 tumors (Lugade et al., 2005), and comparable radiation doses are less effective at tumor therapy when conducted in immunodeficient mice (Lee et al., 2009). When using aggressive, transplantable tumor models where standard 1.8–2 Gy fractions are less effective, and tumor-bearing survival is too short to complete an extended fractionation schedule. A fundamental issue in stereotactic ablative radiotherapy (SABR) is whether classical radiobiologic modeling with the linear-quadratic (LQ) model is a valid method to assess the biologically effective dose at the high doses typically encountered in radiosurgery. The robustness of the LQ model to predict fractionation and dose-rate effects in experimental models *in vitro* and *in vivo* at doses up to 10 Gy is based on the premise that cell killing is the dominant process mediating the radio-therapeutic response for both early and late effects including vascular effects. However, the administration of a single high-dose of radiation *in vivo* had a much greater effect than predicted by the LQ model; they cited several examples including Leith et al. (1994) who calculated that the dose to obtain a high probability of tumor control for brain lesions would be at least 25–35 Gy using the LQ model, which was much higher than the observed clinically effective radiosurgical dose, which was in the range of 15–20 Gy. The *in vitro* survival curve has goodness of fit in all clinically significant ranges including the ablative range characteristic of SABR (Guerrero and Li, 2004).

RADIATION THERAPY AND ACTIVATION OF STRESS-RESPONSE PATHWAYS

The delivery of an ablative dose of radiation of 15–25 Gy was found to cause a significant increase in T cell priming in draining lymphoid tissue, leading to reduction or eradication of the primary tumor or distant metastasis in a $CD8^+$ T cell-dependent fashion in an animal model. While conventional 2 Gy doses seem inferior at generating such responses, higher sized dose fractions may be better than single doses (Dewan et al., 2009). Radiation cannot only kill tumor cells releasing tumor antigens and molecules with what are collectively called damage-associated molecular patterns (DAMPs) that exert various immunomodulatory effects including induction of the expression of cytokines, chemokines, and release of inflammatory mediators (**Figure 1**; Gattinoni et al., 2005). Radiation also increases the permeability of the local vasculature either directly or through cytokine production that leads to recruitment of circulating leukocytes into surrounding tissues including APCs and effector T cells (Ganss et al., 2002). Thus, a radiation-induced pro-inflammatory microenvironment within irradiated tumors could provide DCs with maturation inducing stimuli critical for eliciting effective antigen presentation (**Figure 1**). The introduction of cytokines, in particular IL-2 for cancer treatment was a major clinical effort that had modest success. This situation changed with the molecular cloning of human TAAs that could be recognized by T cells, the ability to culture powerful APCs in the form of DCs and to assess immune responses to specific tumor epitopes using tetramer and enzyme-linked immunosorbent spot (ELISPOT) assays (Yee et al., 2001). The "danger" model of immunity suggests that pathogens with associated molecular patterns (PAMPs) and DAMPS engender an inflammatory milieu that promotes the development of antigen-specific immunity through DC maturation that allows internalization of apoptotic and necrotic cellular debris and presentation of processed antigen to T cells. Thus, administration of radiation may therefore be considered to create an inflammatory setting via DC maturation, induction of apoptosis, necrosis, cell surface molecules, and secretory molecules (**Figure 1**). As with many other challenges, radiation upregulates expression of immunomodulatory surface molecules (MHC, costimulatory molecules, adhesion molecules, death receptors, heat shock proteins) and secretory molecules (cytokines, inflammatory mediators) in tumor, stromal, and vascular endothelial cells. Important amongst these may be the upregulation of TNF family members that could promote cell killing, not only by TNF in the microenvironment but also by radiation-induced TNF.

Activation of Fas-mediated cell death is a mechanism by which immune cells eliminate damaged cells, including those damaged by RT. Thus, while whole-body radiation is "immunosuppressive" due to triggering widespread apoptosis of immune cells via Fas, focal radiation such as that used for treatment of many types of solid tumors instead has limited immunosuppressive side effects, and may actually promote changes in the local tumor microenvironment that paradoxically enhance infiltration and activation of multiple immune cell types that may either foster, and/or suppress tumor development (de Visser et al., 2006). At the most simplistic level, a main mechanism by which ionizing radiation mediates a biologic effect is via generation of free radicals that lead to genotoxic (DNA) damage, and subsequent activation of stress-response pathways through activation of the DNA damage pathway ataxia telangiectasia mutated (ATM). Activation of the ATM protein pathway following RT involves activation of p53 and nuclear factor (NF)-κB transcription factors. NF-κB can also be activated

independently of DNA damage through radiation-induced activation of TNFR-associated factors (TRAFs; Rashi-Elkeles et al., 2006). NF-κB directly regulates expression of molecules that promote a pro-inflammatory immune response, including TNF-α, IL-1 (Mori and Prager, 1996), chemokines such as CCL5 (Wickremasinghe et al., 2004); ICAM-1, E-selectin and VCAM-1, as well as MHC molecules, and expression of several anti-apoptotic genes including Bax and Bcl-2.

ANTI-TUMOR THERAPY AND TUMOR MICROENVIRONMENT

The tumor microenvironment contains innate immune cells [NK cells, neutrophils, macrophages, mast cells, myeloid-derived suppressor cells (MDSCs), and DC] and adaptive immune cells (T and B lymphocytes) in addition to tumor cells and their surrounding stroma (fibroblasts, endothelial cells, pericytes, and mesenchymal cells; de Visser et al., 2006). Myeloma cells treated with low doses of common therapeutic agents, such as doxorubicin, melphalan, and bortezomib, upregulate DNAM-1 and NKG2D ligands. Azacytidine enhances tumor antigenicity by upregulating MHC class I and tumor antigen expression, increasing the release of pro-inflammatory cytokines and danger signals, and promoting antigen uptake by DC and killing by NK cells. Infiltration of the primary tumor by memory T cells, particularly of the Th1 and cytotoxic types, is the strongest prognostic factor in terms of disease-free and OS at all stages of clinical disease (Pages et al., 2010). A combined therapy of local radiation with Th1 cell could augment the generation of tumor-specific CTL at the tumor site and might also be effective for the treatment of distant metastases. The suppressive activity of MDSCs is associated with the intracellular metabolism of L-arginine, which serves as a substrate for inducible nitric oxide synthase (iNOS/NOS2) that generates NO and arginase 1 (ARG 1) which converts L-arginine into urea and L-ornithine. Tumor-derived exosome-associated Hsp72 could trigger Stat3 activation in MDSCs and determine their suppressive activity in a TLR2/MyD88-dependent manner (Chalmin et al., 2010).

INTERACTION BETWEEN TUMOR CELLS AND THE IMMUNE SYSTEM

The tumor stroma plays an important role in the response to high-dose per fraction radiation treatment because the vascular endothelial cell apoptosis is rapidly activated above 10 Gy per fraction (Garcia-Barros et al., 2003), and that the ceramide pathway orchestrated by acid sphingomyelinase (ASMase) operates as a rheostat that regulates the balance between endothelial survival and death and thus tumor response (Truman et al., 2010). Damage to vascular/stromal elements in tumors has also been observed around 2 weeks after radiation exposure that was less dependent on size of dose per fraction (Chen et al., 2009). Pathological observations show profound changes in vasculature after radiosurgery and from studies on arteriovenous malformations, where obliteration of abnormal vasculature occurs months after irradiation, but is rarely seen below single doses of 12 Gy climbing steeply with increasing doses above this threshold (Szeifert et al., 2007). Although lymphocyte radiosensitivity is well recognized, the effects of different doses and delivery methods on systemic and locoregional naive, effector, or Treg or other immunologically relevant populations is still the subject of debate. Several authors have investigated the potential immunomodulatory effects of localized RT on tumors resulting in conflicting reports as to whether these responses promote or interfere with tumor reduction. This dualism is something that is to be expected and is inherent in a system that has to promote both destruction of pathogens and tissue healing while regulating anti-self-reactivity. Apetoh et al. (2007) showed that radiation can trigger signals that stimulate TLR4 on antigen-presenting DCs. Liao et al. (2009) have shown that irradiation of DC can enhance presentation of antigenic peptides by the exogenous pathway and is a maturation signal, while inhibiting internal antigen processing, and Merrick et al. (2005) have shown a decrease in IL-12 production that has a negative effect on presentation. Several reports have shown increased expression of MHC class I and coaccessory molecules after radiation of both tumor and host cells, while Chakraborty et al. (2004) reported a direct effect of radiation on tumors by modifying the phenotype of tumor cells to render them more susceptible to vaccine-mediated T cell killing, and others have shown that radiation-induced changes in the tumor immune microenvironment to promotes greater infiltration of immune effector cells (Lugade et al., 2005).

CELL DEATH AND ANTIGEN RELEASE

Effective initiation of adaptive immune responses to cell-associated antigen requires presentation of antigen by professional APCs. Macrophages cross-present antigen from their environment and express an important selection of critical costimulatory molecules; however, for effective presentation of antigens to naive T cells in draining lymph nodes, DCs are particularly critical. For example, apoptotic cells efficiently load DCs with tumor antigen, but do not cause DC maturation (Melero et al., 2000). By contrast, antigen from non-apoptotic cells also loads DCs, but also causes DCs to mature and upregulate costimulatory molecules (Basu et al., 2000). These authors demonstrate that CD8 T cell responses play an important role in the therapeutic outcome of RT in animal models (Lee et al., 2009). That immune responses may already be relevant in the success of RT suggests we have an opportunity to increase the therapeutic margin of RT by further enhancing the immune component.

Low-dose RT induces the apoptosis of lymphoma cells. Primary lymphoma cell culture has shown an increase in the number of apoptotic cells after RT (Dubray et al., 1998). Another study using fine-needle cytology was coupled with *in vivo* imaging with 99Tc-Annexin-V scintigraphy in 11 follicular lymphoma patients, out of which, 10 patients were concordant with cytology in both the pre- and post-LDRT evaluation. These studies suggest that LDRT neutralizes the anti-apoptotic properties of the characteristic overexpression of Bcl-2 in follicular lymphoma cells (Langenau et al., 2005). The overexpression of p53 induced by LDRT was confirmed by p53-immunostaining with p53 expression, increasing from 5% of lymphoma cells to >80% after LDRT. This induction of the p53 target was seen as the dominant component of tumor cell apoptosis (Knoops and de Jong, 2008). The investigators showed that LDRT induced both intrinsic and prominently extrinsic apoptosis pathways (Knoops et al., 2007). Therefore,

they found an upregulation of BBC3 (Puma), BAX, PMAIP1 (Noxa), and a significant overexpression of cleaved caspase-9 after LDRT. Death receptor genes, including TNFRSF10B (TRAIL-R2) and FAS, were also upregulated after LDRT. Knoops et al. (2007) also demonstrated that LDRT induced an upregulation of macrophage activation-related genes, indicating that macrophage activation was probably induced by signals from apoptotic cells. No increase of $CD68^+$ cells was observed after LDRT. These data indicate that LDRT induces an apoptosis of follicular lymphoma cells that could activate innate and adaptive immunity and contribute to the therapeutic effect observed in clinical practice.

TLR4 expression by DCs also appears to be a prerequisite for efficient antigen presentation of tumor antigens furnished by dying cancer cells (Apetoh et al., 2007). Th1-related genes, including IL-18-, COP1-, and IFN-induced chemokines such as CXCL9, CXCL10, and CXCL11, were also induced after LDRT (Knoops et al., 2007). The response of RT outside of the radiation field, rarely observed in many malignancies including lymphoid malignancies due to the abscopal effect (Robin et al., 1981). The abscopal effect is not often observed after RT alone (Kusmartsev and Gabrilovich, 2002). Evidence supporting the role of RT in promoting cross-priming and the induction of anti-tumor T cell responses was suggested by at least one experimental model (Chakravarty et al., 1999). When Fms-like tyrosine kinase receptor 3 ligand (Flt3-L), a growth factor that stimulates the production of DCs were administered after the treatment of a mouse lung carcinoma by local RT, the treated mice experienced significant prolonged disease-free survival. In contrast, Flt3-L alone induced moderate delayed growth only. Mice bearing a syngeneic mammary carcinoma, 67NR, in both flanks were treated with Flt3-L after local irradiation with a single dose of 2–6 Gy to only one of the two tumors. The growth of the non-irradiated tumor was also impaired by the combination of RT and Flt3-L; moreover, growth of a non-irradiated A20 lymphoma in the same mice harboring an irradiated 67NR tumor was not affected. Demaria et al. (2004) also showed that no such effect was observed in athymic mice. With a murine colon adenocarcinoma model, Reits et al. (2006) showed that radiation enhances MHC class I expression and that anti-tumor immunotherapy with adoptive CTL cells is active only when preceded by RT (8–10 Gy) of the primary tumor. Similar observations were more recently described by Shiraishi et al. (2008) with ECI301 (a chemokine secreted by various leukocytes, including T lymphocytes and activated macrophages and recruiting certain cells such as monocytes and DCs) after local irradiation of 6 Gy. Marked infiltration of $CD4^+$ and $CD8^+$ cells was observed, not only in the irradiated site, but also at the non-irradiated site. In a recent animal model study, Lee et al. (2009) observed that local RT on grafted tumors generates $CD8^+$ T cell immunity to lead to tumor reduction, reduces local relapse, and even eradicates metastasis in some settings. An increase of infiltrating T cells in the tumor microenvironment and the draining lymphoid tissues was seen 1–2 weeks after treatment with higher radiation dose (15–20 Gy in one to four fractions).

Additional approaches to induce a CTL-mediated tumor cell killing are based on the *in vivo* activation of DCs, which should take up tumor antigens and consecutively present tumor peptides to T cells to achieve co-stimulation. However, macrophages recognize and phagocytose dying tumor cells swiftly and silently and thereby remove tumor antigens (Gaipl et al., 2007), which are recruited by find-me signals such as lysophosphatidylcholine (LysoPC) secreted by RT-induced apoptotic cells. Moreover, the latter may even cause caspase 3-dependent tumor cell repopulation by generating potent growth-stimulating signals (Huang et al., 2011). In order to enable enhanced access of DCs to RT-induced apoptotic and necrotic tumor cells is to block their clearance by macrophages with the PS-binding protein Annexin A5 (AnxA5; Bondanza et al., 2004; Frey et al., 2009). The growth of syngeneic tumors is significantly retarded by a single injection of AnxA5 around the tumor. The combination of RT with AnxA5 resulted in the most effective inhibition of tumor growth (Frey et al., 2009). *In vivo* experiments with immune-competent mice bearing syngeneic tumors have proven that AnxA5 increases the immunogenicity of tumor cells. The injection of irradiated tumor cells pre-incubated with AnxA5 cured established tumors in about 50% of the animals, while the injection of irradiated tumor cells only resulted in $<10\%$ of tumor free mice (Bondanza et al., 2004). Targeting of PS on therapy-induced dying and on viable metastatic cells could therefore both lead to efficient anti-tumor immune responses by promoting uptake of the tumor cells by DCs, to mention here one of multiple possible modes of action resulting from the shielding of PS (Frey et al., 2012).

RADIATION THERAPY-INDUCED IMMUNOGENIC CELL DEATH

Radiation of tumor cells generally produces two responses: proliferative arrest (which in the case of senescence is indefinite) or cell death, which occurs by several mechanisms including apoptosis, necrosis, autophagy, or mitotic catastrophe (Galluzzi et al., 2007). Autophagy is marked by sequestration of large parts of the cytoplasm in autophagic vacuoles typically before cells undergo apoptosis. Finally, mitotic catastrophe is described by prolonged mitotic arrest with associated micro- and/or multinucleation prior to undergoing death. Radiation-mediated cell death is generally thought to occur primarily through either apoptosis or mitotic catastrophe. Immunization with tumor cells treated with either chemotherapy or RT prevented regrowth of tumors in $\sim$30% of mice as compared to mice immunized with untreated tumor cells. When cells were harvested from draining lymph nodes in immunized mice, and re-challenged *ex vivo*, only lymph node cells from mice immunized with tumor cells treated with RT produced IFN-γ in response to re-challenge. Protective immunization in this scenario was dependent on the presence of TLR4 on DCs and its ligand HMGB1, both released by tumor cells following RT (Apetoh et al., 2007). Two other factors, calreticulin and ATP, also significantly contribute to immunogenic cell death, in a manner similar to HMGB1, where cell death triggers rapid translocation of calreticulin to the surface of cells thereby promoting antigen presentation by dying cells and DCs (Perez et al., 2009). Cytotoxic therapies (chemotherapy and RT) induce rapid release of ATP from cells. ATP acts on the P2X(7) purinergic receptor expressed by DCs, leading to activation of the NOD-like receptor family, pyrin domain containing-3 protein

(NLRP3)-dependent caspase-1 activation complex also known as the inflammasome. Inflammasome activation leads to release of pro-inflammatory cytokines such as IL-1β, which are important for priming T cells. When components of this pathway (NLRP3, caspase-1 or IL-1R) are absent, reduced T cell responses toward cells killed by chemotherapy or RT are observed (Ghiringhelli et al., 2009), thus indicating that release of ATP from dying cells is a critical aspect of immunogenic cell death and anti-tumor immunity. Innate leukocytes, including DCs, macrophages, NK cells, and mast cells, are referred to as "first responders" to inflammatory mediators, largely based on the fact that they are often pre-stationed in tissues. RT induces opposing responses in tumors with regards to DCs: directly irradiated DCs are less effective APCs, however, the tumor microenvironment generated by RT enhances APC capabilities of DCs. Tumor-infiltrating macrophages, derived from circulating monocytes, make up a substantial component of the leukocyte infiltrate in solid tumors (Mantovani and Sica, 2010). Using human macrophage-derived cell lines, Lambert and Paulnock (1987) observed that RT enhanced macrophage cytolytic activity. Other groups have reported that low-dose whole-body RT increased expression of TLR4/MD2 and CD14 expression on murine peritoneal macrophages, leading to increased secretion of anti-tumor cytokines including IL-12 and IL-18, thus indicating that RT increases anti-tumor potential of macrophages (Shan et al., 2007).

Mast cells are pre-stationed in many tissues where they act as important sentinel cells capable of mounting rapid responses to tissue damage. Heissig et al. (2005) reported that LDRT fostered mast cell-dependent vascular regeneration in a limb ischemia model where RT promoted vascular endothelial growth factor (VEGF) production by mast cells in a matrix metalloproteinase-9 (MMP-9)-dependent manner. RT, through MMP-9 upregulated by VEGF in stromal and endothelial cells, induced release of Kit-ligand (KitL) and promoted migration of mast cells from bone marrow to the ischemic site similar to RT effects in the thoracic cavity where mast cell density increased in bronchoalveolar lavage fluid (Heissig et al., 2005). RT and adaptive immunity in experimental rodent models of cancer development, e.g., brain, sarcoma, lung, and breast, RT alone or in combination with DC or immunostimulatory therapies enhanced generation of anti-tumor responses mediated by CTL cells (Matsumura et al., 2008). RT alone can also stimulate anti-tumor T cell-based immunity when given at high doses by increasing the number of activated $CD8^+$ T cells. In 4T1 mammary tumors, recruitment of CTL cells is dependent on CXCR6, a receptor for CXCL16. RT in combination with anti-CTLA-4 mAB increases recruitment of $CXCR6^+$ $CD8^+$ T cells (Matsumura et al., 2008). In orthotopically transplanted sarcoma and carcinomas, presence of macrophages was inversely correlated with tumor regression following RT (Milas et al., 1987). In melanoma, local RT of implanted tumors increased the number of APCs in draining lymph nodes and increased the number of $CD11b^+$ cells in tumors (Lugade et al., 2005). $CD11b^+$ myeloid cells (a portion of which are macrophages) contribute growth factors such as VEGF and MMP-9 that supports angiogenic programs in growing tumors. Preventing influx of $CD11b^+$ myeloid cells following RT results in enhanced RT effects likely due to their increased expression of T cell suppressive molecules iNOS and ARG 1 (Meng et al., 2010). Examination of tumor cells exposed to ionizing radiation *in vitro* indicates that RT induces expression of NKG2D ligands, an activating receptor for NK cells (Kim et al., 2006).

Robust acute inflammation could be triggered by sterile cell death which induces DAMP exposed on the plasma membrane or secreted extracellularly. These cell-derived DAMP, such as uric acid, DNA (specifically unmethylated CpG-rich regions), HMGB1, SAP130, S100 proteins, and Hsp could stimulate an IL-1- and inflammasome-dependent response (Osterloh et al., 2008). Other pro-inflammatory stress molecules released by dying cells include Hsp70, a stress-response protein with a role in binding defective proteins and presenting them on the surface of cells. When exposed to RT, pancreatic and colon carcinoma cell release Hsp70, thereby targeting them for lysis by NK cells (Gehrmann et al., 2005). That NKG2D ligands and Hsp 70 render cells more susceptible to NK cell-mediated cytolysis indicates that RT-stimulated NK activity may be an important component of RT-induced immune responsiveness. Apoptotic microparticles could transfer chemokine receptors and arachidonic acid between cells, activates complement, promote leukocyte rolling, and stimulate the release of pro-inflammatory mediators. LysoPC, but none of the LysoPC metabolites or other lysophospholipids, represents the essential apoptotic attraction signal able to trigger a chemotactic response through phagocyte receptor G2A (Peter et al., 2008). The prototypical DAMP-HMGB1 is released with sustained autophagy, late apoptosis, and necrosis. HMGB1 could act as chemotactic and/or activating factors for macrophages, neutrophils, and DC (Apetoh et al., 2007). Forced expression of CD39 (NTPDase-1, an ecto-apyrase responsible for the degradation of NTP) could abrogate the chemoattractant activity of apoptotic cells (Elliott et al., 2009). Myeloid leukemia, migrating hematopoietic progenitors, and also solid tumors were found to overexpress CD47, resulting in a reduced uptake by SIRPα-expressing macrophages.

CONCLUSION

To design effective therapies for the future, it remains to be determined to what degree this endogenous response can be relied upon to clear tumors once they have successfully emerged from immune control. The radiation is cytotoxic to lymphocytes, and it is possible that prolonged fractionation limits the capacity of adaptive immunity to influence the outcome of RT. On the other end of the radiation dose scale, the advents of hypofractionated RT may permit more direct translation of the lessons learnt in animal models into clinical research and minimize the negative effects of fractionated radiation on tumor-site immune responses. With the continuing evolution of technology in RT it may become more feasible to optimize the cytotoxic component of radiation while simultaneously taking into account optimal immune activation. LDRT is now widely acknowledged to be very active against indolent lymphomas and is a useful tool in the management of this disease with virtually no toxicity. In clinical practice, the best methods to develop active combinations with RT and current treatments such as rituximab, immunotherapy, and chemotherapy remain to be studied. Combination immunotherapy and radiation

approaches are being translated into the clinic where intratumoral DCs injection with coordinated irradiation and introduction of autologous, unmanipulated DCs have been the subject of anti-tumor therapy. At present, SABR represents an exciting, effective, yet empirically designed RT. In addition, SABR could be optimized for use with immunotherapeutic approaches so as to better generate tumor antigen-specific cellular immunity.

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